

Importance de la variabilité verticale dans un lac méromictique profond: diversité et activité lysogène des communautés virales

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Importance de la variabilité verticale dans un lac méromictique profond: diversité et activité lysogène des communautés virales

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RESUME

Les objectifs de ce travail visaient à déterminer l'importance qualitative, quantitative et fonctionnelle des virus dans le Lac Pavin, en tenant compte des gradients liés à la profondeur. Il s'agissait, plus spécifiquement, d'examiner (i) la dynamique saisonnière et spatiale de la biodiversité des virus, (ii) l'importance des gradients liés à la profondeur dans les variations saisonnières de l'abondance et de l'activité lytique des virus, et (iii) l'importance du mode de vie lysogène. Grâce à la mise au point d'un protocole original de concentration, nous montrons que la dynamique de la diversité du virioplancton du lac est étroitement associée à celle des communautés microbiennes. Il en est de même pour les variations d'abondance et d'activité lytique. La prise en compte de l'ensemble de la colonne d'eau indique que la structure des réseaux trophiques microbiens et la place des virus dans ces réseaux dépendent de la profondeur, avec une simplification des modes trophiques, et des niveaux d'organisation biologique des communautés, et des interactions trophiques potentielles, avec la profondeur. Ainsi le fonctionnement trophique des couches profondes, réduites aux virus et procaryotes, serait essentiellement régi par la boucle virale. Finalement, jusqu'à 16 % du bacterioplancton serait lysogène. Ce mode de vie, antagoniste à celui lytique, dépend de la disponibilité de l'hôte et serait plus un mécanisme de survie que de prolifération virale, pouvant par ailleurs avoir une incidence sur l'évolution adaptative des hôtes. Les résultats acquis montrent que les virus seraient essentiels dans la diversification et l'écologie des communautés microbiennes, et dans les flux de matière et d'énergie circulant dans la colonne d'eau.

Mots clés : Lac Pavin - Virioplancton - Réseaux trophiques microbiens - Gradients verticaux - Lyse virale – Lysogénie

ABSTRACT

The main goal of this thesis was to assess qualitative, quantitative and functional significance of viruses in Lake Pavin, by taking into account the depth-related gradients in the water column. Specifically, we have examined the (i) spatio temporal dynamics of viral community diversity, (ii) significance of depth-related gradients in seasonal variations of viral abundance and lytic activity and (iii) relative significance of lysogenic 'life style'. From an original concentration protocol, we have shown that the dynamics of viral diversity in the lake was strongly linked to those of microbial communities. Similar for variations in viral seasonal abundance and lytic activity. Through the entire water column, microbial trophic network structure and the role of viruses in this network depended strongly on depth, with a simplification of trophic modes, of biological organization levels of communities, and of trophic interactions with depth. Biological limnology of the deep layers is reducte to viruses and prokaryotes, and is thus governed by the viral loop processes. Finally, up to 16 % of bacterioplankton could be lysogens. This way of life, antagonist with lytic one, depended on host availability and could be more a mechanism for the maintenance of viral traits, than can further have significant incidences on the evolutionary ecology of host communities. Overall, our results show that viruses are essential in the diversification and ecology of microbial communities, and in the matter and energy flows in the lake water column.

Key Words : Lake Pavin - Virioplankton - Microbial loop - Depth related gradients- Viral lysis- Lysogeny

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INTRODUCTION GENERALE, ELEMENTS BIBLIOGRAPHIQUES

Avec la découverte, il y a une vingtaine d'années, de fortes abondances virales dans les milieux aquatiques (**Bergh et al. 1989**), généralement plus d'un milliard de particules virales par litre d'eau, l'écologie virale aquatique a connu un développement considérable. Les virus, parasites obligatoires, forment l'entité biologique la plus abondante des écosystèmes naturels où ils sont capables d'infecter, potentiellement, toutes les cellules vivantes. Cela explique leur omniprésence dans l'environnement aquatique, y compris dans les systèmes les plus extrêmes (sources hydrothermales profondes, milieux hypersalés, déserts…) (**Prangishvili et al. 2006, Le Romancer et al. 2007**). Par ailleurs, malgré les difficultés liées à l'observation de particules nanométriques et à l'absence de traceurs évolutifs comme l'ARN ribosomal, on considère que les virus représentent un des plus grands réservoirs de la diversité génétique non caractérisée de notre planète (**Suttle 2007**). Ils contiennent des gènes codant pour des fonctions biologiques, dont la circulation fait des populations hôtes de puissants vecteurs d'échanges génétiques dans l'environnement (**Hambly et Suttle 2005, Suttle 2007**). Les virus aquatiques seraient essentiels dans divers processus structurant la dynamique de la biodiversité microbienne aquatique : mortalité cellulaire, cycles biogéochimiques, transferts horizontaux de gènes… (**Sime-Ngando 1997, Wommack and Colwell 2000, Sime-Ngando et al. 2003, Weinbauer 2004, Suttle 2005 et 2007, Hamilton 2006**). Ainsi, l'écologie virale suscite, aujourd'hui, un intérêt croissant dans le cadre des sciences de l'environnement. De manière générale, les recherches récentes en écologie virale sont sources de nouvelles connaissances, non seulement dans le domaine de la diversité du vivant et du fonctionnement des écosystèmes, mais également dans le domaine de l'évolution (**Prangishvili et al. 2006, Forterre 2006**).

Dans ce chapitre introductif, nous examinons l'état des connaissances en écologie virale aquatique, en insistant particulièrement sur la diversité des virus et leurs rôles dans les réseaux trophiques microbiens, en relation avec les gradients physiques, chimiques et biologiques liés à la profondeur. Les principaux objectifs du travail de thèse et l'organisation du présent mémoire seront également présentés.

1. Les réseaux trophiques microbiens

Dans les milieux aquatiques, la principale source de carbone autochtone provient de la photosynthèse microbienne, processus par lequel le carbone atmosphérique est transformé en matière organique qui est à la base de la mise en place des réseaux trophiques. Le devenir de

cette matière a fait l'objet de nombreuses recherches dans le cadre de la compréhension et de la gestion des écosystèmes aquatiques. Historiquement, avant les années 1980s, il était convenu que l'essentiel de la production primaire était particulaire (biomasse algale), laquelle servait de principale source nutritive pour les herbivores, et de base pour une chaîne alimentaire simple et linéaire, dite classique (phytoplancton → zooplancton métazoaire → poissons) (**Steele 1974**). Les microorganismes hétérotrophes étaient alors largement méconnus, en raison, essentiellement, des difficultés méthodologiques liées à leur étude. En effet, les bactéries hétérotrophes, essentiellement appréhendées à l'aide de méthodes culturales, n'étaient pas considérées comme étant suffisamment abondantes pour jouer un rôle significatif, autre que celui de la minéralisation de la fraction phytoplanctonique détritique non consommée par le zooplancton métazoaire.

Au cours des trois dernières décennies, les progrès réalisés au niveau des techniques d'identification, de dénombrement et de mesure d'activité métabolique, notamment en microscopie à épifluorescence et en biologie moléculaire, ont permis d'entrevoir l'extraordinaire diversité des microorganismes aquatiques, l'étendue de leurs conditions de vie et leurs abondances jusqu'alors largement sous-estimées. De plus, l'amélioration sensible des méthodes séparatives a permis de décrire la composition biochimique des communautés et d'aborder les transferts de matière au sein des réseaux trophiques sous un angle qualitatif. C'est grâce à ces progrès techniques que les organismes de plus petite taille qui, on le sait aujourd'hui, représentent le plus grand réservoir de diversité, de carbone et de fonctions dans le plancton, ont été pris en compte dans les écosystèmes aquatiques (**Pomeroy 1974**). De ce point de vue, l'élaboration du concept de '*boucle microbienne*' (**Azam et al. 1983**) a été structurant pour le développement de l'écologie microbienne aquatique. Dans ce concept, une fraction importante de la matière organique élaborée lors de la photosynthèse phytoplanctonique (10-60 %) est excrétée sous forme dissoute dans le milieu (**Larsson and Hagström 1982, Obernosterer and Herndl 1995**). Par leurs capacités assimilatrices liées à un rapport surface sur volume élevé, les bactéries hétérotrophes deviennent alors un élément majeur dans les écosystèmes aquatiques (**Kirchman 1997**). La biomasse bactérienne hétérotrophe ainsi produite est utilisée par le nano- et le microzooplancton qui, par leur taille, deviennent des proies accessibles au zooplancton macrobien (Figure 1). Récemment, la notion de '*boucle microbienne*' s'est enrichie de la notion de '*réseau trophique microbien*', par la prise en compte des microorganismes pico- et nanoplanctoniques autotrophes qui forment l'essentiel des producteurs primaires aquatiques, et sont également des proies indiquées pour les protozoaires (**Rassoulzadegan 1993, Amblard et al. 1998**).

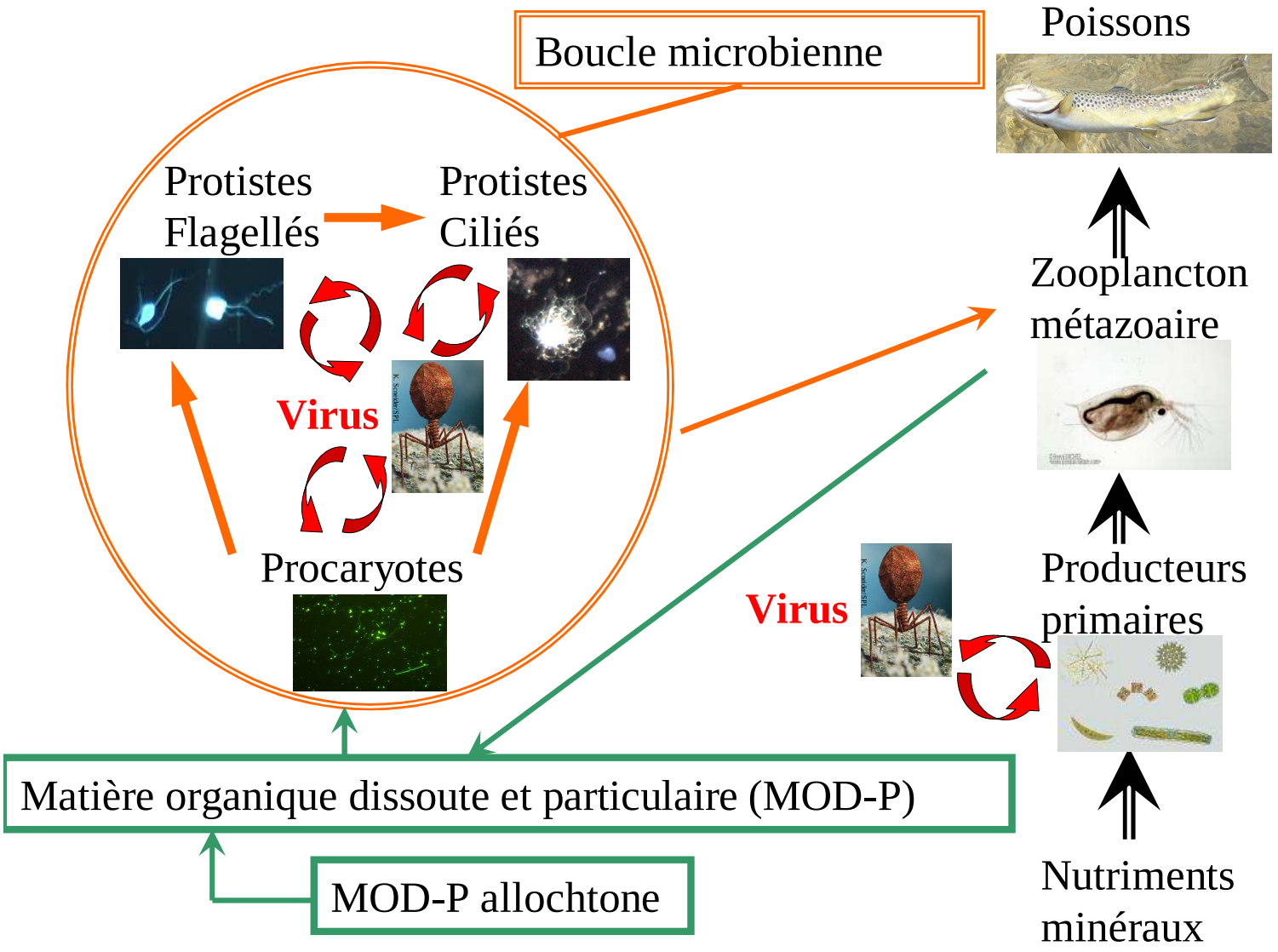


Figure 1. Schématisation des réseaux trophiques en milieux aquatiques pélagiques (modifié d'après **Amblard et al. 1998, Bettarel 2002**)

L'importance fonctionnelle et quantitative des bactéries hétérotrophes au sein des réseaux trophiques microbiens aquatiques a donc posé la question des facteurs de régulation de ce compartiment prépondérant dans les flux de matières et d'énergie. Il a d'abord été admis que cette régulation était le fait des ressources (*bottum-up control*) et de la prédation (*top-down*), notamment par les protozoaires nanoflagellés, dont les effets dépendaient des conditions environnementales (**Ducklow & Carlson 1992**). Cependant, il est apparut que la seule prédation ne pouvait suffire à expliquer l'ensemble des pertes bactériennes dans les écosystèmes aquatiques (**McManus & Fuhrman 1988**). C'est en partie pour cette raison que les hydrobiologistes se sont intéressés aux rôles des virus dans les processus écologiques en milieu pélagique (Figure 1).

2. Les virus dans les écosystèmes aquatiques

A. Définition

Les virus (ou phages) sont des entités biologiques constituées au minimum d'un acide nucléique (ADN ou ARN simple ou double brins) entouré d'une capside de nature protéique (**Valentine et al. 1966**). Le diamètre de cette capside est généralement inférieur à 250 nm, et les virus possèdent également ou non une queue de longueur variable. Les particules virales sont infectieuses mais inaptes à présenter un métabolisme autonome. Ce sont des parasites intracellulaires obligatoires qui ont besoin d'une cellule hôte sensible et métaboliquement active, pour tous processus requérant de l'énergie, y compris la reproduction.

B. Cycle biologique

Les virus présentent trois principaux types de 'cycles de vie', s'amorçant tous par l'adsorption ou fixation du virus sur des récepteurs spécifiques localisés à la surface de la cellule cible. En fonction de la nature du virus, peut alors se produire l'un des trois 'cycles de vie'.

(1) Le cycle de type 'lytique' dans lequel le génome viral provoque la synthèse par la cellule hôte des différents constituants viraux, ainsi que la réplication du génome viral. Il y a ensuite auto-assemblage des constituants viraux dans la cellule infectée,

encapsidation de l'acide nucléique et formation de néovirus qui sont libérés dans l'environnement par lyse de la cellule hôte.

(2) Le cycle de type 'chronique', où les virus néoformés ne provoquent pas la lyse de la cellule hôte, mais sont expulsés par bourgeonnement ou par extrusion de filaments, sans que le métabolisme et la reproduction de la cellule hôte soient affectés.

(3) Le cycle de type 'lysogénique', où le génome viral s'intègre au génome de la cellule hôte. Le provirus ainsi formé se reproduit lors de la réplication de l'ADN de la cellule hôte et peut ainsi être transmis à de multiples générations. Dans ce cas, des changements environnementaux ou un stress peuvent induire l'activation du provirus et provoquer la mise en place du cycle lytique.

Il existe un quatrième type de cycle de reproduction des virus proche de la lysogénie : la pseudo-lysogénie au cours duquel le provirus ne s'intègre pas au génome de la cellule hôte mais reste en état de latence dans le cytoplasme.

C. Notions de classification

Les virus ne sont pas considérés comme des êtres vivants, au sens propre du terme. L'absence de traceurs évolutifs comme l'ARN ribosomal fait que leur classification ne peut être intégrée à celle réalisée pour les êtres vivants. Il a donc été nécessaire de mettre au point une nomenclature particulière pour la classification des virus. Les phages sont classés en fonction de leur hôte et des caractéristiques physiques du virion libre, incluant la taille de la capside, la forme, la structure, la résistance aux solvants organiques et le type et la taille de l'acide nucléique (ARN ou ADN simple ou double brins) (**Murphy et al. 1995**). Récemment, il a été proposé de regrouper les virus et autres agents acellulaires infectieux possédant un génome composé d'acides nucléiques dans un domaine à part (*Akamara*), afin de les distinguer des organismes appartenant aux trois grands domaines de la vie : *Bacteria, Archaea* et *Eukaryota* (**Hurst 2000**). Les virus peuvent donc ainsi être classés à partir du domaine, du règne, de l'embranchement, de la classe, de l'ordre, de la famille, du genre et de l'espèce. Le concept d'espèce virale peut être défini comme « une classe polythétique de virus qui constitue une lignée réplicative et occupe une niche écologique particulière » (**Regenmortel 1992**). Un exemple de la classification d'un virus est donné sur la figure 2. Un descriptif complet ainsi que les limites de la classification actuelle et de la nomenclature des virus sont fournis par l'ICTV (*International Committee on Taxonomy of Viruses*) (**Fauquet et al. 2005**).

Enfin, une classification basée sur des séquences protéomiques de 105 phages a été dernièrement proposée, et constitue la dernière avancée en matière de classification virale (**Rohwer & Edwards 2002**).

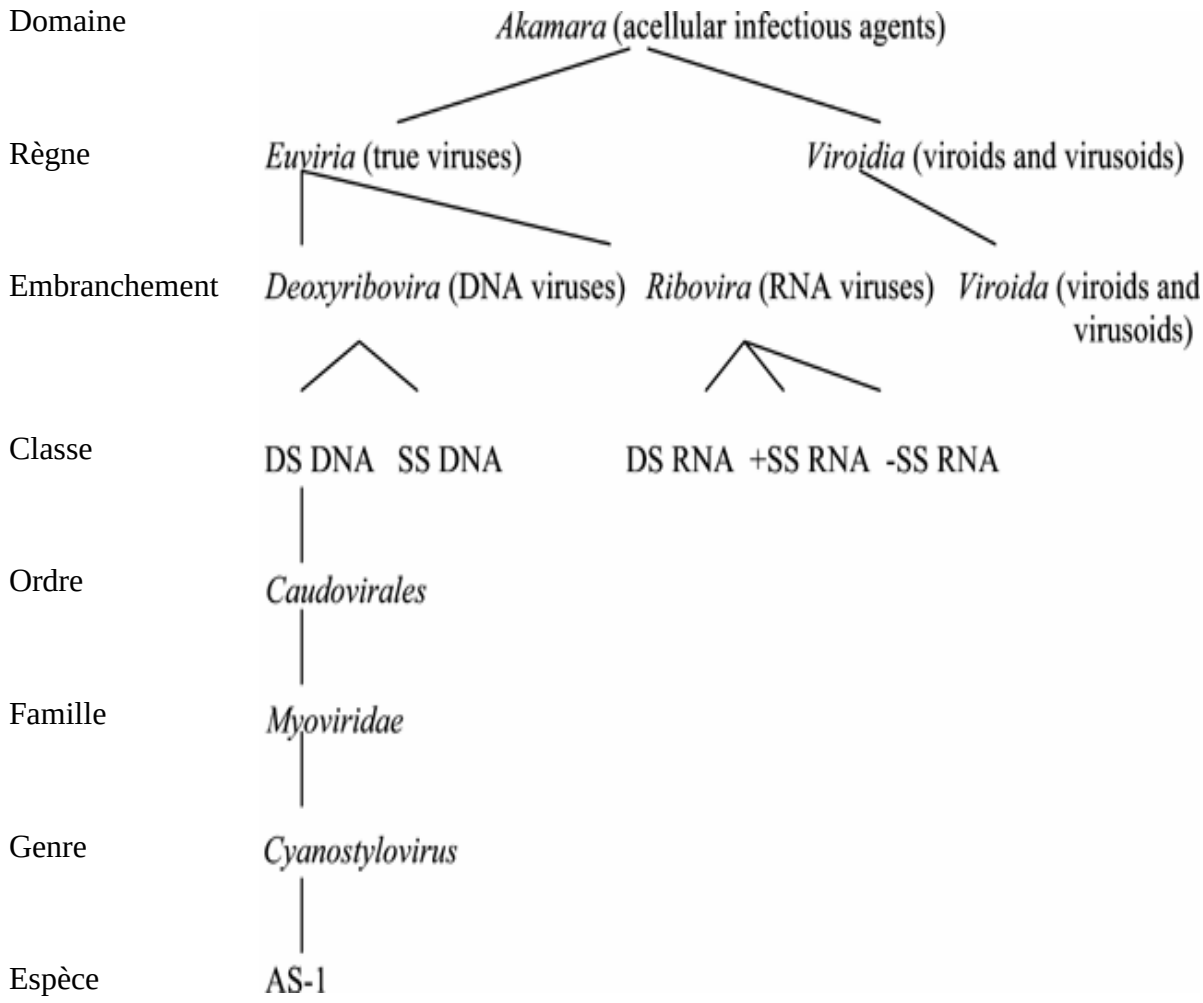


Figure 2. Affiliation taxonomique potentiel du cyanophage AS-1(**Weinbauer 2004**). −SS RNA, *negative-sense single-stranded RNA genome*; +SS RNA, *positive-sense, single-stranded RNA genome*; DS RNA, *double-stranded RNA genome*; SS DNA, *single-stranded DNA genome*; DS DNA, *double-stranded DNA genome*.

D. Diversité des virus

Aujourd'hui on estime qu'il existe sur notre planète près de 10^{31} particules virales (**Rohwer & Edwards 2002**), pour la plupart aquatiques, ce qui représente une diversité largement supérieure à celle de l'ensemble des organismes des trois règnes du vivant (*Bacteria, Archaea, Eukaryota*). Or, seulement quelques milliers 'd'espèces virales' sont actuellement recensés par l'ICTV. Les paramètres utilisés pour classer les virus nécessitent l'isolation des phages, isolation qui n'est possible qu'à la condition de connaître l'hôte spécifique qui doit être cultivable. Malheureusement, à ce jour, on estime que moins de 1 % des procaryotes sont cultivables (**Hugenholz et al. 1998**), ce qui limite la possibilité d'isoler des phages bactériens. Des mesures holistiques de la diversité virale dans les écosystèmes aquatiques ne peuvent donc être faites à partir des seules espèces virales définies par les classifications établies.

Les premières descriptions de la diversité virale aquatique ont été faîtes à partir de la forme générale des particules virales, observée par microscopie électronique à transmission. Les résultats ont révélé une large diversité de formes avec la dominance de phages à capside isométrique, avec présence ou non d'une queue de longueur variable (**Torella & Morita 1979, Frank & Moebus 1987, Demuth et al. 1993, Børsheim 1993, Proctor 1997, Liu et al. 2006**). Les virus possédant une queue sont classés dans l'ordre des *Caudovirales* ou virus à queue (ICTV, **Ackermann 1999**), lesquels sont des virus à ADN double brins (Figure 2). Leur importance quantitative en milieu aquatique semble être dépendante des milieux étudiés et s'échelonne en moyenne de 8 à 43 % en milieux salés ou d'eaux douces (Tableau 1), valeur pouvant atteindre 65 % dans certains milieux comme le lac Donghu (**Liu et al. 2006**). Cependant, le faible nombre d'études quantitatives ne permet pas de dégager une généralisation satisfaisante.

Parmi l'ordre des *Caudovirales*, trois familles émergent du point de vue quantitatif: les *Siphoviridae* avec une longue queue flexible non contractile, les *Podoviridae* avec une courte queue, et les *Myoviridae* avec une queue contractile de longueur moyenne (**Ackermann 1999, Hendrix et al. 1999**, Figure 3A). Les *Myoviridae* constituent la majorité des phages marins isolés (**Børsheim 1993, Proctor 1997**), toutefois dans les systèmes aquatiques salés ou d'eaux douces, leur importance numérique reste à être vérifiée, notamment par rapport aux *Siphoviridae*. Les *Podoviridae* semblent être quantitativement moins importants que les deux autres familles de caudovirus (**Auguet et al. 2006)**. Dans la plupart des études, les phages sans queues sont, très souvent, largement dominants d'un point de vue quantitatif. Sachant que 96 % de tous les bactériophages connus (soit plus de 5100 'espèces')

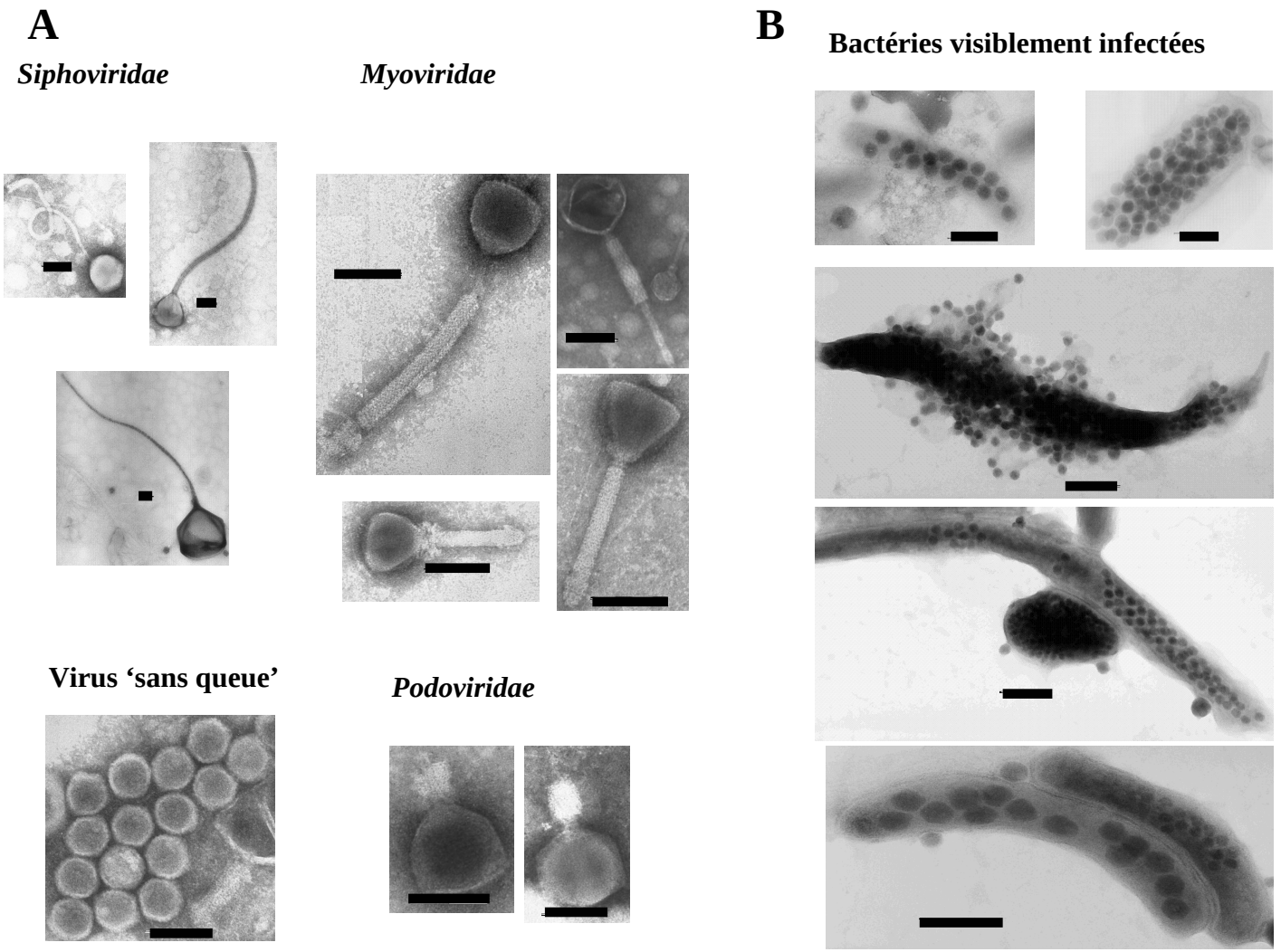


Figure 3. Exemple de phages appartenant aux familles des *Myoviridae, Siphoviridae, Podoviridae* et des phages ‘sans queue’ (A), et cellules bactériennes visiblement infectées (B) (Microscopie électronique à transmission). Barre d’échelle pour A=100 nm, pour B=200 nm.

ont une queue (**Ackermann 1996**), il est probable que la plupart des virus aquatiques appartenant à l'ordre des *Caudovirales* soient des bactériophages. L'absence de queue n'étant pas un critère de classification, en l'absence d'études approfondies des caractéristiques structurales et génomiques des virus sans queue, ces virus sont regroupés dans la catégorie des virus 'indéfinis'. Il est important de spécifier que l'utilisation de l'ultracentrifugation, utilisée en routine pour concentrer les virus avant observation microscopique (**Sime-Ngando et al. 1996**), peut provoquer la dislocation des queues et de ce fait altérer les résultats de la diversité morphologique des virus.

La fréquence de distribution du diamètre de la capside des virus est également couramment utilisée comme traceur de la diversité virale dans les écosystèmes aquatiques. Cette approche est basée sur l'hypothèse d'une absence de pleiomophisme chez les bacteriophages. Qualitativement, il apparaît que les virus aquatiques présentent des tailles de capside très variées, s'échelonnant de moins de 30 nm à plus de 700 nm. Des virus géants (de 200 à plus de 700 nm) ont été répertoriés dans de nombreux systèmes aquatiques (**Sommaruga et al. 1995, Gowing 1993, Peduzzi & Weinbauer 1993, Kepner et al. 1998, Pina et al ; 1998, Auguet et al. 2006**). Quantitativement, il apparaît que la majorité des virus ont un diamètre de capside compris entre 30 et 60 nm et que leur importance en fonction des classes de tailles définies varie d'un milieu à l'autre (Tableau 1). Les tailles moyennes s'échelonnent de 51 à 64 nm dans les milieux marins et estuariens, et de 51 à 89 nm en eaux douces (Tableau 1).

Le développement de techniques basées sur la PCR a permis d'estimer la diversité virale d'un point de vue génomique, mais seulement pour certains groupes spécifiques de virus. En effet, les virus ne possèdent pas de marqueurs moléculaires communs comme l'ARN ribosomal chez les organismes cellulaires (**Hendrix et al. 1999**). Seuls les cyanophages ou d'autres groupes restreints comme les virus *picorna* par exemple (**Culley et al. 2003**), possèdent des marqueurs moléculaires communs, comme, par exemple, le gène g20 codant pour la formation de la capside chez 'certains' myovirus.

Pour obtenir une estimation de la diversité virale génomique de l'ensemble des communautés virales, indépendamment de la PCR, les chercheurs ont eu recours à l'électrophorèse sur gels en champs pulsés (PFGE) (**Wommack et al 1999 a et b, Steward & Azam 2000**). Une concentration préalable des virus afin d'obtenir une quantité adéquate d'ADN est nécessaire. L'ADN viral est extrait des capsides et les génomes viraux sont séparés en fonction de leur taille sur un gel d'agarose. Le nombre de bandes sur le gel donne une estimation du nombre 'd'espèces virales' dominantes dans le milieu étudié. En outre, les

Tableau 1. Distribution de taille des particules virales et pourcentage de phages 'à queue' dans l'environnement (Adapté de **Weinbauer, 2004**).

Environnement	Classes de taille de la capside des virus					Moy.[a]	Phages 'à queue' (%)	Références
	<30 nm	30-60 nm	60-80 nm	80-100 nm	> 100 nm			
Marin et estuarien								
Océan Atlantique Nord	0	69	28	1	2	59		Bergh et al. 1989
Baie de Chesapeake (USA)	0	41	25	25	10	64		Bergh et al. 1989
Fjord (Norvège)	0	47	26	19	7	64		Bergh et al. 1989
Baie de Chesapeake (USA)	5	65	32 (> 60 nm)			NA	43	Wommack et al. 1992
Pacifique (Californie sud)	12	63	22	<1	<1	56		Cochlan et al. 1993
Golfe de Bothnie (Finlande)	20	64	17	14	3	55		Cochlan et al. 1993
Mer Adriatique	5	63	20	8	5	59		Weinbauer et al. 1993
Eaux côtières (Californie sud)	15	45	36	4	1	58		Cochlan et al. 1993
Mer Alboran		73	27			NA		Alonso et al. 2001
Bassin à huitres (France)		76 (< 64 nm)	16 (64-105 nm)		8 (>105 nm)	NA		Montanié et al. 2002
Golfe de Trieste (Italie)	13	50	25 (60-90 nm)	12 (> 90 nm)		53	26	Stopar et al. 2004
Estuaire Charente (France)		71 (< 65 nm)	29 (> 65 nm)			59-61	8	Auguet et al. 2006
Eau douce								
Lac Plußsee (Allemagne)	0	65	17	14	3	62		Bergh et al. 1989
Lac Plußsee zone oxique						68		Demuth et al. 1993
Lac Plußsee thermocline						84		Demuth et al. 1993
Lac Plußsee zone anoxique						89		Demuth et al. 1993
Danube (bras mort)		16 (< 60 nm)	84 (> 60 nm)			NA		Mathias et al. 1995
Lac Constance		50 (< 60 nm)	<50 (> 60nm)			NA		Hennes & Simon 1995
22 lacs (Québec)		> 80 (< 70nm)	<20 (> 70nm)			NA		Maranger and Bird 1995
Lac Supérieur (USA)	53	45	3 (60-110 nm)			NA		Tapper & Hicks 1998
Danube		74 (< 60 nm)	21 (60-90 nm)	5 (>90 nm)		NA		Fischer & Velimirov 2002
Charente (France)						51	9	Auguet et al. 2006
Lac Donghu (Chine) (6 sites)	13.6	57.9	15.6	12.9 (> 80nm)		NA	37 (de 14 à 65)	Liu et al. 2006

[a] Valeurs calculées à partir des données de distribution de taille en assumant une valeur moyenne pour chaque classe de taille et en excluant les virus appartenant à une classe de taille non délimitée (<30, >100 nm par exemple). Dans l'étude de **Demuth et al. (1993)** les virus ont une taille comprise entre 41 et 117 nm dans les eaux oxygénées, 53–106 nm dans la thermocline, et 48–117 nm dans les eaux anoxiques. NA, non applicable. Les valeurs moyennes pour la Charente sont celles fournies par **Auguet et al. (2006)**.

bandes peuvent être analysées en densitométrie ou par l'intensité de leur fluorescence, après marquage à l'aide d'un fluorochrome. Ainsi, la numérisation de l'intensité de différentes bandes permet d'estimer la concentration relative des différentes populations virales dans l'échantillon. Les résultats de la littérature indiquent que les virus ont une taille de génome fluctuant de 10 à 850 kb dans les milieux aquatiques (Tableau 2). A notre connaissance, seules 4 études ont été menées en eaux douces et 3 en lacs (Tableau 2). La fréquence de distribution de la taille du génome viral laisse apparaître une distribution multimodale, avec des pics majeurs dans des régions de tailles inférieures à 70 kb. La taille moyenne du génome viral, déterminée par deux auteurs, est de 50 kb en milieu marin et de 50 à 59 kb dans l'estuaire de la Charente (Tableau 2). Aucune donnée de taille moyenne n'est disponible pour des lacs d'eaux douces; une valeur moyenne de 52 kb a été rapportée pour la rivière Charente, France (Tableau 2).

Les limites de l'utilisation de la PFGE pour étudier la diversité virale dans les milieux aquatiques sont nombreuses. En effet, seuls les virus dominant et possédant un ADN double brins sont pris en compte. D'autre part, la résolution de la PFGE ne permet pas, dans la plupart des cas, de distinguer des génomes viraux différents de seulement quelques kilobases. Par conséquent, les données sur la diversité virale par PFGE doivent être discutée en tenant compte de ces limites. Récemment une approche métagénomique utilisant le pyroséquençage (**Breitbart et al. 2002, Edwards et al. 2006**) a été développée en milieu marin et devrait fournir de nouvelles données permettant d'estimer la diversité virale en milieu aquatique. Les premiers résultats suggèrent une très grande diversité en milieu marin, probablement plusieurs centaines de milliers d'espèces virales différentes (**Angly et al. 2006**). Cependant, le succès d'analyses comparatives de données métagénomiques dépendra du développement d'infrastructure et de moyens analytiques pour traiter l'énorme jeu de données généré par ces approches (**Seshadri et al. 2007**).

Les quelques recherches menées ont permis de montrer que la diversité génomique des communautés virales fluctuait avec le temps (**Wommack et al. 1999a, Castberg et al. 2001, Larsen et al. 2001 et 2004, Sandaa & Larsen 2006, Tijdens et al. 2007, Lymer et al. 2008**) et dans l'espace (**Wommack et al. 1999b, Steward et al. 2000, Riemann & Middelboe 2002, Jiang et al. 2004, Angly et al. 2006, Auguet et al. 2006, Fillipini & Middelboe 2007**). Il en est de même si l'on considère les critères morphologiques comme indicateurs de la biodiversité au sein des communautés virales (**Børsheim et al. 1990, Bratbak et al. 1990, Cochlan et al. 1993, Maranger et al. 1994, Maranger & Bird 1995,**

Tableau 2. Distribution de la taille du génome viral et estimation du nombre de types viraux dans les milieux aquatiques (Adapté de **Weinbauer 2004**).

Environnement	Taille du génome (kb)		No. de bandes[a] (par empreinte)		Références
	Moy.	Gamme	Moy.	Gamme	
Marin, hypersalé ou estuarien					
Baie de Chesapeake (USA)		12-314	11	7-16	Wommack et al. 1999a
Eaux côtières Pacifique nord, Scripps pier (USA)				8-16	Steward et al. 2000
Eaux côtières Pacifique nord, Baie de Monterey (USA)			<35		Steward & Azam 2000
Moyenne d'environnements marins variés	50	26-350		8-35	Steward et al. 2000
Mésocosmes marins, Fjord (Norvège)		19-415	11	5-16	Castberg et al. 2001
Mésocosmes marins, Fjord (Norvège)		19-485	20		Larsen et al. 2001
Eaux côtières (Danemark)				14-29	Riemann & Middelboe 2002
Marais salant (Espagne)		10-533	8	4-12	Sandaa et al. 2003
Mésocosme, Fjord (Danemark)		25-630	13	5–21	Ovreas et al. 2003
Lac Mono (USA)		14->400		up to 27	Jiang et al. 2004
Eaux côtières (Norvège)		up to 560			Larsen et al. 2004
Eaux côtières (Norvège)		26-500	13	5-18	Sandaa & Larsen 2006
Estuaire de la Charente (France)	50-59	21-331	4	< 7	Auguet et al. 2006
Eaux côtières (Danemark)		30-242		10-15	Filippini & Middelboe 2007
Eau douce					
Charente (France)	52	21-180	4	< 7	Auguet et al. 2006
Lac eutrophe Loosdrecht		30-200	8	6-10	Tijdens et al. 2007
Lacs d'eau douce (Danemark)		12-360		10-20	Filippini & Middelboe 2007
Lacs d'eau douce (Suède)		15-661		6-13	Lymer et al. 2007

[a] Le nombre de bandes par empreinte donne une estimation du nombre de types viraux ('espèces'). Toutes les empreintes sont obtenues par électrophorèse sur gels en champs pulsés, indiquant que seuls les virus à ADN double brins sont analysés.

Montanié et al. 2002, Liu et al. 2006, Auguet et al. 2006). Toutefois, les facteurs contrôlant la diversité des virus restent largement méconnus. Différents facteurs de nature biologique (diversité et dynamique des communautés d'hôtes, prédation des protozoaires sur les hôtes et sur certains types viraux…) ou physico-chimiques (adsorption des virus sur des particules, UV, Température…) sont capables d'influencer la richesse et la diversité virales dans les milieux aquatiques (**Weinbauer 2004**).

E. Importance quantitative et fonctionnelle (lyse et lysogénie) des virus

a. Importance quantitative

Différentes méthodes sont utilisées pour estimer l'abondance des virus dans les milieux aquatiques : les plages de lyses ou '*Plaque assays*' (PA), la méthode du nombre le plus probable (MPN), la microscopie électronique à transmission (TEM), la microscopie à épifluorescence (EFM), et la cytométrie de flux (FC). Les approches PA et MPN sont utilisées pour quantifier l'abondance d'unités infectieuses, qui causent la lyse d'un hôte particulier en culture solide (sur gélose, PA) ou liquide (MPN). Les méthodes TEM, EFM et FC sont utilisées pour déterminer l'abondance totale des particules virales dans un échantillon d'eau. Les avantages et les inconvénients de ces méthodes sont discutés en détail par **Weinbauer (2004)** et par **Suttle (2007)**.

Les résultats obtenus au cours des 20 dernières années montrent que l'abondance des virus dans les écosystèmes aquatiques fluctue de 10^4 à plus de 10^8 virus ml^{-1}, excédant donc clairement celle des bactéries. **Suttle (2007)** estime que les océans contiennent environ 4 x 10^{30} virus, soit, mis bout à bout, l'équivalent en distance de 10 millions d'années lumière. En termes de carbone, ces virus représenteraient 200 Mt de carbone. Ainsi, les virus représentent 94 % de l'abondance totale des particules contenant des acides nucléiques, mais ils représentent moins de 10 % de la biomasse carbone de ces particules, en raison de leur taille nanométrique. En général, l'abondance virale augmente avec la productivité des systèmes aquatiques. Par conséquent, dans les océans elle décroît des côtes vers le large et des couches euphotiques vers les couches aphotiques (**Wommack & Colwell 2000, Weinbauer 2004**). Plus en profondeur (y compris au niveau des sources d'eaux chaudes profondes océaniques), les virus et les procaryotes sont souvent très abondants, et peuvent même représenter les seules formes vivantes (**Ortmann & Suttle 2005**). Dans les eaux douces, les abondances virales sont plus fortes que dans les systèmes marins (**Maranger & Bird 1995**) et augmentent

avec le degré d'eutrophisation. Dans ces milieux dulçaquicoles, l'abondance virale peut être plus élevée en surface (**Tapper & Hicks 1998**), mais aussi en profondeur (**Weinbauer & Höfle 1998**). Des variations temporelles allant de la minute à l'année ont également été mises en évidence en écologie virale aquatique (**Wommack & Colwell 2000**). Ces fluctuations sont, dans la plupart des cas, associées à celles des bactéries qui forment, de part leur abondance, le plus grand réservoir d'hôtes pour la prolifération virale (**Wommack & Colwell 2000**).

Cependant, des différences marquées, notamment en termes du rapport virus/bactéries, ont été répertoriées dans différents environnements. Par exemple, dans les eaux de surface des océans Arctique et Pacifique, les virus sont, respectivement, 40 et 10 fois plus abondants que les bactéries, alors que dans les lacs, ce rapport est souvent inférieur à 5 (**Clasen et al. 2007**). Par ailleurs, ce rapport virus/bactéries est variable en fonction de la profondeur, de la localisation géographique, et du temps (**Weinbauer 2004**). Des variations d'un ordre de magnitude dans les abondances virales et le rapport virus/bactéries à l'échelle du centimètre ont été mises en évidence dans des environnements aquatiques (**Seymour et al. 2006**). Les raisons de ces variations ne sont pas claires. Le taux d'élimination et/ou d'inactivation des virus en suspension dans l'eau, variable selon les facteurs environnementaux physico chimiques (température, rayonnement UV, …) et/ou biologiques (digestion par des exo- ou ectoenzymes, consommation par les protozoaires, adsorption sur des particules vivantes ou détritiques, …) (**Sime-Ngando 1997, Fuhrman 1999, Weinbauer 2004**), peut être une explication. Cependant, ces facteurs restent très mal connus. D'autre part, l'importance numérique des virus non bactériophages peut expliquer les variations du rapport virus/bactéries. En effet, **Maranger & Bird (1995)** rapportent une meilleure corrélation entre les abondances virales et les teneurs en chlorophylle *a*, comparée aux corrélations entre virus et bactéries, ce qui suggère qu'une fraction significative de virus peut être associée aux algues ou aux cyanobactéries. Dans le lac Bourget ou en mer Méditerranée, **Dorigo et al. (2004)** et **Bettarel et al. (2002)** ont, respectivement, montré que la dynamique des virus est plus étroitement corrélée à celles des picocyanobactéries qu'à celle des bactéries hétérotrophes. Ces résultats empiriques sont confirmés par plusieurs études en milieux marins montrant que la concentration des virus infectant des cyanobactéries ou des espèces picoplanctoniques eucaryotes autotrophes comme *Micromonas pusilla*, pouvait atteindre et dépasser 10^5 virus ml^{-1} (**Waterbury & Valois 1993, Suttle & Chan 1993 et 1994, Cottrell & Suttle 1995**).

Le déterminisme et les facteurs de contrôle des variations de l'abondance virale et du rapport virus/bactéries dans les écosystèmes aquatiques restent donc méconnus. Cela, même s'il ne fait plus aucun doute que les virus interviennent, de manière significative, dans les

processus de perte affectant l'ensemble des communautés microbiennes, et pas seulement bactériennes.

b. Importance fonctionnelle

- Le virioplancton : agent de la mortalité microbienne

Par l'intermédiaire de leur cycle lytique, les virus représentent une des principales causes de la mortalité microbienne dans les écosystèmes aquatiques. La mesure de leur impact, notamment sur les populations procaryotiques, compartiment central des réseaux trophiques, se fait par le biais de différentes approches méthodologiques reposant sur différents principes de base.

(i) L'observation de cellules visiblement infectées en microscopie électronique à transmission (TEM) (**Proctor & Fuhrman 1990, Sime-Ngando et al. 1996, Weinbauer et al. 2002**) (Figure 3B).

(ii) L'énumération de virus après une lyse provoquée de cellules bactériennes infectées (**Heldal & Bratbak 1991**).

(iii) L'estimation du taux d'élimination (*decay rate*) des virus par utilisation d'un inhibiteur des hôtes procaryotiques. Sachant que les virus ne peuvent théoriquement subsister longtemps à l'état libre en l'absence d'hôtes spécifiques, leur présence dans l'eau indique qu'il s'agit de virus néoformés. Le taux d'élimination des virus après empoisonnement des hôtes (généralement au KCN) serait donc équivalent au taux de production virale (**Heldal & Bratbak 1991**).

(iv) L'utilisation de traceurs viraux fluoromarqués et inertes (virus fluoromarqués à chaud, microbilles fluorescentes…). Pendant l'incubation, l'importance relative des virus marqués diminue, contrairement à celle des virus naturels, du fait de la production virale nouvelle qui peut, de cette façon, être estimée (**Suttle & Chen 1992, Noble & Fuhrman 2000**).

(v) La mesure du taux de synthèse de l'ADN viral en présence d'un précurseur radioactif (par exemple la thymidine tritiée), méthode semblable à celle permettant de mesurer la production bactérienne (**Steward et al. 1992**).

(vi) L'utilisation d'une méthode dite de dilution, dont le principe vise à réduire progressivement la probabilité de rencontre entre virus libres et leurs hôtes spécifiques dans un échantillon naturel, de manière à supprimer la possibilité de nouvelles infections. L'apparition de néovirus étant la résultante de la lyse

cellulaire des hôtes déjà infectés avant la dilution, on peut ainsi déduire une mesure de la production virale (**Wilhelm et al. 2002**).

Cependant, toutes ces approches méthodologiques ont recours à des facteurs de conversion ou autres algorithmes théoriques provenant de cultures en laboratoire. On parle de facteurs non-contraints (**Suttle 2005**), par rapport aux écosystèmes naturels fluctuants. Il est à noter qu'il s'agit d'une critique relativement générale des méthodes en sciences de l'environnement. A titre d'exemples, l'estimation de la production virale par TEM nécessite l'utilisation d'un facteur pour convertir le nombre de bactéries visiblement infectées (les virus intracellulaires ne sont visibles qu'à la fin du cycle lytique) en nombre de bactéries réellement infectées. L'extrapolation à partir de mesures d'augmentation d'abondances virales après une lyse provoquée ne donne toujours pas de mesures précises du nombre de virus produit par cellule et par événement lytique (*burst size*), paramètre crucial pour la détermination de la production virale. La déduction de la production virale à partir du taux d'élimination des virus ou de l'utilisation de traceurs, considère que les pertes et productions virales sont en équilibre (*steady state*), que les traceurs sont représentatifs des communautés naturelles, et que le *burst size* est connu. L'extrapolation de la production virale à partir du taux de synthèse viral d'un précurseur de l'ADN comme la thymidine, requiert des facteurs de conversion et est sensible à la contamination par l'ADN bactérien. L'utilisation de méthodes de dilution nécessite des manipulations de l'échantillon et l'estimation du *burst size*. Par conséquent, les mesures de mortalité microbienne induite par les virus ou de la production virale dans la littérature restent des estimations.

D'un point de vue quantitatif, ces estimations montrent que les virus détruisent entre 10 et 50 % de la production bactérienne dans les eaux de surfaces marines, et en moyenne de 10 à 20 % de la production bactérienne dans l'ensemble des systèmes aquatiques (**Wommack & Colwell 2000**). Ces valeurs fluctuent de quasi nulles à 100 %, en fonction de l'environnement étudié (**Weinbauer 2004**). A l'échelle des communautés, plusieurs études ont montré que le degré de lyse virale était dépendant de l'abondance et de l'activité de l'hôte, et de façon plus générale de la productivité du système (**Weinbauer 2004**). Il apparaît également que les facteurs physico chimiques tels que les radiations solaires (**Suttle & Chen 1992, Noble & Fuhrman 1997, Höfer & Sommaruga, 2001**), la température (**Pradeep Ram et al. 2005**) ou le degré d'anoxie (**Weinbauer & Höfle 1998**) peuvent également affecter le niveau de lyse virale dans un écosystème. Pour autant, l'importance relative des différents facteurs reste à être déterminée.

En écologie virale aquatique, l'importance des cellules lysogènes (cellule possédant un génome virale ou prophage intégré dans son propre génome et en état de dormance, on parle de phage tempéré) et l'impact de la lysogénie, ont été bien moins étudiés que la lyse virale. Ces cellules présentent la caractéristique d'être un réservoir considérable de lyse virale, en cas d'induction massive. Elles ont également l'avantage de conférer aux cellules hôtes de nouvelles propriétés métaboliques ou géniques, comme l'immunité vis-à-vis de nouvelles infections virales ou de la superinfection par des phages homologues (**Marsh & Wellington 1994, Clore et Stedman 2006**). La lysogénie représente donc une stratégie de survie des virus dans l'environnement, notamment en cas de limitation par la disponibilité en hôtes spécifiques (**Colombet et al. 2006**). Le rôle des virus comme agent de mortalité bactérienne dépend donc du type de virus en présence (phage lytique vs phage tempéré), et est étroitement lié à l'importance quantitative des cellules lysogènes (conversion lysogénique) et des facteurs potentiels d'induction. D'un point de vue méthodologique, l'estimation de la fraction de cellules lysogènes est basée sur une induction artificielle du cycle lysogène en cycle lytique, en utilisant un agent inducteur, couramment la mitomicyne C (**Jiang & Paul 1996**). L'efficacité de cet antibiotique a été considérée comme étant discutable (**Ackermann & Dubow 1987**) et il existerait, selon les milieux, des agents inducteurs plus efficaces (**Cochran et al. 1998**). Plus récemment, une approche génomique a été proposée pour identifier la présence de prophages dans le génome bactérien (**Casjens 2003, Chen et al. 2006**). Cependant, à l'échelle des communautés, cette approche n'est pas applicable puisqu'elle nécessite la connaissance du génome de l'hôte.

Les estimations quantitatives des cellules lysogènes dans les systèmes aquatiques (Tableau 3) sont largement moins documentées que celles des cellules lytiquement infectées. Les quelques résultats disponibles montrent que la fréquence des cellules lysogènes (FLC) fluctue entre 0 et 100 % (de l'abondance bactérienne totale) dans les environnements marins (Tableau 3). Encore moins étudiée dans les eaux douces, la FLC y fluctue entre 0,1 et 7,4 % des communautés bactérioplanctoniques de systèmes tempérés ou tropicaux, et entre 0 et 73 % dans les lacs Antarctiques (Tableau 3). Les facteurs affectant la lysogénie (facteurs d'induction) restent controversés (**Weinbauer 2004**). Différents facteurs physico-chimiques (pH, température, radiations solaires, statut trophique, pollutions…) et biologiques (abondance et activité des hôtes…) pourraient être essentiels (**Weinbauer 2004**).

L'ensemble des données de la littérature et nos propres recherches montrent donc que les virus sont des agents importants dans la mortalité bactérienne aquatique, soit directement par l'intermédiaire de la lyse, ou indirectement par induction de la fraction lysogène au sein

Tableau 3. Etendue de l'importance relative des cellules lysogènes et comparaison avec la fréquence des cellules lytiquement infectées (FIC) en milieu aquatique. La fréquence des cellules lysogènes (FLC) est estimée après induction, soit à partir du nombre de bactéries lysées (méthode bactérienne) soit à partir du nombre de virus produits (méthode virale) pondéré par le *burst size*. (ND : non déterminée)

Environnement aquatique	**FLC**	**Méthode d'estimation**	**FIC**	**Références**
Eau douce				
Lac Supérieur	0.1-1.2	Virale	ND	Tapper et Hicks 1998
	0.5-7.4	Virale		
Lac Bonney	7.9-52.8	Bactérienne	ND	Lisle et Priscu 2004
(Antarctique)	2.2-62.5	Virale		
Lac Hoare	18.5-54.1	Bactérienne	ND	
(Antarctique)	2.0-18.6	Virale		
Lac Fryxell	0-36.6	Bactérienne	ND	
(Antarctique)	0-17	Virale		
Lac Vanda	0-30.6	Bactérienne	ND	
(Antarctique)	0-48	Virale		
Lac Druzhby	0-22.3	Virale	ND	Säwström et al. 2006
(Antarctique)	0-3.1	Virale		
Lac Crooked	0-73.0	Virale	ND	
(Antarctique)	0-11.7	Virale		
Systèmes tropicaux	0.1-7.1	Virale	2-8	Bettarel et al. 2006
Marin				
Estuaires	10.5-67.3	Bactérienne	ND	Jiang et Paul 1996
	2-38	Virale		
Zones côtières	26.5-78.8	Bactérienne	ND	
	1.5-3.5	Virale		
Pleine mer (oligotrophe)	13.2-25	Bactérienne	ND	
	2.5-7	Virale		
Golfe du Méxique	0.07-4.4	Virale	ND	Weinbauer et Suttle 1996
Baie de Tampa	0-41	Virale	ND	Cochran et Paul 1998
Baie de Tampa	0.92-126	Virale	ND	Williamson et al. 2002
	1.5-210	Virale		
	0-51.2	bactérienne		
Fjord (Colombie britannique)	80	Virale	ND	Ortmann et al. 2002
Mer Méditérannée	0-84	Virale	0.7-12	Weinbauer et al. 2003
Mer Baltique	0-79	Virale	4-33	
Mer Adriatique	2-16		ND	Bongiorni et al. 2005
Cultures marines				
Atlantique, Pacifique	25-62.5	Virale	ND	Jiang et Paul, 1998
Golfe de Trieste	71		ND	Stopar et al. 2004

des communautés bactériennes. Ces deux processus viraux doivent donc être pris en compte dans les flux de matières et d'énergie qui transitent par les écosystèmes aquatiques.

- *Le Virioplancton : catalyseur des cycles biogéochimiques*

Par leur implication dans la mortalité microbienne, les virus jouent un rôle important dans les cycles biogéochimiques. Les modèles théoriques élaborés (**Wilhelm & Suttle 1999, Fuhrman 1999, Middelboe et al. 2003**) démontrent que les virus catalysent et accélèrent la transformation des nutriments de l'état particulaire (organismes vivants, matières détritiques…) à l'état dissous (produits de la lyse virale : virus, débris et organites cellulaires, molécules et éléments organiques et inorganiques divers…), directement assimilable par la fraction non infectée des communautés procaryotiques (**Wilhelm & Suttle 1999, Middelboe & Lyck 2002**), des protistes phagotrophes (**Gonzales & Sutlle 1993**), et des producteurs primaires. Ainsi, dans certains cas, le fer contenu dans les produits de la lyse virale peut combler les besoins métaboliques des organismes planctoniques (**Poorvin et al. 2004**).

Les virus court-circuitent donc une partie des transferts trophiques s'effectuant par le biais des chaînes alimentaires en transformant les particules énergétiques en éléments dissous (Figure 4), ce qui a pour effet d'augmenter les processus d'oxydation respiratoire et de baisser l'efficacité du transfert de carbone aux niveaux trophiques supérieurs (**Suttle 2005**). Ainsi, la matière organique dissoute est convertie en nutriments inorganiques non seulement par la respiration mais aussi par photodégradation (**Weinbauer 2004**). D'après un modèle proposé par **Fuhrman (1999)**, dans un système où les virus détruiraient 50 % de la production bactérienne, la respiration et la production bactérienne augmentent de 33 %, alors que la prédation des protistes et du zooplancton métazoaire serait réduite respectivement de 37 % et de 7 %. En outre, la conversion, par l'action de la lyse virale, de la matière organique particulaire en agrégats organiques à fort potentiel de sédimentation peut, potentiellement, influencer la proportion de carbone qui est exporté de la zone photique au fond des océans et des lacs (pompe biologique) (**Suttle 2007**). Par ailleurs, en tant que particules riches en azote et en phosphore, la production virale correspond à une séquestration de ces éléments dans la colonne d'eau (**Suttle 2005, 2007**). Cette séquestration change les rapports des éléments minéraux dans la colonne d'eau (C, N et P), ce qui peut affecter la production primaire essentielle au fonctionnement des réseaux trophique aquatiques. Les effets de la lyse virale peuvent aussi, potentiellement, affecter les processus d'émission du dimethyl sulphide (**Evans et al. 2007**), un gaz servant de noyau de condensation des nuages, et produit massivement par les communautés microbiennes des glaces polaires en réponse à l'accroissement des

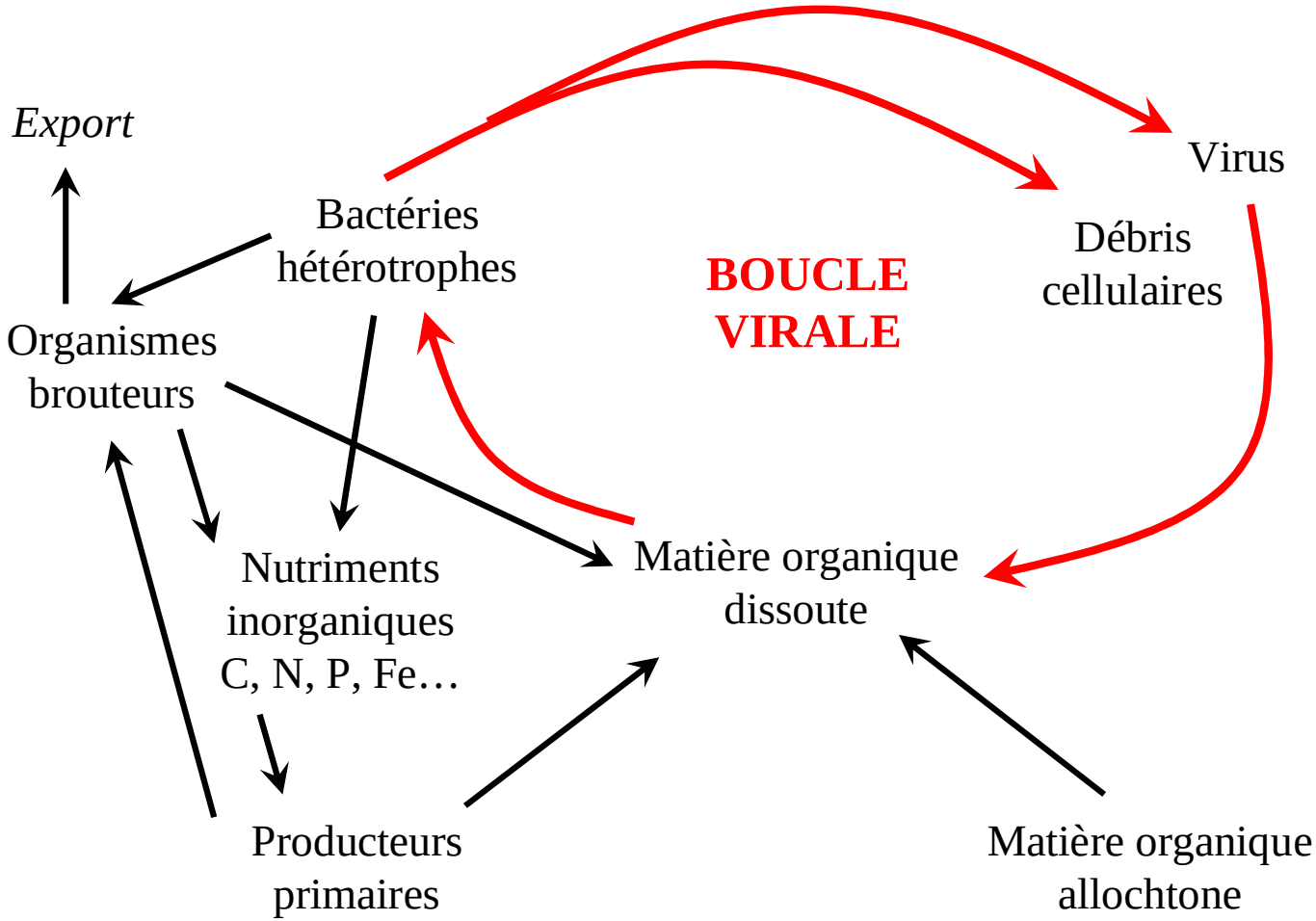


Figure 4. Représentation schématique de la boucle virale planctonique (Fuhrman 1999).

rayonnements UV. Ainsi, les produits de la lyse virale sont structurants pour les communautés microbiennes, aussi bien par leur qualité/nature que par leur importance quantitative.

- *Le virioplancton : agent structurant de la diversité des communautés microbiennes*

La diversité moléculaire des communautés procaryotiques dans les milieux aquatiques est importante. Elle joue un rôle fondamental dans les flux de matières et d'énergie des écosystèmes aquatiques. Toutefois, les mécanismes contrôlant cette diversité sont encore relativement méconnus (**Polz et al. 2006**). L'une des hypothèses est que le degré de spécificité entre virus et hôtes, souvent réduit à la souche (**Baross et al. 1978, Koga et al.1982**), fait des virus des agents potentiels dans le contrôle de la composition des communautés microbiennes (**Weinbauer & Rassoulzadegan 2004**). Ceci est à la base d'un modèle densité-dépendant, le modèle du '*phage kills the winner*', proposé et développé par **Thingstad & Lignell (1997)** et par **Thingstad (2000)**. Dans ce modèle, la diversité et la diversification au sein des communautés microbiennes seraient maintenues par l'infection virale qui s'attaque, préférentiellement, aux espèces/souches dominantes et, donc, aux populations les plus compétitives pour l'acquisition des ressources ambiantes. En effet, étant donné que le contact virus-hôtes se fait par diffusion aléatoire et est fonction de la densité de l'hôte, les espèces hôtes les plus abondantes sont les plus exposées aux infections virales, ce qui implique que les virus contrôlent les souches ou groupes les plus compétitifs une fois qu'ils deviennent abondants, dégageant ainsi des niches écologiques pour le développement et la co-existence de nombreuses autres souches moins compétitives. Cette hypothèse a été vérifiée, notamment lors d'efflorescences algales ou cyanobactériennes (**Bratbak et al. 1993, Nagasaki et al. 1994, Tarutani et al. 2000, Brussaard et al. 2005, Martinez et al. 2007**). Quelques rares études ont pu également mettre en évidence des changements dans la composition des peuplements bactériens en liaison avec l'infection virale (**Hennes et al. 1995, Simek et al. 2001, Sime-Ngando et Pradeep Ram 2005, Pradeep Ram et Sime-Ngando 2008**). Il semblerait donc que les virus, par leur activité lytique, ont un impact déterminant dans la dynamique des communautés microbiennes et, à ce titre, peuvent intervenir dans la stabilité, la résilience et la résistance des écosystèmes aquatiques suites à des perturbations naturelles ou provoquées.

L'abondance de cellules lysogènes dans l'environnement peut également affecter la biodiversité microbienne (**Weinbauer & Rassoulzadegan 2004**). En effet, grâce à l'intégration du génome viral au génome de la cellule hôte, la cellule alors lysogène est non seulement immunisée contre d'autres infections virales, mais peut également acquérir des

propriétés métaboliques, morphologiques ou immunogéniques de la part du phage. On parle de conversion phagique. L'acquisition de nouvelles propriétés affecte alors indubitablement le niveau de compétitivité de la cellule hôte et donc, à terme, la diversité microbienne. Quelques exemples confirment cette hypothèse. Certaine souches de *Vibrio cholerae*, agent du cholera, ne sont toxiques que lorsqu'elles sont lysogènes, le phage transportant le gène codant pour la toxine responsable de la maladie (**Waldor & Mekalanos 1996**). Il a été montré que les souches d'*Escherichia coli* lysogènes se reproduisent plus rapidement que des souches non lysogènes (**Edlin et al. 1975; Lin et al. 1977**), ce qui peut influencer la composition des communautés en permettant la dominance et la survie des cellules capables de conversion lysogénique.

Enfin, les virus peuvent également intervenir comme vecteurs de transferts génétiques entre les microorganismes à travers, notamment, deux types de mécanismes : la transduction (**Jiang & Paul 1998**) et la transformation (**Paul et al. 1993**). Deux types de transduction existent (**Weinbauer 2004**). Pendant la transduction 'classique', le matériel génétique de l'hôte peut être empaqueté par erreur dans la capside du phage virulent (responsable du cycle lytique) ou tempéré (responsable du cycle lysogène) et être transféré à un autre hôte récepteur lors de son infection. Une transduction dite 'spécialisée' implique uniquement les phages tempérés. En effet, lors de l'infection lysogène, une séquence ADN de l'hôte est excisée avec le prophage et peut être transférée à une cellule hôte réceptrice. Ce mécanisme de transduction entre microorganismes pose le problème de la spécificité de l'infection virale qui serait, en réalité, moins restrictive, ce qui suggère que certains virus seraient non plus des 'spécialistes', mais plutôt des 'généralistes' (**Weinbauer 2004**). Par ailleurs, le mécanisme de transformation concerne le processus d'intégration de gènes microbiens libres provenant de la la lyse virale, dans le patrimoine génétique d'autres microorganismes. L'importance quantitative de ces mécanismes aléatoires de transferts horizontaux de gènes entre microorganismes par l'action virale reste encore largement méconnue en milieu aquatique, cependant on suppose qu'ils jouent un rôle significatif dans la régulation de la diversité microbienne.

L'ensemble des éléments bibliographiques résumés ci-dessus montre, clairement, que les virus ont une importance fonctionnelle majeure dans les écosystèmes aquatiques. Cette importance dépend étroitement du type de 'cycle de vie' des virus. En effet, le cycle lytique et le cycle lysogénique ont, chacun, un mode d'action aux conséquences différentes pour le fonctionnement de l'écosystème. Ces deux 'cycles de vie' sont liés, puisque l'on considère

que les cellules lysogènes peuvent, sous certaines conditions, devenir lytiquement infectées. Pourtant, il est surprenant de constater que les études comparant l'importance relative de ces deux cycles sont très rares (Tableau 4), et concernent uniquement des campagnes d'échantillonnages ponctuels, conduites pour la plupart en milieu marin. La question de l'existence de virus typiquement lytiques et de virus typiquement lysogènes reste entièrement posée, et n'a jamais été explorée dans le contexte de l'écologie virale aquatique.

3. Importance de la variabilité verticale en écologie aquatique : le lac Pavin, un model unique

Les milieux aquatiques pélagiques sont des interfaces fondamentales dans les flux de matière entre l'atmosphère et les sédiments aquatiques. Les milieux aquatiques jouent le rôle de piégeur et de stockeur de CO_2 atmosphérique, gaz essentiel à l'évolution de notre climat. Ce rôle est étroitement lié aux processus biologiques (notamment microbiens) qui se passent dans la colonne d'eau et au fond des systèmes aquatiques. Ainsi, le concept de pompe biologique a été avancé. La pompe biologique est une combinaison de processus qui conduit à la séquestration de matière organique particulaire (donc riche en carbone) au fond des milieux aquatiques. Le fonctionnement de ce processus biologique d'exportation et de séquestration de la matière est intimement dépendant des caractéristiques physico-chimiques de la colonne d'eau. On sait que l'intensité de la lumière incidente et son atténuation dans la colonne d'eau sont des paramètres essentiels dans le fonctionnement des écosystèmes aquatiques. Par ailleurs, ces écosystèmes se caractérisent par la mise en place temporaire ou permanente d'une stratification thermique verticale, essentiellement due à la faible conductivité thermique de l'eau. Cette stratification thermique conduit à l'établissement d'un gradient de densité qui, en raison du confinement de différentes masses d'eau, est à l'origine de l'apparition d'une stratification chimique. La distribution verticale des propriétés physiques et chimiques est un paramètre fondamental dans la structure et le fonctionnement des communautés biologiques aquatiques (**Pourriot & Meybeck 1995**). Par exemple, la pénétration de la lumière délimite une zone euphotique où la photosynthèse domine, d'une zone aphotique profonde dominée plutôt par la respiration. Sachant que la photosynthèse est responsable de la transformation du carbone atmosphérique en matière organique et que la respiration minéralise ces composés organiques, cette ségrégation spatiale a un impact certain sur les flux de matières. D'autre part, la mise en place d'une thermocline est souvent associée à une concentration de

nombreux microorganismes, bénéficiant de la stabilité physique de la thermocline et de la richesse en microhabitats pour leur prolifération (**Sime-Ngando et Hartmann 1991**, **Sime-Ngando et al. 2008**).

La distribution verticale des propriétés physiques, chimiques et biologiques associées à la colonne d'eau est naturellement plus marquée dans les lacs méromictiques profonds comme le lac Pavin, où le brassage des eaux, par mélange turbulent vertical et par refroidissement convectif, n'affecte que la partie superficielle du lac (le mixolimnion). La couche profonde du lac (le monimolimnion) contient en général une concentration en substances dissoutes nettement plus importante que dans le mixolimnion. Entre le mixo- et le monimolimnion, se trouve une zone de transition permanente de fort gradient de densité (pycnocline) et de composition chimique (chimiocline) et, parfois, une thermocline profonde ou une zone de gradient de matière en suspension minérale et / ou bactérienne (**Lemmin & Imboden 1987**). Il est donc évident que l'efficacité des processus biologiques d'exportation ou de recyclage de la matière dépend du fait que l'activité biologique dans la colonne d'eau est hétérogène (**Tanaka & Rassoulzadegan 2002, 2004, Tanaka et al. 2004**). Compte tenu de l'importance fonctionnelle des virus (développée précédemment) et de leur impact potentiel sur la pompe biologique (**Suttle 2007**), il est surprenant de constater que toutes les études saisonnières concernant les virus considèrent seulement quelques points d'échantillonnage dans la zone euphotique. La contribution bien connue des gradients liés à la profondeur comme facteurs de forçage dans les écosystèmes aquatiques reste donc à établir.

Le lac Pavin, par ses caractéristiques physico-chimiques et biologiques particulières, offre un modèle d'étude unique pour étudier ces interactions microbiennes et écologiques, en prenant en compte la variabilité verticale des écosystèmes lacustres. Il s'agit en effet d'un lac oligomésotrophe méromictique et dimictique. C'est un lac de cratère de type maar caractérisé par une profondeur maximale de 92 m, une faible surface (44 ha) et un bassin versant réduit (50 ha). L'une des caractéristiques importantes du lac Pavin est qu'il présente une ségrégation verticale nette des gradients physico chimiques. La couche euphotique se limite aux alentours des 20 premiers mètres de la colonne d'eau. La thermocline, temporaire et séparant l'épilimnion de l'hypolimnion, se situe aux alentours de 12 m. La chémocline, permanente, séparant le mixolimnion du monimolimnion se trouve entre 60 et 70 m et est surmontée par une oxycline entre 55 et 60 m. Nous avons donc à faire à différentes masses d'eaux, aux caractéristiques physico chimiques et biologiques contrastées (**Aeschbag Hertig et al. 2002, Olive & Boulègue 2004, Devaux 1980**).

D’un point de vue biologique, la plupart des études n’ont considéré que la partie soumise au brassage du lac (le mixolimnion), au dessus de 40 m de profondeur, ce qui représente seulement environ la moitié du volume total d’eau contenu dans le lac Pavin. Dans cette zone, il apparaît que les variables biologiques, que ce soit les virus (**Bettarel et al. 2004, Jardillier et al. 2005, Colombet et al. 2006**), les bactéries (**Bettarel et al. 2004, Boucher et al. 2005, Lepère et al. 2006**), le phytoplancton (**Devaux 1980, Amblard 1986, Amblard & Bourdier 1990, Lepère et al. 2006**), ou les protozoaires (**Carrias et al. 1996 et 1998, Bettarel et al. 2004, Lepère et al. 2006, Lefèvre et al. 2007**), varient avec la profondeur et le temps, en termes de composition, d’abondance et d’activité. Dans la zone non brassée, où règne une anoxie permanente, les études se limitent à des échantillonnages ponctuels relatifs à la diversité et aux abondances des procaryotes (**Lehours et al. 2005**). Il apparaît donc nécessaire d’intégrer l’ensemble de la colonne d’eau dans les études écologiques aquatiques, plus particulièrement en ce qui concerne les virus et les microorganismes dont l’étendue des conditions de vie est la plus importante dans le monde vivant.

4. Objectifs et présentation des études

L’analyse bibliographique montre que l’état de nos connaissances concernant le rôle des virus dans les processus écologiques aquatiques, notamment dans les eaux douces, souffre d’une stratégie d’échantillonnage restrictive et/ou d’un nombre de variables étudiées restreint, l’importance des gradients verticaux étant jusque là négligée. De ce fait, la connaissance de la dynamique des variables de structure, de l’abondance et de l’activité des virus et des microorganismes, et des facteurs de régulation associés, reste partielle.

C’est dans ce cadre scientifique que se situent les objectifs de ce travail dont le site d’étude est le lac Pavin. Il s’agit, plus particulièrement, d’examiner (i) la dynamique saisonnière et spatiale de la diversité des communautés virales lacustres, (ii) l’importance des gradients liés à la profondeur dans les variations de l’abondance et de l’activité lytique des virus, et (iii) l’importance du mode de vie lysogénique dans la structure et le fonctionnement des communautés virales. La dynamique virale lacustre est étudiée en tenant compte de l’environnement physique, chimique et biologique, notamment par la prise en compte de la composition, de l’abondance et de l’activité de différentes communautés microbiennes.

Ce mémoire de thèse se présente sous la forme d’un recueil d’articles scientifiques publiés, sous presses, soumis ou en préparation, regroupés dans deux grands chapitres. De

plus, deux articles scientifiques publiés, concernant l'importance quantitative et fonctionnelle des virus dans d'autres environnements, sont donnés, à titre d'information, en annexe. Ces travaux on été effectués aux cours des années de thèse, dans le cadre de collaborations avec des équipes extérieures ayant eu recours à notre expertise en écologie virale.

Chapitre 1 : Etude de la dynamique de la diversité des communautés virales

Partie 1 : Etude méthodologique

L'évolution des connaissances en écologie virale, notamment sur la diversité des communautés virales, est étroitement dépendante des méthodes de concentration des virus. Les méthodes de concentration courantes sont, pour la plupart, consommatrices en temps, onéreuses, et d'une efficacité qui reste à être éprouvée. C'est dans ce contexte que nous avons entrepris des recherches méthodologiques qui nous ont permis de mettre au point un protocole rapide, peu onéreux, et efficace, pour la concentration de particules virales planctoniques à des fins d'étude de la diversité morphologique, morphométrique et génomique des virus aquatiques. Cette étude a fait l'objet d'un manuscrit publié dans la Revue '*Journal of Microbiological Methods*'.

Partie 2 : Etude de la dynamique de la diversité des communautés virales

Cette partie se présente sous la forme d'un manuscrit préparé pour publication, portant sur l'étude de la dynamique saisonnière et spatiale de la diversité des virus du lac Pavin. Différents traceurs de cette diversité ont été mesurés (familles morphologiques, fréquence de distribution du diamètre de la capside, taille du génome déterminée par électrophorèse), en relation avec les facteurs physico chimiques du milieu, et différentes communautés microbiennes.

Chapitre 2 : Etudes des gradients liés à la profondeur des abondances et activités virales

Ce travail vise à compléter les études précédentes menées dans le lac Pavin, où quelques variables virales ont été mesurées mais uniquement dans le mixolimnion (Bettarel et al. 2004, Jardillier et al. 2005). Il s'agissait pour nous de prendre en compte tous les gradients majeurs liés à la profondeur, dans la distribution saisonnière et spatiale de l'abondance et de l'activité virales, en relation avec les facteurs physico-chimiques (température, oxygène, profondeur, chlorophylle *a*) et microbiens (i.e. bactéries hétérotrophes, picocyanobactéries,

picoplancton eucaryote, protistes flagellés auto- et hétérotrophes) d'intérêt écologique pour la dynamique virale.

Partie 1 : Etude préliminaire

Avant une campagne saisonnière, une étude préliminaire, concluante, a été menée sur la variabilité verticale fine des variables virales (diversité, abondance, activité lytique, activité lysogénique) dans le lac Pavin. 15 profondeurs différentes, représentatives des différentes strates de la colonne d'eau (de la surface à 80 m), ont été échantillonnées au cours de cette étude préliminaire et ponctuelle. Les résultats acquis ont été publiés dans la Revue *Applied and Environmental Microbiology*.

Partie 2 : Etudes saisonnières

Cette partie comprend 3 manuscrits soumis ou en préparation, reposant sur une campagne d'étude saisonnière menée d'Avril à Décembre 2005 dans le lac Pavin, où 12 profondeurs différentes (de la surface à 80 m) représentatives de la colonne d'eau, ont été échantillonnées. Cette campagne et les modalités d'échantillonnage ont été motivées et justifiées par les résultats préliminaires obtenus (voir ci-dessus). Le premier manuscrit examine les variations d'abondances virales en relation avec les communautés microbiennes et l'environnement physico chimique. Le second analyse les variations de l'activité lytique en comparaison avec les procaryotes et les microorganismes potentiellement bactérivores. Enfin, le troisième manuscrit étudie les fluctuations de l'importance relative des cellules lysogéniquement infectées et des cellules lytiquement infectées.

CHAPITRE I - ETUDE DE LA DYNAMIQUE DE LA DIVERSITE DES COMMUNAUTES VIRALES

.

Partie 1: Etude méthodologique

Virioplankton 'pegylation' : use of PEG (polyethylene glycol) to concentrate and purify viruses in pelagic ecosystems

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Abstract

We have described the use of Polyethylene glycol (PEG) for the precipitation of natural communities of aquatic viruses, and its comparison with the usual concentration method based on ultracentrifugation. Experimental samples were obtained from different freshwater ecosystems whose trophic status varied. Based on transmission electron microscope observations and counting of phage-shaped particles, our results showed that the greatest recovery efficiency for all ecosystems was obtained when we used the PEG protocol. On average, this protocol allowed the recovery of > 2-fold more viruses, compared to ultracentrifugation. In addition, the diversity of virioplankton, based on genomic size profiling using pulsed field gel electrophoresis, was higher and better discriminated when we used the PEG method. We conclude that pegylation offers a valid, simple and cheaper alternative method to ultracentrifugation, for the concentration and the purification of pelagic viruses.

Introduction

Phages are now recognized as ubiquitous, abundant, and diverse biological entities in world aquatic ecosystems where their activity is crucial to several processes, including microbial mortality, potential gene transfer, and lysogenic conversion **[see Suttle, 2005; Casas and Rohwer, 2007 for review]**. Metagenomic analysis of viral communities collected in four major oceanic regions over a decade showed a high global diversity, presumably several hundred thousands of 'species' **(Angly et al., 2006)**. However, the genetic and biological diversity of aquatic viruses remains largely unexplored, mainly because of the methodological difficulties related to the observation of environmental nanoparticles and the weakness of environmental viral gene bank.

The study of free-floating viruses in aquatic systems requires a concentration method for estimating their number and their diversity, i.e. based on both morphologic and genomic features. The first generation of methods used came from the study of human, animal and plant pathogenic viruses. These methods are based on physicochemical approaches such as adsorption of phages onto microporous filters charged positively or negatively **(Singh and Gerba, 1983; Shields et al., 1986)**, the use of precipitation agents like calcium phosphate, aluminium or magnesium **(American Public Health Association, 1989)** or acid precipitation **(Sobsey et al., 1978)**, and the use of organic flocculation **(Katzenelson et al., 1976)**. The main disadvantage of these techniques for natural samples is their selectivity. Indeed, various viruses may have different adsorptive properties **(Seeley and Primrose, 1982)**, and electrostatic interactions may affect the viability of concentrated viruses. Chemical precipitation methods generally require low pH that may also compromise the viability of some viruses **(Lewis and Metcalf, 1988)**. **Børsheim *et al*. (1990)** have proposed a concentration method based on the ultracentrifugation of viruses contained in few millilitres of samples directly onto grids, followed by staining before observation under transmission electron microscope (TEM). Disadvantages of this method include the risk of viral disruption due to the high centrifugation speed, the effects of suspended particles and 'virucidal' substances, and the low initial volume of experimental samples that may render TEM observations tedious according to the high magnifications used. The method is thus time-consuming (for large volume of samples) and costly, and therefore cannot be recommended as a routine procedure **(Bettarel et al., 2000)**. An alternative approach is the one currently used for aquatic microbes, based on low pressure membrane-concentration, fluorochrome staining, and the use of epifluorecence microscope **(Noble and Fuhrman, 1998; Bettarel et al., 2000)**. A protocol using fluorochrome staining and flow cytometer **(Marie et al., 1999)** was

optimized for rapid counting of virus-like-particles (VLPs) in the plankton **(Brussaard, 2004)**. However, these fluorochrome-based methods are well known to provide accurate counts but poor description of microbial/viral diversity.

An accurate description of the diversity of pelagic viruses usually needs highly concentrated aliquots. The current method available to date is ultrafiltration that allows concentrating viruses contained in several tens or hundreds of litres in small volume of some tens of millilitres **(Suttle et al., 1991; Wommack et al., 1995)**. However, the application of this procedure must be followed by ultracentrifugation to obtained viral pellets necessary for TEM observations and for estimating viral diversity **(Wommack et al., 1999a; Diez et al., 2000; Auguet et al., 2006)**. To avoid ultracentrifugation and the underlined disadvantages, we herein propose a simple, cheaper and efficient alternative protocol using polyethylene glycol (PEG) reconcentration to obtain virioplankton concentrates (i.e. pegylation) that can be used for different purposes, primarily for TEM observations, electrophoretic plugs, and the following cloning/sequencing possibilities. To our knowledge, this study is the first application of the PEG-concentration method to typical pelagic environmental samples. PEG was first used by **Albertson and Frick (1960)** to precipitate viruses between two immiscible aqueous polymer phases. At the same time, **Philipson et al. (1960)** have applied this method to concentrate and purify particular viruses (i.e. bacteriophage T2, adenovirus, and ECHO virus prototype 7 and 19), and found it to be rapid, inexpensive, and non-destructive of viruses. Since then, PEG has been used, usually in combination with salts such as NaCl, to recover various viruses in different growing medium **(Lewis and Metcalf, 1988)**. However, comparison of PEG method with current methods, primarily ultracentrifugation, has not been done for pelagic viruses. It is thus worth asking if PEG precipitation is the best method for concentrating virioplankton in natural samples. PEG and monovalent salts are well known as good inductors of attractive interactions that preferentially crystallize biological macromolecules, such as DNA and viruses, in the interpolymer spaces between PEG molecules **[see Tardieu et al., 2002 for the logic behind]**. Effective pegylation occurs when the concentration of DNA and viruses exceeds their solubility **(Polson, 1977)**. Advantages of the method for pelagic samples, in addition to its gentle effect on viruses, include the ability to obtain precipitation at various conditions, e.g. neutral pH, high ionic concentrations, and in the absence of other organic materials **(Atha and Ingham, 1981)**.

Materials and methods

Origin of experimental samples. Samples were obtained from the following freshwater ecosystems, which differed in trophic status and were located in the Massif Central region of France (45°29N, 2°56E): (i) an oligotrophic mountain lake (Godivelle d'en haut), (ii) a moderate-altitude oligomesotrophic lake (Lake Pavin), (iii) an eutrophic lake (Lake Aydat) and (iv) in the output water from a domestic sewage works lagoon (the Cournon plant), which was considered as hypereutrophic environment. For each sampling site, a first series of 20 L of surface water (ca 0.5 – 1 m depth) samples were collected during April 2006, for viral counts. A second series of the same amount of samples were also collected on a different occasion during the same month in Lake Pavin, for pulsed field gel electrophoresis (PFGE). PFGE samples were collected in three contrasted depths, i.e. epi. (1 m), meta. (15 m) and hypolimnion (30 m). After sampling, all these raw natural samples were successively prefiltered by gravity through 100, 50, 25 and 10 µm pore-size nylon fiber filters, and stored in insulated dark sterile boxes. Back to the laboratory, prefiltered samples were immediately filtered under low pressure (< 0.2 bar) using two successive size-pore filters (1.2 and 0.2 µm, 142 mm diameter, Sartorius). Viral concentration and reconcentration experiments were then conducted followed by viral counting for the first series of samples collected in all sampling sites, and by PFGE analysis for the second series of samples collected in Lake Pavin.

Viral concentration and reconcentration. The protocol design used for the viral concentration and reconcentration experiments is shown on Fig. 1. Viruses contained in 20 L experimental samples were concentrated by ultrafiltration to a volume of approximately 1 L, using a high performance concentration/diafiltration system (Model DC 10LA, Amicon®) equipped with a reusable hollow fiber cartridge of 30-kDa cut-off and surface area of 0.45 m² (Amicon®, Epernon, France). Recirculation rate was at 10 L min^{-1} with an entry pressure of 0.9 bar. Retentates were then reconcentrated by two approaches: ultracentrifugation and PEG precipitation.

For ultracentrifugation, 3 runs of 3 h 30 min each at 120,000 x *g* (i.e. 28,000 rpm), 4 °C with a SW 40 Ti rotor (LE 80K, Beckman) were necessary to ultracentrifuged 210 mL (6 tubes of 12 mL filled with 11.7 mL of samples per run) of ultrafiltered retentate. Viral pellets were then resuspended and incubated under agitation during at least 10 h at 4 °C in SM buffer [0.1 M NaCl, 8 mM $MgSO_4$-$7H_2O$, 50 mM Tris-HCl, and 0.005 % (wt/vol) glycerol) adjusted at neutral pH] before analysis (final volume equal to about 400 µL) before counts or PFGE analysis. For the PEG method, we adapted the protocol proposed in Lech et al. (1990).

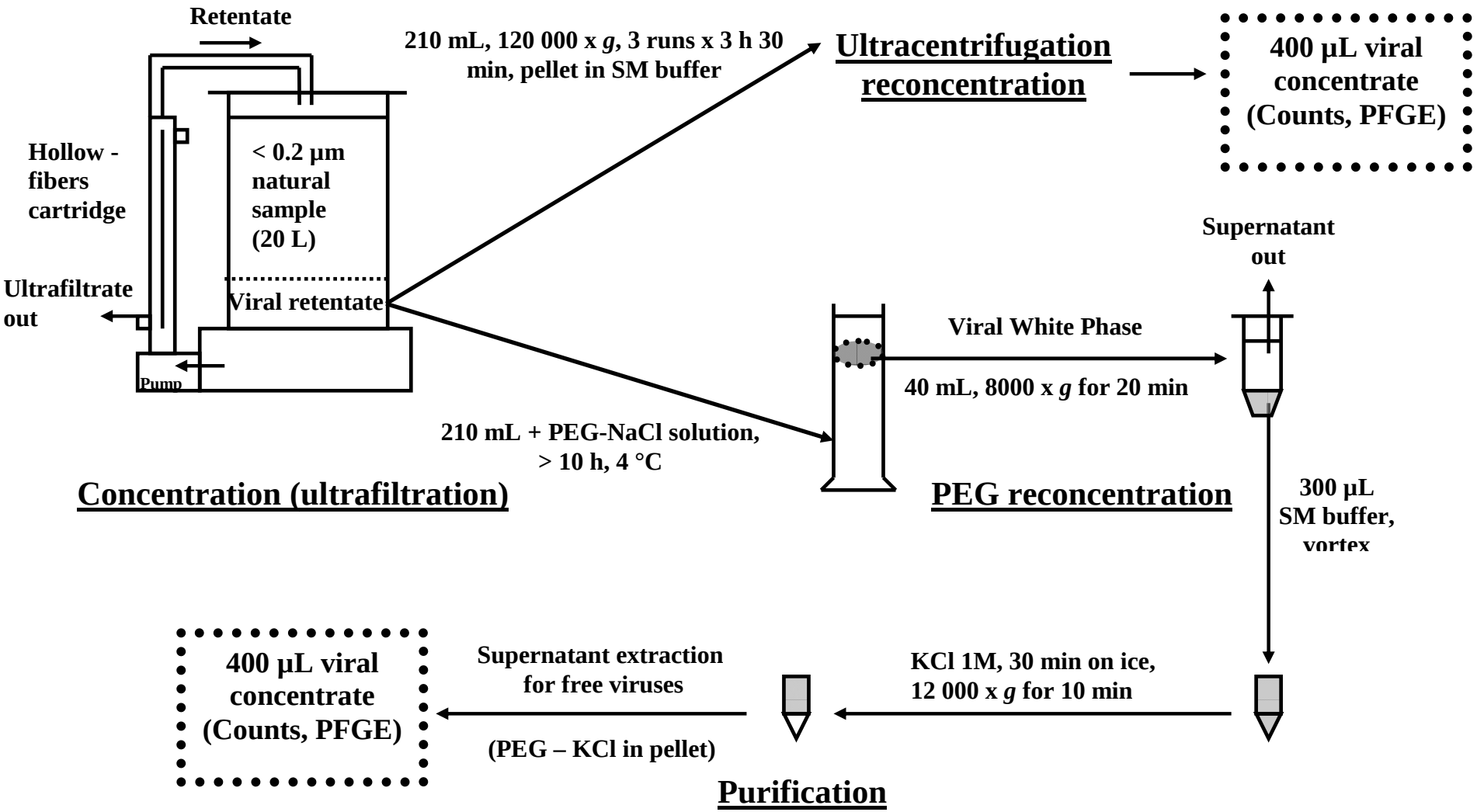


Figure 1. Sample partitioning and experimental design used for ultrafiltration and reconcentration of freshwater samples using polyethylene glycol (PEG) and ultracentrifugation. SM buffer is a mixture of 0.1 M NaCl, 8 mM MgSO4-7H2O, 50 mM Tris-HCl, and 0.005 % (wt/vol) glycerol, adjusted at neutral pH. PFGE, pulsed field gel electrophoresis

Polyethylene glycol 8,000 (catalog no. 81268; Sigma) together with NaCl were added to 210 mL of viral ultrafiltered retentates to final concentrations of 10 and 0.6 %, respectively, and incubated at 4 °C in the dark, in a transparent cylindrical glass container of 400 mL, for at least 10 h. PEG molecules of molecular weight (mw) lower than 6000 are less effective in precipitating macromolecules, while molecules equalling or above 6000 mw appear to behave identically **(Yamamoto et al., 1970)**. The incubation time was determined during preliminary experiments and was over the minimal interval required to obtain a constant and stable white layer of viral precipitates, whatever the sampling site was. At the end of incubations, the white phase containing crystallized viruses (approximately 40 mL) was pipetted, transferred into a 50 mL sterile tube (BD Falcon™, Le Pont de Claix, France), centrifuged at 8,000 x *g* for 20 min at 4 °C, and resuspended in 300 µL of SM buffer. Viral solution was transferred into a 2 mL sterile tube (Eppendorf®, Le Pecq, France). 1 M KCl was then added, the mixture incubated on ice for 20 min and centrifuged (12,000 x *g*, 10 min at 4 °C), in order to precipitate the PEG solution slowly while leaving the purified free viruses behind. The supernatant with clean viruses was finally pipetted, yielding a final volume of about 400 µL viral concentrate (Fig. 1) for viral counts or PFGE analysis.

Viral counts. Aliquots were taken in the initial experimental samples and after ultrafiltration and reconcentration steps and fixed with glutaraldehyde (final concentration 2 %), for viral counts. Viral abundances were determined in initial samples using two different methods, transmission electron microscopy (TEM) and epifluorescence microscopy (EFM), following protocols described previously in **Sime-Ngando *et al.* (1996)** and in **Noble and Fuhrman (1998)**, respectively. For concentrated samples, only TEM was used in order to make sure that typical phage-shaped particles (but not virus-like particles that may include non viral entities, **Bettarel et al., 2000**) were concentrated and counted. We considered these counts as underestimates because TEM is known to be much less sensitive than EFM **(Bettarel et al., 2000)**. For each experimental samples analysed for viral counts, counts were done in triplicates for within sample variability.

Pulsed field gel electrophoresis (PFGE). To further assess the recovery and purification efficiencies of the two reconcentration methods compared (i.e. PEG and ultracentrifugation), a second series of samples were collected in three contrasted depths in Lake Pavin and analysed for the genomic size structure of viral communities using PFGE **(Wommack et al., 1999b; Auguet et al., 2006)**. Viral concentrates were first heated in a waterbath at 60 °C for

10 min for nucleic acid extraction **(Jiang et al., 2003)**. Plug molds were prepared by mixing equal volumes of the viral concentrate (50 µL) and 1.5 % molten (50 °C) agarose (Incert agarose, FMC®, Rockland, USA) in 0.5 % TBE (50 mM Tris, 45 mM Boric acid, 0.5 mM EDTA, pH 8.4). Solidified plugs were punched out from the molds into 1mL of TE buffer (20 mM Tris et 50 mM EDTA, pH 8) and stored at 4 °C. Additional plugs containing phage lambda concatemers (MidRange PFG Markers, Biolabs) were used as molecular markers. Viral and marker plugs were place into wells of 1 % agarose (New England Eurobio®) prepared in 0.5 % TBE buffer, and PFGE performed with a CHEF-DRIII system (Bio-Rad) at 14 °C, 6 V cm^{-1}, a 120° angle, and a switch time of 1 to 15 s for 22 h, using 0.5 % TBE as running buffer. After electrophoresis, the gel was stained for 30 min in ethidium bromide (concentration 0.05 µg mL^{-1}) and rinsed for 1 h in distilled water. The gel was digitally scanned for fluorescence with a gel imager (UVT-28MP, Herolab, Germany) and analysed for digital picture (i.e. densitometry) with the software analysis program Image J (Scion Corporation®, Washington DC, USA).

Statistical analysis. For experimental samples that were concentrated (ultrafiltration) or reconcentrated (ultracentrifugation, PEG-concentration), the recovery efficiency of viruses was calculated as the mean percentage (i.e. for triplicates) of the number of viruses in a given concentrate, compared to the number of viruses in a specified original sample determined using TEM. Viral TEM counts in reconcentrated samples were compared by performing a one-way analysis of variance, for difference between ultracentrifugation and PEG methods. The null hypothesis was that there was not a significant difference between the results obtained with the two protocols. Statistical analyses were performed using MINITAB 12.

Results and discussion

Initial viral concentrations. In order to assess and validate the use of polyethylene glycol (PEG) as an effective chemical precipitant for planktonic viruses, we concentrate viral particles with PEG and the usual ultracentrifugation methods, using ultrafiltered samples originating from diverse freshwater ecosystems. From our initial experimental samples (i.e. < 0.2 µm size fraction), these systems ranged in viral contents from 1.09 ± 0.03 to 13.37 ± 1.3 x 10^7 VLPs mL^{-1} when analysing with EFM, and from 0.73 ± 0.02 to 7.77 ± 1.32 x 10^7 VLPs mL^{-1} under TEM. Overall, EFM counts were significantly higher (ANOVA, $p < 0.05$) than TEM ones (although Lake Pavin appeared as an exception), and within sample variability (n = 3) was the highest in the more productive systems (Table 1).

Quite similar results were reported and discussed previously in an EFM vs TEM comparative study of freshwater VLPs, agreeing well with the related literature in marine systems (cf Bettarel et al., 2000). For both counting methods, viral abundances naturally increased from the oligotrophic Lakes (Godivelle and Pavin) to the output water from the domestic sewage works lagoon considered as hypereutrophic waters. Compared to raw samples, our initial VLP abundances are likely underestimates as prefiltration through 0.2 µm pore size membranes are known to decrease, at times significantly (i.e. up to 60 %), the numerical abundance of viruses in pelagic systems **(Paul et al., 1991)**. For example, viral abundances in < 0.2 µm samples reported using EFM in Lakes Pavin and Aydat during this study (Table 1) represented about 45 and 75%, respectively, of those reported previously in the same lakes and during the same sampling period (i.e. in April) but in unfiltered raw samples **(Bettarel et al., 2003)**.

Table 1. Initial abundances of viruses obtained by epifluoresecnce microscopy (EFM) and transmission electronic microscopy (TEM), and viral recovery efficiencies obtained from TEM counts after concentration of initial pelagic samples (< 0.2 µm) by ultrafiltration, followed by ultracentrifugation and pegylation (PEG-method) of ultrafiltrate retentates. In all cases, the differences between the two latter methods were significant (ANOVA, $p < 0.001$). Mean values and standard deviations were obtained from triplicate counts (i.e. within sample variability).

Source of sample	Initial conc. (10^7 viruses mL^{-1} ± SD) EFM	Initial conc. (10^7 viruses mL^{-1} ± SD) TEM	Recovery efficiency (% ± SD)		
			Ultrafiltration£	Ultracentrifugation#	Pegylation#
Godivelle	* 2.48 ± 0.09	* 0.73 ± 0.02	11.40 ± 0.74	28.06 ± 2.08	81.38 ± 8.41
Pavin	* 1.09 ± 0.03	* 1.32 ± 0.03	98.20 ± 4.71	22.24 ± 1.03	37.85 ± 2.05
Aydat	* 3.74 ± 0.20	* 2.03 ± 0.25	36.90 ± 1.32	28.06 ± 2.12	70.68 ± 4.05
Cournon	* 13.37 ± 1.30	* 7.77 ± 1.32	62.19 ± 5.60	13.38 ± 0.93	28.40 ± 1.03
Mean			**52.17 ± 3.09**	**22.94 ± 1.54**	**54.57 ± 3.89**

* Significant differences between the four sampling sites (ANOVA, $p < 0.05$)
£ Calculated from the initial viral abundances in natural samples
Calculated from the viral abundances in ultrafiltrated retentates

Ultrafiltration of viruses. In viral diversity studies, a first step concentration of viruses in large volumes of experimental samples is necessary before a second step reconcentration adapted to small volumes such as ultracentrifugation (e.g. our total rotor capacity is limited to 72 mL, i.e. 6 tubes x 12 mL). In general, ultrafiltration methods are used for large volume of

initial samples **(Suttle et al., 1991; Wommack et al., 1995; Wommack et al., 1999a; Diez et al., 2000; Auguet et al., 2006)**. In this study, our viral recovery efficiency of ultrafiltration averaged 52 % (range, 11 – 98%) and increased according to the following respective sampling sites: Godivelle, Aydat, Cournon and Pavin (Table 1). There was thus no clear recovery pattern according to the trophic status of the sampling sites, mainly because of low recovery rates in the most productive systems compared to those in the oligomesotrophic Lake Pavin. This indicates that concentration of pelagic viruses using ultrafiltration may be more efficient in systems with intermediate productivity and particle load. Very few studies where pelagic viruses are concentrated by ultrafiltration have provided recovery rates for an accurate comparison. Applying a double filtration method (i.e. concentration of 0.1 µm filtrates with 10,000 molecular weight cut-off ultrafilters), **Wommack et al. (1995)** reported recovery rates that ranged from 23 to 72 % for a mixture of two cultured phages (named CB 7 and CB 38), and from 58 to > 100 % for natural communities of VLPs collected in the Chesapeake Bay. Wommack and co-authors explained recovery rates > 100 % by an underestimation of their initial viral abundances counted under a TEM. **Suttle et al. (1991)** reported recovery rates from 75.1 to 99.6 % for two specific bacteriophages (named PWH3-P1 and LMG1-P4) added to ultrafiltered virus-free seawater from Gulf of Mexico and counted using plaque essay method. **Morales-Morales et al. (2003)** reported recovery rates of 38 and 45 % for T1 and PP7 phages, respectively, based on plaque essay counting method after concentration using hallow-fiber ultrafiltration (50,000 molecular weight cut-off). These rates increased to 73 and 62 %, respectively, after elution with 0.05 M glycine containing retentate. Our ultrafiltration recovery rates of planktonic viruses in < 0.2 µm freshwater samples thus fell within the few ones reported.

Comparison of PEG and ultracentrifugation methods. For the second step concentration, PEG precipitation appeared largely more efficient than ultracentrifugation, in terms of recovering free-floating phages in ultrafiltered freshwater samples examined under TEM. For all the sampling sites, recovery efficiency averaged 55 % (range 28 – 81 %) for the PEG method and only 23 % (13 – 28 %) for the ultracentrifugation method (Table 1). The rates obtained with the former chemical method were significantly higher than those obtained with ultracentrifugation ($p < 0.001$, ANOVA) for all sampling sites. Viral abundances in PEG treated samples were 1.7 to 2.9 fold (mean, 2.3 fold) higher than in ultracentrifuged samples. These differences were obvious on TEM photomicrographs where viruses reconcentrated

from the same initial volumes of ultrafiltered samples visibly were well crystallized and more abundant in PEG-treated than in ultracentrifuged samples (Fig. 2).

Compared to our initial < 0.2 µm samples, our overall recovery rates, i.e. 10 – 28 % (mean 17 %) for PEG method and 4 – 16 % (means 8 %) for ultracentrifugation method, were lower than a single value of 45 % reported recently in coastal waters by **Auguet et al. (2006)** using ultrafiltration followed by ultracentrifugation. To our knowledge, the lowest overall recovery rates of viruses after tangential flow filtration before ultracentrifugation were reported in a solar saltern system in Alicante, Spain. We calculated from provided data that these rates ranged from 0.2 to 1.3 % **(Diez et al., 2000)**. The experimental procedure and conditions applied by Diez and coauthors, i.e. tangential flow filtration using 100-kDa cut-off filter, 6 h ultracentifugation at 45,000 rpm using a Kontron TFT 70 rotor (Kontron Instruments, Cumbernauld, UK) followed by filtration through 0.45 µm disposable filter coupled to a syringe, together with the type of ecosystem (see below), likely may help explain their low recovery rates compared to those in **Auguet and coauthors** and in our study. Yet, accurate comparison still remains difficult because very few studies in aquatic viral ecology have provided recovery rates from ultracentrifugation.

The weak recovery efficiency from the ultracentrifugation method was not related, at least theoretically, to an insufficient rate of sedimentation of phages. Indeed we have calculated, according to **Suttle (1993)**, that the conditions we applied for our filled centrifuge tubes and the Beckman rotor SW 40 (i.e. 120,000 x *g*, 3.5 h, 4 °C) were sufficient to allow 100 % sedimentation of particles of 80 Svedbergs, i.e. corresponding to the smallest planktonic viruses **(Suttle, 1993)**. It is likely that ultracentrifugation provokes mechanical disruption of a significant number of viruses in natural communities. This may be more marked for samples with high particle contains such as in our output water from the Cournon domestic sewage works lagoon (Table 1) or in solar salterns **(Diez et al., 2000)** where, to our knowledge, the lowest recovery rates after ultracentrifugation were obtained. The ultracentrifugation speed and time (see above) applied by Diez and co-authors were among the highest ones used in aquatic viral ecology **(Metcalf, 1975; Wommack et al., 1999a; Zhong et al., 2002; Auguet et al., 2006)**. From the above comparisons, we hypothesize that (i) mechanical and velocity-related shocks are significant viral loss factors during ultracentrifugation (i.e. the more widely used although costly technique in aquatic ecology for the concentration of virioplankton), and (ii) the potential effects of these shocks increase with particle contains and are to be added to other potential loss factors related to viral decay, e.g. enzyme digestion and other virucidal substances.

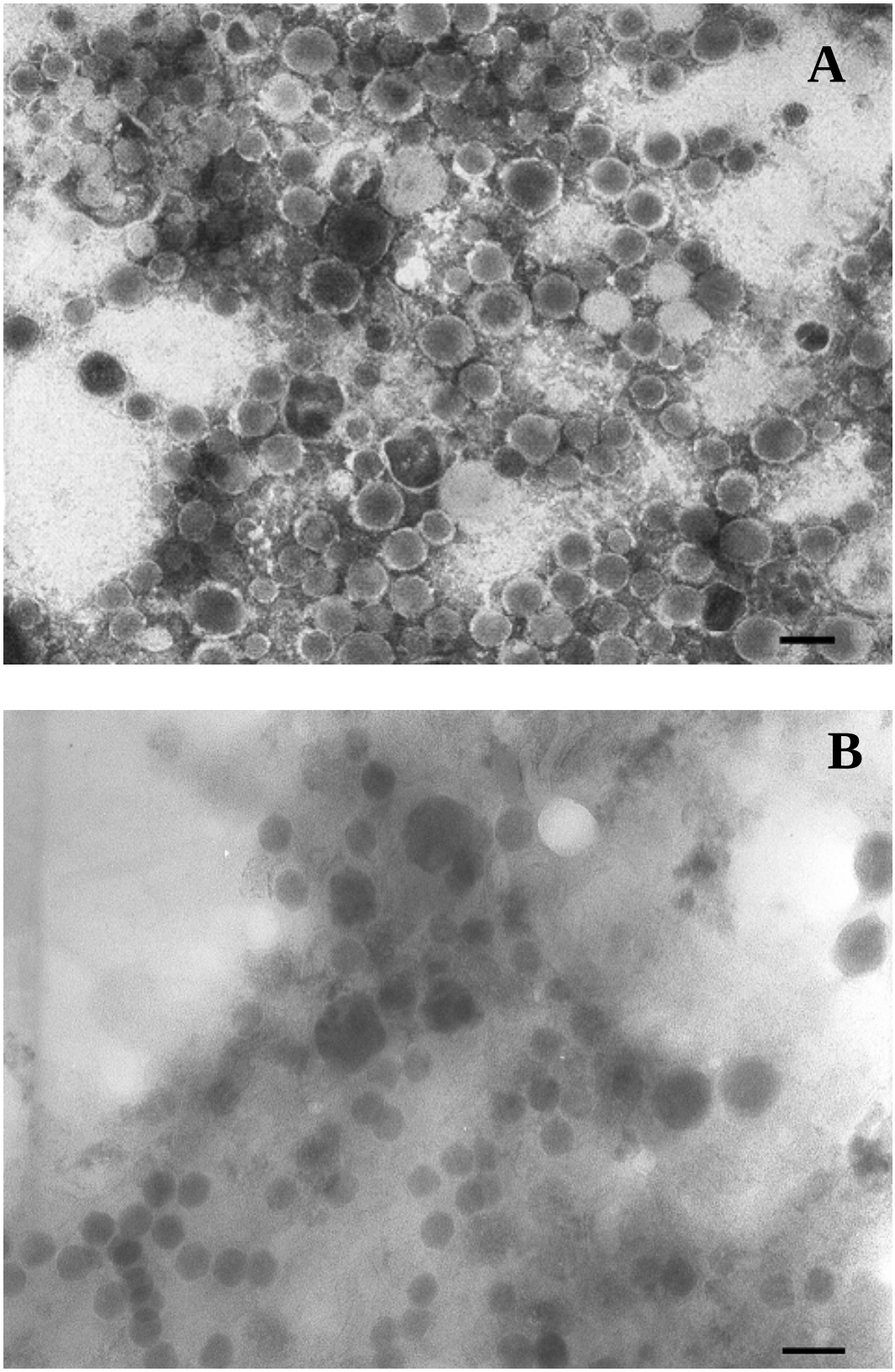


Figure 2. TEM micrographs of uranyl acetate stained viruses collected in the output waters from the Cournon domestic sewage works lagoon obtained after reconcentration using polyethylene glycol (PEG, A, note the crystallization of viral particles) and ultracentrifugation (B) for the same sample. Scale bar = 100 nm.

The more effective recovery of natural viruses by pegylation compared to ultracentrifugation reported herein agrees well with the sole available study comparing the two methods but for the recovery of particular viruses, i.e. cytomegaloviruses in human urine (**Kimpton et al., 1990**). Using PEG precipitation, recovery rates ranging from 40 to 100 % have been reported by **Lewis and Metcalf (1988)** in oyster tissues and environmental samples that were seeded with particular viruses (i.e. hepatitis A virus, human rotavirus Wa, simian rotavirus SA11, and poliovirus). Our recovery rates from simple viral pegylation in ultrafiltered natural samples also agree with those (typical range, 11 – 97 %) reported using other but more elaborate physical/chemical methods **(Ramia and Sattar, 1980; Singh and Gerba, 1983; Morales-Morales et al., 2003; Cashdollar and Dahling, 2006)**. The minimal viral concentration needed for the application of PEG method was shown to be at 10^5 pfu mL^{-1} based on plaque-cultured phages **(Yamamoto et al., 1970)**. We have applied the PEG method to our 0.2 µm filtered natural samples, i.e. without ultrafiltration, but the viral precipitation time was long and depended on the viral load, i.e. from 10 days for the output waters from the domestic sewage lagoon, to 20 days for Lake Pavin surface waters. However, 'pegylation' of viruses directly in natural plankton still needs proper testing.

***Application to PFGE*.** After pegylation of viruses, the use of KCl to remove PEG molecules allows to obtain purified viral pellets that can be directly used in molecular ecology of viruses after DNA extraction, through both PCR-based or non-PCR-based approaches **(Kimpton et al., 1990; Lech et al., 1990)**. Viral concentrations in our final pellets were all above the optimal value of 5 x 10^9 VLPs mL^{-1} for genome size profiling using PFGE **(Riemann and Middelboe, 2002)**. For both Peg-treated and ultracentrifugated samples, genomes in virioplankton fractions from the three contrasted depths sampled in Lake Pavin ranged in size from about 15 to 290 kpb, with a bulk around 35 kpb and in the metalimnion (Fig. 3A), corroborating data from the literature **(e.g. Wommack et al., 1999a; Riemann and Middelboe, 2002; Auguet et al., 2006)**. In all depths, the number of viral DNA-based populations estimated from PFGE was clearly superior in PEG-treated than in ultracentrifuged samples, as determined from visible electrophoretic bands in our agarose gel (Fig. 3A), from computer-generated bands (Fig. 3B), as well as from densitometry profiles, (Fig. 3C). The minimal numbers of bands discriminated visibly were at 9, 8, and 7 for PEG concentration, and only at 6, 5, and 6 for ultracentrifugation, in the epi-, meta-, and hypolimnion of Lake Pavin, respectively. In addition, PFGE-derived genomic diversity of viruses appeared even well discriminated when PEG method was used, compared to

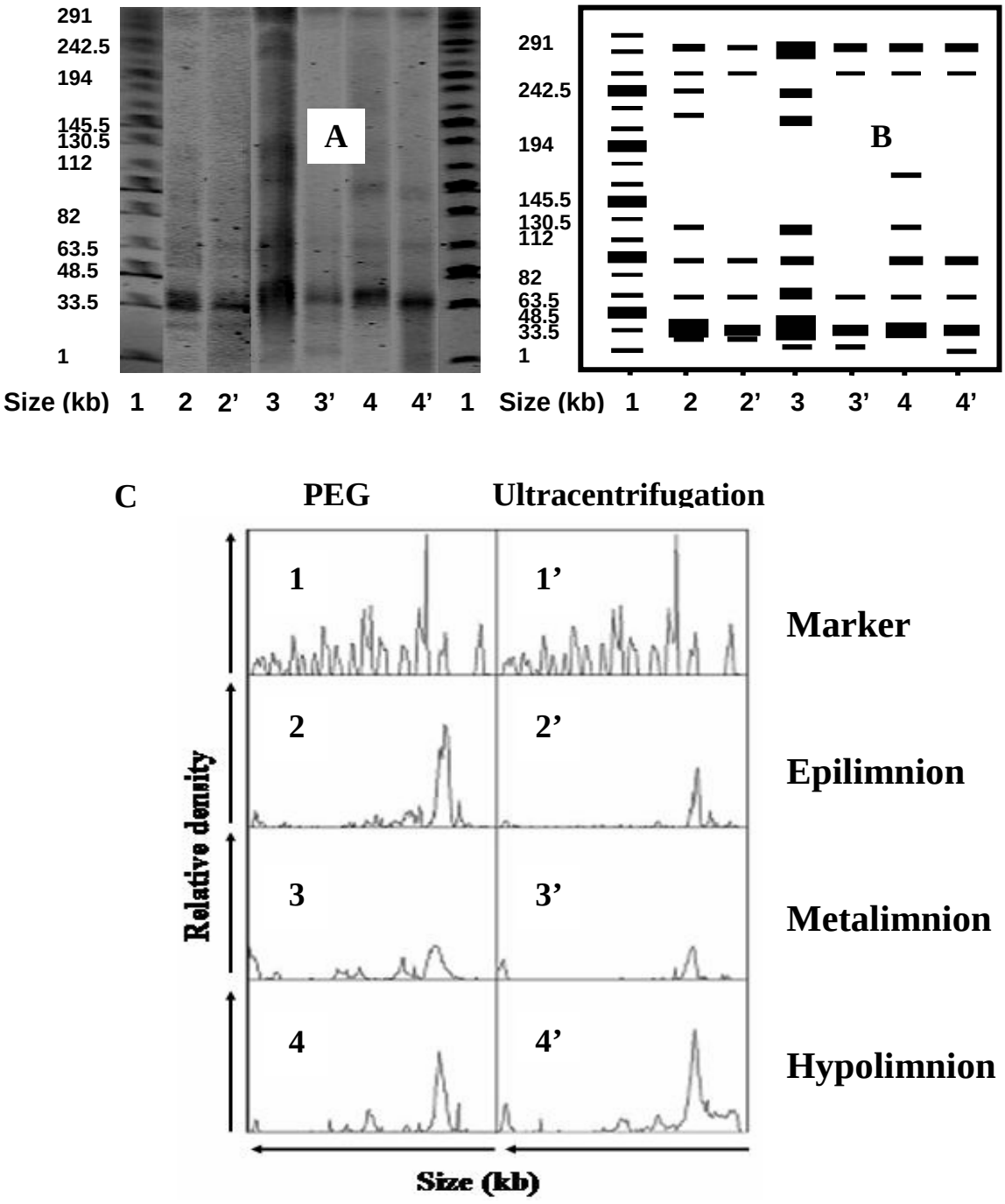


Figure 3. Comparison of polyethylene glycol (PEG) and ultracentrifugation reconcentration methods for pulsed field gel electrophoresis (PFGE) fingerprinting of natural viral communities collected in three contrasted depths in Lake Pavin. Fingerprints are shown on panel (A), the derived schematic computer-generated banding patterns on panel (B), and the corresponding densitometry profiles on panel (C). In panels A and B, bands 1 corresponds to the MidRange PFGE marker; bands 2, 3, and 4 to PEG-treated samples collected in the epi-, meta, and hypolimnion, respectively, and bands 2', 3' and 4' to the same samples but concentrated by ultracentrifugation. The identification numbers of bands in A and B are inserted in the corresponding subpanels in C.

ultracentrifugation. We considered this as the result of the significant higher efficiency of the chemical method over ultracentrifugation, for concentrating and purifying free-floating viruses in the plankton.

Conclusions

This study provides a simple, cheaper and more efficient alternative protocol to ultracentrifugation, for chemical concentration and purification of pelagic viruses using polyethylene glycol. The PEG method is less labour intensive, can be applied to large volumes and greater numbers of samples at any one time, and do not require access to expensive centrifuge facilities. The effects of potential virucidal compounds (e.g. enzymes, antibodies, and other inhibitory substances) are probably minimized or avoided during pegylation. This property of PEG molecules is currently used in medicine to protect drug-carrier viruses, interferons of immunitary system, or antibodies **(O'Riordan et al., 1999; Harris and Chess, 2003; Le et al., 2005; Dominguez et al., 2006)**. PEG could thus also be used as a conservative for planktonic viruses, thereby avoiding the use of toxic fixatives and the related disadvantages such as losses during storage of samples. This needs proper testing and training, as do the direct use of PEG method with raw natural samples, i.e. without a first step concentration by ultrafiltration. In summary, pegylation is suitable for concentrating and purifying virioplankton in a variety of natural samples, a prerequisite for the study of viruses with modern molecular biology approaches such as PFGE, currently used to assess the diversity of natural viral communities. It is a valid, practical, and economical method that offers a strong alternative to the more expensive and harmful ultracentrifugation.

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Partie 2 : Etude de la dynamique de la diversité des communautés virales

Virioplankton biodiversity: pheno- and genotypic size distributions in a deep freshwater lake

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Running title: Diversity of viruses in Lake Pavin

En préparation

Abstract

Very little is known about the viral diversity and diversification in freshwaters. Herein, we examined changes in morphological and genomic diversities of viruses by means of transmission electron microscopy and pulsed field gel electrophoresis (PFGE), respectively; over a nine-month period (Avril-December 2005) in four contrasting depths in the oligomesotrophic Lake Pavin. Morphologically, we found that viruses in this Lake were dominated by untailed phages (61 % of total abundance) and by *Siphoviridae* (33.5 %), with capsid sizes ranging from 30 to 60 nm (66.5 %). Genomically, the majority of viruses exhibited genome sizes ranging from 15 to 45 kb (61% of total relative abundance calculated from densitometric analysis). On average, 12 different genotypes dominated each of the PFGE fingerprints. The highest genomic viral richness was recorded in summer (mean = 14 bands per PFGE fingerprint) and in the epilimnion (mean = 13 bands per PFGE fingerprint). Spatio-temporal changes observed in the structure (capsid sizes, morphological traits and genome sizes) of viral community appeared to be linked mainly to microbial communities. Empirical relationships suggested the existence of three different viral communities based on the sizes of phenotypes and genotypes: (i) viruses with large capsid (> 80 nm) and genome (up to > 290 kb) sizes were related to eukaryotes, (ii) viruses with intermediate capsid (30-80 nm) and genome (< 105 kb) sizes appeared to be linked to picocyanobacteria, while (iii) the smallest viruses with capsid size < 60 nm and genome size < 75 kb probably were specific to

heterotrophic bacteria. Moreover, the dynamics of *Myoviridae* and *Siphoviridae* showed a tendency to be forced by autotrophic microorganisms, whereas the dynamics of untailed viruses were linked to bacteria. Finally, temperature also appeared a potential driving factor for viral diversity and diversification, but the host availability was obviously prevalent over the physico-chemical environment in regulating viruses in the plankton.

Introduction

It is now widely accepted that viruses are the most abundant entities in aquatic ecosystems, where their abundance is about an order of magnitude greater than the number of bacteria **(Weinbauer 2004, Suttle 2007 for the most recent reviews)**. Viruses are ubiquitous entities in natural environments infecting virtually every living form of life, from cellular prokaryotes to animals and plants. A given host has probably a range of different co-infectious viruses, resulting of enhanced gene exchanges between phages and hosts **(Moineau *et al.* 1994, 1995)**. For example, it was suggested that bacteria and their co-infecting phages function as 'phage factory' and produce a variety of chimeric or mosaic phages which increase viral diversity **(Ohnishi *et al.* 2001)**. Undoubtedly, environmental viruses are the largest reservoir of genetic diversity on the planet **(Suttle 2005, Suttle 2007)**. Excepted for algal viruses, cyanophages or picorna-like viruses **(Culley *et al.* 2003)**, there is no common molecule for viruses that could be used to access their diversity, similar to molecular RNA genes for cellular organisms **(Hendrix *et al.*, 1999)**. This renders the study of viral diversity difficult by PCR-based methods. Recently, in addition to the frequently used transmission electronic microscopy (TEM) for estimating viral diversity based on phenotypic traits **(Wommack and Colwell 2000, Weinbauer 2004 and references therein, Liu *et al.* 2006, Auguet *et al.* 2006)**, few metagenomic and other molecular techniques have been developed **(see Wommack and Colwell 2000, Breitbart *et al.* 2002, 2003, Hambly and Suttle 2005, Angly *et al.* 2006, Williamson *et al.* 2008)**. Among these, the most frequently used is the pulsed field gel electrophoresis (PFGE), which allows to easily and rapidly estimate the whole-genome fingerprinting of natural communities of viruses **(Wommack *et al.* 1999 a and b, Steward and Azam 2000)**. Few results obtained with this method showed that between 7 and 35 bands can be distinguished by applying PFGE to individual samples, as a minimum viral richness, with a dominance of < 70 kb genome in both marine **(see Weinbauer 2004)** and freshwater plankton **(Auguet *et al.* 2006, Filippini and Middelboe 2007)**.

Some studies have shown that viral genomic diversity, known from PFGE and the relative intensity of individual bands, can vary seasonally **(e.g. Wommack *et al.* 1999b, Castberg *et al.* 2001, Larsen *et al.* 2001, 2004, Sandaa and Larsen 2006)** and spatially **(Wommack *et al.* 1999a, Steward *et al.* 2000, Riemann and Middelboe 2002, Jiang *et al.* 2004, Auguet *et al.* 2006, Filippini and Middelboe 2007)**. However, little is known about the determinism of these variations, because very rare works have studied simultaneously the viral genomic composition (VGC) and the microbial community structure and environmental factors. Few authors have hypothesized that VGC changed with the structure of host communities **(Steward *et al.* 2000, Auguet *et al.* 2006). Sandaa and Larsen (2006)** have shown that seasonal changes in viral community composition can be related to changes in the abundances of cyanobacteria, autotrophic nano- and picoeukaryotes, and heterotrophic bacteria, whereas **Riemann and Middelboe (2002)** reported no direct link between changes in VGC and bacterial community composition and/or phytoplankton abundance. These few available studies were conducted in marine waters but without taking into account phytoplanktonic species and protistan flagellates. Potential links between VGC and microbial community structure are thus to be established, because there are many routes of interactions between viruses and microbial communities **(Sime-Ngando and Pradeep Ram 2005, Pradeep Ram and Sime-Ngando 2008)**. For example, in addition to represent a potential host reservoir for viruses **(Massana *et al.* 2007)**, protistan flagellates could also grazed bacteria and/or viruses **(Bettarel *et al.* 2005)**, thereby influencing directly or indirectly the viral diversity **(see Weinbauer, 2004)**. Viral lytic or lysogenic activity could also affect VGC **(Weinbauer, 2004)**. Since the genetic exchanges are probably higher among lysogenic than among lytic phages, the diversity of lysogenic phages is important **(Chen and Lu, 2002)** and higher viral diversity could be recorded during lysogen induction events. However, at the community level, no study to our knowledge has considered simultaneous changes in VGC together with lytic and lysogenic activities and with potential grazers as well. Finally, **Jiang *et al.* (2004)** have shown that VGC changed with oxygen conditions in the hypersaline Lake Mono (California, USA). **Weinbaeur (2004)** suggested that certain physico chemical parameters may define the niches for phages and thus influence viral diversity. However data are not yet available at the community level.

Many biological or physico-chemical factors could thus potentially affect VGC, but very few attempts have been made to study these effects, leaving open many questions of ecological interest concerning the dynamics of the biodiversity within virioplankton communities and the related environmental forcing factors. In this study, we have

investigated, for the first time, the study of the spatio-temporal dynamics of viral community diversity (based on genomic and morphological characteristics) and activity (lytic and lysogenic), concurrently with physico-chemical parameters (temperature, oxygen, chlorophyll a) and microbial community variables (bacteria, picocyanobacteria, autotrophic picoeukaryotes, autotrophic and heterotrophic nanoflagellates). We were seeking for empirical evidences of the potential connections between the diversity of viruses and both abiotic and biotic environmental factors, at consistent seasonal and deep scales in aquatic systems.

Materials and methods

Study site and sample collection. Samples were collected in Lake Pavin (altitude 1197 m), a meromictic and dimictic oligomesotrophic lake located in the French Massif Central, that experiences partial overturn. It is a typical crater mountain lake characterized by a maximum depth of 92 m and low surface (44 ha) and catchment (50 ha) areas. A characteristic feature of the physical structure of Lake Pavin is the existence of an oxic/anoxic interface (i.e. oxycline) between 50 and 60 m depth. Samples were collected monthly (systematically between 09:00 and 10:00 AM) from April to December 2005 from a central location in the lake by using an 8-liter Van Dorn bottle. Four different layers were sampled, corresponding to the epi- (5 m), meta- (12 m) and hypolimnion (30 m), and to the oxycline (57 m). This was done for all biological variables, excepted for pycocyanobacteria and autotrophic picoeukaryotes which were determined in the epi-, meta-, and hypolimnion. All analysis was performed in triplicate, excepted for genomic size profiling samples by PFGE.

Physico chemical variables. Water temperature and dissolved oxygen profiles were measured *in situ* each 0.5 m with an oxycal-SL 197 multiparameter probe (WWT, Limonest, France). Chlorophyll *a* concentrations (Chl) in the 4 sampled depths were determined spectrophotometrically from samples (0.5 to 2 L) collected on Whatman GF/F filters. Pigments were extracted in 90 % acetone overnight in the dark at 4 °C, and concentrations calculated using **SCOR-UNESCO (1966)** equations.

Viral and bacterial concentrations. Subsamples were fixed with 0.02 µm filtered glutaraldehyde (final concentration 2 % v/v) immediately after sampling, and filtered (<15 kPa vacuum) through 0.02 µm pore size Anodisc filters (Whatman, Maidstone, UK) which were mounted between microscope slides and glass cover slips using a mixture of 80 % AF1

Citifluor (Citifluor, London, England) and 20 % Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, U.S.A.) as antifading mounting medium. Counts were done under a Leica DC 300F epifluorescence microscope following staining with SYBR Green I fluorochrome (Molecular Probes Europe, Leiden, Netherlands) as described by **Noble and Fuhrman (1998)**. When not analysed immediately, slides were stored at –20 °C until counting. Bacteria were distinguished from virus-like particles (VLPs) on the basis of their relative size and brightness. A blank was routinely examined to control for contamination of the equipments and reagents.

Picocyanobacteria and autotrophic picoeukaryote concentrations. Picocyanobacteria and autotrophic picoeukaryotes collected on 0.2 µm polycarbonate black filters (Whatman, Maidstone, UK) were mounted between microscope slides and glass cover slips using a non-fluoresecent immersion oil (Olympus optical, Japan). Counts were done under the Leica DC 300F epifluorescence microscope, using two sets of optic filters for distinguishing differences in autofluorescence due to the composition of pigment contain: green light for phycoerythrin-rich picocyanobacteria (Pcy), and blue light for chlorophyll-containing picoeukaryotes (Peu).

Heterotrophic and autotrophic nanoflagellate abundance. Samples for enumeration of heterotrophic (HNF) and autotrophic (ANF) nanoflagellates were fixed immediately after sampling with glutaraldehyde (final concentration 1 %). Primulin stained flagellates collected on 0.8 µm polycarbonate black filters (Whatman, Maidstone, UK) were mounted between microscope slides and glass cover slips using the non-fluorescent immersion oil. Counts were done under the Leica DC 300F epifluorescence microscope, using two sets of optic filters: UV light for HNF and blue light for ANF **(Carrias *et al.* 1998)**. When not analysed immediately, slides were stored at -20 °C until counting. We distinguished, *Kathablepharidae*, Choanoflagellates, Undetermined flagellates, *Monas-like* and *Bodonidae* among HNF community, and *Chrysidalis*, *Chloromonas*, *Rhodomonas* and O*chromonas* among ANF, based on morphological characteristics (for details see **Carrias *et al.* 1998**).

Frequency of infected cells and burst size. For viral lytic infection, the frequency of infected cells (FIC) was calculated from the frequency of visibly infected cells (FVIC) obtained, together with burst size (i.e. mean number of viruses per infected cell), from observations under a JEOL 1200EX transmission electron microscope, following ultracentrifugation and uranyl acetate staining. The procedure is detailed elsewhere **(Bettarel *et al.* 2004)**. FIC was

calculated from FVIC (as a percent) using the formula FIC = 9.524 FVIC – 3.256 **(Weinbauer *et al.* 2002)**.

Frequency of lysogenically infected cells. The frequency of lysogenically infected cells (FLC) was determined from the induction of prophages using mitomycin C **(Jiang and Paul 1996)**. Mitomycin C was added to samples (final concentration 1 µg mL^{-1}) in 20 mL sterile serum bottles, and untreated samples served as controls. The incubation time (24 hours *in situ*) was fixed from a 76-hour time series preliminary experiment conducted on 25 March 2004. Subsamples were removed with syringes at t0 and t24 hours and fixed with glutaraldehyde for viral and bacterial counts. FLC was estimated from viral abundances in mitomycin C treated (VAm) and control (VAc) incubations, bacterial abundance (BAt0) and maximal burst size (MaxBSt0) in original samples: FLC = 100[(VAm – VAc)/(MaxBSt0 x BAt0)] **(Weinbauer *et al.* 2003)**.

Potential grazing. Among all 'species' of nanoflagellates determined based on their morphology, we have considered the abundances of target taxonomic groups known as typical bacterivores in Lake Pavin **(Carrias et al. 1996)**, for the estimation of potential grazing activity. These groups or bacterivores were as follows: choanoflagellates, *Monas-like* cells, and a fraction of undetermined small flagellates (see below) for HNF, and *Ochromonas* spp. for ANF (i.e. mixotrophs). For the group of 'undetermined flagellates', cells with straight and long single flagellum, typical of fungal zoospores (i.e. chytrids) based on both morphological and molecular characterization (cf. **Lefèvre et al. 2007**) and known as non-phagotrophs from previous bacterivory experiments in Lake Pavin **(Carrias 1996, Carrias et al. 1996)**, were excluded from comparisons. These forms represented on average about 25% of the undetermined HNF group. Potential grazing rates from the different groups of bacterivores were estimated as the product of bacterial concentration, the abundance of flagellates of interest, and mean clearance rates determined previously in a seasonal study in Lake Pavin **(Carrias *et al.* 1996)**. These rates were as follows: 2.5, 6.1, 7.1 and 7.8 nL $flagellate^{-1}h^{-1}$ for undetermined flagellates, *Monas-like* cells, choanoflagellates, and *Ochromonas*, respectively. These values fell within the range of values published for freshwater systems **(Sanders *et al.* 1989)**. Total grazing rate was estimated by adding specific grazing rates from the different groups of potential bacterivores.

Virioplankton concentration. For the diversity study of viruses, viral concentrates are required. Strategy employed for viral concentration was based on the use of Polyethylene glycol (PEG), an approach that we have recently described in detail in **Colombet *et al.* (2007)**. Briefly, following successive prefiltration steps, viruses contained in 20L of < 0.2 µm lake water were first concentrated by ultrafiltration, using a high performance concentration/diafiltration system (Model DC 10LA, Amicon®) equipped with a reusable hollow fiber cartridge of 30-kDa cut-off (Amicon®, Epernon, France). Concentrated viruses in the retentate were then precipitated by 'pegylation', i.e. PEG-treated. Polyethylene glycol 8,000 (catalog no. 81268; Sigma) together with NaCl were added to 210 mL of viral ultrafiltered retentates to final concentrations of 10 and 0.6 %, respectively, and incubated at 4 °C in the dark for at least 10 h. At the end of incubations, the white phase containing crystallized viruses was pipetted, centrifuged at 8,000 x *g* for 20 min at 4 °C, and resuspended in SM buffer [0.1 M NaCl, 8 mM $MgSO_4$-$7H_2O$, 50 mM Tris-HCl, and 0.005 % (wt/vol) glycerol) adjusted at neutral pH]. 1 M KCl was then added, the mixture incubated on ice for 20 min and centrifuged (12,000 x *g*, 10 min at 4 °C), in order to precipitate the PEG solution slowly while leaving the free viruses behind. The supernatant with clean viruses was finally pipetted and used for diversity analysis (cf. **Colombet *et al.* 2007**).

Morphological and morphometric diversity of phages. The shape and size of viruses were analyzed by transmission electronic microscopy (TEM). Viruses in concentrates were collected directly onto 400-mesh Cu electron microscopy grid with carbon–coated Formvar film (Pelanne Instruments, Toulouse, France). 60 µL of viral concentrate (stored at 4 °C) was diluted in 6 mL of 0.02 µm filtered distilled deionised water, and centrifuged at 120,000 x g for 3 h 30, 4 °C with a SW 40 Ti rotor (LE 80K, Beckman). Three grids were observed at a magnification of 50,000X, and at least 300 viruses were observed and counted on each grid. During observations, viruses were classified based on two morphological criteria: the size of the capsid and the general shape of viral particles. For capsid-size classification, viruses were categorized into five size classes: < 30, 30-60, 60-80, 80-100, and > 100nm. For shape classification, four morphotypes were distinguished, untailed viruses and three families in the order *Caudovirales*, distinguished according to the morphological criterion defined by the International Commitee on Taxonomy of Viruses. These families were as follows: *Siphoviridae* (viruses with noncontractile long tails), *Myoviridae* (with a contractile long tails, presence of a neck), and *Podoviridae* (with short tails).

Genomic size diversity of virioplankton. Viral genomic composition (VGC) was analyzed by pulsed field gel electrophoresis (PFGE). Viral concentrates were first boiled in a waterbath at 60 °C for 10 min for nucleic acid extraction **(Jiang *et al*. 2004)**. Plug molds were prepared by mixing equal volumes of the viral concentrate (50 µL) and 1.5 % molten (50 °C) agarose (Incert agarose, FMC®, Rockland, USA) in 0.5 % TBE (50 mM Tris, 45 mM Boric acid, 0.5 mM EDTA, pH 8.4). Solidified plugs were punched out from the molds into 1mL of TE buffer (20 mM Tris et 50 mM EDTA, pH 8) and stored at 4 °C. Additional plugs containing phage lambda concatemers (MidRange PFG Markers, Biolabs) were used as molecular markers. Viral and marker plugs were place into wells of a 1 % agarose (Eurobio, France) prepared in 0.5 x TBE buffer, and PFGE performed with a CHEF-DRIII system (Bio-Rad) at 14 °C, 6 V cm^{-1}, a 120° angle, and a switch time of 1 to 15 s for 22 h, using 0.5 x TBE as running buffer.

After electrophoresis, the gel was stained for 30 min in ethidium bromide (concentration 0.05 µg mL^{-1}) and rinsed for 1h in distilled water. The gel was digitally scanned using a gel imager (UVT-28MP, Herolab, Germany) and quantitative analysis of digital gel pictures (molecular weight and fluorescence intensity of bands) was performed with the software analysis program Image J (Scion Corporation®, Washington D.C., USA). Each gel was normalized using molecular weight ladder run on both sides of the gel. The region bracketed by the largest and smallest DNA size standards was divided into 19 subregions (<15, 15-30, 30-45, 45-60, 60-75, 75-90, 90-105, 105-120, 120-135, 135-150, 150-165, 165-180, 180-195, 195-210, 210-225, 225-240, 240-255, 255-290, >290 kb). Genome copy number in a subregion was determined by calculating the density within each subregion divided by an average molecular size in the subregion **(Riemann and Middelboe, 2002)**. To facilitate comparison among samples, the copy number present in one subregion was divided by the total copy number present in the same sample according to **Steward *et al.* (2000).**

Statistical analysis. Normal distribution of data was checked by Kolmogorov-Smirnov test. Because all the data sets did no follow normal distribution, we applied log transformation to meet the requirements for parametric statistics. One way analysis of variance was used to test for differences in viral diversity parameters between seasons [i.e., spring (April to June), summer (July to September), and autumn (October to December)] and sampling depths. Potential relationships among variables were tested by Pearson correlation analysis, with the

significance level considered at $p < 0.01$. All statistical analysis was performed using MINITAB 12 and SYSTAT 10.

Results

Temperature, dissolved oxygen, chlorophyll a. The changes in water temperature and oxygen contents were typical of a deep meromictic dimictic temperate lake. Thermal stratification started in May and ended in November, with partial overturns and homogenization of the mixolimnic water column in April and December (Fig 1A). The oxic/anoxic interface was located between about 55 and 59 m (Fig. 1B). The temperature and oxygen profiles confirmed that the depths sampled were in the epi-, meta-, hypolimnion and oxycline. Large spatial and seasonal differences in the distribution of chlorophyll were observed. Values were higher in the three uppest layers than in the oxycline, with peaks in spring (Table 1).

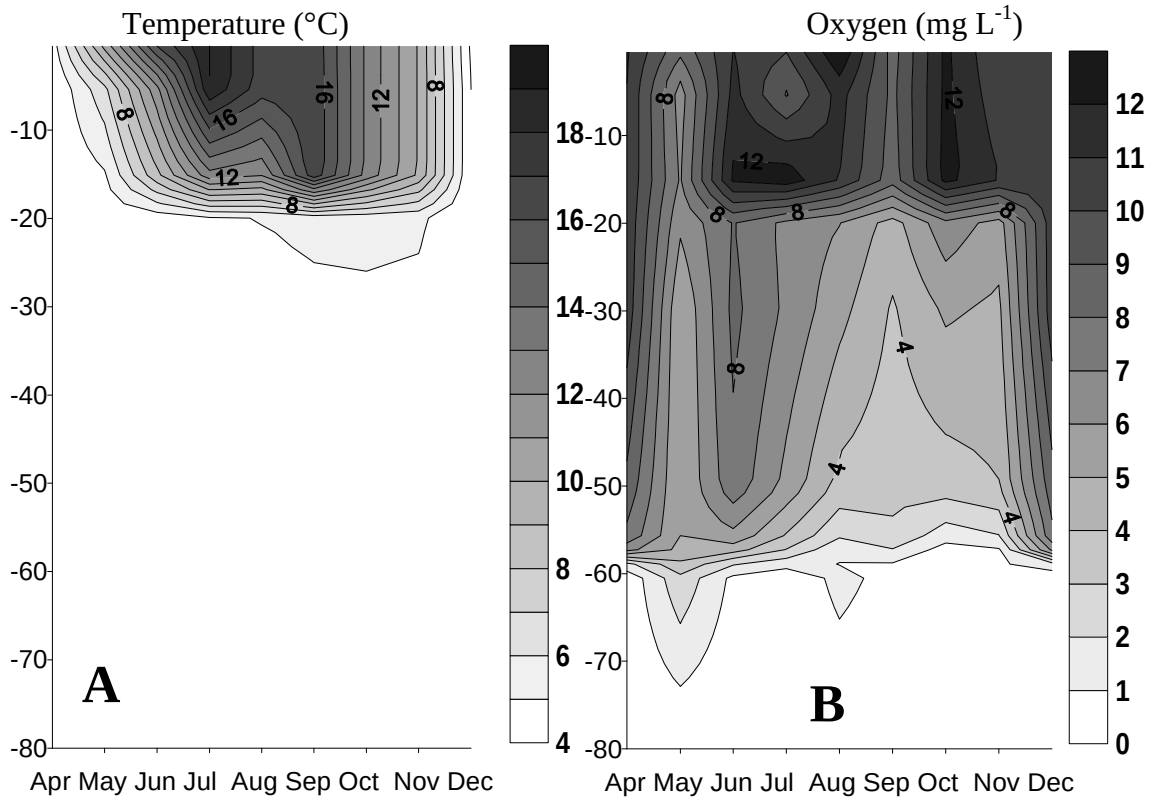


Figure 1. Seasonal and vertical variations of temperature (A) and oxygen concentrations (B) in Lake Pavin, April – December 2005.

Table 1. Mean concentrations (coefficients of variation) of planktonic communities for each layer and season sampled in Lake Pavin. Epi., Epilimnion; Meta., Metalimnion; Hypo., Hypolimnion; Oxy., Oxycline.

		Chl. (mgm^{-3})	aBacteria ($10^6 mL^{-1}$)	VLP ($10^7 mL^{-1}$)	Picocya. ($10^3 mL^{-1}$)	Picoeu. ($10^3 mL^{-1}$)	ANF ($10^2 mL^{-1}$)	HNF ($10^3 mL^{-1}$)
Layer	**Epi.**	2.2 (77)	6.1 (34)	2.7 (32)	20.3 (114)	3.1 (155)	5.1 (64)	0.9 (44)
	Meta.	3.4 (108)	8.3 (73)	3.0 (39)	22.4 (125)	2.3 (142)	4.6 (58)	1.0 (39)
	Hypo.	3.5 (59)	4.4 (36)	1.8 (19)	8.9 (84)	2.5 (80)	1.5 (94)	0.7 (78)
	Oxy.	1.3 (123)	3.9 (33)	1.6 (23)	ND	ND	0.1 (112)	0.5 (71)
Season	**Spring**	4.7 (69)	5.2 (17)	1.7 (15)	2.2 (111)	6.3 (55)	1.4 (93)	0.9 (51)
	Summer	1.5 (54)	8.2 (65)	2.7 (45)	30.0 (97)	1.2 (139)	3.0 (94)	0.8 (53)
	Autumn	1.6 (66)	3.6 (37)	2.4 (36)	19.2 (73)	0.4 (87)	4.0 (95)	0.6 (75)

Chl a, Chlorophyll *a*; VLP, Virus-Like-Particles; Picocya., Picocyanobacteria; Picoeu., Picoeukaryotes; ANF, autotrophic nanoflagellates; HNF, heterotrophic nanoflagellates; ND, not determined.

Viral abundance (VA) and microbial community composition. Complete detailed description of seasonal VA and microbial community composition variations through the whole water column of the lake is presented elsewhere **(Colombet *et al. submitted*)**. Briefly, VA fluctuated from 0.96 to 5.09 x 10^7 virus like particles (VLPs) mL^{-1} and peaked in the photic depths (epi- and metalimnion) in summer and autumn (Table 1). Heterotrophic bacteria and picocyanobacteria also peaked in the surface photic waters in summer, while auotrophic picoeukaryotes peaked in spring in the epilimnion. Similarly, nanoflagellates peaked in the photic depths, in summer/autumn for autotrophs and in spring/summer for heterotrophs (Table 1). The species *Crysidalis* and/or *Chloromonas* dominated ANF communities, whereas undetermined species and *Monas-like* cells dominated HNF communities (Table 2). The Structure of microbial communities was submitted to seasonal variations, with different groups of species presenting abundance peaks in spring (*Chloromonas, Ochromonas, Monas like, Kathablepharidae*), in summer (*Crysidalis*) and in autumn (*Crysidalis, Choanoflagellate*). The abundances of other groups (*Bodonidae*, undetermined HNF) remained relatively constant during the study period. The ratio between heterotrophic and autotrophic microbial communities, in terms of abundance, decreased with seasons, from 336 in spring, to 169 in summer, and 156 in autumn.

Table 2. Relative abundances (% of total) of different autotrophic and heterotrophic nanoflagellated species for each layer and season sampled in Lake Pavin. Epi., Epilimnion; Meta., Metalimnion; Hypo., Hypolimnion; Oxy., Oxycline.

		% of Autotrophic nanoflagellates				% of Heterotrophic nanoflagellates				
		Cry	**Rh**	**Ch**	**Oc**	**Ka**	**Mo**	**Co**	**Bo**	**In**
Layer	**Epi.**	67	11	15	7	5	19	3	2	71
	Meta.	70	10	16	5	4	18	3	2	73
	Hypo.	47	18	24	10	7	19	3	1	71
	Oxy.	54	12	20	14	4	23	2	1	71
Season	**Spring**	0	27	46	27	7	30	0	1	61
	Summer	63	9	22	6	3	15	2	2	78
	Autumn	89	8	4	0	5	9	7	1	78

Cry, *Chrysidalis*; Rh, *Rhodomonas*; Ch, *Chloromonas*; Oc, *Ochromonas*; Ka, *Kathablepharidae*; Mo, *Monas*-like cells; Co, Choanoflagellates; Bo, *Bodonidae*; In, undetermined cells.

Viral activities and protistan grazing potential. The frequency of infected cells (FIC) fluctuated from 0.0 to 22.3 % and was lower in the oxycline than in the mixolimnic layers, with no marked seasonal pattern (Table 3). However, sporadic peaks were observed in April in photic area and in October in all layers. The frequency of lysogenically infected cells (FLC) fluctuated from 0.0 to 2.4 %, with no particular vertical or seasonal trend (Table 3), at the exception of a sporadic peak recorded in May in the metalimnion. Estimated flagellate grazing (FG) fluctuated from 0.08 to 6.65 x 10^7 bacteria $L^{-1}h^{-1}$ and was significantly (ANOVA, $p < 0.05$) influenced by depth with highest values recorded in the photic zone, and by seasons with highest values occurring during summer (Table 3), particularly in August.

Table 3. Importance of lysis, lysogeny and potential grazing activities for each layer and season sampled in Lake Pavin. Epi., Epilimnion; Meta., Metalimnion; Hypo., Hypolimnion; Oxy., Oxycline. Coefficients of variation are shown in brackets.

		FIC (%)	**FLC (%)**	**Grazing (10^6 bacteria $liter^{-1}h^{-1}$)**
Layer	**Epi.**	8.9 (56)	0.4 (114)	16.5 (55)
	Meta.	7.5 (47)	0.6 (134)	22.4 (80)
	Hypo.	7.2 (52)	0.5 (93)	9.2 (110)
	Oxy.	3.1 (79)	0.5 (82)	5.5 (89)
Season	**Spring**	6.2 (93)	0.6 (120)	15.1 (61)
	Summer	6.0 (77)	0.3 (96)	19.0 (92)
	Autumn	7.7 (85)	0.6 (90)	6.2 (108)

FIC, frequency of lytically infected cells; FLC, frequency of lysogenically infected cells.

Morphological diversity of viruses.

The frequency of distribution of caspid size. Mean (± standard deviation) capsid size for all samples was at 49 ± 4 nm, and fluctuated weakly from 47 ± 4 nm in the metalimnion to 50 ± 5 nm in the oxycline. No clear vertical trend was recorded (Table 4). Seasonal mean values were relatively lower in spring and summer than in autumn (Table 4). Particles with capsid size ranging from 30 to 60 nm largely dominated the viral communities, representing from 55 to 78 % of the total viral abundance (Fig. 2). Viruses in the capsid size classes of < 30 nm and 60-80 nm were in equal proportion (from 6 to 26 % and from 6 to 25 %, respectively) (Fig. 2). Viruses with large capsid size were less abundant, ranging from 1 to 11 % and from 0 to 3 % of the total abundance, for 80-100 nm and > 100 nm size classes, respectively (Fig. 2). According to depths, only viruses with capsid size < 60 nm presented significant vertical changes (ANOVA, $P < 0.001$), with lowest values in the oxycline (Fig. 2). According to seasons, only viruses > 80 nm presented significant changes (ANOVA, $P < 0.007$) with lowest values in summer for the size classe > 100 nm, and in spring and summer for the size class 80-100 nm (Fig. 2).

Table 4. Mean (coefficients of variation) genome and capsid sizes for each layer and season sampled in Lake Pavin. Epi., Epilimnion; Meta., Metalimnion; Hypo., Hypolimnion; Oxy., Oxycline.

		Mean genome size (kb)	Mean capsid size (nm)
Layer	**Epi.**	55 (16)	49 (6)
	Meta.	52 (10)	47 (8)
	Hypo.	50 (10)	48 (5)
	Oxy.	48 (12)	50 (10)
Season	**Spring**	50 (8)	48 (9)
	Summer	55 (16)	48 (4)
	Autumn	49 (7)	50 (5)

Morphological families. Based on general morphology, viral communities were dominated by the uncertain family of untailed phages (from 54 to 68 % of the total abundance), followed by *Siphoviridae* (27 - 40 %), *Myoviridae* (2 - 6 %), and *Podoviridae* (0.2 - 2.4 %) (Fig. 2). *Myoviridae, Siphoviridae* and untailed viruses presented significant variations with depths (ANOVA, $P < 0.001$), with values lowest in the hypolimnion for *Myoviridae*, in the epilimnion for *Siphoviridae*, and in the hypolimion and oxycline for untailed viruses (Fig. 2). No significant seasonal pattern was recorded (ANOVA, $P > 0.05$),

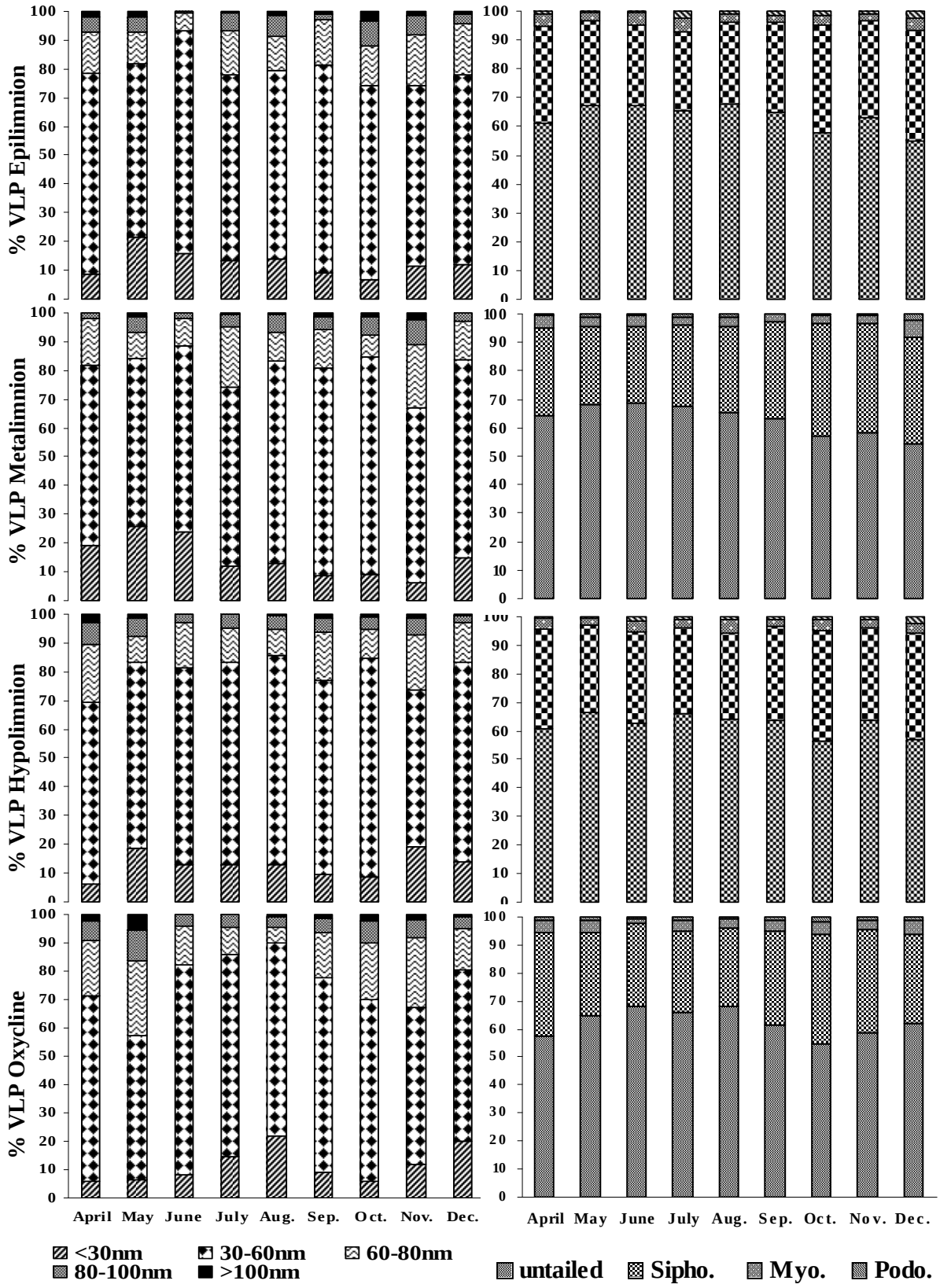


Figure 2. Seasonal and vertical variations of viral morphological diversity based on capsid size classes and on general morphology, in Lake Pavin, April – December 2005.

although the relative abundance of *Myoviridae* and *Siphoviridae* were highest in autumn, contrasting with untailed viruses (Fig. 2).

Viral genomic composition

*Qualitative analysis***.** Analysis of viral genomic composition by pulsed field gel electrophoresis revealed the presence of 34 different viral genotypes in the sampled depths and during the entire study period (Fig. 3). Three sampling months were excluded from qualitative analysis due to insufficient discrimination between bands, i.e. the month of June in the hypolimnion, and the months of August and October in the metalimnion. On average (± standard deviation), 12 ± 3 different bands per lane were recorded. The maximal mean number of bands per lane was recorded in summer (14 ± 3 bands), with highest mean values in August (16 bands) and in September (16 bands). The lowest values were observed in autumn (12 ± 1 bands) and in spring (10 ± 2 bands) (Fig. 3). Mean number of bands per lane increased weakly from oxycline (11 ± 2 bands) to the epilimnion (13 ± 3 bands), with intermediate values in the hypolimnion (12 ± 3 bands) and in the metalimnion (12 ± 2 bands) (Fig. 3).

The analysis of the distribution of genome size classes based on presence/absence of bands showed some similarities and differences according to the sampling time or depths. Five dominant genome size classes (25-40, 55-65, 80-100, 155-180 and 250-292 kb) were common in all sampling depths and dates (Fig. 3). Differences in fingerprinting were located in the intermediate size classes. Appearance of new bands occurred in summer and autumn, around 20 kb, in the 40-50 kb size classes and around 75 kb (Fig. 3). Interestingly, we have also observed appearance of new bands in 100-115 kb and 130-160 kb regions in summer-autumn and in summer, respectively, particularly in the epi- and metalimnion (Fig. 3). Finally we have observed an heterogeneous distribution of bands in the 190-230 kb region (Fig. 3).

Quantitative analysis. Mean (± standard deviation) genome size for all samples was at 51 ± 7 kb. Mean genome size decreased clearly with depth, from 55 ± 9 kb in the epilimnion to 52 ± 5, 50 ± 5, and 48 ± 6 kb in the metalimnion, hypolimnion, and oxycline, respectively (Table 4). Mean genome size was higher in summer (55 ± 9 kb) than in spring (50 ± 6 kb) and autumn (49 ± 3 kb) (Table 4). Viruses with a genome size ranging from 15 to 30 kb and from 30 to 45 kb were dominant, averaging 32 % and 29 % of total abundance, respectively (Fig. 3). These high proportions were due to the bulk of viruses at approximately 33 kb for all sampling dates (Fig. 3). Viruses with genome sizes < 15 kb represented the smallest

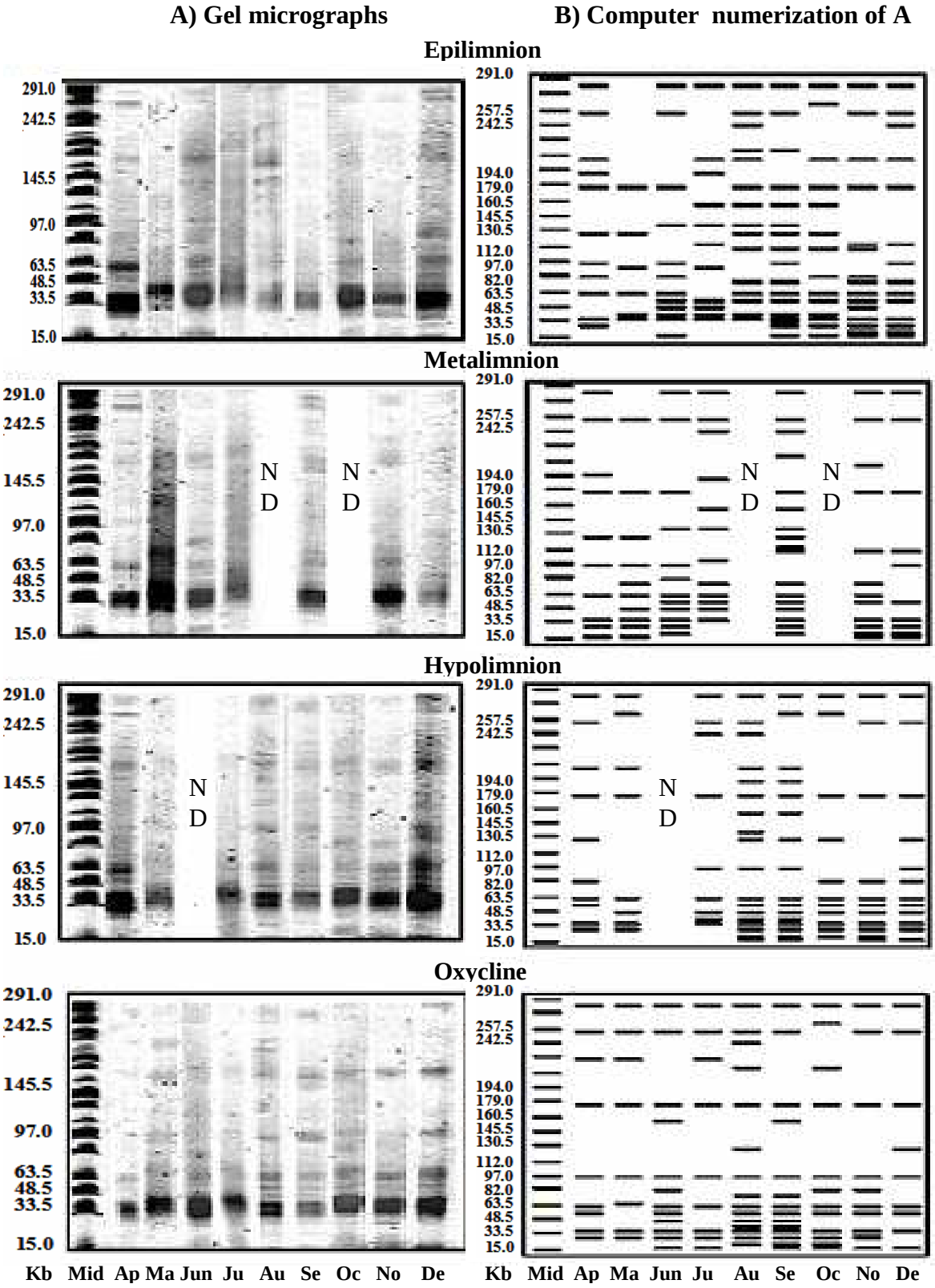


Figure 3. Virioplankton PFGE fingerprints (A) and the corresponding computer-generated representations of presence/absence of bands (B) for samples obtained from April to December 2005 in the Epilimnion, Metalimnion, Hypolimnion and Oxycline of Lake Pavin. Mid = MidRange PFG Markers, Ap = April, Ma = May, Jun = June, Jul = July, Au = August, Se = September, Oc = October, No = November, De = December, ND = not determined.

Table 4. Mean (coefficients of variation) genome and capsid sizes for each layer and season sampled in Lake Pavin. Epi., Epilimnion; Meta., Metalimnion; Hypo., Hypolimnion; Oxy., Oxycline.

		Mean genome size (kb)	Mean capsid size (nm)
Layer	**Epi.**	55 (16)	49 (6)
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Season	**Spring**	50 (8)	48 (9)
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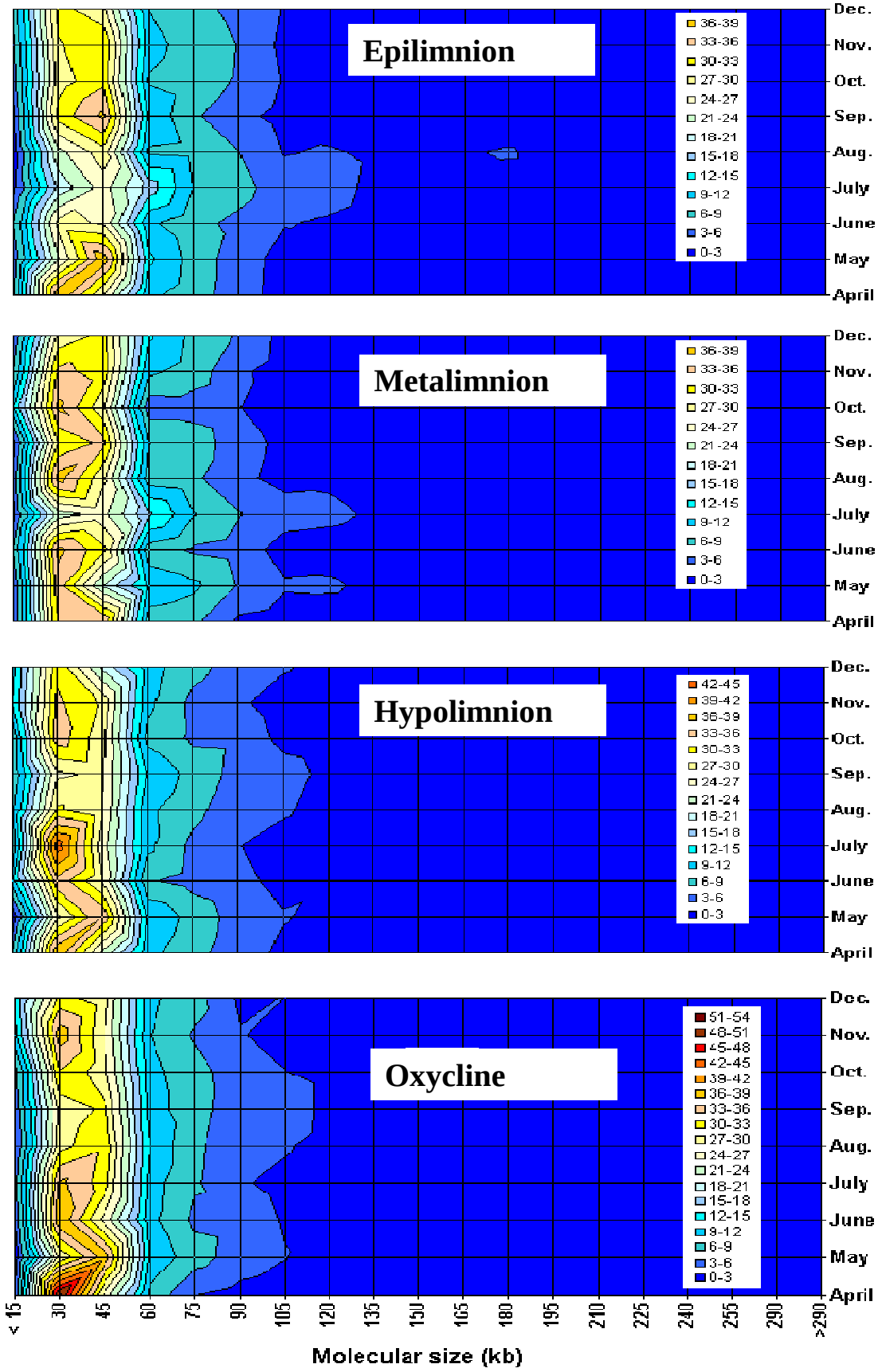


Figure 4. Densitometric analysis of gels shown in Fig. 3 converted to relative percentage of viruses in arbitrarily defined size regions (see methods for details).

proportion of viral abundance (5 %), while those with genome sizes ranging from 45 to 90 kb and > 90 kb represented an intermediate fraction (21 and 12 %, respectively) (Fig. 4). Interestingly, the proportion of viruses with genome sizes > 60 kb decreased with depth and peaked in summer, whereas that of viruses with genome sizes < 60 kb increased with depth, and peaked in spring and autumn (Fig. 4).

Correlation analysis. Correlation coefficients ($p < 0.01$) between the different tracers of viral diversity and the other variables measured are given in Table 5. Viruses presenting large capsid and large genome sizes were mainly correlated with the abundances of eukaryotes. Autotrophic picoeukaryotes and *Chrysidalis* species were correlated with about all genome and capsid size classes, but indeed were the sole microorganisms which were correlated with viruses in the largest genome (> 105 kb) and capsid (> 80 nm) size classes. In contrast, the abundances of prokaryotes, i.e. heterotrophic procaryotes and picocyanobacteria, were about exclusively correlated with small size viruses, both in terms of genome (15-105 kb) and capsid (30-80 nm) sizes. Regarding the morphological families of viruses, the dominant group of 'untailed viruses' was correlated with the abundances of all picoplankton categories, and with the *Chrysidalis* species. *Podoviridae* were correlated only with these species, while *Myoviridae* and *Siphoviridae* were correlated with the autotrophic groups of picocyanobacteria, picoeukaryotes and *Chrysidalis*. All correlation coefficients with picoeukaryotes were negative, in contrast to the other coefficients. Finally, very few correlations were noted between viruses and the biological activity variables (i.e. FIC, FLC, grazing potential), while temperature appeared as a significant forcing factor for the dominant < 60 nm capsid size classes of viruses, and for almost all the morphological and genome size categories distinguished, due to peaks in summer/autumn (Tables 4, 5).

Discussion

Methodological considerations. Critical assumptions for the use of pulsed field gel electrophoresis (PFGE) to assess viral diversity have previously been made in various papers **(Wommack *et al.* 1999b, Steward *et al.* 2000, Riemann and Middelboe 2002, Weinbauer 2004, Filippini and Middelboe 2007)**. Among these, it is important to keep in mind that only quantitatively dominant viral genome size may be analysed, because of the sensitivity of the method ($\geq 10^6$ viruses per band, **Wommack *et al.* 1999b**), that makes that some information about the total number of viral genotypes in a given sample can be biased. In addition, a close

Table 5. Significant Pearson correlation coefficients ($p < 0.01$) between viral descriptors and environmental parameters.

		Abundances				Activities		
Viral diversity	**Temp.**	**Bacteria**	**Picocya.**	**Picoeu.**	**Cry.**	**FIC**	**Grazing**	**FLC**
Capsid size								
>100nm								
80-100nm				**-0,67**	**0,52**			
60-80nm			**0,647**	**-0,73**	**0,542**			*-0,5*
30-60nm	**0,759**	**0,558**	**0,627**	**-0,75**	**0,713**			
<30nm	**0,527**				0,355			
Morphological families								
Myo.	*0,489*		*0,55*	*-0,5*	**0,552**			
Sipho.	**0,603**		**0,629**	**-0,82**	**0,719**	*0,45*		
Podo.					**0,595**			
Untailed	**0,753**	**0,629**	**0,582**	**-0,75**	**0,631**		*0,431*	
Genome size								
< 15kb					0,403			
15-30kb	*0,453*	0,372	**0,775**	**-0,67**	**0,61**			
30-45kb	**0,628**	*0,44*	*0,57*	**-0,7**	**0,571**			
45-60kb	**0,688**	**0,55**	*0,6*	**-0,72**	**0,654**			
60-75kb	**0,655**	*0,48*	*0,59*	**-0,75**	**0,64**			
75-90kb	**0,692**		*0,53*	**-0,71**	**0,631**			
90-105kb	**0,622**		*0,52*	**-0,64**	**0,568**			
105-120kb	**0,699**			**-0,67**	**0,586**			
120-135kb	**0,663**			*-0,53*	*0,48*	*0,45*		
135-150kb	**0,672**	**0,571**			*0,48*		*0,466*	
150-165kb	**0,606**			*-0,55*				
165-180kb	**0,655**			**-0,61**	**0,593**			
180-195kb	**0,631**			*-0,6*	**0,642**			
195-210kb	**0,57**			*-0,57*	*0,51*			
210-225kb	**0,591**			*-0,56*	**0,567**			
225-240kb	**0,603**				*0,52*			
240-255kb	**0,667**	**0,557**		*-0,52*	*0,53*			
255-290kb	*0,482*			*-0,57*	*0,51*	**0,53**		
>290kb	**0,703**			**-0,61**	*0,51*			

[a] In bold $p<0.001$, in italic $p<0.01$; Temp., temperature; Picocya., Picocyanobacteria; Picoeu., Picoeukaryotes; Cry, *Chrysidalis*; FIC, frequency of lytically infected cells; FLC, frequency of lysogenically infected cells

succession of high density-bands, that differed only by few kb, results sometimes on a smear making difficult the distinction of individual bands in a given region (in 33 kb region of our gels for example). Instead of providing a realistic estimation of total viral diversity, based on the distribution of dominant genome sizes, PFGE approach provides an efficient, rapid and easy way to detect major differences in viral communities between environments or time-points, and gives an estimation of minimum number of different viral genotypes **(Filippini and Middelboe 2007)**.

These estimations were, in this study, completed by estimation of viral phenotypic diversity by transmission electron microscopy (TEM). The viral concentration method used allowed to obtain purified viral concentrates, avoiding major interferences from organic or inorganic particles during PFGE migration or TEM observations **(Colombet *et al.* 2007)**. However, during preparation of the grids for ultracentrifugation, the tails of phages may be dislocated from the capsid, resulting in an underestimation of tailed phages. In addition, positive staining may not be an optimum procedure for detecting fine structures such as the small tailed *Podoviridae* **(Proctor 1997)**. Finally, in addition to the above inconvenients of PFGE and TEM methods, preparation of viral concentrates can introduce a bias by removing large sized viruses during prefiltration (onto 0.2 µm) of natural samples **(Paul *et al.* 1991, Auguet *et al.* 2006)**.

Viral diversity and relationships with microbial communities

General morphology. According to general morphological traits, virioplankton in Lake Pavin appeared to be dominated by untailed viruses (from 54 to 68 %), followed by *Siphoviridae* (from 27 to 40 %). The proportion of untailed viruses fell in the range of those previously reported in freshwater lakes **(Colombet *et al.* 2006, Liu *et al.* 2006)**, although higher proportion has been reported by **Auguet *et al.* (2006)** in estuarine waters. However, because (i) 96 % of all known bacteriophages are tailed, and (ii) exhaustive documentation of virioplankton diversity in aquatic environments has shown that the majority of virioplankton are tailed **(Ackermann 2001)**, it is surprising to have such high proportion of untailed viruses during our study. A possible explanation is the disruption of tails during ultracentrifugation of samples prior to TEM observations. The dominance of *Siphoviridae* among tailed viruses (88 % of total tailed viruses) was in agreement with the study by **Auguet *et al.* (2006)** but was not found in other studies **(Wommack *et al.* 1992, Ackermann 2003)**.

Temporal variations in the abundances of untailed viruses and of *Sipho-*, *Podo-*, and *Myoviridae* were weak, suggesting a seasonal stability in viral community composition.

However the highest proportions of *Myoviridae* and *Siphoviridae* were recorded in autumn and these morphotypes were correlated with picocyanobacteria and whith *Chrysidalis* species. This is in agreement with the data from the literature showing that almost all viruses infecting phytoplankton, primarily cyanobacteria, were *Myoviridae* or *Siphoviridae* **(Wilson *et al.* 1993, Suttle and Chan 1993, Waterbury and Valois 1993, Lu *et al.* 2001, Mann 2003, Yoshida *et al.* 2006)**. Other interesting correlation was the one between untailed viruses and bacteria, which apparently contrasts with the fact that bacteriophages generally are considered to be tailed particles. A significant tail loss during sample preparation could explain this observation, suggesting that the tails of bacteriophages could be more fragile than those of algal viruses. Hence, the majority of untailed viruses in our samples could be bacteriophages. Spatial variations showed contradictory results with lowest proportion of *Myo*- and *Siphoviridae* recorded in the surface waters (epi-, metalimnion), and the lowest proportion of untailed viruses in deep layers (hypolimnion, oxycline) were bacteria dominate. Direct or undirect (through infected cells) sedimentation can be evoked, with *Sipho*- and *Myoviridae* probably presenting a higher sedimentation rate compared to the untailed viruses.

Capsid size. Based on the assumptions (i) that typical pelagic phages in temperate aquatic systems lack pleiomorphism, and (ii) that differences in the capsid size reflects difference in viral community composition, results obtained during the present study showed that virioplankton was dominated (55 to 78 % of total abundance) by viruses with capsid size diameter comprised between 30 and 60 nm. Dominance of VLPs with capsid size in this range have been previously recorded in marine and freshwater environments **(Hennes and Simon 1995, Alonso *et al.* 2001, Stopar *et al.* 2004, Weinbauer 2004, Auguet *et al.* 2006, Liu *et al.* 2006)**. One exception is results reported by **Mathias *et al.* (1995)** in the Kühwörte backwater system of the Danube River where viruses with capsid size diameter from 60 to 90 nm dominated. Our mean capsid diameter was at 49 ± 4 nm and fell in the range (51 ± 18) of results reported by **Auguet *et al.* (2006)**, but was slightly lower than those (62-68 nm) reported by **Demuth *et al.* (1993)** in freshwater environments. In various environments, some studies have shown that the diversity of viruses estimated based on the distribution frequency of the capsid size can vary with time and space, whereas others have reported no significant variations **(see Weinbauer 2004)**. Herein, low variability (47 to 50 nm) of mean capsid size suggested spatio-temporal stability in viral community.

Few interesting variations were recorded, e.g. those in viruses with capsid size > 80 nm which presented peak abundance in autumn. **Brussaard (2004)** and **Dunigan *et al.* (2006)**

have reported that algal virus presented higher capsid diameter (> 100 nm) than bacteriophages. So it is no surprising to find high proportion of virus > 80 nm in autumn, concomitant or following peak in phytoplanktonic species and characterized by the lowest ratio between total abundances of heterotrophic and photoautotrophic species. This was reinforced by the positive correlation between viruses with capsid size > 80 nm and *Chrysidalis* species. Thus we can hypothesize than viruses with capsid sizes > 80 nm could be algal viruses. In contrast, bacteria abundance was exclusively correlated with small capsid size viruses (30-60 nm) and picocyanobacteria with both small and intermediate capsid size viruses (30-80 nm). These results corroborate the findings by **Weinbauer and Peduzzi (1994)** and by **Hennes and Suttle (1995)** on the predominance of viruses with small capsid size between 30 and 60 nm within bacterioplankton, while picocyanobaceria which are larger in size than heterotrophic bacteria harbour larger size (40-90 nm) viruses **(cf. Lu *et al.* 2001, Yoshida *et al.* 2006)**. Spatial variations showed contradictory results, with lowest proportion of small viruses (30-60 nm) recorded in the oxycline, whereas this environment appeared to be dominated by heterotrophic bacteria. One again, this could be explained by the sedimentation of large viruses or of infected hosts from the surface waters **(Danovaro *et al.*, 2001)**.

Genome size. Results recorded showed that the viral genomic composition (VGC) was dominated by five size classes (25-40, 55-65, 80-100, 155-180 and 250-292 kb) common to all sampled depths, the two genome size classes in the range 25 - 65 kb being the highly dominant populations. Distribution in several classes of VGC in freshwater viral communities was also demonstrated by **Auguet et al. (2006)** and by **Filippini and Middelboe (2007)**. Auguet and coauthors reported five dominant molecular size groups (12-17, 24-29, 30-48, 50-70, > 90 kb) while Filippini and Middelboe reported two dominant size classes (30-41, 53-64 kb), with the majority of viruses exhibiting genome size < 65 kb in both cases. Our results suggest that the five dominant classes in Lake Pavin were permanent members of the mixolimnic layer of the lake. In this lake, we have found a total of 34 different genomic bands (i.e. all sampling depths and dates combined), ranging in size from 15 up to 292 kb. In marine waters, between 5 and 35 different bands can be distinguished **(Weinbauer, 2004)**. Herein, we have recorded between 6 and 18 bands per lane (mean, 12 bands per lane). **Filippini *et al.* (2007)** reported between 10 and 20 clearly visible bands per lane in marine coastal waters, estuarine waters and in freshwater lakes of Denmark, with highest values in the eutrophic

Lake Frederiksborg Slottsø. **Auguet *et al.* (2006)** reported only 4 ± 3 bands per lane on average, along a salinity gradient of the Charente Estuary, France.

Spatio-temporal variability in VGC was obvious from the appearance of new bands in summer and/or in autumn in the region 15-60 kb for all sampled depths, and in the regions 100-115 kb and 130-160 kb in the epi- and metalimnion. With the assumption that bands < 60 kb represented bacteriophages **(Ackermann *et al.*, 1992)**, occurrence of new bands in 15-60 kb region could reflect seasonal changes in bacterial community composition (BCC). **Riemann and Middelboe (2002)** in a study of VGC and BCC at a seasonal scale, have failed to show significant covariations of these two parameters, probably due to the fact that they found high spatio-temporal stability in both VGC and BCC, which is not the case in Lake Pavin for BCC **(Boucher *et al.*, 2006)**. More interesting is the appearance of new bands in 100-115 kb and 130-160 kb regions in summer-autumn and in summer respectively, concomitant to peaks in the abundances of autotrophic species, primarily picocyanobacteria and *Chrysidalis* species. It appears that variations in VGC could be linked to those in microbial communities, as additionally supported from densitometric analyses of PFGE bands. Indeed, the highest mean genome sizes were recorded during summer in the surface epi- (55 ± 9 kb) and metalimnion (52 ± 5 kb) due to peaks in viruses with genome sizes > 60 kb, which were concomitant to peaks in the abundances of autotrophic species. Moreover, about all genome size classes (up to > 290 kb) were correlated with *Chrysidalis* species, but only the sizes classes within 15-105 kb and 15-75kb genomic regions were correlated with picocyanobacteria and with heterotrophic bacteria, respectively.

This indicates that not only the phenotype size classes but also the genome size classes within viral communities increased with the increasing size and biological organization of their potential hosts, with viral genome size populations vs potential hosts suggested as follows: > 290 kb vs eukaryotes, 15-105 kb vs picocyanobacteria, and 15-75 kb vs heterotrophic procaryotes. Similar indication was suggested by **Sandaa and Larsen (2006)** in marine waters, agreeing well with the data from the literature showing that the viral genome sizes decreased from bacteriophages (< 60 kb, cf. **Ackermann *et al.* 1992**) to cyanophages (40.9 - 252 kb, **Yoshida *et al.* 2006, Pope *et al.* 2007, Xinyao *et al.* 2007**), and to eukaryotic algal viruses such as phycodnaviruses (100 - 560 kb, **Brussaard 2004, Dunigan *et al.* 2006**). The approach used in this study thus provides new and original information on the link between viral diversity assessed based on the genome size and the composition and structure of microbial communities, although a single PFGE band could not be firmly linked to a given host population.

Conclusions

In the present study, we have shown that the main viruses in Lake Pavin have a capsid size diameter comprised between 30 and 60 nm, belonged to the family of *Siphoviridae* or were untailed, with a genome size comprised in two major size classes: 25-40 and 55-65 kb. Although the composition of viral community based on these criteria show tendency to be relatively stable, interesting spatio-temporal variations and empirical correlations were recorded. The composition of viral community was more strongly linked to the microbial community structure than to activities (i.e. lysis, lysogeny, potential grazing) or to the chemical environment (i.e oxygen, chlorophyll). Empirical relationships suggested the existence of three different viral communities based on the sizes of phenotypes and genotypes: (i) viruses with large capsid (> 80 nm) and genome sizes (up to > 290 kb) were related to eukaryotes, (ii) viruses with intermediate capsid (30-80 nm) and genome sizes (< 105 kb) appeared to be linked to picocyanobacteria, and (iii) the smallest viruses with capsid size < 60 nm and genome size < 75 kb probably were specific to heterotrophic procaryotes. The dynamics of *Myoviridae* and *Siphoviridae* showed a tendency to be forced by autotrophic microorganisms, whereas the dynamics of untailed viruses were linked to bacteria. Finally, temperature appeared as a strong forcing factor in the dynamics of viral diversity, may be as the result of indirect interactions between temperature and the viral host availability. Overall, the present study supports previous statement **(Colombet et al. 2006)** that the host availability is crucial to viral diversity, diversification, seasonal abundances, and activities, than do the physico-chemical environment.

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CHAPITRE II - ETUDES DES GRADIENTS LIES A LA PROFONDEUR DES ABONDANCES ET ACTIVITES VIRALES

Partie 1 : Etude préliminaire ponctuelle

Depth-related gradients of viral activity in Lake Pavin

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Abstract

High resolution vertical sampling of viral and prokaryotic parameters in a deep volcanic lake shows that in the absence of thermal stratification but within light, oxygen and chlorophyll gradients, host availability empirically is prevalent over the physical and chemical environments and favors lytic over lysogenic "viral life cycles".

Introduction

Viral activity is crucial to microbial mortality, diversity, potential gene transfer, and lysogenic conversion in aquatic systems **(recent reviews in 18, 19)**. Viruses can impact ecological processes and nutrient cycles **(23)** but this, ultimately, depends on whether the virus is lytic or temperate. Studies examining large data sets have revealed that prokaryotes form the major host reservoir for viruses in pelagic systems **(cf. 2)**. Protistan grazing and viral lysis are major sources of bacterial mortality in these systems. In general, bacterivory dominates but losses of bacteria from viral lysis at times are comparable to those from protistan bacterivory **(cf. 13)**. In conditions such as anoxia, bacteriolysis generally is much higher than bacterivory based on evidence from theoretical **(12)** and empirical field

investigations **(2)**. In contrast to bacterivory and lytic viral infection which have been examined in a variety of marine systems, only two studies have investigated the two viral 'life cycles' simultaneously but not together with bacterivory **(11, 21)**. In freshwaters, only one study has examined temperate phages in the surface layer of Lake Superior **(17)**.

Pelagic viral ecology thus lacks studies where potential prophage induction, lytic viral infection, and bacterivory are investigated simultaneously. In addition, most of the studies on these processes have ignored the well known contribution of depth-related gradients as a major forcing factor in aquatic systems. Such contribution is needed to fully assess the potential roles of viruses for microbial food-web processes, and to address hypotheses that (i) in suboxic and anoxic waters, the importance of lytic viral infection increases relative to bacterivory **(2, 13, 20)**, and (ii) lysogeny increases as lytic viruses decreases with their host densities and production **(21)**. Herein, we address these questions by examining the vertical distribution of the frequencies of lytically and of lysogenically infected cells together with the potential bacterivory from heterotrophic nanoflagellates in a deep meromictic lake. Samples were collected in early spring when the isothermal water column showed gradients of light, oxygen and chlorophyll, in order to avoid false interpretations of correlations that often result from the co-variation of two variables with respect to a third factor, usually temperature.

Materials and Methods

Lake Pavin is a deep (Z_{max} = 92 m) meromictic mountain lake located in the French Massif Central (2°56'E, 45°29'N). Samples were taken in triplicates on 20 April 2004 at the reference station (located in the middle and the deepest part of the lake) using a 8-L Van Dorn bottle. A total of 15 depths were sampled in three distinct layers, comprising 7 depths in the mixolimnion (0.5, 5, 15, 20, 30, 40 and 50 m), 5 depths in the oxycline, (56, 57, 57.5, 58 and 59 m), and 3 depths in the anoxic monimolimnion (60, 70, 80 m). Water temperature and dissolved oxygen were measured *in situ* with an oxycal-SL 197 multiparameter probe (WWT, Limonest, France). Secchi depth (Z_s) measurements were used to estimate the euphotic depth (Z_{eu}) according to the relationship $Z_{eu} = 2.42\ Z_s$ **(22)**. Chlorophyll *a* concentrations (Chl) were determined spectrophotometrically following extraction in 90% acetone **(16)**. For viral and bacterial abundances, subsamples were fixed with 0.02 µm filtered buffered alkaline formalin (final concentration 2% v/v) immediately after sampling, and filtered (<15 kPa vacuum) through 0.02 µm pore size Anodisc filters (Whatman, Maidstone, UK). Counts were done under a Leica DC 300F epifluorescence microscope (Episcope) following staining with

SYBR Green I fluorochrome (Molecular Probes Europe, Leiden, Netherlands) as described by Noble and Fuhrman **(10)**. When not analysed immediately, slides were stored at -20°C until counting.

Bacterial production was determined by the incorporation of ^{3}H-leucine (final concentration 40 nm, specific activity = 71 Ci mmol^{-1}, Amersham Biosciences, UK) into bacterial biomass using the microcentrifuge method **(9)**. Incubation time (30 minutes *in situ* in the dark) was fixed from preliminary experiments conducted on 25 March 2004, and protein precipitation in controls (i.e. 5% TCA-killed) and fixed-assays was aided by adding NaCl (final concentration 3.5 % v/v) at 18°C for 30 min. After centrifuge-washings, microbial pellets were dissolved in 0.2 ml 1.2 N NaOH at 80°C for 20 min and scintillation cocktail (1 ml, Ready Safe, Beckman Coulter) added for radioactivity counting with a Beckman LS 6500 liquid scintillation counter. For suboxic and anoxic samples, incubations were done in 5 ml sterile serum bottles sealed with rubber and aluminium crimp caps and flushed with N_2. Leucine incorporation was converted into the number of cells produced by using conversion factors (0.27 x 10^{18} cells mol^{-1} for oxic waters and 0.25 x 10^{18} cells mol^{-1} for suboxic and anoxic waters) determined during preliminary experiments conducted on 25 March 2004.

For viral lytic infection, the frequency of infected cells (FIC) and the viral-induced bacterial mortality were calculated from the frequency of visibly infected cells (FVIC) obtained from observations under a JEOL 1200EX transmission electron microscope, following ultracentrifugation and uranyl acetate staining. The procedure is detailed elsewhere **(2, 13)**. For each sample, mean burst size was estimated from the number of viruses in those infected cells which were filled with phages. In addition, free viruses were examined for the relative abundances of 3 arbitrary divided size classes based on the capsid diameter: < 60, 60-100 and > 100 nm. Viral lytic production was estimated by multiplying bacterial cell production by FIC/100 and the burst size. The frequency of lysogenically infected cells (FLC) was determined from the induction of prophages using mitomycin C **(8)**. Mitomycin C was added to samples (final concentration 1 µg ml^{-1}) in 20 ml sterile serum bottles, and untreated samples served as controls. For the suboxic and anoxic samples, serum bottles were sealed with rubber and aluminium crimp caps and flushed with N_2. The incubation time (24 hours *in situ*) was fixed from a 76-hour time series preliminary experiment conducted on 25 March 2004. Subsamples were removed with syringes at t0 and t24 hours and fixed with glutaraldehyde for viral and bacterial counts. FLC was estimated from viral abundances in mitomycin C treated (VAm) and control (VAc) incubations, and bacterial abundance (BAt0) and burst size (BSt0) in original samples : FLC = 100[(VAm – VAc)/(BSt0 x BAt0) (21).

Samples for enumeration of heterotrophic nanoflagellates (HNF) were fixed immediately after sampling with glutaraldehyde (final concentration 1%). Primulin stained HNF collected on 0.8 µm polycarbonate black filters were counted under UV excitation using the LEICA Episcope **(cf. 2, 13)**. Potential grazing rates of heterotrophic nanoflagellates were estimated as the product of bacterial concentration, flagellate concentration and assumed mean clearance rate of 6.1 nl flagellate^{-1}h^{-1} (range: 0.7 to 11.5 nl flagellate^{-1}h^{-1}) reported by Carrias et al. **(5)** during a seasonal study on Lake Pavin. These values fell within the range of values published for freshwater systems **(15)**.

Differences between mixolimnion, oxycline, and monimolimnion were compared with standard analysis of variance (ANOVA). Potential relationships among variables were tested by Pearson correlation analysis. All data were analyzed after logarithmic transformations.

Results and discussion

The vertical environment. During the study, the isothermal water column temperature was at about the point of maximum density (4.3°C). The water transparency (Secchi depth = 6 m) represented half of the annual maximum **(5)** and the euphotic zone extended over one third of the mixolimnion **(22)**. Oxygen concentrations in the water column reflected the annual range, i.e. from anoxia to 12 mgL^{-1} **(2, 5)**. Based on these concentrations, the three layers of the water column were characterized as oxic (O_2 > 8 mgL^{-1}), suboxic (O_2 between 0.1 and 8 mgL^{-1}) and anoxic (O_2 between 0 and 0.1 mgL^{-1}), corresponding to mixolimnion (0-50 m depth), oxycline (50-59 m), and monimolimnion (60-92 m), respectively (Table 1, Fig. 1). Pigment concentrations (2 - 13 µgL^{-1}) also reflected the typical annual range and were related to the development of large size diatoms *Aulacoseira italica* and *Asterionella formosa* which, at the sampling period, typically accounted for 50 to 98% of the total phytoplankton biomass in Lake Pavin. Deep Chl peaks in Lake Pavin, observed at 40 and 57.5 m depths during this study, typically are known as the result of the sedimentation of these diatoms **(1)**.

Viral size classes. Phage polyhedral head sizes in our samples ranged from 10 to 130 nm and tail sizes from 10 to 180 nm. Tailed viruses accounted for 75 % of the total free viruses, indicating that most of the viruses were bacteriophages. We considered this to be conservative because they may have been nontailed bacteriophages, or tailed bacteriophages that lost their tails during ultracentrifugation or with low to noncontrasted tails during the staining procedure. Phages with head sizes ≤ 60 nm clearly dominated the viral community in

Table 1. Mean values (± CV) for samples collected in the three main layers of Lake Pavin. Between-layer comparisons (ANOVA, $p < 0.05$): pairs of * or of ^{x} indicate significant differences between two of the three layers, and $^{+}$ significant differences between the three layers.

	O_2^+	Chl	VA	BS	FIC$^+$	VBM$^+$	FLC	BA	BP	HNF$^+$
Mixolimnion	10.5 (7.8)	10.3 (17.8)*^{x}	6.22 (30.2)^x	13.2 (22.3)^x*	9.8 (58.8)	11.8 (43.4)	12.0 (15.7)^x*	2.7 (26.8)^x	10.6 (25.0)^x*	1.6 (101.5)
Oxycline	4.7 (55.7)	4.5 (60.7)*	7.7 (30.1)*	25.9 (13.6)*	33.9 (36.5)	39.9 (40.7)	2.4 (107.5)*	3.8 (32.5)*	20.0 (35.9)*	0.7 (57.5)
Monimolimnion	0.3 (5.7)	2.7 (34.0)^x	29.9 (33.7)^x*	27.7 (8.4)^x	58.9 (22.7)	82.5 (46)	2.2 (56.4)^x	11.4 (42.8)*^{x}	19.8 (35.9)^x	0.005 (100)

O_2, oxygen concentration (mgL^{-1}); Chl, chlorophyll *a* concentration (μgL^{-1}); VA, viral abundance (10^9 particles L^{-1}); BS, burst size (viruses $bacteria^{-1}$); FIC, frequency of lytically infected cells (% of VA); VBM, viral mediated bacterial mortality (% of BP); FLC, frequency of lysogenically infected cells (% VA); BA, bacterial abundance (10^9 cells L^{-1}); BP, bacterial production (10^7 cells L^{-1} $hour^{-1}$); HNF, abundance of heterotrophic nanoflagellates (10^6 cells L^{-1}).

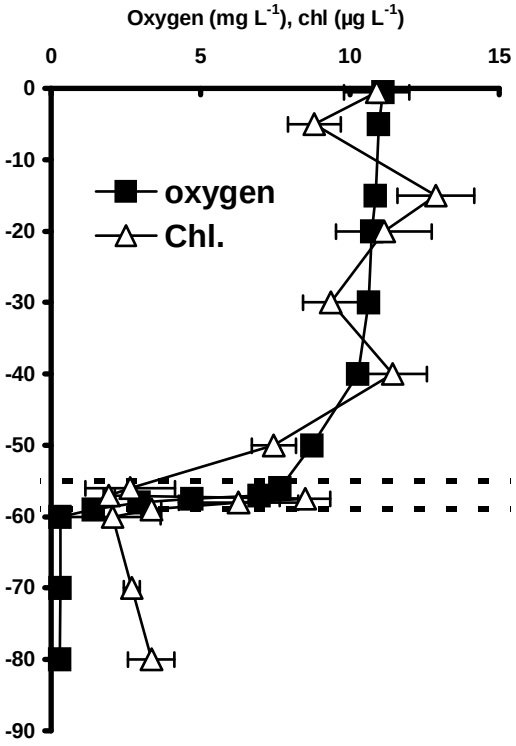


Figure 1. Vertical variations of oxygen and chlorophyll *a* concentrations in Lake Pavin. Error bars represent standard deviations for triplicates. The three layers (mixolimnion, oxycline, and deep monimolimnion) of the sampled water column are shown (same in other figures)

oxygenated waters (Fig. 2), corroborating reports from the world aquatic systems were the size class 30-60 nm is normally dominant within the virioplankton **(19, 24)**. However, this generalisation is weakened herein because the three viral size classes were about equally abundant in hypoxic and anoxic deep waters, suggesting that the composition of viral communities may differ markedly according to vertical gradients in aquatic systems. For example, the presence of eukaryotic (algae, metazooplankton, fish) and allochthonous (from catchment, atmosphere) viruses may be prevalent in the mixolimnion than in the monimolimnion of deep meromictic lakes such as Lake Pavin.

***Standing stocks*.** Viral abundances (range 3 to 37 x 10^6 particles mL^{-1}) peaked in the anoxic monimolimnion and were roughly lower than the seasonal abundances (10 to 50 x 10^6 cells mL^{-1}) reported by **Bettarel et al. (3)** in the thermally stratified mixolimnion of Lake Pavin. Our bacterial abundances (7 – 25 x 10^6 cells mL^{-1}) were up to one order of magnitude higher than in Bettarel and coauthors (2 – 9 x 10^6 cells mL^{-1}), due to the very high bacterial stock in the monimolimnion. Compared to the seasonal values (mean = 7.1 ± 1.9) in the stratified mixolimnion of Lake Pavin **(3)**, our virus-to-bacterial ratios, VBRs (mean = 2.3 ± 0.4), were lower but similarly varied little compared to abundances. In Mediterranean and Baltic Seas, low bacterial stocks in suboxic and anoxic deep waters resulted in particularly high VBRs (up to 50) compared to the surface layer **(21)**. The vertical distributions of viral and bacterial abundances during our study were similar and negatively correlated with oxygen (Table 2, Fig. 2). Numerical values and spatial fluctuations in the upper oxic layer were low, followed by an exponential increase towards suboxic and anoxic layers. Together with the low variability noted in VBR, this confirms previous indications that most of the free viruses in the plankton were bacteriophages, and that there is a close coupling between viral and bacterial concentrations **(2, 3)**. Similar vertical trends were reported for viruses in a small eutrophic lake **(20)** and for bacteria in a meromictic lake **(7)**. Surprisingly high viral densities in mesopelagic and deep marine waters (up to 2000 m depth) were also reported **(cf. 6, 21)**. In Lake Pavin, the possibility that monimolimnic viruses come from the surface layers is unlikely because of the chemocline barrier and the indication from the size class analysis that they were indigenous to the deep waters. It has been suggested that high abundances of free viruses in deep marine waters may be due to an increased survival rate of viruses in low temperature waters because viral infectivity decreased with decreasing temperature **(21)**. Our empirical data do not seem to support this hypothesis, at least for the meromictic Lake Pavin which was isothermal during our study. It is thus likely that biological processes, including

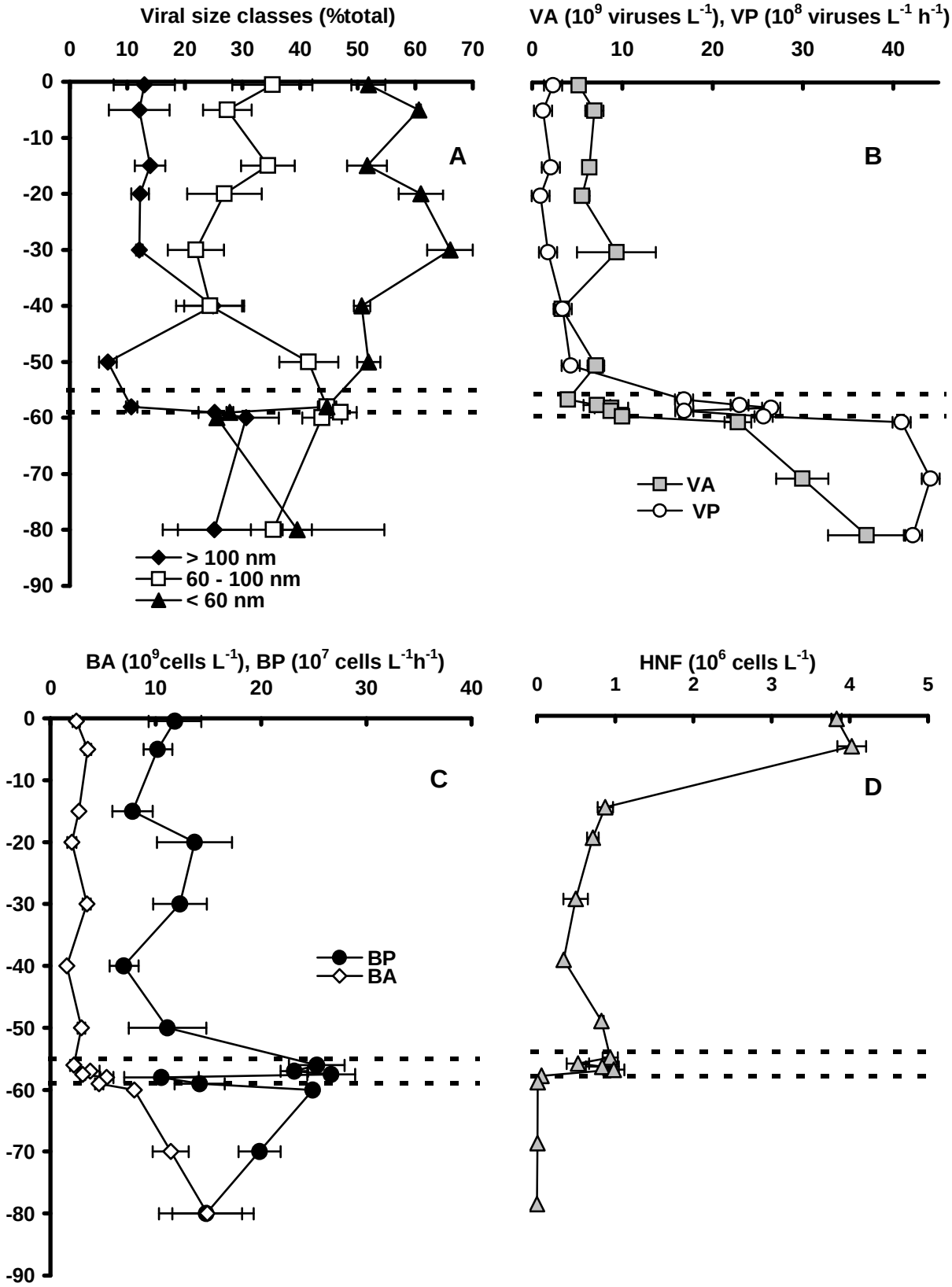


Figure 2. Vertical variations of the relative abundances of viral size classes (i.e. % total observed), of viral abundance (VA) and lytic production (VP), of bacterial abundance (BA) and production (BP), and of the abundance of heterotrophic nanoflagellates (HNF) in Lake Pavin. Error bars represent standard deviations for triplicates.

lytic activity and induction of prophages in conjunction with host availability, were prevalent over the physical environment in the viral proliferation in deep anoxic waters.

Table 2. Correlation relationships of basic parameters in the water column of Lake Pavin. VP, viral production; other abbreviations as in Table 1.

	O_2	Chl	VA	FIC	VP	FLC	BA	BP
Chl.	**0.699**							
VA	**-0.887**	*-0.540*						
FIC	**-0.845**	**-0.690**	**0.656**					
VP	**-0.837**	**-0.825**	*0.633*	**0.943**				
FLC	*0.583*	NS	NS	**-0.743**	**-0.804**			
BA	**-0.900**	*-0.617*	**0.969**	**0.723**	**0.687**	*-0.528*		
BP	NS	**-0.727**	NS	NS	**0.674**	*-0.598*	NS	
HNF	**0.893**	*0.603*	**-0.863**	**-0.716**	**-0.680**	NS	**-0.831**	NS

Italic, $p < 0.05$; **bold, $p < 0.01$**; **underlined bold, $p < 0.001$**; NS, not significant

HNF cell numbers in this study (0 to 4 x 10^3 cells mL^{-1}) were at the lower end of the seasonal ranges (2 to 20 x 10^3 cells mL^{-1}) in the thermally stratified mixolimnion **(3)**, but were similar to those (0.8 – 2.2 x 10^3 cells mL^{-1}) reported during early spring in the same lake **(4)**.The spatial pattern in HNF abundances clearly contrasted with those in viral and bacterial abundances (Fig. 2). HNF peaked in the surface, decreased with depth, and were almost absent in the deep anoxic waters. This pattern was negatively correlated with viral and bacterial abundances and positively with oxygen (Table 2), implying that deep anoxic conditions excluded typical bacterivores.

Lytic viral infection vs potential bacterivory. Inclusion of the monimolimnion layer in our study (Table 1) resulted in higher FIC levels (7 to 79%), compared to seasonal values (5 – 40%) reported in the stratified mixolimnion of Lake Pavin **(2)**. In other aquatic systems, this variable generally is < 50% (24). Bacterial lytic mortality in the monimolimnion was about 2 and 8 folds higher than in oxycline and mixolimnion repectively, and almost all the mortality in the anoxic deep waters were through viral lysis (Fig. 3, Table 1). The same vertical pattern was observed for the calculated viral lytic production (Fig. 2). This implies that the high viral densities in the monimolimnion were produced *in situ*. The sharp contrast observed between the vertical patterns of viral bacteriolysis and potential HNF bacterivory supports the idea that in environments such as suboxic and anoxic waters, the importance of lytic viral infection

increases relative to bacterivory (2, 13, 20). FIC and lytic production of viruses were indeed positively correlated with bacterial abundance and production but negatively with oxygen and HNF abundance and potential grazing (Table 2). Increasing lytic mortality with depth has also been reported in the anoxic deep waters of the Baltic Sea with values up to 71% (21), similar to those reported herein for the monimolimnion of Lake Pavin. These values are among the highest reported for marine and freshwater systems (19, 24). We suggested that they are related to the low grazing pressure and to the high host abundance and production perhaps of low diversity in deep anoxic waters.

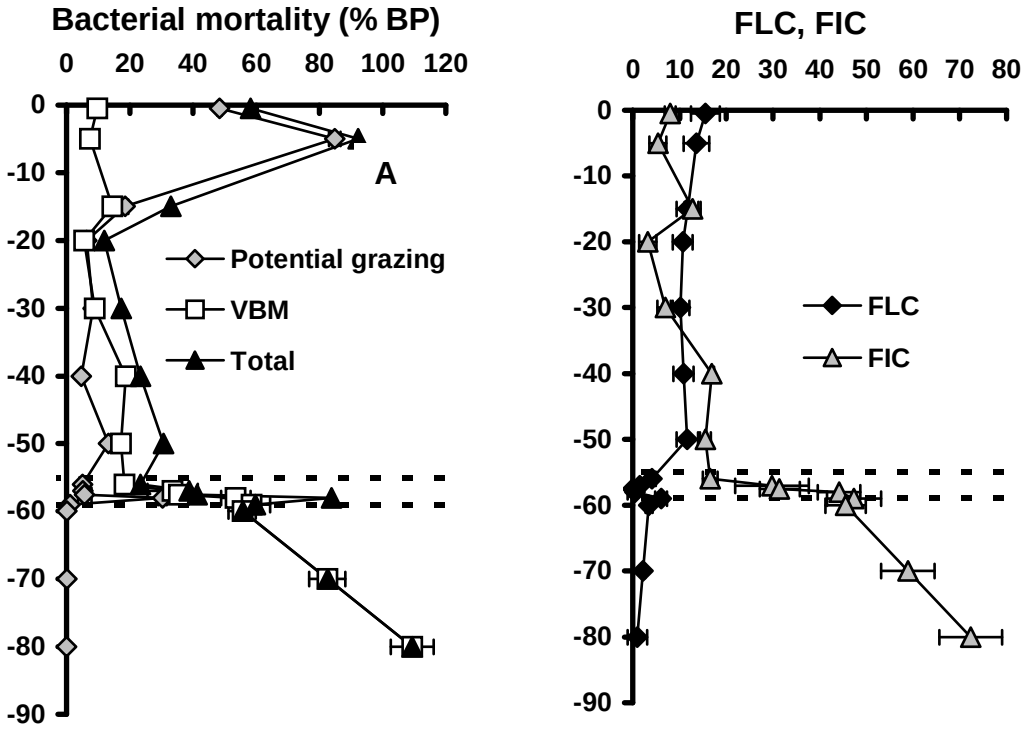


Figure 3. Vertical variations of lytic viral mediated (VBM) and potential grazing mediated mortality in bacterial assemblages, and of the frequencies of lytically (FIC) and of lysogenically (FLC) infected bacterial cells in Lake Pavin. Error bars represent standard deviations for triplicates.

Lysogeny vs lytic infection. Our data suggest that a substantial proportion of bacteria in Lake Pavin contained functional viral genomes. Mitomycin C certainly does not induce all lysogenic bacteria and may also influence non-lysogens, although the concentration we used is known to induce temperate lambda phage whilst having but a small direct effect on its host bacterium, *Escherichia coli* **(14)**. It is thus possible that our frequencies of lysogenic bacteria

(range 0.1 to 16%) are underestimates. Using the same methodological approach, lower values (0.1 to 7.4 %) were reported in the surface layer of Lake Superior **(17)**. Values up to 84% have been reported in deep marine waters, but generally are on average below 15% in estuarine, coastal, and offshore surface waters **(cf. 21)**. In contrast to other viral and bacterial variables under study, lysogens were about 4 fold higher in the oxic and relatively well-illuminated mixolimnion than in suboxic and anoxic waters (Table 1, Fig. 3). This indicates that the level of lysogeny, which was significantly related to depth, apparently was not affected by the surface light environment as expected from current knowledge. Contrary to their original hypothesis, Tapper and Hicks **(17)** also observed higher percentages of lysogenically infected bacteria in the surface than in the subsurface of Lake Superior. In our study, the relative abundances of lysogens were negatively correlated with bacterial abundance and production and with FIC and viral lytic production (Table 2), supporting the hypothesis that lysogeny is a strategy for survival of phages in environments with low host availability. This was also recently supported from a deep profiling study in the Mediterranean Sea, but with inversed vertical patterns compared to our study, i.e. increasing lysogens with decreasing FIC and the host abundance and productivity relative to depth **(21)**. The relationship between the frequencies of lysogenically (y) and of lytically (x) infected bacteria in this study was described best by a power function ($y = -4.5 \ln(x) + 20$, $R^2 = 0.65$), indicating that there are environmental characteristics, in relation with potential host densities and availability, favoring one of the two 'viral life cycles' **(21)**. The contrasting vertical patterns observed between this study and that from Weinbauer and coworkers **(21)** imply that the resource availability for phages (i.e. host environment) may be more important than the physical (i.e. temperature, light) or the chemical (i.e. O_2) environments in favoring one of the two 'viral life cycles'.

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Partie 2 : Etudes saisonnières

A. Seasonal depth-related gradients in virioplankton: standing stock and relationships with microbial communities

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Running heading: Seasonal depth-related gradients in pelagic viruses

Soumis dans FEMS Microbiology Ecology

Abstract

This study presents an original depth-related survey of virioplankton abundance (VA) in the deep (Z_{max} = 92 m) meromictic mountain Lake Pavin (Massif Central, France), in correlation with the abundances of heterotrophic bacteria (BA), picocyanobacteria (Pcy), autotrophic picoeukaryotes (Peu), and of autotrophic (ANF) and heterotrophic (HNF) nanoflagellates. The sampling strategy was designed to be representative of the physico-chemical gradients of the water column of the lake, and the seasonal variability as well, i.e. 12 different depths sampled in triplicates from April to December 2005. The main finding was that the depth-related distributions and the community structures of viruses and the microoorganisms under study differed markedly from those known from previous studies on biological limnology of Lake Pavin, most of which were conducted in the mixolimnic surface waters of the lake. In these waters, all communities were present and viruses were correlated mainly with microautotrophs which exhibited peak abundances in spring, summer and autumn, for Peu, Pcy, and ANF, respectively. Eucaryotes and picoautotrophs were mainly restricted to the

mixolimnion of the lake. From the hypolimnion downward, an apparent shift occurred in the community structure of viruses and heterotrophic bacteria, characterized by an increased in the coefficient of correlation between the two communities from the hypolimnion (0.49) to the oxycline (0.69), and a decrease in virus-to-bacteria ratios from values typical of pelagic systems (2-10) to substantially lower but more stable values (1.6-3.8) in the monimolimnion, due to an increase in BA relative to VA. The permanently anoxic monimolimnion thus appeared as an exclusive habitat for thriving assemblages of viruses and heterotrophic bacteria, where organic matters and nutrients likely are sequestered by the related dark viral loop processes. Overall, the depth-related distribution of the biological items analysed were consistent (i) with their trophic strategies (autotrophs vs heterotrophs) relative to the vertical variability, and with (ii) the decreasing complexity of their biological organization (eukaryotes, prokaryotes, viruses), toward the permanently anoxic deep waters of the lake, considered as an extreme environment.

Keywords: Viruses, Microbial ecology, Population dynamics, Deep variability, Aquatic ecosystems

Introduction

Phages are now widely recognized as the most ubiquitous, abundant, and potentially diverse biological entities in the world aquatic ecosystem **(Fuhrmann & Suttle, 1993; Fuhrman, 1999; Wommack & Colwell, 2000; Weinbauer, 2004; Suttle; 2007)**. Their standing stocks have been shown to vary depending on spatial **(Cochlan *et al.*, 1993; Steward *et al.*, 1996; Weinbauer & Höfle, 1998; Taylor *et al.*, 2003; Brum *et al.*, 2005; Magagnini *et al.*, 2007)** and seasonal/temporal characteristics, in both fresh- **(Mathias *et al.*, 1995; Hofer & Sommaruga, 2001; Bettarel *et al.*, 2003; Vrede *et al.*, 2003; Farnell-Jackson & Ward, 2003)** and marine water masses **(Jiang & Paul, 1994; Weinbauer *et al.*, 1995)**. With the assumption that most of the aquatic viruses are bacteriophages, almost all these studies have considered variations in viral abundance in relation to those in their potential host communities, primarily heterotrophic prokaryotes, without taking into account the other components of the microbial trophic network.

Evidence has been provided that viruses can infect and regulate phytoplankton species **(Mayer & Taylor, 1979; Suttle *et al.*, 1990; Suttle & Chan 1993; Tarutani *et al.*, 2000; Jacquet *et al.*, 2002; Yoshida *et al.*, 2006)**. Few studies in marine and freshwaters have shown that the dynamics of viruses can be more closely related to picocyanobacteria than to

heterotrophic bacteria **(Bettarel *et al*., 2002; Dorigo *et al*., 2004)**. Infection of heterotrophic nanoflagellates (HNF) **(Massana *et al*., 2007)**, consumption of viruses by HNF **(Bettarel *et al*., 2005)** or enhancement of viral lysis by HNF grazing **(Weinbauer *et al*., 2003a; Sime-Ngando & Pradeep Ram, 2005; Weinbauer *et al*., 2007, Pradeep Ram & Sime-Ngando, 2008)** have also been reported. In addition to heterotrophic prokaryotes, viruses are thus probably able to interact directly or indirectly with different components of the microbial food web.

Besides, microbial communities are well known to be highly affected by seasonal forcing and, more particularly, by the vertical segregation of physico-chemical variables through the water column **(Pourriot & Meybeck, 1995)**. This is of a central importance to the carbon flows in aquatic ecosystems **(Tanaka & Rassoulzadegan, 2002, 2004; Tanaka *et al*., 2004 and references therein)**; basically because heat and light penetrations and the related stratification of the water column govern seasonal cycles of primary resources and their physical (e.g. sinking), chemical (photodegradation) and microbial processing. However, the depth-related fluctuations in virioplankton have not yet been explored at a seasonal scale. To our knowledge the rare work studying seasonal changes in viruses and microbial communities in relation to physico-chemical variables in deep lakes have mainly dealt with surface waters **(Bettarel *et al*., 2003)**. This may result in a misreading of relationships linking viruses to microbial communities and to physico-chemical properties of the water column and, therefore, leave open fundamental questions concerning the role of viruses in aquatic systems and the ecological potentials for the whole water column processes. Contribution of deep environments is of a central importance in aquatic microbial ecology **(Karl, 2002)**. Such environments are fundamental for exploring (i) novel viral and microbial diversity **(Vezzi *et al*., 2005; Van Der Wielen *et al*., 2005; Liu *et al*., 2006)**, (ii) new metabolism pathways **(Vezzi *et al*., 2005; Van Der Wielen *et al*., 2005)**, (iii) unknown adaptation strategies in microorganisms **(Minic *et al*., 2006)**, and also (iv) for the characterization of original trophic interactions **(Ortmann & Suttle 2005; Daffonchio *et al*., 2006)**.

Lake Pavin is a deep meromictic lake (Z_{max} = 92m) with a permanently anoxic bottom layer (60 to 92m) and a succession of physico-chemical gradients **(Aeschbag-Hertig *et al*., 2002)**, that offers an environment with low human influences and high annual reproducibility of seasonal dynamics **(Lefèvre *et al*., 2007)**. It is a unique model for studying seasonal depth-related gradients in viral and microbial communities in freshwater ecosystems **(Lehours *et al*., 2005)**. The aim of this study was therefore to examine the depth-related seasonal dynamics in the abundance of viruses and their relationships with microbial communities (i.e.

bacteria, picocyanobacteria, picoeukaryotes, and heterotrophic and phototrophic nanoflagellates), together with the physico-chemical environment (depth, temperature, oxygen and chlorophyll *a* concentrations) in Lake Pavin. The contribution of deep-related gradients is needed (i) to fully examine the potential roles of viruses for microbial food web processes, and (ii) to address the hypothesis that in deep waters, viruses gain competitive advantage over their protistan competitors for bacterial resources, because eukaryotes are constraint by worse environmental conditions such as hypoxia/anoxia **(Colombet *et al.*, 2006)**.

Materials and methods

Study site and sample collection. Samples were collected in Lake Pavin (altitude 1197 m), a meromictic and dimictic oligomesotrophic lake located in the French Massif Central, that experiences partial overturns. It is a typical crater mountain lake characterized by a maximum depth of 92 m and low surface (44 ha) and catchment (50 ha) areas. A characteristic feature of the physical structure of Lake Pavin is the existence of a distinct and permanent chemocline between about 60 and 70 m depth, that separates the seasonally mixed mixolimnion from the monimolimnion. The latter layer is characterized by an elevated conductivity and salinity, and is permanently completely anoxic **(Aeschbach-Hertig *et al.*, 2002)**. Above the chemocline there is an oxic/anoxic interface between 50 and 60 m depths. Samples were collected monthly (systematically between 09:00 and 10:00 AM), from April to December 2005 at a central location in the lake (2°53’12’’E, 45°29’41’’N) by using an 8-liter Van Dorn bottle. A total of 12 depths was sampled in the different physico chemical layers and considered as representative of the whole water column; namely in the epi- (0.5 and 5 m), meta- (12 m), and hypolimnion (20, 30 and 50 m), in the oxycline (56, 57, 58, 59 m), in the chemocline (60 m), and in the monimolimnion (80 m). All samples were collected and analysed in triplicates. For suboxic and anoxic layers, living samples were collected in 20 mL sterile serum bottles sealed with rubber and aluminium crimp caps and flushed with N_2.

Physico chemical parameters. Water temperature and dissolved oxygen profiles were measured *in situ* each 0.5 m from the surface to 80 m with an oxycal-SL 197 multiparameter probe (WWT, Limonest, France). Chlorophyll *a* concentrations (Chl) in the 12 sampling depths were determined spectrophotometrically from particles (0.5 to 2 L) collected on

Whatman GF/F filters. Pigments were extracted in 90 % acetone overnight in the dark at 4°C, and concentrations calculated using **SCOR-UNESCO (1966)** equations.

Viral and bacterial abundances. Subsamples were fixed with 0.02 µm filtered glutaraldehyde (final concentration 1 % v/v) immediately after sampling, and filtered (<15 kPa vacuum) through 0.02 µm pore size Anodisc filters (Whatman, Maidstone, UK) which were mounted between microscope slides and glass cover slips using a mixture of 80 % AF1 Citifluor (Citifluor, London, England) and 20 % Vectashield (Vector Laboratories, Inc., Burlingame, U.S.A.) as antifading mounting medium. Counts were done under a Leica DC 300F epifluorescence microscope following staining with SYBR Green I fluorochrome (Molecular Probes Europe, Leiden, Netherlands) as described by **Noble & Fuhrman (1998)**. When not analysed immediately, slides were stored at -20°C until counting. Bacteria were distinguished from virus-like particles (VLPs) on the basis of their relative size and brightness. A blank was routinely examined to control for contamination of the equipments and reagents.

Picocyanobacteria and autotrophic picoeukaryotes abundance. Picocyanobacteria and autotrophic picoeukaryotes collected on 0.2 µm polycarbonate black filters (Whatman, Maidstone, UK) were mounted between microscope slides and glass cover slips using non-fluorescent immersion oil (Olympus optical, Shinjuku-ku Tokyo, Japan). Counts were done under the Leica DC 300F epifluorescence microscope using two sets of optic filters for differences in autofluorescence due to pigment contains: green light for phycoerythrin containing picocyanobacteria, and blue light for chlorophyll containing autotrophic picoeukaryotes.

Heterotrophic and autotrophic nanoflagellates abundance. Samples for enumeration of heterotrophic (HNF) and autotrophic nanoflagellates (ANF) were fixed immediately after sampling with glutaraldehyde (final concentration 1 %). Primulin stained flagellates collected on 0.8 µm polycarbonate black filters (Whatman, Maidstone, UK) were mounted between microscope slides and glass cover slips using the non-fluorescent immersion oil. Counts were done under the Leica DC 300F epifluorescence microscope using two sets of optic filters: UV light for HNF and blue light for ANF **(Carrias *et al.*, 1998a)**. When not analysed immediately, slides were stored at -20°C until counting. We distinguished the following taxonomic groups: *Kathablepharidae*, Choanoflagellates, undetermined flagellates, *Monas-like* and *Bodonidae* among HNF community, and *Chrysidalis*, *Chloromonas*, *Rhodomonas*

and O*chromonas* among ANF. These identifications were based on morphological characteristics of flagellate assemblages in Lake Pavin (for more details see **Carrias *et al*., 1998a)**.

Statistical analysis. Normal distribution of data was checked by Kolmogorov-Smirnov test. Because not all the data sets followed normal distribution, we applied log transformation to meet the requirements for parametric statistics. Two-way analysis of variance was used to test the potential effects of seasons [i.e. spring (April to June), summer (July to September), and autumn (October to December)] and of depth-locations (epi-, meta-, hypolimnion, oxycline, chemocline, monimolimnion) on the variability in biological variables targeted. In addition, a matrix of data was produced and analysed by means of principal component analysis to identify combination of variables that account for the largest amounts of the total variance observed in different strates of the lake. Potential relationships among variables were tested by Pearson correlation analysis. All statistical analyses were performed using MINITAB 12 and SYSTAT 10.

Results

Temperature, dissolved oxygen, chlorophyll a. The changes in water temperature and oxygen contents were typical of deep dimictic meromictic temperate lakes. Thermal stratification started in May and ended in November, with partial overturns and homogenization of the water column temperature in April and December (Fig. 1A). The oxic/anoxic interface was located between about 55 and 60 m (Fig. 1B). Temperature and dissolved oxygen profiles confirmed that our sampled depths included the different physico chemical layers (i.e. epi-, meta-, hypolimnion, oxycline, chemocline, and monimolimnion) of the water column of the lake (Fig. 1A, B). Chlorophyll *a* concentrations (Chl) exhibited significant differences due to sampling depths or to seasons, both of which interacted in predicting Chl (Table 1) because the highest concentrations were recorded in the three upper layers and during the spring months. Chl peaked in May throughout the water column of the lake (Fig. 1C).

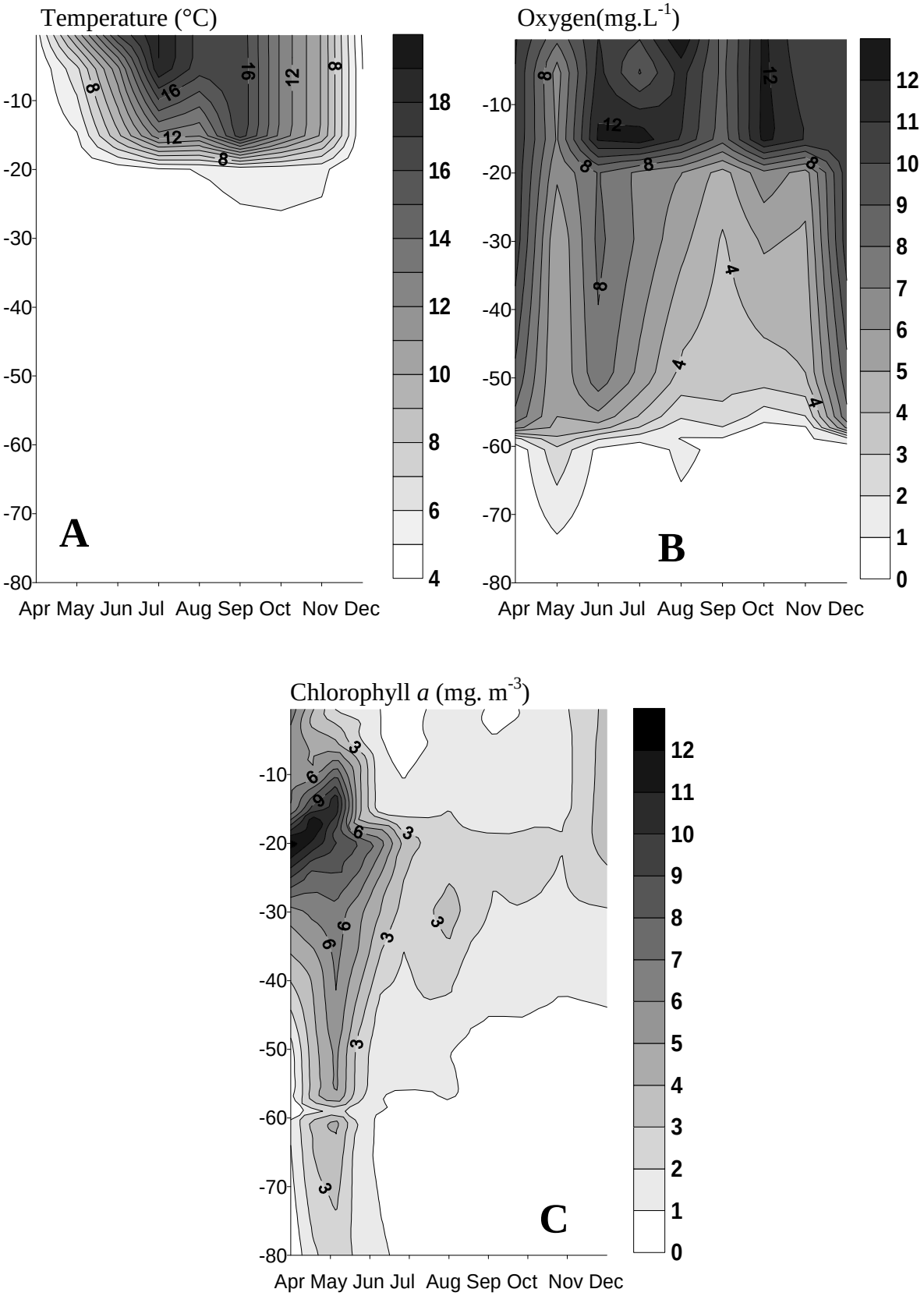


Figure 1. Seasonal variations of temperature (A), oxygen (B) and Chlorophyll *a* (C) in the water column of Lake Pavin, April – December 2005.

Table 1. Results of two-way ANOVA used to test for differences with seasons (S) and depth-layers (L)[a]

	ANOVA Value for											
	Chl		VLP		Bacteria (BA)		Peu		ANF		HNF	
	F	P	F	P	F	P	F	P	F	P	F	P
Season (S)	71.0	0.001	23.7	0.001	72.0	0.001	39.1	0.001	10.3	0.001	1.2	0.317
Layer (L)	38.7	0.001	51.3	0.001	86.6	0.001	4.8	0.001	199.0	0.001	100.0	0.001
Interaction S x L	2.4	0.009	9.1	0.001	12.3	0.001	5.3	0.001	6.0	0.001	1.8	0.070

[a] Data are from samples obtained from April to December 2005 in Lake Pavin. Seasons are defined as spring (April-June), summer (July-September), and autumn (October-December). Depth layers correspond to samples collected in the epilimnion (0.5 and 5m), metalimnion (12 m), hypolimnion (20, 30, 50m), oxycline (56, 57, 58, 59m), chemocline (60m) and the monimolimnion (80m) of the lake. Samples were collected in triplicate (n = 36). Chl, chlorophyll a; VLP, abundance of virus-like particles; Pcy, abundance of picocyanobacteria; Peu, abundance of autotrophic picoeucaryotes; ANF, abundance of autotrophic nanoflagellates; HNF, abundance of heterotrophic nanoflagellates; F, F-value; P, probability.

Virus-like particles (VLPs) and bacteria. Viral abundance (VA) fluctuated from 0.8 to 5.1 x 10^7 VLPs mL^{-1} (mean = 2.2 x 10^7 VLPs mL^{-1}). Differences between layers were significant (Table 1), mainly due to the highest values recorded in the metalimnion and in the monimolimion (Fig. 2A). These values occurred in summer and in autumn, explaining the significant effects of seasons on VA and the interaction between seasons and depths as well (Table 1). However, the seasonal variability in VA was visibly weaker from the lower hypolimnion to the oxycline and chemocline (i.e. from 30 to 60 m depth), compared to the upper layers and to the monimolimnion of the lake (Fig. 2A).

Bacterial abundance (BA) fluctuated from 2.0 to 28.3 x 10^6 cells mL^{-1} (mean = 6.0 x 10^6 cells mL^{-1}) and peaked in August in the metalimnion and in the monimolimnion where the highest seasonal abundance was noted, largely higher than the metalimnic peak. The above patterns in BA accounted for the significant effects of seasons and depths and their interaction (Table 1). Interestingly, the August peaks in BA coincided with similar ones in VA. In addition, low seasonal variability was also obvious in BA between about 20 and 60 m depths, similar to the pattern observed in VA (Fig. 2A,B). Virus-to-bacteria ratio (VBR) fluctuated from 1.6 to 10.0 (mean = 4.4) and peaked during autumn in about the whole water column, due to the increase in viral abundance (Fig. 2C). The lowest values were recorded in the monimolimnion (range 1.6 – 3.8), due to the increase in BA relative to VA in this layer.

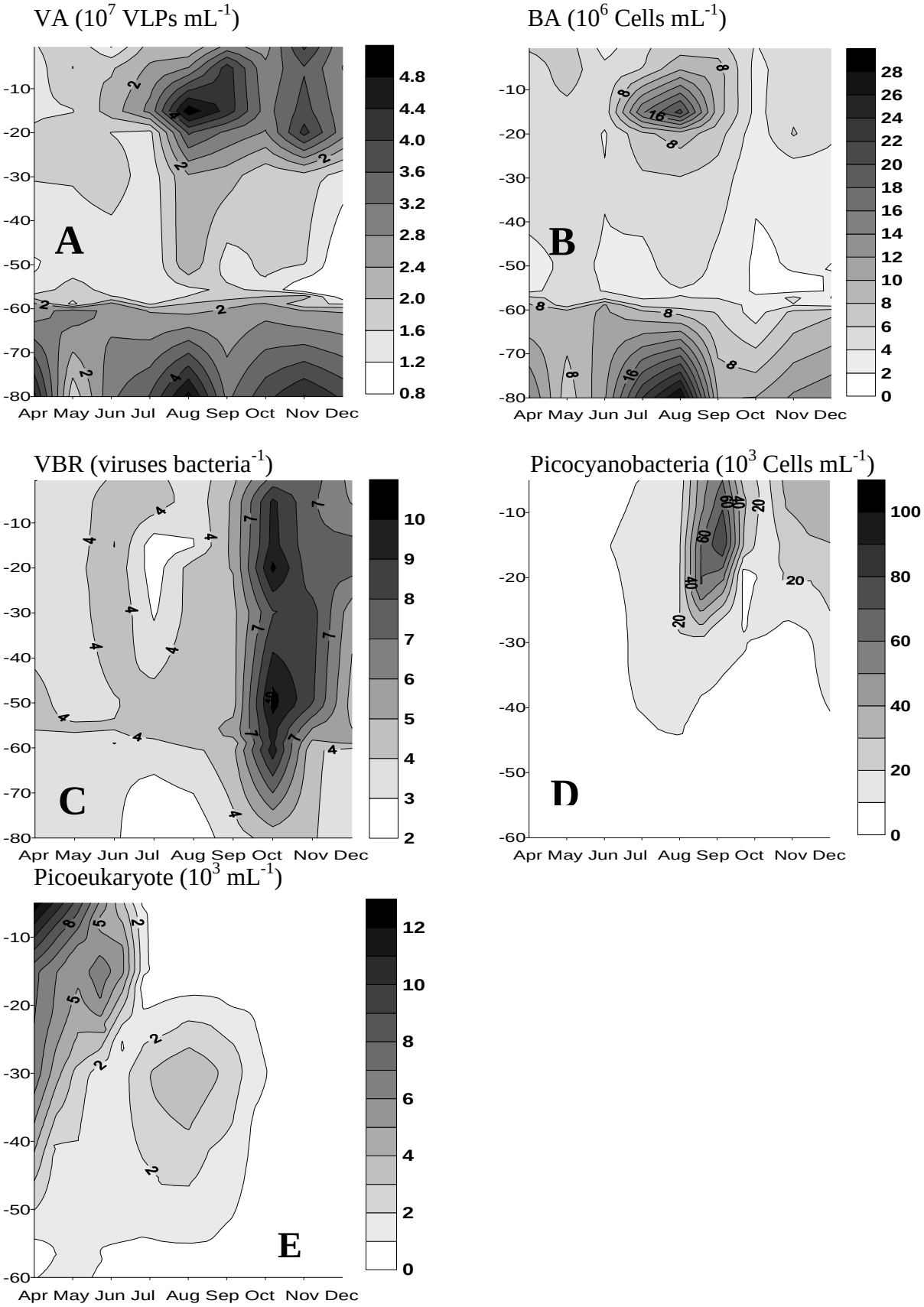


Figure 2. Seasonal variations in the abundances of viruses (VA, A) and bacteria (BA, B), in virus-to-bacteria ratio (VBR, C), and in the abundances of picocyanobacteria (D) and autotrophic picoeukaryotes (E) in the water column of Lake Pavin, April-December 2005.

Picocyanobacteria (Pcy) and autotrophic picoeukaryotes (Peu). Pcy and Peu abundances fluctuated from 0.1 to 91.5 x 10^3 Pcy mL^{-1} (mean = 10.6 x 10^3 Pcy mL^{-1}) and from 0.02 to 12.6 x 10^3 Peu mL^{-1} (mean = 1.82 x 10^3 Peu mL^{-1}). The development of both communities apparently was restricted to the mixolimnion, in spring for eucaryotes and in summer for cyanobacteria. The latter community peaked in the metalimnion and the former in the epilimnion (Fig. 2D,E). The single and combined effects of seasons and depths on both communities were significant (Table 1).

Autotrophic (ANF) and heterotrophic (HNF) nanoflagellates. Nanoautotrophs fluctuated from 0 to 11.9 x 10^2 ANF mL^{-1} (mean = 1.8 x 10^2 ANF mL^{-1}) in abundance, and mainly occurred in the mixolimnic layers. Maximum values peaked in the upper hypolimnion in summer and then shifted upward in the epi- and metalimnion in autumn (Fig. 3A), with significant single and combined effects of seasons and depths (Table 1). ANF assemblages were dominated by *Chrysidalis* species which averaged 67% of the total abundance, with maximum development during summer and in autumn. Other ANF species or taxonomic groups, i.e. *Chloromonas*, *Rhodomonas* and O*chromonas* species, averaged <20% of the total abundance throughout the study but were dominant in spring (Fig. 3C).

Nanoheterotrophs were higher in abundance than autotrophs, ranging from 0 to 18.8 x 10^2 HNF mL^{-1} (mean = 6 x 10^2 HNF mL^{-1}). In contrast to other biological variables, the effect of seasons on HNF variability was not significant and did not interfere with the significant effect of sampling depths (Table 1). The development of HNF in the water column was heterogeneous, with almost no occurrence in the monimolimnion. Maximum abundances moved from the hypolimnion in spring to the oxycline/chemocline in early summer, and then to the epi- and metalimnic layers in late summer and in autumn (Fig. 3B). This was related to the dominant HNF group represented by unknown morphological forms which averaged 73% of total abundance. Other taxonomic categories averaged < 20% of the total abundance throughout the study: *Monas-like* flagellates and *Kathablepharidae* were present in all seasons but in higher relative number in spring, same for *Bodonidae* but with maximum abundance in autumn, while Choanoflagellates apparently occurred only in autumn (Fig. 3D).

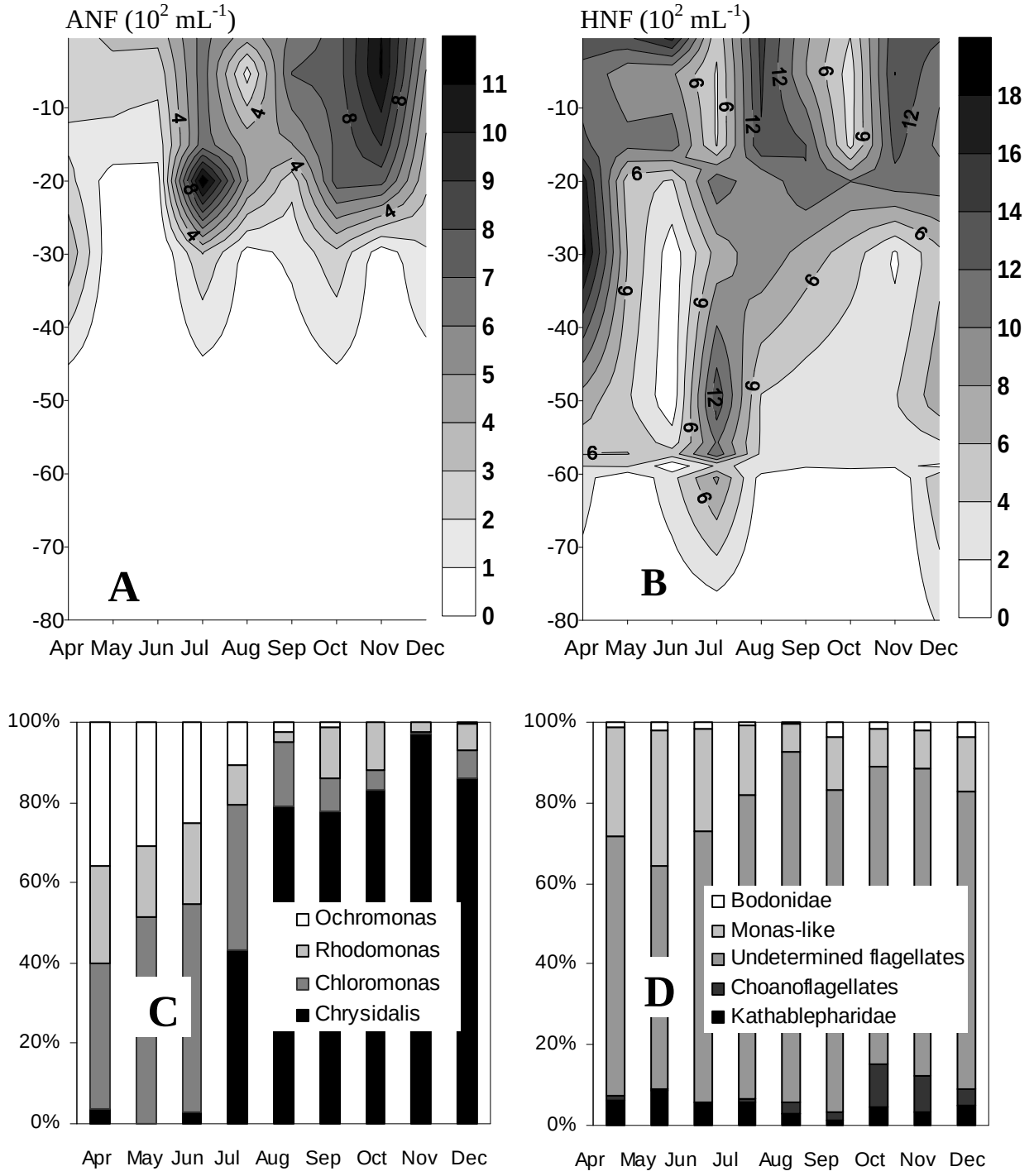


Figure 3. Seasonal variations in the abundances of autotrophic (ANF, A) and heterotrophic (HNF, B) nanoflagellates, and in the relative abundances (as percent of total) of different taxonomic ANF (C) and HNF (D) groups in the water column of Lake Pavin, April-December 2005.

Correlations between viruses and microbial communities. The number of correlations between the abundance of viruses and other variables was higher in the surface than in the

deeper layers of the lake. This was apparently due to a major influence of pigmented communities in the surface waters, although chlorophyll *a* appeared as a strong forcing correlate in the oxycline (Table 2). Viruses were positively correlated to prokaryotes but to picocyanobacteria in the surface waters (i.e. epi- and metalimnion) and to heterotrophic bacteria in the bottom layers (hypolimnion, oxycline, monimolimnion). In the surface waters (epi- and metalimnion), viruses were negatively correlated to autotrophic picoeucaryotes. There were significant positive correlations between viruses and autotrophic nanoflagellates (i.e. total community and the dominant *Chrysidalis* species) in the three mixolimnic layers, while correlation with nanoheterotrophs was not significant in any of the depths sampled. Finally, temperature was a positive correlate for viral abundance in the metalimnic discontinuity layer and more strongly in the hypolimnion, while oxygen depletion in the oxycline contrasted with an increase in viral abundance (Table 2).

Table 2. Results of Pearson correlation analysis used to test for correspondence between temporal changes in the abundance of viruses and the other estimated variables in six different depth-layers of Lake Pavin[a]. Only significant correlations ($p < 0.05$) are given (blanks mean not significant).

Viruses versus :	Epi-	Meta-	Hypolimnion	Oxycline	Chemocline	Monimolimnion
Temperature		0.72*	0.61***			
Oxygen				- 0.48**		
Chlorophyll *a*					0.83***	
Bacteria			0.49**	0.69***		0.72***
Picocyanobacteria	0.78***	0.82***				
Picoeukaryotes	- 0.84***	- 0.88***				
Autotrophic nanoflagellates	0.69***	0.76***	0.48***			

[a] The number of degrees of freedom depends on the number of depths sampled per layer, in triplicates for biological variables (see Table 1 for more details). Asterisks denote significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Blanks = not significant

Principal component analysis (PCA). The plot of field observations from PCA showed that the seasonal distributions of variables under study were dependent to the vertical gradients. Indeed, five different layers were clearly distinguished from this plot [i.e. (epi. and metalimnion), hypolimnion, oxycline, chemocline and monimolimnion], although the number of observations was lower for chemocline and monimolimnion where only one depth was sampled (Fig. 4A). However, the plots of the descriptors were quite similar along the two major PCA axes for oxycline, chemocline and monimolimnic layers. Data from these layers were thus combined in one plot. For the three deep-related PCA plots of the descriptors [i.e. epi. and metalimnion combined (Fig. 4B), hypolimnion (Fig. 4C), and oxycline, chemocline and monimolimnion combined (Fig. 4D)], axes 1 and 2 explained about 55-60% of the total variance. Viruses rotated from the extreme negative end to the extreme positive end of the major axis 1, with the increasing depth. In the mixolimnic layers, this axis distinguished viruses and many other variables (primarily picocyanobacteria and nanoautotrophs), with negative coordinates. This contrast with the deepest lake layers were viruses and heterotrophic bacteria were clearly isolated at the extreme positive end of axis 1, and opposed to all other variables (Fig. 4).

Discussion

***Standing stocks and deep-related community structures*.** The present study is an attempt to analyse the seasonal changes in the standing stock of viruses together with those of microbial communities (i.e. heterotrophic and autotrophic pico- and nanoplankton) in the deep meromictic mountain Lake Pavin, including for the first time the whole water column and the related deep-related gradients. Previous studies on biological limnology in Lake Pavin were restricted to the mixolimnion above 40 m depth **(Carrias *et al*., 1996, 1998ab; Bettarel *et al*., 2003; Lepère *et al*., 2006; Lefèvre *et al*., 2007)**, ignoring the deeper layers which contain about the half of the total volume of water in the lake. In general, biological limnology of deep lakes lacks studies where different communities are investigated simultaneously in the whole water column, although such information is needed to assess the potential roles of viruses and microbial food-web processes in aquatic systems **(Colombet *et al*., 2006)**. Our findings in the mixolimnion of Lake Pavin were quite similar to those reported in previous studies and hereafter, more comparative emphasis will be given to the deeper layers of the lake.

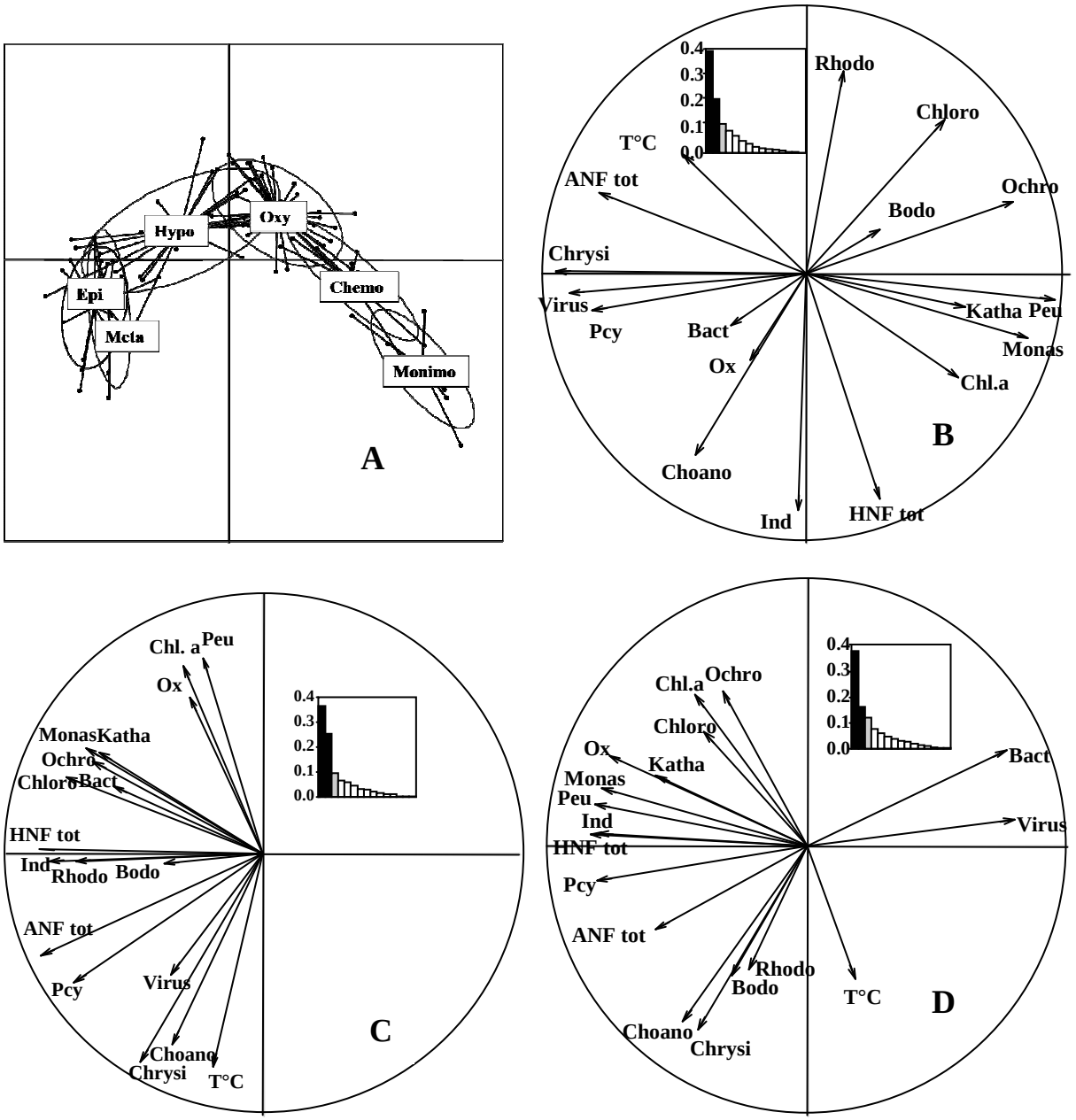


Figure 4. Results of the principal component analysis (PCA) obtained from the observations made for each of the six sampling depth-layers (A), and from the main variables under study in three groups of these depth-layers: epi- and metalimnion combined (B), hypolimnion (C), and oxycline, chemocline and monimolimnion combined (D). Abbreviations are as follows: temperature (T°C), Oxygen concentration (Ox), Chlorophyll *a* concentration (Chl.a), and the abundances of the different communities and the related taxonomic groups: viruses (virus), bacteria (bact), Picocyanobacteria (Pcy), autotrophic picoeukaryotes (Peu), total autotrophic nanoflagellates (ANF tot) and the related taxa (*Chrysidalis* spp, Chrysi; *Chloromonas* spp, Chloro; *Rhodomonas* spp, Rhodo; *Ochromonas* spp, Ochro), and total heterotrophic nanoflagellates (HNF tot) and the related taxa (undetermined flagellates, Ind; *Monas*-like cells, Monas; *Kathablepharidae*, Katha; *Bodonidae*, Bodo). Horizontal X-axis = Axe 1,

Vertical Y-axis = Axis 2. Inset histograms in B-D represent the fractions of variance (Y, %) explained by the different PCA axes (X, from Axis 1 onwards).

Previous data and our proper results related to the mixolimnion of Lake Pavin highlight the importance of light and/or temperature for the seasonal and spatial changes in microbial communities. By including the whole water column, we herein added to these findings the significance of deep oxycline and chemocline gradients and of monimolimnion in these changes. Autotrophic pico- and nanoplankton developed mainly in the surface photic layers, particularly in the epi- and metalimnion in spring, summer or autumn. Compared to these communities, heterotrophic nanoflagellates extended their spatial domain of development through the oxycline and the chemocline with low response to the seasonal forcing, but they were completely inhibited in the anoxic monimolimnion. In contrast, the highest abundances of heterotrophic bacteria and of viruses as well occurred in the latter layer and in the metalimnion, particularly in the summer time. The vertical distributions of the different communities were thus somewhat consistent with their trophic strategies (autotrophic vs heterotrophic) according to the vertical variability, and with the decreasing complexity of their biological organisation (eukaryotes, prokaryotes, viruses) toward the extreme anoxic environment, as known from other aquatic environments **(Pourriot & Meybeck, 1995; Weinbauer & Höfle 1998; Humayoun *et al.*, 2003; Jiang *et al.*, 2003; Taylor *et al.*, 2003)**. However, the originality of Lake Pavin emerges from its deepness and the related physico-chemical gradients and, particularly, from its meromicticity characterized by a permanently anoxic monimolimnion. The complexity of biological communities and the related trophic networks decreased with depth because the monimolimnion layer highly constrained the development of biological communities, excepted viruses and their main potential bacterial hosts which were adapted to the deep anoxic environment.

Its is interesting to note that viral and bacterial communities exhibited an apparent seasonal fluctuations in the monimolimnion with highest abundances in summer and/or in autumn (Fig. 2A,B), although the characteristics of the water masses in this layer are very stable and have been considered in steady state with a residence time that can exceeded 100 years **(Aeschbach-Hertig *et al.*, 2002)**. This suggests the existence of a certain degree of material exchanges between the surface and the deep layers of the lake. High spatial heterogeneity at scale of centimetres has also been reported in the monimolimnion of Lake Pavin for prokaryotes **(Lehours *et al.*, 2005)** and in the deep sea sediments for viruses **(Middelboe *et al.*, 2006)**. In our study, the range of viral abundances (0.77 to 5.09 x 10^7

VLPs mL^{-1}) parallels that of a previous study conducted in the mixolimnion of Lake Pavin **(Bettarel *et al*., 2003)** and was characteristic of oligo- to mesotrophic environments **(Hennes & Simon, 1995; Wilhelm & Smith, 2000)**, as was the peaks noted in the metalimnion in summer and in autumn **(Weinbauer *et al*., 1995; Drake *et al*., 1998; Bettarel et al. 2003)**. Thus the abundance maxima noted for viruses in the deep anoxic monimolimnion of Lake Pavin was not enough to significantly change the overall seasonal range over the whole water column.

This was not the case for bacteria which exhibited maxima abundances in the monimolimnion, particularly during summer (ca 10^7 cells mL^{-1}), i.e. about an order of magnitude higher than the typical bacterial seasonal abundances in the mixolimnic layers of Lake Pavin **(Bettarel *et al*., 2003)**. As a consequence, virus-to-bacteria ratios (VBR) in the monimolimnion (range, 1.6 - 3.8) were significantly lower than in other layers (range, 2 - 10), and than the typical values (range, 3 to 10) known from other pelagic environments **(Wommack & Colwell, 2000)**. These findings corroborate those from **Maranger & Bird, (1996)** in a comparative study of oxic and anoxic freshwater sediments. In our study, the temporal variability in VBR in the monimolimnion (CV < 30%) was further substantially lower than in the mixolimnion (CV > 40%), supporting a changing relationships between viruses and their main potential hosts between the two layers **(Middelboe *et al*., 2006)**. The low values and low variability of VBR in the monimolimnion may suggest a constant level of virus production and losses and that most of the free viruses in the monimolimnion were bacteriophages from anaerobic *Bacteria* or *Archaea* **(Tuomi *et al*., 1997, Hara *et al*., 1991)**.

The mechanisms behind the spatial shift in viruses vs prokaryote interactions are unclear but we believe that the deep, cold, and anoxic characteristics of monimolimnic waters are significant factors. For example, cold temperature can inhibit viral infectivity per see **(Pradeep Ram *et al*., 2005 and references therein)**. The main bacterial grazers, i.e. heterotrophic nanoflagellates, were absent in the monimolimnion likely because of the constraint from the prevailing anoxic conditions, a situation with, together with the abundant availability of resources **(Lehours *et al*., 2005)**, is advantageous to prokaryote growth. However, because of thermodynamic considerations, the growth efficiencies of anaerobic microbes are generally substantially lower that those of aerobes **(Fenchel & Finlay, 1994)**. This might imply that anoxic bacterial hosts would produce proportionately smaller viral quantities than oxic hosts, although this was partially tested in both marine and freshwater bacterioplankton **(Weinbauer & Höfle, 1998; Taylor *et al*., 2003; Weinbauer *et al*., 2003b)**. A recent study in Lake Pavin has unveiled highly diverse prokaryote communities in the

monimolimnion **(Lehours *et al.*, 2005)**, a situation that can reduce the probability of contacts between viruses and their specific hosts, and the related viral proliferation as well. An important fraction of active bacteria in the monimolimnion could also be lysogens, resistant, or non specific to the surrounding viruses.

The absence of grazers in the monimolimnion and the different virus vs prokaryote interactions compared to the mixolimnion could thus have a significant influence on nutrient cycling and the related microbial and viral processes in Lake Pavin, although the mechanisms involved still are to be properly investigated. We consider that the trophic network in the monimolimnion is reduced to viruses and prokaryotes whose interactions can influence the cycling of organic matter in the lake. For example, organic matters and nutrients can be sequestered and fully recycled several times in the monimolimnic DOM- prokaryotes- viruses trophic loop, before incorporation into the sediments or back to the upper water layers, in relation to the water residence time that can exceeds 100 years in this deepest layer of the lake **(Aeschbach-Hertig *et al.*, 2002)**.

Coupling of viruses with microbial communities. Statistical analyses indicated that empirical relationships between viruses and microorganisms under study were more numerous and complex in the surface than in the deep waters, with a dominance of various microautrotrophic variables as strong predictors in the surface photic layers of the lake (Table 2, Fig. 4). No correlation was calculated between viruses and heterotrophic nanoflagellates, parly because these communities exhibited no clear seasonality (Fig. 3B). This agrees with previous findings in Lake Pavin, both in terms of absence of correlation between viruses and HNF **(Bettarel *et al.*, 2003)** and of low direct consumption of viruses by HNF **(Bettarel *et al.*, 2005)**. In contrast, the significant correlations between viruses and microautotrophs (i.e. picocyanobacteria, picoeukaryotes, and nanoflagellates) in the mixolimnic layers were not found in a previous study conducted by **Bettarel *et al.* (2003)** in these layers, but picoautotrophs were not quantified in this study. **Dorigo *et al.* (2004)** reported a correlation between viral and picocyanobacterial abundances in the mesotrophic Lake Bourget (France) and attributed their finding to the prevalence of cyanophages. It is thus tempting to consider that cyanophages and perhaps other 'phytophages' represented a significant pool of viral standing stocks in the mixolimnion of Lake Pavin. A seasonal succession of the different groups of microautotrophs was noted, from picoeukaryotes in spring (Fig. 2E) to picocyanobacteria in summer (Fig. 2D) and to nanoflagellates in autumn (Fig. 3A), corresponding to the main peak development in viral abundance in the mixolimnion.

Although similar peaks of viruses also occurred in the monimolimnion (Fig. 2A), the present study highlights the importance of autotrophic communities in the dynamics of viral populations in the surface waters of Lake Pavin.

As the abundances of autotrophic components decreased from below the metalimnion toward the deep waters, heterotrophic bacteria became the most significant predicting variable for viruses. Both communities were coupled, opposed to other variables, and isolated at the extreme positive end of the principal component analysis plot for the deeper layers of the lake (Fig. 4D). Indeed, the correlation coefficients between viruses and bacteria increased from 0.49 in the hypolimnion to 0.69 in the oxycline, supporting the changing relationships between viruses and their main potential hosts between the surface and the deep layers of the lakes, as previously suggested by the vertical distribution of VBR (see above). Our findings thus corroborate preliminary results (i.e. one day sampling) on depth-related gradients of viral activity in Lake Pavin, where heterotrophic prokaryotes appeared as the main regulating factor, if not the sole mortality source, for viruses in the anoxic monimolimnion **(cf. Colombet *et al.*, 2006)**, although VBR levels in this layer were atypically low.

However, we have observed no correlation between viruses and bacteria in the anoxic chemocline, i.e. beneath the oxycline (Table 2) where one depth was sampled. A recent spatial study has shown that there is a complete shift in the composition of the prokaryotic assemblages in the chemocline of Lake Pavin **(Lehours *et al.*, 2005)**. This layer is characterized by particular geochemical properties that act as a key interface in the vertical flow and recycling of matter through the water column of the lake **(Aeschbach-Hertig *et al.*, 2002)**. Analyses of viral diversity based on capsid size also have suggested that the chemocline represent a physical 'barrier' that traps sedimenting particles in Lake Pavin **(Colombet *et al.*, 2006)**. This may help explain why viral abundance was correlated with chlorophyll *a* in this layer (Table 2), known as an accumulator of particles from the surface waters **(Aeschbach-Hertig *et al.*, 2002; Lehours *et al.*, 2005)**, i.e. a known source of adsorbed viral inputs **(Danovaro *et al.*, 2001)**.

Conclusions

Overall, this study presents an original deep-related survey of a lake virioplankton in relation to microbial communities, in a site that offers a unique environment with low human influences and high annual reproducibility of seasonal dynamics in the water column. The sampling strategy adopted, with 12 different depths sampled through the 92 m deep water column, was considered representative of the whole water column of Lake Pavin, and the

related physico-chemical gradients. The deep-related distributions and community structures of viruses, prokaryotes and microbial eukaryotes under study differed markedly from those known from previous studies on biological limnology of Lake Pavin which were restricted to the mixolimnic surface layers of the lake, i.e. above 40 m depth. In these surface waters, viruses were empirically related to several of the complex communities present, with an apparent dominance of autototrophic prokaryotes and eucaryotes in predicting viral abundances. In contrast, the monimolimnion of the lake clearly appeared as an exclusive habitat for viruses and heterotrophic bacteria, where their interactions markedly differed from those in the surface waters. We thus consider that the ecology of the deepest waters of Lake Pavin is essentially driven by the dark viral loop (DOM-prokaryotes-viruses) which can sequester organic matters and nutrients for a long-live turnover time.

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B. Seasonal depth-related gradients in virioplankton: lytic activity and comparison with prokaryotes and potential bacterivores

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Running tittle: Seasonal depth-related gradients in viral lysis

En préparation

Abstract

This study presents an original deep-related survey of virioplankton lytic activity in relation to prokaryotes and potential bacterivores in the deep (Z_{max} = 92 m) meromictic volcanic Lake Pavin (Massif Central, France), complimenting a companion paper on the seasonal abundances and vertical community structure of viruses and microorganisms in the same site. The sampling strategy was designed to be representative of the physico-chemical gradients of the water column of the lake, and of the seasonal variability as well, i.e. 12 different depths sampled in triplicates from April to December 2005. In the space, viral lytic activity estimated from the frequency of visibly infected prokaryotic cells and from burst size over the study period generally decreased with depth. This was viewed as a paradox compared to the abundances of viruses and prokaryotes and to the prokaryotic production which increased with depth. According to the seasonal variability, almost all correlations with lytic activity occurred from the hypolimnion downwards, implying bacterivores (in spring in the hypolimnion) but mainly prokaryotes as dominant correlates and as exclusive microorganisms in the deepest layers of the lake. Compared to previous studies known from the mixolimnion, we conclude that deep waters in Lake Pavin represent an exclusive environment for heterotrophic prokaryotes whose seasonal activity offers an optimal and

unique resource for thriving viral communities, some of which may be typical, endemic to the ambient dark, cold and stable deep-water masses. Overall, the present study on the functional responses of viruses and of prokaryotes to the depth gets well around a previous statement that the ecology of the deepest waters of Lake Pavin is essentially driven by the dark viral loop (DOM-prokaryotes-viruses) processes, which can sequester organic matters and nutrients for a long-live turnover time. This is in agreement with recent demonstrations from marine systems that meso- and bathypelagic waters are optimal environments for viral survival and proliferation.

Keywords: Viruses, Microbial ecology, Population dynamics, Deep variability, Aquatic ecosystems

Introduction

It is now widely accepted that bacteria play a major role in the cycling of energy and matter in aquatic ecosystems. The related concepts of 'microbial loop' in which nutrients are recycled through bacteria-grazer interactions **(Azam et al. 1983)**, and of 'microbial food web' that includes the relationships between heterotrophic and autotrophic microorganisms and its relations to biogeochemical cycles **(Cole et al. 1988, Rassoulzadegan 1993)**, have stimulated a large amount of researches on the mechanisms which regulate bacterioplankton biomass and activities. Some authors have argued that bacteria are mainly controlled by resources **(**Billen **et al. 1990, Bird and Kalff 1984, Cole et al. 1988)**, and others that bacterial mortality is largely due to protistan grazing **(Fenchel 1982, Sherr and Sherr 1987). Ducklow and Carlson (1992)** have shown that the control mechanisms of bacterioplankton may change seasonally. The effects of grazers vs those from viruses may also change with time and space as well **(Pradeep et al. 2005)**.

In the late 1980s it was shown that in marine and freshwaters, viral particles occur in great numbers, often exceeding bacterial numbers **(Bergh et al. 1989, Proctor and Fuhrman 1990, Suttle et al. 1990)**. Most of these viruses are considered as bacterial viruses (bacteriophages) and their lytic activity as an additional mechanism that significantly contributes to the regulation of bacterial production. On average, 10-40 % of bacterial production is lysed by viruses in both marine and freshwaters **(Wommack and Colwell 2000,**

Suttle 2005). The loss of bacterial biomass caused by grazing or by virus-mediated lysis has different consequences for organic matter fluxes. If a major part of bacterial loss is due to viruses, carbon is burned into a dissolved organic carbon (DOC)-bacteria-DOC loop **(Bratbak et al. 1992)**, while protozoan grazing transfers part of bacterial carbon up to the higher trophic levels **(Carrick et al. 1991, Dolan and Gallegos 1991)**.

Only few studies have investigated the effects of viral bacteriolysis and potential bacterivores within prokaryotic communities in aquatic systems **(Fuhrman and Noble 1995, Guixa-Boixereu et al. 1996, Weinbauer and Höfle 1998a, Guixa-Boixereu et al. 1999, Simek et al. 2001, Almeida et al. 2001, Fischer and Velimirov 2002, Choi et al. 2003, Bettarel et al. 2004, Pradeep Ram et al. 2005, Jacquet et al. 2005, Colombet et al. 2006)**. Almost all these studies were done at a short time scale, with only those from **Bettarel et al. (2004)** and from **Pradeep Ram et al. (2005)** which have integrated month to month seasonal variability in lakes. However, these authors have considered only few points in the surface water column although representative of the thermal stratification gradient. In viral and microbial ecology, data on seasonal depth-related gradients are lacking, primarily in freshwater lakes. The little available information on viral core parameters (abundance, lysis, lysogeny) in both fresh- **(Colombet et al. 2006)** and marine systems **(Weinbauer et al. 2003)** are known from sporadic sampling. These authors reported highly significant changes with depth, particularly unregardless of oxygen concentrations. Vertical gradients in physico-chemical variables are essential in the vertical partitioning of biological variables **(Pourriot and Meybeck, 1995)**. In a companion paper, we have demonstrated that density relations between viruses and microbial communities changed significantly from photic area to aphotic and permanently deep anoxic monimolimnion of the meromictic Lake Pavin **(Colombet et al. submitted)**, similar to recent findings concerning prokaryotic community composition **(Lehours et al. 2005)**. Vertical segregation is of central importance in carbon flows within aquatic ecosystems; the ecology of these systems is dependant on the processes that happen through the whole water column **(Tanaka and Rassoulzadegan 2002, 2004, Tanaka et al. 2004, and references herein)**.

In this study, we examine the seasonal variations of viral lytic activity related to fine vertical changes in physico-chemical and pertinent biological variables (i.e. prokaryote production and potential bacterivores) in the deep meromictic Lake Pavin. Such contributions are needed to fully assess the impact of viruses in microbial food-web and the related biogeochemical cycling in aquatic systems. The deep, meromictic and dimictic Lake Pavin exhibits a typical vertical succession of physico-chemical conditions **(Aeschbag-Hertig et al.**

2002), offering a unique model to study virus-microbial interactions in relation to physico-chemical and biological depth-related gradients.

Materials and methods

Study site and sample collection. Lake Pavin (altitude 1197 m) is a meromictic and dimictic oligomesotrophic lake located in the French Massif Central that experiences partial overturns. It is a typical crater mountain lake characterized by a maximum depth of 92 m and low surface (44 ha) and catchment (50 ha) areas. A characteristic feature of the physical structure of the lake is the existence of a distinct and permanent chemocline between about 60 and 70 m depths, that separates the seasonally mixed layer (i.e. mixolimnion) from the monimolimnion. The latter layer is characterized by an elevated conductivity and salinity as well as complete and permanently anoxic conditions **(Aeschbach-Hertig et al. 2002)**. Above the chemocline there is an oxic/anoxic interface that occurred between 50 and 60 m depths (Fig. 1).

Samples were collected monthly (systematically between 09:00 and 10:00 AM), from April to December 2005 at a central location in the lake by using an 8-liter Van Dorn bottle. Sampling was realized over the whole water column at 12 different depths considered as representative of the different physico-chemical particularities of the water column. Indeed, in addition to surface depths characteristic of the thermal stratified mixolimnion (i.e. epi-, meta and hypolimnion) where almost all studies done to date in Lake Pavin were conducted **(Bettarel et al. 2003, Bettarel et al. 2004, Jardillier et al. 2005)**, the deepest layers of the lake were also sampled. Indeed, the six different layers of the water column were sampled for a total of 12 different depths located in the epi- (0.5 and 5 m), meta- (12 m), and hypolimnion (20, 30 and 50 m) for the mixolimnic layers, and in the oxycline (56, 57, 58, 59 m), the chemocline (60 m) and the monimolimnion (80 m) for the deep layers (Fig. 1). All samples were collected and analysed in triplicates. For suboxic and anoxic layers, live samples were taken and processed (i.e. for bacterial production measurements, see below) in sterile serum bottles sealed with rubber and aluminium crimp caps and flushed with N_2.

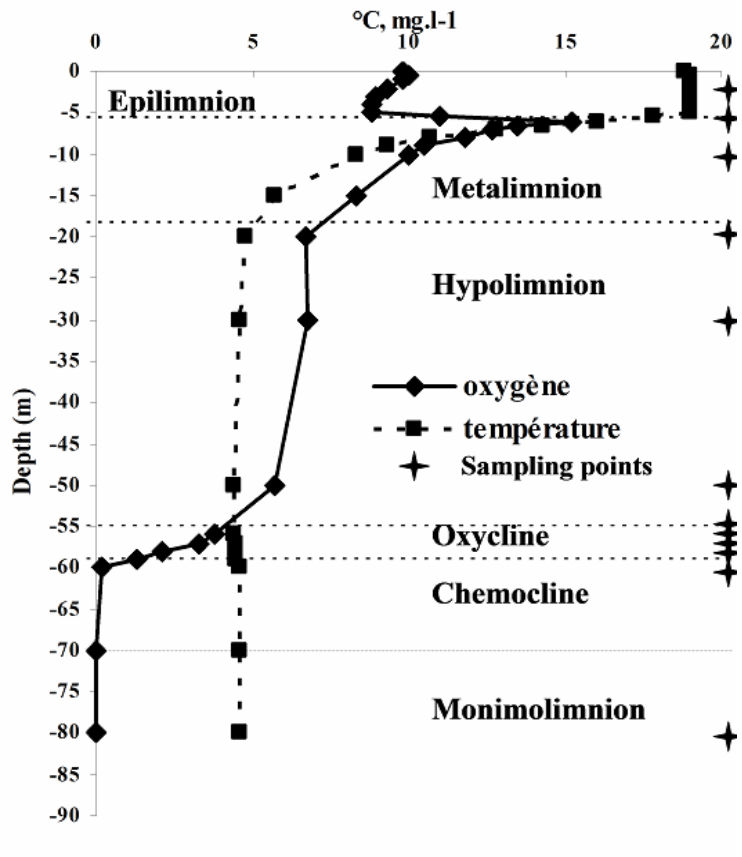


Figure 1. Sampling depths and sampling strategy in the water column of Lake Pavin, including the different depth-related gradients as delineated by the vertical structure of temperature and oxygen concentration profiles during period of strong thermal stratification.

Bacterial Production. Bacterial production (BP) was determined by incorporation of ^{3}H-leucine (final concentration 40 nmol, specific activity = 71 Ci mmol^{-1}, Amersham Biosciences, UK) into bacterial biomass using the microcentrifuge method **(Kirschner and Velimirov 1999)**. Incubation time (30 minutes *in situ* in the dark) was fixed from preliminary experiments conducted on 25 March 2004 **(Colombet et al. 2006)**, and protein precipitation in controls (i.e. 5 % TCA-killed) and fixed essays was aided by adding NaCl (final concentration 3.5 % v/v) at 18 °C for 30 min. After centrifuge-washings, microbial pellets were dissolved in 0.2 mL 1.2 N NaOH at 80 °C for 20 min and Scintillation cocktail (1 mL, Ready Safe, Beckman Coulter) added for radioactivity counting with a Beckman LS 6500 liquid scintillation counter. Leucine incorporation was converted into the number of cells produced by using conversion factors (0.27 x 10^{18} cells mol^{-1} for oxic waters and 0.25 x 10^{18} cells mol^{-1} for suboxic and anoxic waters) determined during preliminary experiments conducted on 25 March 2004 **(Colombet et al. 2006)**.

Phage-infected bacteria and burst size. For viral lytic infection, the frequency of visibly infected cells (FVIC) was obtained from observations under a JEOL 1200EX transmission electron microscope, after ultracentrifugation and uranyl acetate staining. The procedure is detailed elsewhere **(Bettarel et al. 2004, Pradeep Ram et al. 2005)**. For each sample, mean burst size (BS) was estimated from the mean number of viruses in those infected cells which were filled or not with phages.

Potential bacterivores. Samples for enumeration of nanoflagellates (NF) were fixed immediately after sampling with glutaraldehyde (final concentration 1 %). Primulin stained NF collected on 0.8 µm polycarbonate black filters were counted under a Leica DC 300F epifluorescence microscope, using two sets of optic filters: UV light for heterotrophic NF (HNF) and blue light for pigmented NF **(Carrias et al. 1998a)**. The abundances of the overall NF communities have been described and discussed in detail in a companion paper **(Colombet et al., submitted)**. In the present paper, we have considered the abundances of target NF taxonomic groups known as typical bacterivores in Lake Pavin **(Carrias et al. 1996)**. These groups of bacterivores were as follows: choanoflagellates, *Monas-like* cells, and a fraction of undetermined small flagellates (see below) for HNF, and *Ochromonas* spp. for pigmented NF (i.e. mixotrophs). For the group of 'undetermined flagellates', cells with straight and long single flagellum, typical of fungal zoospores (i.e. chytrids) based on both morphological and molecular characterization **(cf. Lefèvre et al. 2007)** and known as non-phagotrophs from previous bacterivory experiments in Lake Pavin **(Carrias 1996, Carrias et al. 1996)**, were excluded from comparisons. These forms represented on average about 25% of the undetermined HNF.

Other variables. The temporal and vertical variations in the water temperature, dissolved oxygen and concentrations of chlorophyll *a*, as well as those in the abundances of viral and microbial communities (i.e. heterotrophic bacteria, picocyanobacteria, autotrophic picoeukaryotes, and heterotrophic and autotrophic nanoflagellates,) have been described and discussed in detail in a companion paper **(Colombet et al. *submitted*)**.

Statistical analysis. Normal distribution of data was checked by Kolmogorov-Smirnov test. Because not all the data sets followed normal distribution, we applied log transformation to meet the requirements for parametric statistics. Two-way analysis of variance was used to test for the effects of seasons [i.e., spring (April to June), summer (July to September), and

autumn (October to December)] and depth-related gradients as represented by the different layers sampled (i.e., epi-, meta-, hypolimnion, oxycline, chemocline, and monimolimnion). In addition, a matrix of data was produced and analysed by means of principal component analysis to identify combination of variables that account for the largest amounts of the total variance observed in different strates of the lake, with particular emphasis on viral lytic activity. Potential relationships among variables were tested by Pearson correlation analysis for each sampled layer. All statistical analyses were performed using MINITAB 12 and SYSTAT 10.

Results

Heterotrophic bacterial production (BP). BP fluctuated from 3.8 to 73.7 x 10^6 bacteria L^{-1} h^{-1} (mean = 21 x 10^6 bacteria L^{-1} h^{-1}). The lowest mean value of BP occurred in the hypolimnion (16.8 x 10^6 bacteria L^{-1} h^{-1}) and the highest ones in the deep layers (i.e. from the oxycline downwards, > 20 x 10^6 bacteria L^{-1} h^{-1}), but the effect of depth-layers was not significant (ANOVA, $p > 0.05$). This contrasts with the effect of sampling time which was highly significant (ANOVA, $p < 0.001$, F-value = 99), with a decreasing production with seasons, from spring (mean = 40 x 10^6 bacteria L^{-1} h^{-1}) to summer (mean = 13 x 10^6 bacteria L^{-1} h^{-1}) and autumn (mean = 9 x 10^6 bacteria L^{-1} h^{-1}) (Fig. 2A). There was no significant interaction between seasons and depth-layers.

Viral activity. The frequency of visibly infected cells (FVIC) fluctuated from 0 to 3.1 % (mean = 0.8 %), corresponding to a viral-induced bacterial mortality of 0 to 43.2 % of bacterial production, according to the formula proposed by **Weinbauer et al. (2002)** and **Binder (1999)**. In contrast to BP, the effects of seasons on FVIC were not significant (ANOVA, $p > 0.05$) while the effects of depth-layers were (ANOVA $p < 0.001$, F-value = 22). Mean FVIC decreased with depth, from > 1 % in the surface waters to < 0.5 % in the monimolimnion. Maxima occurred in April in the hypolimnion and in October in the epilimnion (Fig. 2B), accounting for the significant interaction (ANOVA, $p < 0.001$, F-value = 7) between season and depth-layer effects.

Infected cells contained between 7 and 127 viruses $bacteria^{-1}$, with an overall mean burst size of 31 viruses $bacteria^{-1}$. The effects of seasons (ANOVA, $p < 0.01$, F-value = 4) and depth-layers ($p < 0.001$, F-value = 10) were significant, although both factor did not interacted in predicting variance in burst size measurements. Highest seasonal values occurred

in April in the meta- and in the hypolimnion, in July in the hypolimnion, and in October from the hypolimnion to the chemocline (Fig. 2C).

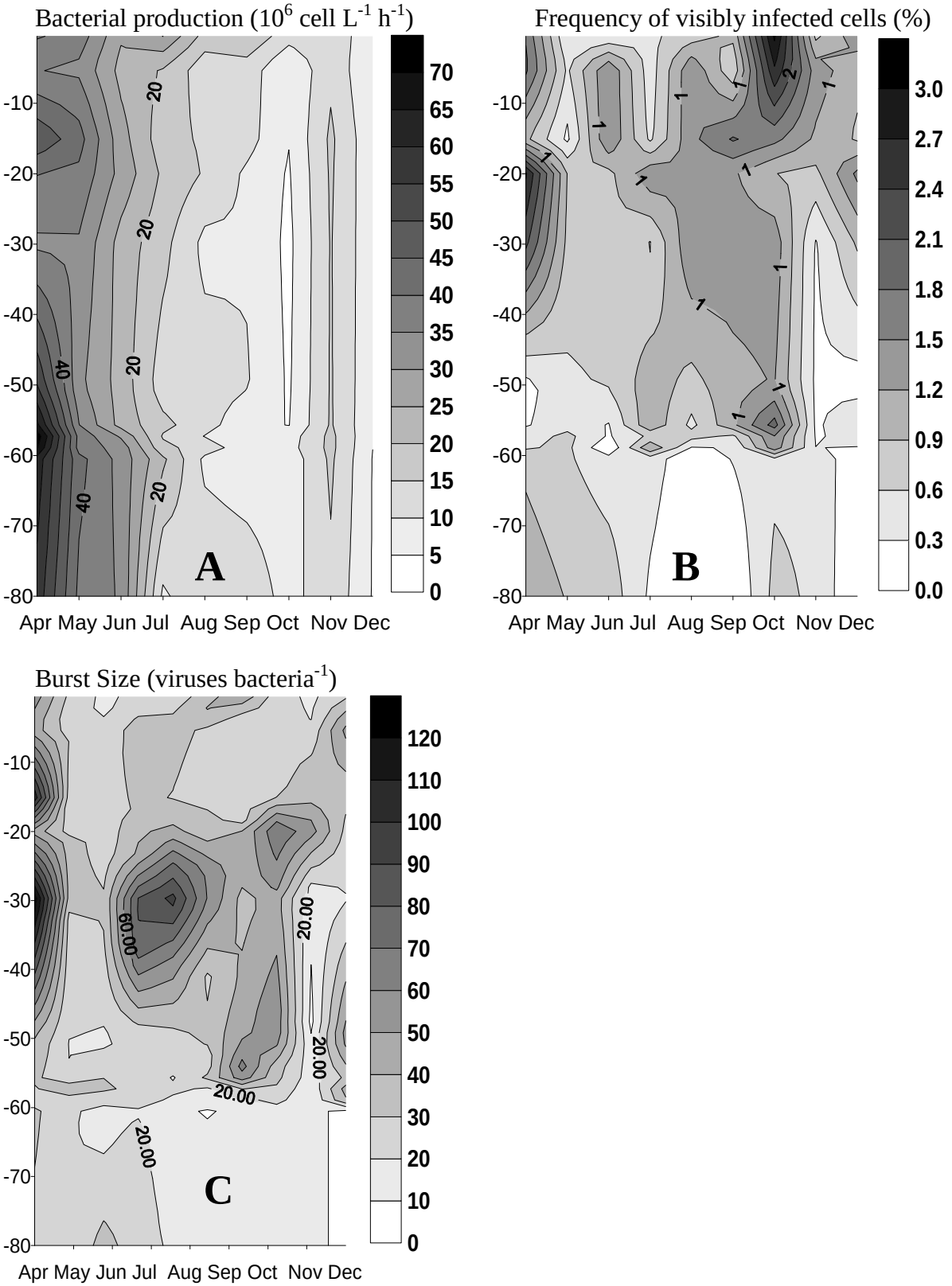


Figure 2. Seasonal and depth-related variations in heterotrophic bacterial production (A), frequency of visibly infected cells (B) and burst size (C) in Lake Pavin, April-December 2005.

Potential bacterivores. The seasonal and depth-related abundance of heterotrophic flagellates considered as bacterivores based on previous studies **(Carrias 1996, Carrias et al. 1996)**, and the relative seasonal abundances (as percent of total) of the target taxonomic groups, are depicted in Figure 3. Total abundances fluctuated from 0 to 1.5 x 10^3 cells ml^{-1} (mean = 0.5 x 10^3 cells ml^{-1}), with very few or almost no cells occurring in the deepest layers of the lake, i.e. from the chemocline. The seasonal abundances of the potential bacterivores indeed peaked in the mixolimnic layers, particularly during the spring time (Fig. 3A). Both the effects of seasons (ANOVA, $p < 0.03$, F-value = 0.6) and of depth-layers ($p<0.001$, F-value = 33) were significant and there was no interaction between the two parameters in explaining the variance in the abundance of potential bacterivorous communities.

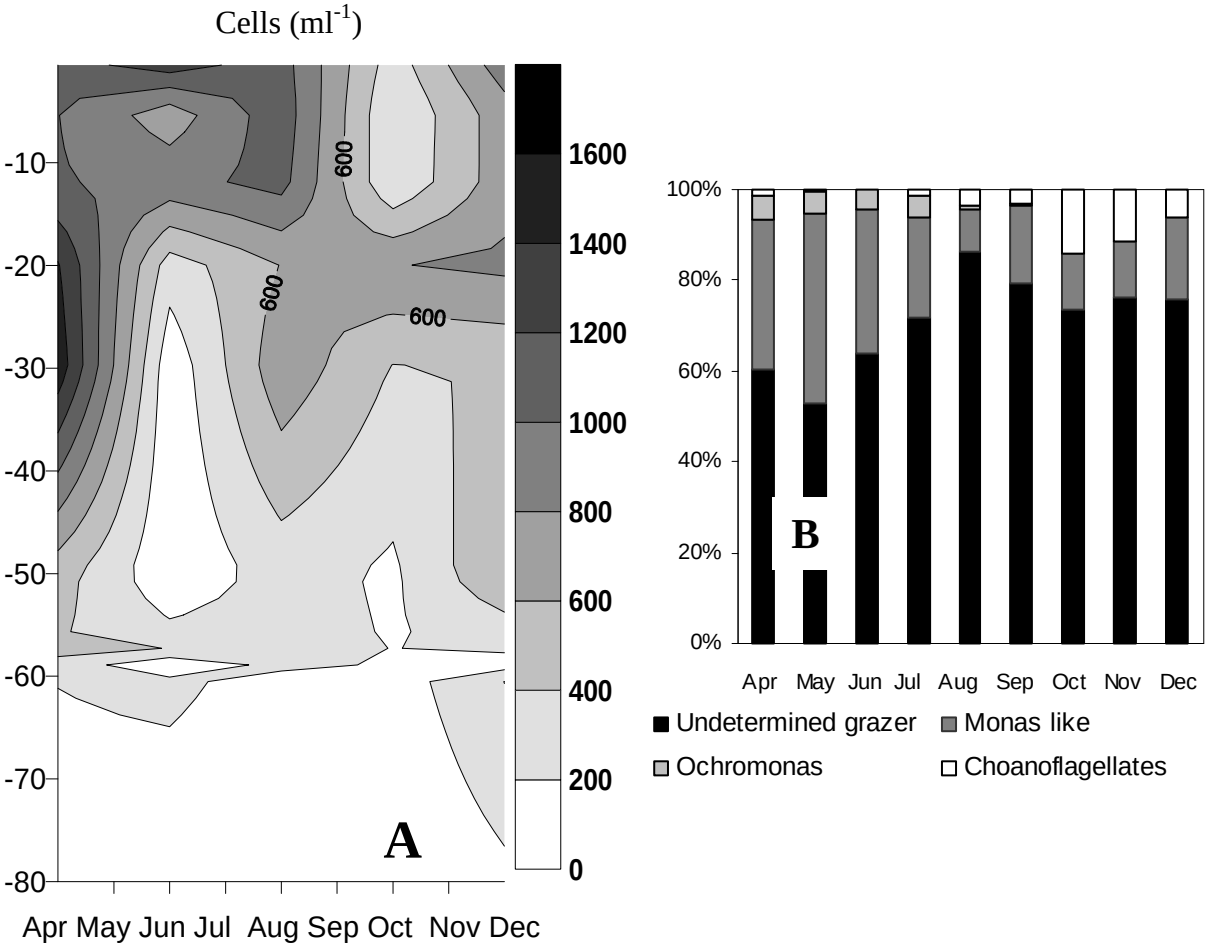


Figure 3. Seasonal and depth-related variations in the abundance of heterotrophic nanoflagellates specifically targeted as potential bacterivores (A), and in the relative abundances (as percent of total) of different taxonomic groups in Lake Pavin, April-December 2005. The characterization of potential bacterivores was based on a previous seasonal study on protistan bacterivory in Lake Pavin **(Carrias et al. 1996)**.

For all sampled depths where they occurred, these communities were largely dominated by the group of 'undetermined grazers' which represented 50 to 85 % of the total abundance. The dominance of this category of grazers was accentuated in summer and autumn, while in spring *Monas*-like cells were relatively abundant, representing around 40 % of the total abundance. The minor grazer groups (< 20 % of total abundance) succeeded each other seasonally, from *Ochromonas* spp. in spring-summer to choanoflagellates in summer-autumn (Fig. 3B).

Correlations and principal component analysis (PCA). According to depth-layers, 80 % of the total number of significant correlation coefficients between the measured viral lysis parameter (i.e. FVIC) and other biological variables of interest were calculated in the deep layers of the lake, from the Hypolimnion downwards. Particularly, FVIC was correlated with bacterial abundance in the hypolimnion and in the oxycline, and with bacterial production in the oxycline, chemocline and monimolimnion. These correlations with bacteria variables were negatives in the oxycline but positives in the other layers. FVIC was also positively correlated with burst size in the hypolimnion, oxycline and chemocline, and similarly with the abundance of potential bacterivores and with chlorophyll *a* concentration in the hypolimnion (Table 1).

Table 1. Results of Pearson correlation analysis used to test for correspondence between temporal changes in the frequency of visibly infected cells (FVIC) and the other estimated variables in six different depth-layers of Lake Pavin[a]. Only significant correlations are given (blanks mean not significant).

FVIC versus :	Epilimnion	Metalimnion	Hypolimnion	Oxycline	Chemocline	Monimolimnion
Bacterial abundance			0.44	- 0.35		
Bacterial production				- 0.34	0.78	0.71
Burst size			0.43	0.41	0.91	
Bacterivores			0.51			
Chloropyll a			0.47			

[a] The number of degrees of freedom depend on the number of depths sampled per layer, in triplicates for biological variables. $P < 0.001$, Blanks = not significant.

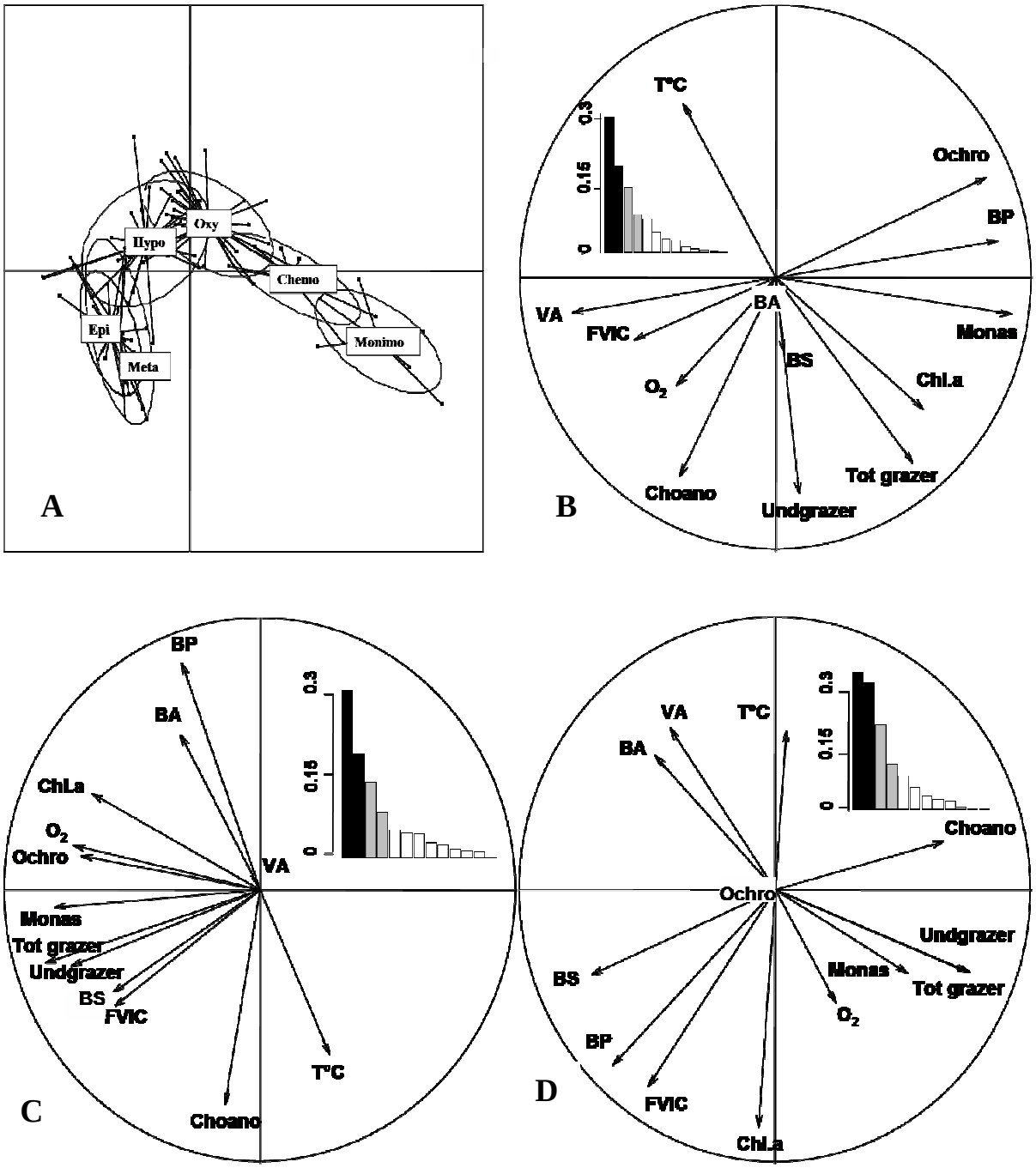


Figure 4. Results of the principal component analysis (PCA) obtained from the observations made for each of the six sampling depth-layers (A), and from the main variables under study in three groups of these depth-layers: epi- and metalimnion combined (B), hypolimnion and oxycline combined (C), and chemocline and monimolimnion combined (D). Abbreviations are as follows: temperature (T°C), Oxygen concentration (O_2), Chlorophyll *a* concentration (Chl.a), the abundances of viruses (VA), bacteria (BA), potential bacterivores (Tot grazer) and the related taxonomic groups [*Monas*-like cells (Monas), Choanoflagellates (Choano), *Ochromonas* (Ochro), and undetermined grazers (undgrazer)], bacterial production (BP), burst size (BS), and the frequency of visibly infected cells (FVIC). Horizontal X-axis = Axe

1, Vertical Y-axis = Axis 2. Inset histograms in B-D represent fractions of variance (Y, %) which are explained by the different PCA axes (X, from Axis 1 onwards).

The plot of field observations from PCA showed that the seasonal distributions of variables under study were dependent to the vertical gradients. According to the locations of the different depth-related layers along the two major axes of this plot, we have distinguished three groups of two layers each along the water column: epi. and metalimnion, hypolimnion and oxycline, and chemocline and monimolimnion (Fig. 4A). In the related plots of the descriptors, the depicted axes 1 and 2 explained about 50-65 % of the total variance and FVIC was located in between the negative ends of the two axes in all plots. However, the associated variables differed markedly from one plot to another, with bacterial production rotating from the extreme positive end of axe 1 in the surface waters (i.e. epi- and metalimnion plot), to the extreme positive end of axe 2 in the intermediate layers (hypilimnion and ocycline), before closely approaching the FVIC position in the deepest waters (chemocline, monimlimnion). Major explaining variables for FVIC mainly included viral abundance in the surface layer plot (Fig. 4B), burst size and grazers in the intermediate layer plot (Fig. 4C), and burst size and bacterial production in the deepest layers (Fig. 4D).

Discussion

General considerations.The present study is an attempt to analyse the seasonal changes in viral bacteriolysis together with those in prokaryotic production and potential bacterivores in the deep meromictic mountain Lake Pavin, including for the first time the whole water column and the related deep gradients. Previous studies on biological limnology in Lake Pavin were restricted to the mixolimnion above 40 m depth **(Carrias *et al*., 1996, 1998ab; Bettarel *et al*., 2003; Lepère *et al*., 2006; Lefèvre *et al*., 2007)**, ignoring the deeper layers which contain about the half of the total volume of water in the lake. These studies include a study conducted in 2000 in Lake Pavin on similar topic as herein, but with a sampling strategy restricted to only three depths (i.e. sampled depths: 5, 10, and 35 m) located in the mixolimnion of the lake **(Bettarel et al. 2004)**. In the present study, our mains results in the mixolimnion generally were quite similar to those reported and discussed in **Bettarel and coauthors**. Accordingly, in the following discussion, emphasis will be given to the undersampled deeper layers of the lake and the related original, additional information in the context of aquatic viral ecology.

Viral infectivity and the deep-related variability

The frequency of visibly infected cells (FVIC). We have used the whole cell method for the determination of FVIC. This approach has been submitted to critics and can yields estimates with relatively large errors **(Bettarel et al. 2004)**. For example, at least 600 and up to 1800 bacterial cells were inspected per grid during the present study, for a number of visibly infected cells between 0 and 50 cells. However, our FVIC levels (i.e. 0 - 3.1 %) were within the typical range (i.e., < 5 %) reported in pelagic systems using the same methodological approach **(Wommack and Colwell 2000)**. In **Bettarel et al. (2004)**, mixolimninic FVIC varied between 0.3 and 4.1% and peaked at 10 m in spring and at 5 and 35 m in autumn but with no significant differences between the three sampled depths. This differed from the findings in the present study where more depths were sampled in the mixolimnion, and the depth-related gradients considered in the whole water column. Indeed, in the present study, the effects of depth-layers but not of seasons on FVIC were highly significant, although the FVIC range (< 3.1 %) was relatively narrower than that (< 4.1 %) reported previously in the study conducted in 2000 in the mixolimnion by **Bettarel and coauthors**. This comparison indicates an apparent mismatch in the seasonal and depth-related variability of viral infectivity in Lake Pavin between the two studies, which can be related to interannual differences in viral proliferation, or to differences in sampling strategies adopted in the two studies.

The latter explanation is most likely because for each sampling time, the coefficients of variations around FVIC mean values in this study (range 40 – 81 %) were largely higher than those (< 25%) in **Bettarel et al. (2004)**, due to the sharp decreased in FVIC with depths, i.e. as revealed by the present study where the entire water column was sampled. Although a contrasted pattern in the vertical distribution of FVIC was occasionally reported on 20 April 2004 in Lake Pavin (**Colombet et al. 2006)**, the above explanation is further strengthened by the facts that the seasonal depth-related patterns reported in **Bettarel et al. (2004)** in the mixolimnion of Lake Pavin, particularly the peaks noted in spring and autumn with no significant depth-related difference, were also found in the present study, and in other lakes as well **(cf. Bettarel et al. 2004)**. Furthermore, FVIC values in this study were relatively lower in the subsurface waters at 0.5 m depth, primarily during spring and summer times (Fig. 2B), corroborating the hypothesis on UV-induced inactivation of surface viruses that is also discussed in **Bettarel et al. (2004)**, based on supporting literature in the context of world aquatic ecosystems **(e.g. Hofer and Sommaruga, 2001; Suttle and Chen 1992, Noble and Fuhrman 1997 and Garza and Suttle 1998)**. Above comparisons thus clearly highlight the

importance of the deepest layers in viral activity and ecology, and the related processes in deep lake ecosystems such as Lake Pavin.

Burst size (BS). The mean numbers of intracellular viruses observed per infected bacterial cell in the present study (range 7 – 127, median value = 67) were apparently higher compared to those (range 10 – 55, median value = 32) reported in the mixolimnic study by **Bettarel et al. (2004)**. However, the overall mean BS in both studies were more comparable than the range and median values, with a substantially higher coefficient of variation around mean in the present study (mean ± CV = 31 ± 58) than in **Bettarel and coauthors** where seasonal BS were about constant (mean ± CV = 25 ± 4). This was clearly due to the inclusion of deep waters in the present study where BS were significantly (ANOVA, $p < 0.001$) lower than in the mixolimnic waters. No clear seasonal patterns occurred in both studies, corroborating similar results reported in Lake Mono where BS varied from 10 to 55 viruses bacteria^{-1} (**Brum et al. 2005**). Our findings on the spatial variability of viruses in Lake Pavin contrast with reports from **Weinbauer and Höfle (1998a)** and **Weinbauer et al. (2003a)** where BS were higher in the anoxic waters than in the surface waters of Lake Pluβsee and of the Baltic Sea, respectively. Unexpectedly, the general occurrence of higher BS in the surface than in the deep waters of Lake Pavin also contrasted with the vertical pattern in bacterial production which was, on average, higher in deep that in surface waters. This seems unreasonable because BS is related to bacterial cell biovolume (**Weinbauer and Peduzzi 1994, Hennes and Simon 1995, Weinbauer and Höfle 1998b**) and both variables are known to increase with increasing bacterial growth rate (**Middelboe 2000**). As previously given in **Bettarel et al. (2004)**, this assumption is realistic only if the viral populations have about the same capsid size.

From both the present study and a preliminary one conducted in Lake Pavin (cf. **Colombet et al. 2006**), evidence is provided than this is untrue because of the high morphometric diversity of pelagic viruses in this lake, particularly in relation to the depth-related gradients. Indeed, viral communities in the permanently anoxic monimolimnion of Lake Pavin comprised a substantial number of atypical bacteriophage forms, larger in capsid size (> 60 nm), and with more complex spatial conformation (Fig. 5), compared to the mixolimnic viruses which were more typical of the world aquatic free-occurring bacteriophages dominated by small (capsid size < 60 nm) Sipho-, Podo- and Myoviridae (**Sime-Ngando et al. 2003**). In addition, an analysis of viral diversity based on the distribution frequency of capsid sizes through the whole water column of Lake Pavin has indicated that viruses were

apparently typical and more diversified in the monimolinion than in the surface waters, although the study was based on one-date sampling (see Figure 2A in **Colombet et al. 2006**). This finding corroborates a recent study in Lake Pavin which has unveiled highly diverse prokaryote communities in the monimolimnion of Lake Pavin (**Lehours et al., 2005**), i.e. the sole potential hosts for viral proliferation in this particular habitat (**Colombet et al., submitted**). Because prokaryotic specific cell volume is similar through the water column at about 0.1 μm^3 (**Sime-Ngando et al. 1991**, unpublished data), it is thus likely that deep anoxic viruses in Lake Pavin are dominated by endemic, typical populations, with different morphometry and diversity characteristics compared to surface viruses. This may help explained why burst sizes in this lake decreased significantly in the deep layers, and further stress the ecological importance of these layers.

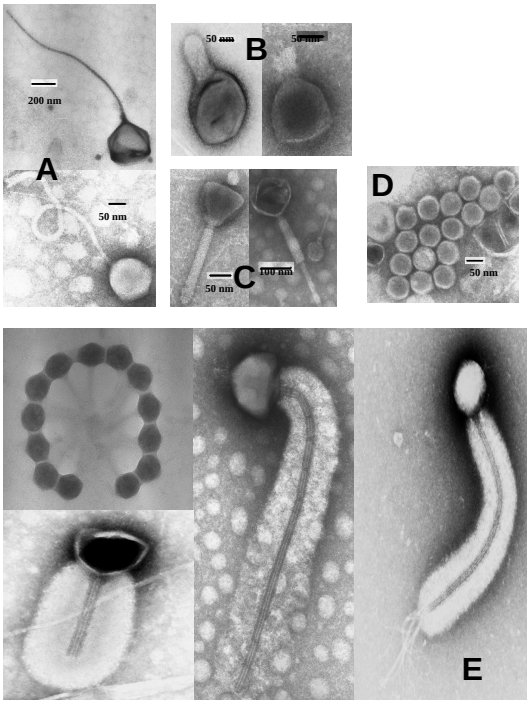


Figure 5. Surface and deep water viruses are apparently different in Lake Pavin: overview of the general morphotypes of viruses in the water column, including the characteristic free-occcuring pelagic forms [Sipho- (A), Podo- (B), Myoviridae (C), and untailed phages (D)], which are dominant, primarily in the mixolimnion, and the more atypical forms or complex spatial arrangement/conformation (E) which occurred mainly in the permanently deep anoxic waters.

***Empirical forcing of depth-related gradients in viral lytic activity*.**

In a companion paper analysing the seasonal and spatial abundances of viruses and based on multivariate and correlative statistical analyses, an apparent shift in the viral interactions with various autotrophic and heterotrophic microbial components is suggested between the mixo- and the monimolimnion of Lake Pavin. In this paper, viruses were correlated mainly with microautotrophs in the mixolimnion but exclusively with heterotrophic prokaryotes in the monimolimnion where the other microbial communities were apparently constraint by the dark anoxic conditions **(Colombet et al. submitted)**. In the present paper and based on the same type of analyses, the differential functional responses of lytic viruses to the depth-related gradients in their biological environment were confirmed. Indeed, assuming that burst size (BS) and the frequency of visibly infected bacterial cells (FVIC) within prokaryotic communities reflected the lytic activity of viruses, the results of correlation and principal component analyses (PCA) have highlighted the ecological importance of deep waters in the context of aquatic viral ecology.

Almost all significant correlations with BS and FVIC occurred from the hypolimnion downwards, implying mainly prokaryotic variables (abundance, production) as dominant correlates. Indeed, the relative importance of bacterial production in explaining variance in BS and FVIC increased with depth, and prokaryotic variables were exclusive correlates for FVIC and BS from the oxycline downwards. All correlations were positives, at the exception of those between FVIC and prokaryotic variables in the oxycline (Table 1, Fig. 4). This corroborates the hypothesis that this layer is a key transitive microhabitat in Lake Pavin, where the complex mixolimnic biological communities and the related interactions are replaced by a simple viral loop system (i.e. DOM > prokaryote > viruses > DOM) towards the dark and permanently anoxic monimolimnion **(Colombet et al. 2006, submitted)**. In general, oxycline/chemocline layer in Lake Pavin is well known as an important barrier, characterized by particular geochemical properties, that act as a key interface in the vertical flow and recycling of matter through the water column **(Aeschbach-Hertig *et al*., 2002)**. A recent spatial study has shown that, in this layer, there is a complete shift in the composition of the prokaryotic assemblages **(Lehours *et al*., 2005)**, the main and exclusive hosts for the thriving viral communities in the monimolimnion of the lake.

However, the mean seasonal FVIC decreased with increasing depth while the mean seasonal values for the prokaryotic host production increased with depth. In terms of abundances, viruses and bacteria peaked in the metalimnion but also in the monimolimnion **(Colombet et al. submitted)**, a pattern that somewhat also contrasted with that in FVIC. This

was unexpected from the literature data **(Steward et al. 1996, Peduzzi and Schiemer 2004, Pradeep Ram et al. 2005)**, and from the single-date preliminary study conducted in Lake Pavin where viruses and prokaryotes increased with depth in both terms of abundance and activity **(Colombet et al. 2006)**. The above comparison implies that, at a seasonal scale, viral proliferation as estimated from FVIC method in the deepest layers was at its lower potential and do not fully exploited the abundant and rapidly renewing ambient host resources. Otherwise, part of the free-occurring abundant viruses in theses layers was not lytically infective for their ambient hosts, and the fate of free viruses through grazing was unlikely because of the general absence of grazers in the monimolimnion. Similar finding was recently reported and termed 'infection paradox', as a typical feature of freshwater benthic viruses **(Fillipini et al. 2006)**. The mechanisms behind an apparent low infectivity in freshwater benthoviruses are still unknown **(cf. Fillipini et al. 2006)**.

In this study, the discrepancy between high seasonal viral abundance and low FVIC in the deepest planktonic layers of Lake Pavin also appeared difficult to be reconciled. We have shown that deep viruses in Lake Pavin are typical, and there is a probability of the coexistence of different styles of viral proliferation, including chronic, lysogenic or pseudolysogenic production. Evoking inputs of viruses from the surface waters and/or from the sediments is also tempting, although (i) oxycline and chemocline are strong barriers for surface vs deep vertical exchanges within the lake, (ii) the monimolimnion is considered as a physically stable and steady-state environments **(Aeschbach-Hertig *et al*., 2002)**, (iii) where viruses are likely typically different from those in the surface water **(Colombet et al. 2006)**. Because free viruses can maintain their physical integrity and even their infectivity for long time, up to several tens of years **(Suttle 2005)**, it is also likely that a pool of 'long-live' allochthonous viruses may cohabiting with the endemic typical deep viruses which depend on the ambient prokaryotic production. It has been shown that viruses in deep, cold marine waters exhibit particularly low decay rate **(Parada et al. 2007)** and this may be the case for the so-called 'long-live' deep viruses. Greater resistance to infection due to low temperature mediated modifications of viral receptors on the host membrane or of intracellular defence responses can also be prevalent in deep cold waters **(Bohannan and Lenski 2000)**. It is indeed rational that short host generation times promote rapid evolution of phage resistance. A number of plausible reasons could thus account with the observed high abundances of viruses and prokaryotes concomitant with high prokaryotic production but with low lytic activity of viruses in the deepest waters of Lake Pavin.

Compared to the previous studies in Lake Pavin and with other sites where no correlation has been observed between viruses and HNF assemblages considered as the main grazers of pelagic prokaryotes **(cf. Bettarel et al. 2004, Colombet et al. submitted)**, FVIC in the present study was significantly correlated to the 'specific abundance' of potential grazers in the hypolimnion where peak in FVIC coincided with peak in the abundance of *Monas*-like grazers. The later organisms, together with burst size, indeed appeared as strong explaining factors for the seasonal variability in FVIC recorded in the hypolimnion/oxycline layer (Fig. 4). We now know that HNF assemblages in pelagic ecosystems comprise non-bacterivorous species such as fungal zoospores **(Lefèvre et al. 2007)**. In the present study, the interference from these non-bacterivores was minimized by targeting specific groups or taxa known as strict bacterivores or mixotrophs **(Carrias et al. 1996)**. The approach probably has contributed to the observed correlation between FVIC and the abundance of potential bacterivores, although this was observed only in the hypolimnic layer where many of the correlations involving FVIC were calculated (Table 1). It is well known that there are many routes of interactions between viruses and grazers. This has been widely tested from experimental studies **(cf. Pradeep Ram and Sime-Ngando 2008 and references therein)** but only partially from empirical and trophodynamic seasonal data **(cf. Bettarel et al. 2003)**.

Conclusions

This study presents an original deep-related survey of virioplankton lytic activity in relation to prokaryotes and potential bacterivores in a volcanic deep lake, complimenting a companion paper on the abundances and vertical community structure of viral and microorganisms **(Colombet et al. submitted)**. The sampling strategy adopted, with 12 different depths sampled through the 92 m deep water column, was considered representative of the whole water column of Lake Pavin, and the related physico-chemical gradients. Compared to previous studies known from the mixolimnic layers in Lake Pavin, the main findings have highlighted the importance of the deepest layers in viral activity and ecology, and the related processes in deep freshwater ecosystems such as Lake Pavin. Deep waters indeed appeared as an exclusive environment for heterotrophic prokaryotes, whose seasonal activity offers an optimal and unique resource for thriving viral communities, some of which may be typical, endemic to the ambient dark, cold and stable deep-water masses. The exclusive seasonal coupling between viruses and prokaryotes was not obvious from the sole depth-related gradients, because high abundances of viruses and prokaryotes concomitant

with high prokaryotic production but with low lytic activity of viruses, occurred in the deepest waters of the lake. Overall, the main findings based on the functional responses of viruses and prokaryotes to depth gets well around a previous statement that the ecology of the deepest waters of Lake Pavin is essentially driven by the dark viral loop (DOM-prokaryotes-viruses) processes, which can sequester organic matters and nutrients for a long-live turnover time **(Colombet et al. submitted)**. This is in agreement with recent demonstrations from marine systems that meso- and bathypelagic waters are optimal environments for viral survival and proliferation in North Atlantic **(Parada et al. 2007)** and Mediterranean Seas **(Magagnigni et al. 2007)**.

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C. Cryptic phages and lysogenic infections in a deep freshwater lake bacterioplankton

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Running title : Lysogenic infections in Freshwaters

En préparation

Abstract

The imprint of viruses on aquatic microbial ecology ultimately depends on whether the virus is lytic or temperate. However, few attempts have been made to investigate the importance of temperate phages in aquatic ecosystems, primarily in freshwaters. In this study, high resolution vertical sampling was conducted in a deep meromictic lake during nine-month period (April to December 2005), for evidence of experimentally inducible cryptic phages, i.e. prophages, integrated into the genetic material of bacterioplankton. Temperate viruses responded to mitomycin-C used to induce prophages into lytic cycle, i.e. the burst size method. Prokaryotic lysogens were restricted to the oxygenated layers of the water column, with a frequency of lysogenically infected cells (FLC) representing < 0.1 to 11.5 % of the total abundance. No consistent correlation was found between FLC and the physico-chemical parameters (light, salinity, temperature, oxygen, chlorophyll) but with the abundances of bacteria and free viruses, and with the frequency of lytically infected cells (FIC) which was higher (range <0.1 to 26 %). These correlations were negatives and FLC was detected at a significant level only from under an apparent threshold bacterial abundance of about 5 x 10^6 cells mL^{-1}. In conjunction with the finding that the potential viral production from spontaneous induction of all lysogens represented less that 2 % of viral lytic production, our study provides empirical indications that lysogeny mainly is a strategy for the maintenance of viral genotypic and phenotypic traits in the environment, and can further have significant incidences on the evolutionary ecology of host communities. This is in agreement with recent finding from a sporadic sampling that host availability is prevalent over the physical and chemical environments and favour lytic over lysogenic 'viral lyfe cycles'. Although, we

believe that few available studies on lysogens in aquatic systems preclude any simple extrapolation to the natural conditions, and that further investigations are warranted.

Introduction

The significant importance of viral processes in natural and aquatic environments is now well accepted and increasingly investigated. Basically, viruses have three major 'life' cycles: chronic, lytic and lysogenic infections **(Fuhrman 1999, Sime-Ngando 1997)**. In the chronic cycle, production of progeny viruses is not lethal to the host cell, viruses being released in the ambient environment by extrusion or budding over several host generations. The importance of this viral reproductive strategy in aquatic microbial ecology is completely unknown. In the lytic cycle, phages force the host metabolism to produce numerous progeny viruses that are released in the environment by fatal bursting of the host cell. In lysogenic infection, the lysogenic phage either enters the lytic cycle directly, or integrates into a host genetic material as replicating prophages for several generations, until the lytic cycle is induced, generally by environmental stimuli.

The quantitative importance of lytically infected cells is relatively well documented at the community level in both marine and freshwater pelagic systems **(Sime-Ngando *et al.* 2003, Weinbauer 2004, and references herein)**. Less attention has been given to the importance of lysogens, i.e. cells in the prokaryotic community containing inducible viral genomes known as prophages. In a review paper, **Ackermann and Dubow (1987)** have calculated that nearly 50 % of 1200 bacterial strains examined contained inducible prophages. A sequencing of 82 bacterial genomes, mostly enteric bacteria, has revealed that 62 % are prophage carriers, and that these cryptic prophages can constitute as much as 10-20 % of a bacterium's genome **(Casjens 2003)**. In aquatic environments, the incidence of lysogenic cells has been inferred from induction experiments, typically using mitomycin C **(Jiang and Paul 1996)**. The few studies conducted to date have shown that the frequency of lysogenically infected cells (FLC) ranged from not detectable to over 100 % in marine bacterioplankton communities **(Weinbauer 2004 and references herein)**, from 0.1 to 16 % in tropical and temperate freshwaters **(Tapper and Hicks 1998, Colombet *et al.* 2006, Bettarel *et al.* 2006)**, and from 2 to 89 % in Antarctic lakes **(Lisle and Priscu 2004, Säwström *et al.* 2006)**.

Almost all these studies were sporadic (i.e. based on few sampling dates or stations), including, to our knowledge, only three studies on long-term seasonal variations **(Cochran and Paul 1998, Williamson *et al.* 2002, Säwström *et al.* 2006)**, and two on depth-related

gradients **(Weinbauer *et al.* 2003, Colombet *et al.* 2006)**. As a consequence, our knowledge of the spatio-temporal variability in lysogenic infections and the potential regulating factors in aquatic systems is limited. The available data in the literature is even somewhat contradictory. For example, in the Tampa Bay (Florida, USA), **Cochran and Paul (1998)** have detected lysogeny only when the water temperature exceeded 19°C, while in the same site **Williamson *et al.* (2002)** have successfully induced lysogens during winter months at temperature < 5 °C. **Weinbauer and Suttle (1996, 1999)** found no correlation between the percentage of lysogens and temperature. **Tapper and Hicks (1998)** recorded highest FLC in UV-exposed subsurface layer of Lake Superior, while **Weinbauer and Suttle (1999)** showed that solar radiation is a strong inducing factor for prophages in coastal and offshore marine bacterioplankton. **Colombet *et al.* (2006)** have reported that FLC decreased with depths but with increasing viral resources (i.e. prokaryote abundance and production), and **Wilcox and Fuhrman (1994)** and **Jiang and Paul (1996)** did not observed any evidence of solar-mediated induction of lysogens in aquatic bacterial communities. **Jiang and Paul (1994, 1998)** reported maximal FLC in oligotrophic environments whereas **Ortmann *et al*. (2002)** found that the induction of lysogens was independent of the trophic status. Finally, **Weinbauer *et al.* (2003)** and **Colombet *et al.* (2006)** provided evidence that FLC rose in conditions of low host abundance and productivity, whereas **Ortmann *et al*. (2002)** reported high induction of lysogens at high host growth rate.

The question of a better understanding of the contribution of lysogens to bacterial standing stocks and the related processes is thus critical. This question is particularly worth because, in the general context of aquatic microbial ecology, the functional importance of lysogeny is fundamentally different to that of lytic viruses. Lytic cycle, as a cause of bacterial mortality, releases and increases the retention times of organic substrates and nutrients in the water column **(cf. Middelboe and Lyck 2002)**, and influences greatly the structure of the host communities **(see reviews in Wommack and Colwell 2000, Weinbauer 2004)**. Lysogeny rather allows a cryptic maintenance and dissemination of viral phenotypic and genetic characters and provides competitive advantages to the host cells, including resistance to antibiotics, changes of antigenic traits or acquisition of virulence factors **(Waldor and Mekalanos 1996)**. The impact of viruses on ecological and biogeochemical processes **(Wilhelm and Suttle 1999)** ultimately thus depends on whether the virus is lytic or temperate. To assess this, we need studies that integrate long-term seasonal and spatial variations of lysogenic cells compared to lytically infected cells. To our knowledge, only short-term **(Ortmann *et al.* 2002, Weinbauer *et al.* 2002, Bettarel *et al.* 2006)** or spatial

(Weinbauer *et al.* 2003, Colombet *et al.* 2006) attempts have investigated simultaneously these two viral 'life' cycles in natural waters. Such information is essential to understand the whole ecological significance of viruses in aquatic environments.

The general objective of this study was to examine, for the first time, the seasonal variations of lysogenically infected cells across the vertical physico-chemical and biological gradients in the deep freshwater Lake Pavin (France). Specifically, we aimed (i) to analyse the fluctuations in the quantitative importance of lysogens, (ii) to tackle the potential empiric factors capable of influencing this dynamics, and (iii) to compare the FLC dynamics to those of lytically infected cells. The deep meromictic Lake Pavin (Zmax=92m) has a permanently anoxic bottom layer (60 to 92m) and presents a succession of physico-chemical (**Aeschbag-Hertig et al. 2002)** and biological **(Colombet et al. submitted a and b)** vertical gradients that offer a unique model for such studies.

Materials and methods

Study site and sample collection. Samples were collected in Lake Pavin (altitude 1197 m), a meromictic and dimictic oligomesotrophic lake located in the French Massif Central, that experiences partial overturn. It is a typical crater mountain lake characterized by a maximum depth of 92 m and low surface (44 ha) and catchment (50 ha) areas. A characteristic feature of the physical structure of the lake is the existence of a distinct and permanent chemocline between about 60 and 70 m depth, that separates the seasonally mixed mixolimnion from the monimolimnion. The latter layer is characterized by an elevated conductivity and salinity as well as complete anoxic conditions **(Aeschbach-Hertig *et al.* 2002)**. Above the chemocline there is an oxic/anoxic interface between 50 and 60 m depths.

Samples were collected monthly (systematically between 09:00 and 10:00 AM), from April to December 2005 at a central deepest location in the lake by using an 8-liter Van Dorn bottle. A total of 12 depths was sampled in the different physico-chemical layers and considered as representative of the whole water column; namely in the epi- (0.5 and 5 m), meta- (12 m), hypolimnion (20, 30 and 50 m), and in the oxycline (56, 57, 58, 59 m) for the mixolominion, as well as in the chemocline (60 m) and in the monimolinmion (80 m). All samples were collected an analysed in triplicates. For suboxic and anoxic layers, living samples were contained and processed (i.e. for bacterial production and FLC incubations) in sterile serum bottles sealed with rubber and aluminium crimp caps and flushed with N_2.

Physico chemical variables. Water temperature and dissolved oxygen profiles were measured *in situ* each 0.5 m from the surface to 80 m with an oxycal-SL 197 multiparameter probe (WWT, Limonest, France). Chlorophyll *a* concentrations in the 12 sampling depths were determined spectrophotometrically from particles (0.5 to 2 L) collected on Whatman GF/F filters. Pigments were extracted in 90 % acetone overnight in the dark at 4 °C, and concentrations calculated using **SCOR-UNESCO (1966)** equations.

Bacterial and viral abundances. Subsamples were fixed with 0.02 µm filtered glutaraldehyde (final concentration 2 % v/v) immediately after sampling, and filtered (<15 kPa vacuum) through 0.02 µm pore size Anodisc filters (Whatman, Maidstone, UK) which were mounted between microscope slides and glass cover slips using a mixture of 80 % AF1 Citifluor (Citifluor, London, England) and 20 % Vectashield (Vector Laboratories, Inc., Burlingame, U.S.A.) as antifading mounting medium. Counts were done under a Leica DC 300F epifluorescence microscope following staining with SYBR Green I fluorochrome (Molecular Probes Europe, Leiden, Netherlands) as described by **Noble and Fuhrman (1998)**. When not analysed immediately, slides were stored at –20 °C until counting. Bacteria were distinguished from virus-like particles (VLPs) on the basis of their relative size and brightness. A blank was routinely examined to control for contamination of the equipments and reagents.

Bacterial Production. Bacterial production (BP) was determined by the incorporation of ^{3}H-leucine (final concentration 40 nm, specific activity = 71 Ci mmol^{-1}, Amersham Biosciences, UK) into bacterial biomass using the microcentrifuge method **(Kirschner and Velimirov 1999)**. Incubation time (30 minutes *in situ* in the dark) was fixed from preliminary experiments conducted on 25 March 2004. Protein precipitation in controls (i.e. 5 % TCA-killed) and fixed-essays was aided by adding NaCl (final concentration 3.5 % v/v) at 18 °C for 30 min. After centrifuge-washings, microbial pellets were dissolved in 0.2 mL 1.2 N NaOH at 80 °C for 20 min and Scintillation cocktail (1 mL, Ready Safe, Beckman Coulter) added for radioactivity counting with a Beckman LS 6500 liquid scintillation counter. Leucine incorporation was converted into the number of cells produced by using empirical conversion factors (0.27 x 10^{18} cells mol^{-1} for oxic waters and 0.25 x 10^{18} cells mol^{-1} for suboxic and anoxic waters) determined during preliminary experiments conducted on 25

March 2004, by regressing bacterial numbers against ^{3}H-leucine incorporation rates from a dilution-growth experiment, i.e. the derivative method **(cf. Jugnia *et al.* 2000)**.

Frequency of infected cells and maximal burst size. For viral lytic infection, the frequency of infected cells (FIC) was calculated from the frequency of visibly infected cells (FVIC) obtained from observations under a JEOL 1200EX transmission electron microscope, following ultracentrifugation and uranyl acetate staining. The procedure is detailed elsewhere **(Bettarel *et al*. 2004)**. FIC was calculated from FVIC (as a percent) using the formula FIC = 9.524 FVIC – 3.256 **(Weinbauer *et al.* 2002)**. For each sample, maximal burst size (MaxBS) was estimated from the mean number of viruses in infected cells which were totally filled with phages. Lytic viral production, i.e. the number of lytic phages produced by bacterial communities per time and volume units, was estimated by multiplying bacterial cell production by FIC/100 and MaxBS **(Weinbauer *et al.* 2003)**.

Frequency of lysogenically infected cells. The frequency of lysogenically infected cells (FLC) was determined from the induction of prophages using mitomycin C **(Jiang and Paul 1996)**. Mitomycin C was added to samples (final concentration 1 µg mL^{-1}) in 20 mL sterile serum bottles, and untreated samples served as controls. The incubation time (24 hours *in situ*) was fixed from a 76-hour time series preliminary experiment conducted on 25 March 2004. Subsamples were removed with syringes at t0 and t24 hours and fixed with glutaraldehyde for viral and bacterial counts. FLC was estimated from viral abundances in mitomycin C treated (VAm) and control (VAc) incubations, and bacterial abundance (BAt0) and MaxBSt0 in original samples: FLC = 100[(VAm – VAc)/(MaxBSt0 x BAt0) **(Weinbauer *et al.* 2003)**. Prophage replication rates, i.e. the number of prophages produced by bacterial communities per time and volume units, were estimated as the bacterial cell production times FLC/100 **(Weinbauer *et al.* 2003)**, assuming there is one additional viral genome per cell division **(Marsh and Wellington 1994)**.

Statistical analysis. Normal distribution of data was checked by Kolmogorov-Smirnov test. Because not all the data sets followed normal distribution, we applied log transformation to meet the requirements for parametric statistics. Two-way analysis of variance was used to test for the potential effects of seasons [i.e. spring (April to June), summer (July to September), and autumn (October to December)] and of depth-related gradients (epi-, meta-, hypolimnion, oxycline, chemocline and monimolimnion) on the different biological variables. All

significant differences given to the readers thereafter were at $p < 0.001$. Potential relationships among variables were tested by Pearson correlation analysis for each sampled layer. All statistical analyses were performed using MINITAB 12 and SYSTAT 10. Data on viral abundance and bacterial abundance and production are fully presented and discussed in detail elsewhere **(cf. Colombet et al. submitted a and b)**. They are briefly presented and used herein as accompanying variables in relation to the variability in lysogens.

Results

Temperature, dissolved oxygen, chlorophyll a. The changes in water temperature and oxygen contents were typical of a deep meromictic dimictic temperate lake. Thermal stratification started in May and ended in November, with partial overturns and homogenization of the water column temperature in April and December (Fig 1A). The oxic/anoxic interface was located between about 55 and 59 m (Fig. 1B). The temperature and oxygen profiles confirmed that the sampled depths included the different physico-chemical layers (i.e. epi-, meta-, hypolimnion, oxycline, chemocline, and monimolimnion) and were thus considered representative of the whole water column of the lake (Fig. 1AB). Chlorophyll *a* concentrations (Chl) fluctuated from about 1 to 14 $\mu g L^{-1}$, with a significant effect of depth due to the fact that values in the three uppest layers of the lake were higher than in the three deepest layers (Fig. 1C). The effect of seasons on Chl was also significant, with peak concentrations in spring months. Pigment concentrations were particularly high in the month of May, in the whole water column (Fig. 1C).

Virus-like particles (VLPs) and bacterial abundance. Viral abundance (VA) fluctuated from 0.8 to 5.1 x 10^7 VLPs mL^{-1}, and the depth-related mean values ranged from 1.4 to 3.7 x 10^7 VLPs (overall mean = 2.2 x 10^7 VLPs mL^{-1}). Differences between layers were significant, mainly due to the highest values recorded in the metalimnion and in the monimolimion (Table 1, Fig. 2A). These values occurred in summer for both layers and in autumn for the monimolimnion, explaining the significant effects of seasons on VA. However, the seasonal variability in VA was visibly weaker from the lower hypolimnion to the oxycline and chemocline (i.e. from 30 to 60 m depth), compared to the upper layers and to the monimolimnion of the lake (Fig. 2A). Bacterial abundance (BA) fluctuated from 2.0 to 28.3 x 10^6 cells mL^{-1} (mean = 6.0 x 10^6 cells mL^{-1}) and peaked in August in the metalimnion and in the monimolimnion where the highest seasonal abundance was noted, largely higher (about 2 fold) than the metalimnic peak (Tables 1, Fig. 2B).

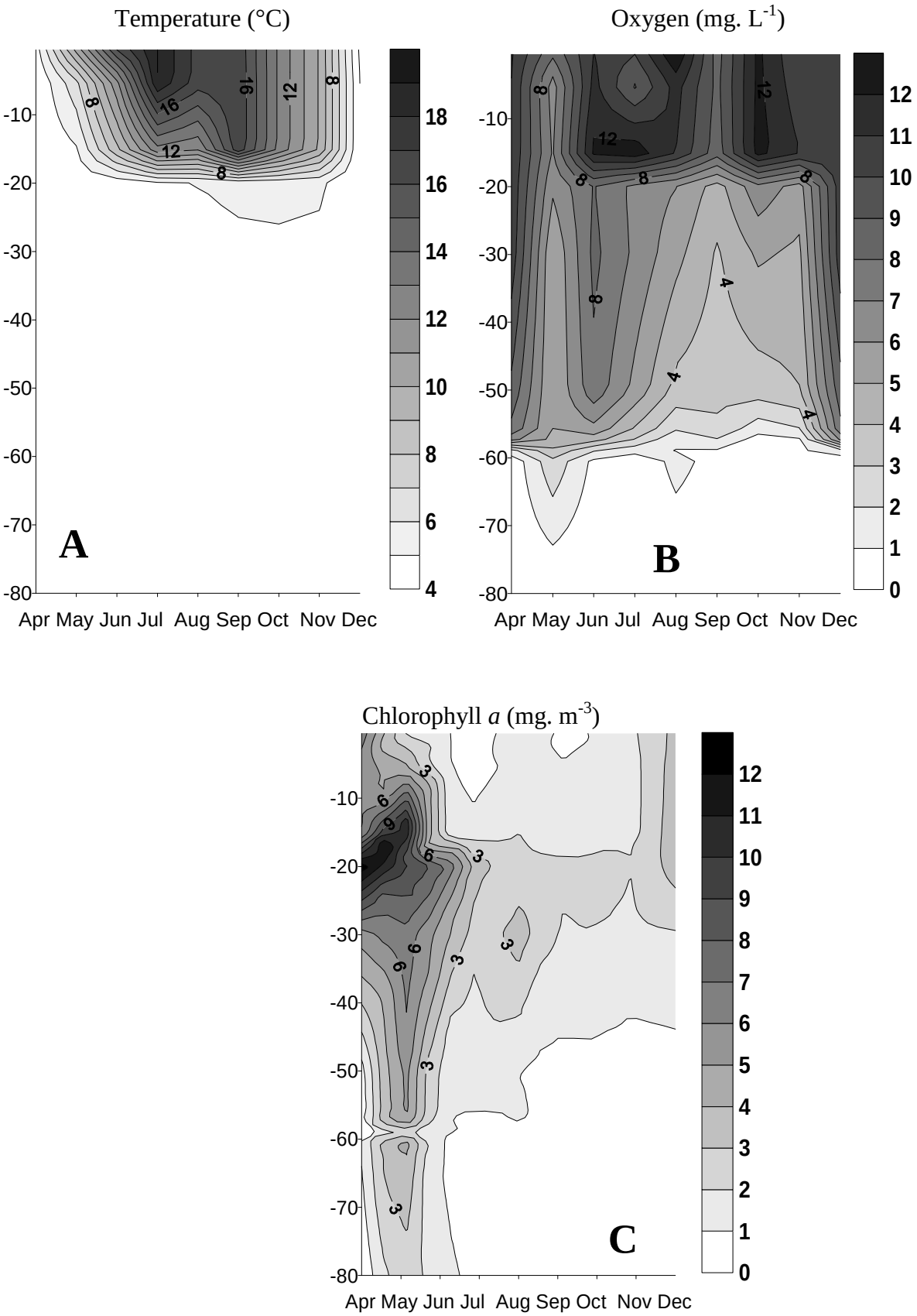


Figure 1. Seasonal variations of temperature (A), oxygen (B) and Chlorophyll *a* in the water column of Lake Pavin, April – December 2005.

Table 1. Mean (CV) of biological variables under study in Lake Pavin, April to December of 2005[a]

Layer	Depth	BA (10^6 bact. mL^{-1})	VA (10^7 VLP mL^{-1})	BP (10^6 bacteria L^{-1} h^{-1})	MaxBS	FIC (%)	Lytic viral production (10^7 VLP L^{-1} h^{-1})	FLC (%)	Prophage replication rate (10^7 VLP L^{-1} h^{-1})	FIC/FLC
E	**-0,5**	5.7 (24)	2.4 (36)	21.6 (52)	49.7 (42)	7.3 (78)	9.1 (161)	0.5 (178)	0.012 (194)	14.6
	-5	6.1 (34)	2.7 (32)	17.8 (62)	53.2 (31)	8.9 (56)	8.2 (138)	0.4 (114)	0.008 (128)	22.3
M	**-10**	8.3 (73)	3.0 (39)	21.0 (74)	69.9 (55)	7.5 (47)	10.5 (131)	0.6 (134)	0.016 (200)	12.5
H	**-20**	5.7 (34)	2.6 (38)	19.6 (68)	76.6 (41)	9.0 (47)	16.2 (158)	0.5 (164)	0.010 (206)	18.0
	-30	4.4 (36)	1.8 (19)	16.8 (68)	100.8 (103)	7.2 (52)	28.2 (249)	0.5 (93)	0.06 (99)	14.4
	-50	3.2 (38)	1.4 (30)	18.9 (84)	56.0 (52)	3.5 (62)	2.0 (86)	1.5 (76)	0.025 (94)	2.3
O	**-56**	3.1 (37)	1.4 (25)	22.7 (96)	50.5 (52)	4.0 (79)	2.3 (121)	2.5 (137)	0.042 (135)	1.6
	-57	3.9 (33)	1.6 (23)	22.3 (99)	53.3 (28)	3.1 (77)	2.6 (120)	0.5 (82)	0.007 (79)	6.2
	-58	4.9 (53)	1.8 (26)	23.5 (92)	42.1 (34)	1.7 (29)	1.4 (153)	0.6 (134)	0.011 (120)	2.8
	-59	5.6 (42)	2.0 (22)	23.0 (92)	39.1 (32)	2.7 (87)	2.4 (124)	0.2 (170)	0.003 (132)	13.5
C	**-60**	7.2 (30)	2.5 (10)	22.5 (89)	33.0 (26)	1.4 (56)	2.4 (201)	0.4 (154)	0.006 (108)	3.5
Mo	**-80**	14.3 (47)	3.7 (26)	22.7 (83)	43.4 (41)	2.4 (90)	4.2 (159)	0.1 (124)	0.002 (161)	24.0

[a] BA, bacterial abundance; VA, viral abundance; VLP, virus-like particles; BP, bacterial production; MaxBS, maximal burst size; FIC, frequency of infected cells; FLC, frequency of lysogenically infected cells. E, epilimnion; M, metalimnion; H, hypolimnion; O, oxycline; C, chemocline; Mo, monimolimnion

The above patterns in BA accounted for the significant effects of seasons and depths. Interestingly, the August peaks in BA coincided with similar ones in VA. In addition, low seasonal variability was also obvious in BA between about 20 and 60 m depths, similar to the pattern observed in VA (Fig. 2A,B).

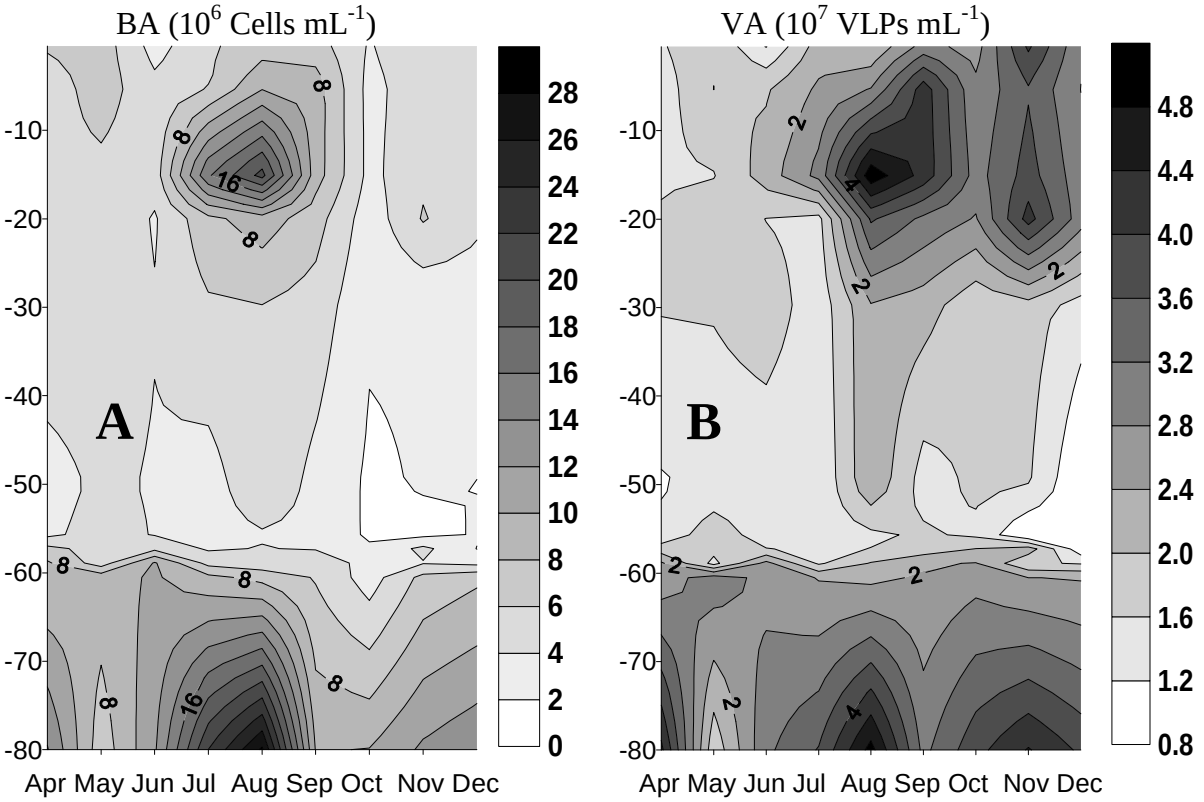


Figure 2. Seasonal variations of Bacterial abundance (BA, A) and of Viral abundance (VA, B) in the water column of Lake Pavin, April – December 2005.

Heterotrophic bacterial production (BP). BP fluctuated from 3.8 to 73.7 x 10^6 cells L^{-1} h^{-1} and the mean values for the different sampling depths were relatively similar to each other, varying from 16.8 x 10^6 cells L^{-1} h^{-1} in the hypolimnion to 23.5 x 10^6 cells L^{-1} h^{-1} in the oxycline (overall mean = 21 x 10^6 cells L^{-1} h^{-1}) (Table 1). The effect of depth-layers was not significant ($p > 0.05$). This contrasts with the effect of sampling time which was significant, with a decreasing production with seasons, from spring (mean = 40 x 10^6 cells L^{-1} h^{-1}) to summer (mean = 13 x 10^6 cells L^{-1} h^{-1}) and autumn (mean = 9 x 10^6 cells L^{-1} h^{-1}) (Fig. 3A).

Maximal burst size (MaxBS). MaxBS fluctuated from 15 to 363 viruses per infected cell (mean = 56 ± 39 viruses $bacteria^{-1}$). The highest seasonal values occurred in

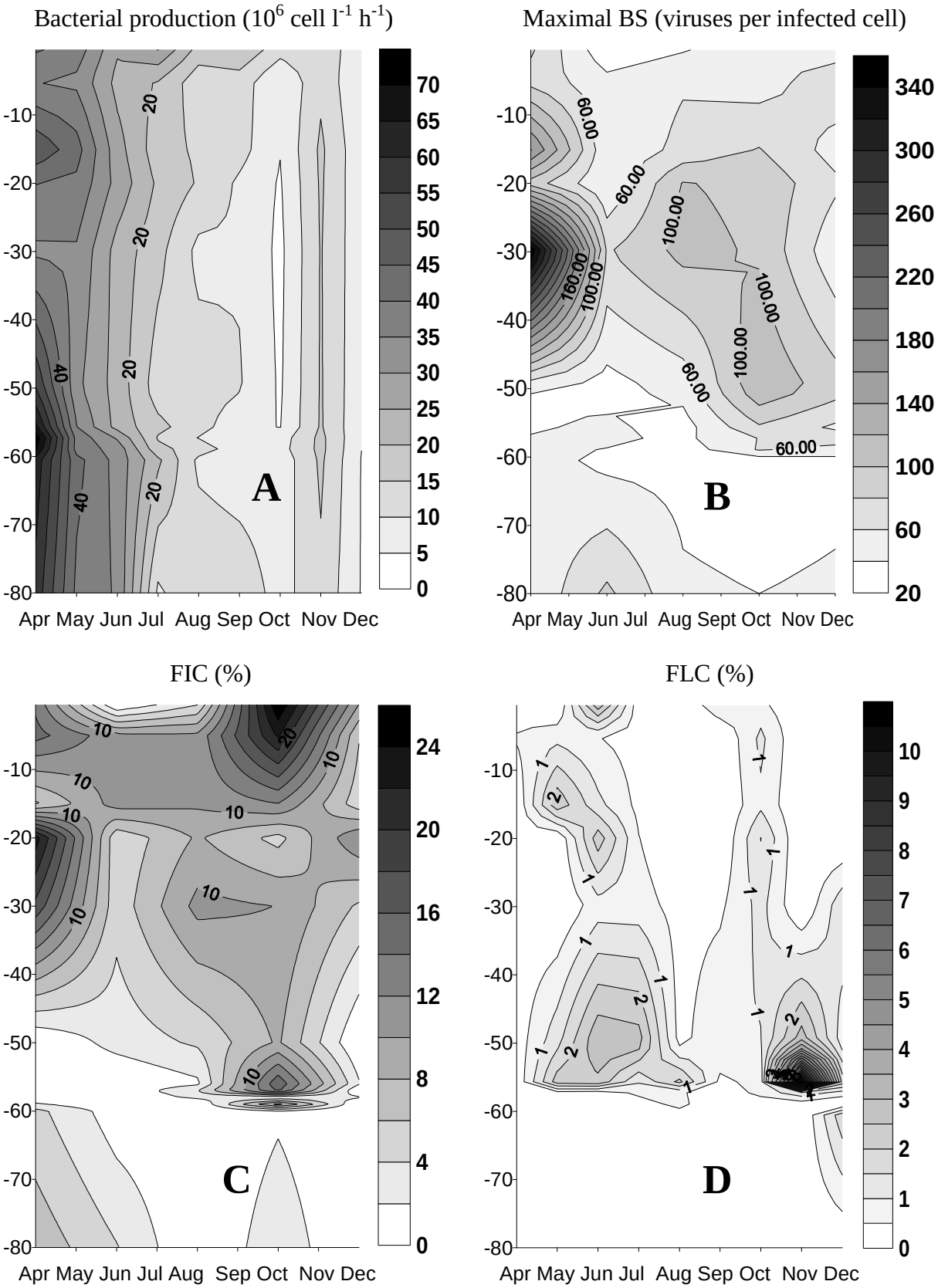


Figure 3. Seasonal variations of bacterial production (A), maximal burst size (B), the frequency of lytically infected cells (FIC, C), and the frequency of lysogenically infected cells (FLC, D) in the water column of Lake Pavin, April – December 2005.

the meta- and hypolimnion (Table 1), particularly in spring (Fig. 3B). This resulted in a significant effect of seasons on the variability of MaxBS. The depth-related effect was also significant, because of the particularly lower MaxBS in the deep chemocline and monimolimnic layers, compared to the mixolimnion (Table 1).

Frequency of infected cells (FIC). FIC fluctuated from < 0.1 to 26 % (overall mean = 4.9 ± 5.4 %) and significantly decreased with depth (Fig. 3C), corresponding to lytic viral production range of 2.14 to 15.4 x 10^7 VLPs L^{-1} h^{-1} (overall mean = 7.4 ± 7.9 x 10^7 VLPs L^{-1} h^{-1}). For both variables, mean values were indeed higher in the oxic layers (epilimnion, metalimnion, hypolimnion, oxycline) than in anoxic ones (chemocline, monimolimnion), similar to the vertical trend noted for burst size (Table 1). The seasonal variability in FIC was also significant, with major peaks in April in the hypolimnion and in October in the surface epilimnic layer (Fig. 3C).

Frequency of lysogenically infected cells (FLC). FLC fluctuated between < 0.1 and 11.5 %, and mean values for the different depths sampled varied from 0.1 % in the monimolimnion to 2.5 % in the oxycline (overall mean = 0.7 ± 1.3 %) (Table 1). This corresponded to prophage replication rates that fluctuated between 0.002 and 0.016 x 10^7 VLPs L^{-1} h^{-1} (overall mean = 0.012 ± 0.011 x 10^7 VLPs L^{-1} h^{-1}). The variability with depth was significant, with higher values in the oxic layers (epilimnion, metalimnion, hypolimnion, oxycline) than in the anoxic ones (chemocline, monimolimnion), similar to the vertical patterns in MaxBS and FIC (Fig. 3B-D). In the oxic layers, lowest mean value was recorded in the epilimnion whereas highest mean value was recorded in the oxycline. Meta- and hypolimion had intermediate mean values (Table 1). Monthly mean FLC varied more than 7-fold but the seasonal variability was not significant ($p > 0.05$). Lowest values were recorded in April, July-October and in December, whereas highest ones occurred in June and November.

Compared to FIC, FLC levels were significantly lower, with FIC : FLC ratios averaging from 1.6 to 24 (overall mean = 10) depending of the sampling depth (Table 1). The lowest differences between the two frequencies were generally noted in the permanent transitive layers, i.e. oxycline and chemocline where FIC : FLC ratios were < 10 (at one exception at 59 m sampling depth). In other layers, these ratios generally exceeded 12 (at one exception at 50 m depth) (Table 1). In terms of viral production vs replication rates, these ratios increased by up to several orders of magnitude, averaging from 54 to 2100 (overall mean = 600) depending of the sampling depth (cf. Table 1).

Among the variables under study, FLC was correlated only with viral and prokaryotic variables. Correlation coefficients between FLC vs viral and bacterial abundances were negative for the whole water column ($r = -0.49$ and -0.48, respectively, $p < 0.001$) and for the oxygenated layers ($r = -0.47$ and -0.49, respectively, $p < 0.001$). This generally contrasted with similar correlations but with FIC, which were positives. FLC and FIC indeed were negatively correlated in the oxygenated water column, due to the coefficient calculated for the oxycline layer ($r = -0.55$, $p < 0.001$).

Discussion

Methodological aspects. Estimation of the frequency of lysogenically infected cells (FLC) in natural microbial community is subject to critics, whatever the method is. In this study, we have used a chemical-mediated prophage induction method, a current method in aquatic microbial ecology **(Jiang and Paul 1996)**. The method is based on the use of mitomycin C as inductor agent and the quantitative importance of lysogens can then be estimated from two different approaches: (i) the reduction in bacterial direct counts after induction of lysogenic bacteria into lytic cycle, or (ii) the difference in viral abundance between treated and control essays divided by burst size. The first approach, termed 'mortality method', requires bacterial mortality rates in control natural samples without mitomycin C addition, in order to calculate the targeted mortality due to prophage induction. Indeed, in this approach, it is important to control for bacterial mortality from sources other than mitomycin (such as lytic viruses, grazers, bacteriocins etc…), in order to estimate the quantitative importance of lysogens **(Jiang and Paul 1996, Williamson *et al.* 2002)**. The second approach, termed 'the burst size method', assumes that the increase in viral abundance due to mitomycin addition is the result of lysogen induction and the subsequent cell lysis, and requires the availability of a transmission electron microscope for direct observation of visibly infected cells. This approach is well known to yield more realistic estimations of FLC, primarily when mean burst size for each single sample is calculated from those visibly cells which were completely filled with viruses, i.e. maximal burst size (MaxBS), as was done in the present study. This is not the case in the majority of pelagic studies on lysogens where theoretical average burst sizes of 30 or 50 viruses bacteria^{-1} (known from the literature and laboratory experiments) are used **(Jiang and Paul 1996, Tapper and Hicks 1998, Cochran and Paul 1998, Williamson *et al.* 2002, Ortmann *et al.* 2002, Säwström *et al.* 2006)**. Other studies have also used average burst size calculated from all cells observed with intracellular viruses, although the number of viruses within an infected but unfilled (i.e. with viruses) cell might still increase

with time **(Weinbauer and Suttle 1996)**. The use of theoretical or average burst size probably results in rough estimates of FLC which are less accurate, compared to our study where MaxBS were calculated for each single sample collected in triplicate. Indeed, we have recently shown in a companion paper that burst size in our study site significantly varied with time and space, partly as a results of the existence of typical viruses in the deep permanently anoxic water masses **(Colombet *et al.* submitted b)**.

From the above comparisons, we conclude that error sources for accurate estimation of FLC were minimized in our study, compared to the available data in the literature. However, the question of the toxicity and of the effectiveness of inductor agents remains a recurrent problem for estimating lysogens in natural waters. To date, mitomycin C is the more frequently used agent and is currently preferred to other agents, such as e.g. the organic pollutants polychlorobiphenyl and Aroclor 1248 which have been successfully used in marine environments **(Cochran *et al.* 1998)**. The effectiveness of mitomycin as an induction agent for natural freshwater bacterioplankton communities is unknown. Different strains of bacteria in the natural community may respond differently to the mitomycin addition. **Ackerman and Dubow (1987)** have shown that mitomycin does not induce all lysogenic isolates. Moreover, it has been shown that induction by mitomycin requires actively growing cells, which can represent only a minor percentage of total lysogens in natural waters **(Williamson *et al.* 2002)**. Consequently, comparison of FLC levels from different environments or spatial / temporal scales must be done with care. A different approach for lysogen estimations was developed by **Weinbauer and Suttle (1996)**, consisting in a reduction of viral abundance by dilution. The approach presents the disadvantage to manipulate bacteria and can alter bacterial growth rates on which viral production is dependent. Detailed description and discussion of this approach are provided by **Paul and Jiang (2001)**. More recently, a genomic approach was employed to identify the presence of prophages directly into bacterial genome **(Casjens 2003, Chen *et al.* 2006)**. However, these integrated prophages may be inducible or not, defective prophages, remnants of intact prophage genomes, or even isolated viral genes **(Canchaya *et al.* 2003)**. Overall, biological experiments designed to test for the nature of the prophages are necessary, and are the only mean to confirm the activity of a specific prophage **(Chen *et al.* 2006)**. An approach developed by **Chen and coauthors (2006)** that couples genomic and induction essays is promising, although the approach required the knowledge of host genome and cannot be employed at a community level.

Quantitative importance of lysogeny. In the present study, our FLC values (<0.1-11.5 %) agree with those (0-16 %) reported in a preliminary study conducted in the same lake on 20 April 2004 **(Colombet *et al.* 2006)**, and also with those (0.1 to 16 %) known from the few previous studies conducted in temperate **(Tapper and Hicks 1998)** and tropical **(Bettarel *et al.* 2006)** freshwater systems, using the same methodological approach. In cold Antarctic lakes, significantly higher percentages of lysogenic prokaryotes (FLC = 2 - 89 %) have been reported **(Lisle and Priscu 2004, Säwström *et al*. 2006)**. In marine waters, FLC vary greatly from 0 to more than 100 % in different sites **(see review in Weinbauer 2004 and reference herein)**. Seasonal FLC levels of 0-37 % **(Cochran and Paul 1998)** and 0- >100 % **(Williamson *et al.* 2002)** have been reported in the same site, i.e. the Tampa Bay, corroborating the difficulties in comparing available data on FLC in the literature, partly because of methodological considerations discussed previously. However, we believe that the variability in the chemically-inducted lysogens in natural waters reflects the reality that a fraction of bacterioplankton contained cryptic viable viral genomes. In the context of the present study, seasonal FLC in Lake Pavin fluctuated 7-fold with time and 20-fold with the depth-related gradients and was detected mainly in spring (June) and in autumn (October-November) in the oxygenated water column, with peak values in the oxycline in November (Fig. 3D). The quantitative importance of lysogens in Lakes Pavin and other sites, and their variability with time and space which, in almost all studies include zero as the minimum FLC level, implies that a significant portion of bacteria in aquatic environments are lysogenic during certain times of the year and/or at certain depths of the water column, perhaps along a continuum from the absence to 'blooms' of lysogens within bacterial communities. The question of the existence of typical lysogens, i.e. bacteria with permanently cryptic and non-inducible prophages in their genome, remains absolutely unexplored in environmental samples. We thus believe that the few existing data on aquatic lysogens preclude any simple extrapolation to the natural conditions, and that further investigations, if not speculations, of the environmental factors that promote the decision for lysogenic conversion within natural communities are warranted.

Lysogeny and the physico-chemical environment. Some physico-chemical factors have been suspected to induce lysogenic events in virus/hosts isolates and in aquatic microbial communities. From our data set, we found no significant correlation between FLC and the physico-chemical variables analysed, i.e. temperature, oxygen and chlorophyll. Furthermore, some of the empirical concomitances observed appeared contradictory according to the within

lake variability or in comparison with the available literature. In relation with the light environment, **Tapper and Hicks (1998)** found high FLC levels in the subsurface lake waters and evoked the inductive impact of solar UV radiation, whereas in our study, there was absence of induction most of the time in the surface waters, including during summer months (Fig. 3D). Regarding temperature, our maximal FLC occurred in November at 4 °C in the dark oxycline (Fig. 3D), getting around the reports from the Antarctic waters where the highest levels (2-89 %) of FLC in lakes were reported **(Lisle and Priscu 2004, Säwström *et al.* 2006)**. However, at different occasions during our study, such as in June where the temperature was at 16 °C in the epilimnion and at 4 °C in the oxycline, similar levels of FLC were observed in both layers (Fig. 3D). Similarly, in the Tampa Bay (Florida, USA), **Cochran and Paul (1998)** have detected lysogeny only when the water temperature exceeded 19 °C, while **Williamson *et al.* (2002)** have successfully induced lysogens during winter months at temperature < 5 °C. **Weinbauer and Suttle (1996, 1999)** found no significant correlation between FLC and temperature in marine waters.

Williamson and Paul (2006) indicated that low oxygenation favoured lysogenic pathway in the marine phage-host system phiHSIC/*Listonella pelagia*. In a preliminary study in Lake Pavin **(Colombet *et al.* 2006)** and in the present study as well, we have similarly recorded higher FLC levels in the oxygenated compared to anoxic waters (although the highest value was recorded in the oxycline). In contrast, **Weinbauer *et al.* (2003)** reported highest FLC in suboxic layers of the Baltic Sea. **Wilson and Mann (1997)** and **Williamson *et al.* (2002)** suggested that low nutrient concentrations favoured lysogeny, as do low salinity **(Williamson and Paul 2006)**. In contrast, **Mc Daniel and Paul (2005)** observed from assays on *Synechococcus* that induction was not nutrient-limited. In the present study, we have recorded low FLC levels in anoxic layers (Chemocline, monimolimnion) where nutrient concentrations **(Michard *et al.* 1994, Viollier *et al.* 1995)** and salinity **(Aeschbach-Hertig *et al.* 2002)** are substantially higher compared to the uppest layers. It is thus likely that the available studies and the related findings on lysogeny prevent from making any satisfactory generalisation on the impact of physico-chemical environment, confirming our previous assumption that the abiotic factors may not be prevalent over the biological environment in promoting lysogenic 'life' style within bacterial communities in aquatic ecosystems **(Colombet *et al.* 2006)**.

Prophage induction and the host availability: ecological implications. The above assumption fits with our finding that FLC was, almost exclusively, correlated with bacterial

and viral abundances in the oxygenated water columns which appeared as a restrictive microhabitat for mitomycin-inducible lysogens. These correlations were negatives, indicating that the development of lysogeny may only be apparent only under certain level of bacterial (and their related phage, **Bettarel *et al.* 2003, Colombet *et al.* submitted a)** abundances. Indeed, in 95 % of our samples where FLC levels were higher than 1 %, bacterial abundances were systematically less than 5×10^6 cell mL^{-1}. More striking were the findings that our maximal value of FLC (11.5 %) at 56 m depth in November coincided with the lowest value of bacterial abundance (1.4×10^6 cells mL^{-1}), and that we failed to induce lysogens in the monimolimnion where the highest bacterial and viral abundances were recorded. Although the depth effect was not significant, higher bacterial production also occurred in the deep oxycline waters (Fig. 3A), and a strong coupling with viral lytic activity was reported and discussed in detail elsewhere **(Colombet *et al.* submitted b)**. In contrast to lytic activity, the importance of lysogens within bacterioplankton may thus be inversely proportional to prokaryotic abundance and growth rate, from under a threshold bacterial density of about 5.7×10^6 cells mL^{-1} as suggested from our case study.

This suggests that one of the crucial factors to the lysogeny decision within natural bacterioplankton is the alteration of physiological and metabolical status of bacterial cells, well known as intrinsic determinants of bacterial abundances in both natural environments **(Del Giorgio *et al.* 1997)** and culture medium **(Créach *et al.* 2003)**. These determinants can also affect viral lytic infections and proliferation, by influencing adsorption, replication, lytic activity and survival of phages **(Moebus 1987, Farrah 1987, Williams 1994)**, indicating that antagonistic interactions between lysogeny and lytic activity may exist in relation to the resource availability (i.e. prokaryotic host abundance and metabolic status). In our study, this was supported by the negative correlation between FLC and FIC, primarily in the oxycline where the highest FLC corresponded to the lowest FIC (Fig. 3D). It has been shown that phage λ can shifts 'life' style from lysogenic to lytic infections depending on the metabolical and physiological status of the host cells, on which directly depends the stability of important proteins repressors which governed lysogenic vs lytic decision **(Ptashne 1992)**. In harsh environments for host growth, lysogenic decision may help struggle the cell morbidity and increase the fitness of the host, as a result of added genes from horizontal transfers **(Klein *et al.* 2001, Filee *et al.* 2003, Abedon and Lejeune 2005)** and/or from immunity acquisition and the related resistance to superinfection **(Marsh and Wellington 1994, Weinbauer 2004, Clore and Stedman 2006)**. Lysogens are more resitant and have more chance to survive in 'unfavourable' conditions than non lysogens. For example, lysogens of *E.coli* have been

shown to reproduce more rapidly than non-lysogens during growth in nutrient-limited chemostat **(Edlin *et al*. 1975, 1977; Lin *et al*. 1977)**. At the community level, there is no such evidence but the negative relationship between FLC and bacteria found in our study supports the proposed hypothesis that lysogeny is a survival strategy for bacteria **(Weinbauer *et al.* 2003, Colombet *et al.* 2006)**, which can this way maintain both their genomic and phenotypic traits in the environment at low host density and growth rate **(Steward and Levin 1984, Freifelder 1987, Williamson et al. 2002, Weinbauer et al. 2003, Säwström et al. 2006)**. In these conditions, lysogenic infections could further represent a forcing factor for the selective evolution of bacterial and viral strains in the environment.

Clearly, our study provides empirical indications that lysogeny mostly is a mechanism for the maintenance of viral traits, which can further have significant incidences on the evolutionary ecology of host communities. Spontaneous induction of lysogens in natural conditions indeed may be a minor source for viral standing stock and production in aquatic systems, less that 2 % of lytic viral production in the case study of Lake Pavin (Table 1).

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DISCUSSION GENERALE

1. Considérations générales

Les écosystèmes aquatiques sont le siège de processus écologiques fondamentaux qui, au-delà des aspects cognitifs et de leur importance pour la qualité de l'eau et les enjeux socioéconomiques associés, ont une influence sur le fonctionnement global de la biosphère et le climat. Au sein de ces écosystèmes, les communautés microbiennes, largement dominées par les procaryotes, représentent la composante biologique majeure des points de vue qualitatif, quantitatif et fonctionnel. Ainsi, la connaissance des facteurs de contrôle et de régulation des communautés microbiennes aquatiques est essentielle, dans le cadre de l'étude des écosystèmes naturels. En dehors des forçages climatiques (vent, température, lumière) et ceux liés aux pollutions, on considère généralement deux principaux types de facteurs de contrôle des communautés microbiennes aquatiques. Les facteurs ascendants ou '*bottom-up*' sont constitués par les ressources et affectent plus les taux de croissance des organismes que leur biomasse. Les facteurs descendants ou '*top-down*' sont représentés par l'ensemble des pressions de prédation (prélèvement de biomasse) (**Ducklow and Carlson 1992, McManus and Fuhrman 1988**). Par extension, tous les facteurs affectant la biomasse microbienne, comme la lyse virale, sont considérés comme des facteurs descendants, même si leurs effets sur la structure et le fonctionnement des réseaux trophiques microbiens sont fondamentalement différents. Selon **McQueen et al. (1989)**, les facteurs ascendants et descendants ne sont pas exclusifs et l'intensité relative de chaque type de contrôle varie dans l'espace et dans le temps.

L'écologie virale aquatique est une discipline en plein développement. Cependant, la grande majorité des études sur le rôle fonctionnel des virus aquatiques reste centrée sur leur impact dans la mortalité microbienne, c'est-à-dire par la seule prise en compte de l'activité lytique. Ces études sont, par ailleurs, très souvent limitées dans le temps et dans l'espace, notamment en ce qui concerne la variabilité verticale des paramètres viraux dans la colonne d'eau. Les objectifs de ce travail de thèse ont donc été conçus afin de prendre en compte les deux principaux modes de prolifération virale (lyse, lysogénie) et la variabilité verticale. Le choix du lac Pavin est lié au fait que ce lac profond d'origine volcanique présente, par son caractère méromictique, la particularité d'avoir un mixolimnion dimictique (stratification saisonnière : thermocline) et un monimolimnion stable en anoxie permanente (présence d'une stratification permanente : oxycline, chemocline). De plus, des données antérieures en écologie virale existent mais seulement pour la strate mixolimnique du lac, ce qui nous a

permis de confronter les données de cette étude aux études antérieures pour cette zone, afin de valider les gradients liés à la profondeur et non à de simples différences interannuelles. Ainsi, les valeurs et la variabilité des paramètres viraux mesurés dans le mixolimnion au cours de la présente étude étaient elles en accord avec les observations de **Bettarel et al. (2003, 2004)**, à l'exception de la diversité virale et de l'activité lysogène qui n'ont pas été prises en compte antérieurement dans le lac Pavin. Ces deux variables et la prise en compte de l'ensemble de la colonne d'eau du lac Pavin (le monimolimnion), constituent donc l'originalité de la présente étude.

2. Diversité et structure du virioplancton dans le lac Pavin

A. Aspects méthodologiques

L'étude de la diversité biologique en milieu aquatique nécessite, en premier lieu, une concentration efficace des particules cibles. Cela est particulièrement vrai pour l'étude de la diversité des virus qui nécessite des concentrats de particules virales ou du matériel nucléique viral, à partir de grands volumes (10-200 litres) de plancton (**Wommack et al. 1999a**). Au début de notre étude, nous avons réalisé que le protocole de routine utilisé, à savoir la filtration tangentielle (ultrafiltration) suivi de l'ultracentrifugation, n'avait jamais été réellement confronté à des approches alternatives. Ce protocole nécessite un matériel onéreux, est consommateur en temps et le rendement de concentration n'est pas satisfaisant. C'est dans ce contexte qu'une étude de la littérature existante en virologie fondamentale et médicale nous a permis d'avoir recours au polyéthylène glycol (PEG) pour la chélation des particules virales en suspension dans le plancton.

Nous avons ainsi pu développer et proposer un protocole couplant une préconcentration par ultrafiltration à une précipitation/cristallisation chimique du virioplancton à l'aide du polyéthylène glycol (PEG). Ce protocole a été comparé à celui, couramment utilisé et nécessitant une ultrafiltration suivie d'une ultracentrifugation (**Wommack et al. 1999, Diez et al. 2000, Auguet et al. 2006**). A partir d'échantillons d'eaux douces, représentatifs du gradient trophique des écosystèmes aquatiques, nous avons mis en évidence que le protocole utilisant le PEG est environ 2 à 3 fois plus efficace, en termes de rendement de concentration, que le protocole utilisant l'ultracentrifugation. Ainsi, quelque

soit le niveau trophique du milieu étudié, ce protocole offre une alternative rapide, simple, peu onéreuse et efficace au protocole usuel.

De plus, l'utilisation du PEG offre non seulement une méthode de conservation des virus (évitant l'utilisation de fixateurs), mais permet également d'obtenir un culot dans lequel les particules virales sont purifiées par précipitation au chlorure de potassium. Cela permet d'obtenir des images d'excellente qualité en microscopie électronique à transmission, et une analyse plus aisée du génome viral par électrophorèse.

B. Dynamique de la diversité virale

La mise en application du protocole PEG nous a permis d'étudier les variations saisonnières de la diversité morphométrique (fréquence de distribution du diamètre de la capside), morphologique (familles phénotypiques) et génomique (taille de l'ADN viral) des communautés virales à 4 profondeurs différentes du lac Pavin. A notre connaissance, aucune autre étude n'a examiné la variabilité saisonnière de la diversité virale sur la base des trois traceurs mentionnés.

Les résultats acquis nous ont permis de montrer que les virus du lac Pavin appartiennent en majorité (environ 61 % de l'abondance totale) aux groupes incertains des virus 'sans queues' et à la famille des *Siphoviridae* (environ 33 %), les *Myoviridae* et *Podoviridae* étant plus faiblement représentés. Du point de vue morphométrique, les particules virales dont le diamètre de la capside est compris entre 30 et 60 nm dominent la communauté virale (environ 66 % de l'abondance totale). Du point de vue de la taille du génome, les virus analysés présentent un génome de taille comprise entre 10 et 300 kb avec une distribution multimodale, dans laquelle apparaissent 5 grandes classes de taille. Parmi ces classes de taille, les virus dont le génome a une taille comprise entre 25 et 65 kb sont les mieux représentés (environ 61 % des génomes viraux). 34 bandes électrophorétiques différenciées ont été distinguées pour la période d'étude, avec en moyenne 12 'génotypes viraux' différents par échantillon. Ces données sont sans doute sous estimées, puisque l'électrophorèse sur gel en champs pulsés (PFGE) ne discrimine que les génomes les plus abondamment représentés dans un échantillon.

Ces résultats sur la diversité saisonnière des virus du lac Pavin corroborent ceux précédemment rapportés dans différents milieux d'eaux douces à partir d'échantillonnages ponctuels, que ce soit d'un point de vue morphologique (**Hennes and Simon 1995, Alonso *et al.* 2001, Weinbauer 2004, Auguet *et al.* 2006, Liu *et al.* 2006**) ou génomique (**Filippini**

and Middelboe 2007). La diversité virale rapportée par **Auguet et collaborateurs (2006)** sur la rivière Charente, d'un point de vue génomique, est inférieure à celle rapportée dans le lac Pavin. De manière générale, la diversité virale aquatique est largement dominée par des particules de petite taille, aussi bien d'un point de vue phénotypique que génomique, ce qui peut être considéré comme une adaptation à la vie planctonique.

Dans l'espace et le temps, des changements originaux dans la structure phénotypique et génomique des communautés virales ont été observés, avec par exemple une plus grande richesse virale enregistrée en été et dans l'épilimnion, en termes de diversité des tailles du génome viral. Ces variations semblent être étroitement liées aux variations de structure des communautés microbiennes. Ainsi, les corrélations enregistrées montrent que l'environnement biologique serait essentiel dans la dynamique de la diversité virale. Plus précisément, ces relations empiriques suggèrent l'existence de trois grands groupes viraux, basés sur les critères phénotypiques et génotypiques : (i) les virus de grande taille (capside > 80 nm et génome > 290 kb) dont la variabilité est mieux prédite par les organismes eucaryotes, (ii) les virus de taille intermédiaire (capside 30-80 nm et génome 75-105 kb) dont le développement semble être lié aux picocyanobactéries, et (iii) les virus de petite taille (capside < 60 nm et génome < 75 kb) qui semble être constitués essentiellement de bactériophages, puisque étroitement corrélés aux bactéries hétérotrophes du milieu. De plus, la dynamique des *Myoviridae* et des *Siphoviridae* semble être associée à celles des microorganismes autotrophes, alors que celle des phages 'sans queues' serait associée plutôt à la dynamique des bactéries hétérotrophes.

Les résultats de diversité virale permettent donc d'affirmer que les virus sont des composantes dynamiques des écosystèmes aquatiques, où ils peuvent, potentiellement, infecter et réguler les différentes composantes microbiennes, non seulement en milieu marin (**Sandaa et Larsen 2006, Auguet *et al.* 2006**), mais aussi dans les écosystèmes lacustres comme le lac Pavin. Parmi les variables de forçage physico chimiques, seule la température semble affecter la variabilité de la structure des communautés virales, probablement de façon indirecte via les effets sur les compartiments microbiens. Il apparaît donc que la disponibilité des hôtes potentiels est cruciale dans la dynamique de la diversité virale, et prévaut sur l'environnement physico-chimique dans la prédiction de cette dynamique.

3. Importance des gradients liés à la profondeur pour l'abondance et l'activité lytique des virus

A. Abondance saisonnière

D'une façon générale, l'étendue de la variation de l'abondance virale dans le lac Pavin (0,8 à 5,1 x 10^7 VLPs ml^{-1}) correspond à celle rapportée précédemment par **Bettarel et collaborateurs (2003)** dans le même lac et est, de manière générale, caractéristique des environnements de statut trophique équivalent (**Wommack et Colwell 2000, Weinbauer 2004**). Cependant, la prise en compte de la totalité de la colonne d'eau au cours de notre étude montre que la distribution spatiale de la structure des communautés, non seulement des virus mais aussi de l'ensemble des microorganismes étudiés, ainsi que les liens trophiques les unissant, diffèrent de manière significative de ceux rapportés dans les études précédentes entreprises jusqu'à présent dans le mixolimnion du lac Pavin.

Nos résultats montrent que la complexité des communautés biologiques et des réseaux trophiques sous jacents décroît avec la profondeur. Cette distribution verticale est en adéquation avec la stratégie trophique des microorganismes, puisque d'une communauté complexe composée de virus et de procaryotes et eucaryotes auto- et hétérotrophes en surface, on passe à une communauté plus simple dans le monimolimnion anoxique, où les procaryotes hétérotrophes et les virus semblent être les seules entités biologiques adaptées à ces conditions contraignantes. Ces entités, notamment les bactéries, sont nettement plus abondantes dans le monimolimnion du lac que dans les couches supérieures. En conséquence, les rapports virus/bactéries (VBR) dans le monimolimnion (1.6 – 3.8) sont significativement plus faibles que dans les autres couches du lac. Ces rapports sont aussi plus faibles que les valeurs typiques connues dans les autres environnements pélagiques (**Wommack et Colwell 2000**), ce qui dénote des différences liées à la profondeur, dans la nature et l'intensité des interactions virus vs procaryotes aquatiques. Cela semble être confirmé par le fait que la variabilité temporelle du VBR dans le monimolimnion du lac Pavin est substantiellement plus faible que dans les couches supérieures du lac. Les relations empiriques (corrélations) entre virus et procaryotes sont, en effet, bien plus étroites et exclusives dans les couches profondes du lac, comparées aux relations en surface où les virus sont associés à de nombreuses variables microbiennes, notamment aux organismes autotrophes (picocyanobactéries, nanoflagellés autotrophes). Cela laisse supposer que les cyanophages et autres phytophages

pourraient être abondants dans les couches supérieures du lac Pavin. L'existence d'une corrélation entre abondance des picocyanobactéries et abondance virale a également été rapportée par **Dorigo et al. (2004)** dans le lac mésotrophe du Bourget.

Nos résultats sur l'importance quantitative des virus sont en adéquation avec l'analyse des variations de la structure des communautés virales et indiquent que non seulement la diversité, mais aussi l'abondance, des communautés virales sont extrêmement dépendantes des communautés d'hôtes potentiels. Il se dégage donc la nécessité d'intégrer l'ensemble de la colonne d'eau dans les études en écologie virale, afin de mieux appréhender le rôle des virus dans la catalyse biogéochimique au sein des écosystèmes aquatiques. Il semble en effet que les flux de matière et d'énergie dans ces écosystèmes soient régis par des interactions complexes entre virus et microorganismes dans les couches mixolimniques. En revanche, dans le monimolimnion anoxique où le temps de résidence des eaux serait supérieur à 100 ans, ces flux se réduisent au fonctionnement de la boucle virale (Matière organique dissoute (MOD) > procaryotes > virus > MOD), où les nutriments peuvent alors être séquestrés et recyclés plusieurs fois avant incorporation dans le sédiment ou retour dans les couches supérieures du lac. La chémocline et l'oxycline apparaissent comme des zones charnières fondamentales de ce point de vue, pour la dynamique lacustre (**Lehours *et al*. 2005**).

B. Activité lytique

Un des résultats marquants de ce travail est la relation entre les paramètres de l'activité lytique des virus, notamment la fréquence de cellules visiblement infectées (FVIC) et le nombre moyen de virus par cellule bactérienne infectée (*burst size*, BS) et la profondeur. Les valeurs enregistrées lors de cette étude sont de 0 à 3,1 % pour la FVIC et de 7 à 127 virus x bactérie^{-1} pour le BS. Ces valeurs sont similaires à celles répertoriées dans la littérature pélagique (**Weinbauer 2004**), et à celles précédemment enregistrées dans le mixolimnion du lac Pavin (**Bettarel et al. 2003**). Cependant, contrairement aux abondances bactériennes et virales et à la production bactérienne, les valeurs les plus faibles de la FVIC et du BS au cours de cette étude ont été, paradoxalement, notées en profondeur. Cela est en contradiction avec les résultats de **Bettarel et ses coauteurs** et de l'étude préliminaire que nous avons menée sur le lac Pavin (**Colombet et al. 2006**) ; également avec la majeure partie des études de la littérature où l'abondance et l'activité lytique des virus sont dépendantes de l'activité des bactéries qui forment le plus grand réservoir d'hôtes pour les virus planctoniques. Il nous a été difficile d'expliquer le paradoxe observé dans la colonne d'eau du lac Pavin, paradoxe qui

semble être caractéristique des milieux benthiques d'eaux douces (**Fillipini et al. 2006**). Nos résultats suggèrent qu'à l'échelle des saisons, (i) la prolifération virale lytique dans les eaux profondes du lac Pavin est contraint par un (des) facteur(s) autre(s) que la seule disponibilité en hôtes, et (ii) qu'une fraction des virus libres présents en profondeur serait d'origine allochthone.

Concernant le déterminisme des variations saisonnières de l'activité lytique, la colonne d'eau peut être, globalement, divisée en 2 parties distinctes : les couches mixolimniques et le monimolimnion. Les couches mixolimniques, bien oxygénées, présentent un réseau trophique complexe avec un environnement physico chimique fluctuant, où la FVIC et le BS, variables liées aux seules bactéries hétérotrophes, ne reflètent sans doute pas l'ensemble de l'activité de toutes les communautés virales présentes. Contrairement à ces couches superficielles, les communautés biologiques du monimolimnion sont réduites aux seuls virus et procaryotes évoluant dans un environnement physico chimique contraignant (anoxie, absence de lumière) mais plus stable. Dans cette zone, la FVIC est clairement et intimement liée à la productivité bactérienne (PB), même si, contrairement à la PB, les valeurs les plus élevées de la FVIC n'ont pas été enregistrées en profondeur. Une augmentation du taux de croissance bactérien dans le monimolimnion, augmentation au moins en partie due à l'absence de prédateurs, s'accompagne sans doute par plus d'expression de récepteurs pour la fixation virale à la surface des cellules hôtes (**Simek *et al.* 2001, Sime-Ngando et Pradeep Ram 2005**). L'absence de bactérivores en profondeur du lac Pavin où les ressources sont abondantes pour le métabolisme des procaryotes, fait donc des virus la principale source de mortalité bactérienne dans le monimolimnion. De plus, les différences verticales enregistrées au niveau du BS et des caractéristiques morphologiques particulières des virus du monimolimnion, laissent supposer l'existence de virus typiques, endémiques, dans cette couche.

Les observations ci-dessus rejoignent donc la principale conclusion tirée par l'analyse des abondances saisonnières des virus, à savoir que le monimolimnion anoxique, où le temps de résidence des eaux serait supérieur à 100 ans, présente un fonctionnement trophique essentiellement régi par la boucle virale (Matière organique dissoute (MOD) > procaryotes > virus > MOD), où les nutriments seraient séquestrés et recyclés plusieurs fois avant incorporation dans le sédiment ou retour dans les couches supérieures du lac. Cela est en accord avec les démonstrations récentes dans des écosystèmes marins, que les eaux méso- et bathypélagiques sont des environnements propices pour la survie et la prolifération virale (**Parada et al. 2007, Magagnigni et al. 2007**).

4. Rôle de la lysogénie dans la colonne d'eau

Le rôle des virus dans les systèmes aquatiques dépend étroitement de leur mode de 'vie', lytique et lysogénique principalement. Très peu de données existent sur l'importance de la lysogénie virale à l'échelle des communautés naturelles par rapport à la prolifération lytique, plus particulièrement dans les eaux douces. C'est dans ce cadre scientifique que nous avons étudié, pour la première fois, les variations saisonnières de l'abondance relative des cellules procaryotes lysogènes, comparées à celles des cellules lytiquement infectées, dans la colonne d'eau du lac Pavin. Les résultats obtenus, par induction à la mitomycine C, indiquent q'une proportion non négligeable de bactéries lacustres contient un génome viral viable, inséré dans le génome bactérien. Ces cellules lysogènes représentent de moins de 0,1 % à 11,5 % de l'abondance totale des bactéries. Les valeurs peuvent atteindre 16 %, en considérant les résultats de l'étude préliminaire menée dans le lac Pavin en Avril 2004. Il s'agit de valeurs tout à fait comparables à celles rapportées dans d'autres écosystèmes d'eaux douces situés en milieu tempéré (**Tapper et Hicks 1998**) ou tropical (**Bettarel et al. 2006**).

Par ailleurs, il est apparu que l'environnement biologique, principalement l'abondance des procaryotes, est un facteur déterminant pour l'apparition de la lysogénie au sein des communautés naturelles. Ainsi, nous avons pu observer que les cellules lysogènes n'apparaissent qu'en dessous d'un seuil de densité bactérienne, qui semble être situé aux alentours de $5x10^6$ bactéries ml^{-1} au lac Pavin. Cette observation, couplée au fait que les variations de la fréquence des cellules lysogéniquement infectées (FLC) est négativement corrélées à celles des cellules lytiquement infectées (FIC), laisse supposer une relation de type antagoniste entre les deux modes de 'vie' des virus. Cela implique que la lysogénie serait une stratégie de survie et de maintien des virus dans leur environnement, lorsque que la disponibilité en hôtes spécifiques (abondance et statut métabolique des procaryotes), facteur limitant par excellence de la prolifération virale, devient limitante. Les valeurs de FLC sont faibles comparées aux valeurs de FIC et la production virale potentielle, dans le cas où toutes les cellules lysogènes seraient induites, reste très insuffisante pour assurer la survie des virus au sein d'un peuplement d'hôtes diversifié. On sait que les cellules lysogènes acquièrent de nouvelles propriétés grâce à la présence de prophages dans leur génome (**Marsh and Wellington 1994, Klein *et al.* 2001, Filee *et al.* 2003, Abedon and Lejeune 2005, Clore and Stedman 2006**). Le mode de vie lysogénique serait donc, pour la communauté virale, plus un mécanisme de survie que de prolifération, pouvant avoir une incidence sur l'évolution adaptative des communautés d'hôtes. Dans la colonne d'eau du lac Pavin, les cellules

lysogènes sont plus abondantes dans les couches mixolimniques, excluant l'hypothèse que la lysogénie pourrait être un réservoir de virus dans les couches profondes du lac, et indiquent que l'évolution écologique des communautés d'hôtes dans ces couches n'est pas liée au mode de vie lysogénique.

CONCLUSIONS GENERALES, PERSPECTIVES DE RECHERCHE

Les objectifs de ce travail de thèse visaient à déterminer l'importance qualitative, quantitative et fonctionnelle des virus dans le lac Pavin, en tenant compte des gradients liés à la variabilité verticale de la colonne d'eau. Plus spécifiquement, il s'agissait (i) d'examiner la dynamique saisonnière et spatiale de la biodiversité au sein des communautés virales, (ii) d'analyser l'importance des gradients liés à la profondeur dans les variations saisonnières de l'abondance et de l'activité lytique des virus, et (iii) d'étudier l'importance relative des cellules lysogènes et du mode de prolifération virale associé.

1. Structure des peuplements et diversité des virus aquatiques

Sur le plan méthodologique, nous avons mis au point un protocole original de concentration des virus aquatiques libres, basé sur une précipitation chimique au polyéthylène glycol couplé à l'ultrafiltration. Ce protocole simple, rapide et peu onéreux, permet d'obtenir une efficacité de concentration virale en moyenne 2 fois supérieure à la méthode utilisant l'ultracentrifugation. De surcroît, le culot viral obtenu subit un traitement de purification qui améliore, considérablement, la qualité des observations en microscopie électronique, et la discrimination électrophorétique de différentes populations virales séparées par la taille de leur génome.

La mise en œuvre de ce protocole nous a permis de montrer (i) que la structure du virioplancton du lac Pavin est dominée par des virus 'sans queue' et par des particules de petite taille (diamètre de la capside compris entre 30 et 60 nm, taille du génome compris entre 25 et 65 kb), et (ii) que la dynamique de la diversité des communautés virales est étroitement associée à la structure des communautés microbiennes, le rôle des variables physico chimiques étant, apparemment, moins important. D'un point de vue empirique, il est apparût que le long d'un gradient de taille de la capside ou du génome viral, le développement des populations virales de petite, moyenne et grande taille semble être associé à différentes communautés microbiennes, respectivement : les bactéries hétérotrophes, les picocyanobactéries et les eucaryotes. Par ailleurs, la dynamique des *Myoviridae* et des *Siphoviridae* serait étroitement associée à celle des microorganismes autotrophes, alors que celle des phages 'sans queues' serait dépendante, plutôt, des bactéries hétérotrophes. La présence de virus non bactériophages en quantité non négligeable, notamment dans le mixolimnion du lac Pavin, est suggérée.

2. Importance des gradients liés à la profondeur pour l'abondance et l'activité lytique des virus

Les résultats obtenus au cours de l'étude saisonnière montrent que la majeure partie de la variance liée à l'abondance virale serait liée à la dynamique des peuplements microbiens, alors que les effets des variables physico chimiques sont faibles. La prise en compte de l'ensemble de la colonne d'eau a changé la vision que nous avions des caractéristiques limnologiques biologiques du lac Pavin, à partir des seules études antérieures qui se sont limitées dans la zone mixolimnique. Ainsi, la structure des réseaux trophiques microbiens et la place des virus dans ces réseaux dépendent de la profondeur. Dans le mixolimnion, les communautés biologiques sont complexes et la dynamique virale suggère l'existence, dans cette zone, de virus des différentes communautés pro- et eucaryotes, auto- et hétérotrophes. En profondeur, notamment dans le monimolimnion anoxique, les virus et les procaryotes, communautés étroitement liées, représentent l'essentiel des peuplements biologiques. L'oxycline et la chémocline sont des zones de transition où s'opèrent les principaux changements dans la structure du réseau trophique microbien du lac. Ainsi, la distribution verticale des communautés biologiques dans le lac Pavin se caractérise par une simplification des modes trophiques (auto-, hétérotrophe) et des niveaux d'organisation biologique des communautés (eucaryotes, procaryotes, virus), et sans doute des interactions et réseaux trophiques associés, avec la profondeur.

D'un point de vue fonctionnel, l'environnement biologique, notamment microbien, est également plus déterminant dans les variations de l'activité lytique des virus, que les facteurs physico chimiques. Les couches mixolimniques, bien oxygénées, présentent un réseau trophique complexe avec un environnement physico chimique fluctuant, où l'activité lytique des bactériophages (fréquence des cellules lytiquement infectées, FVIC ; *burst size*, BS) ne reflète sans doute pas l'ensemble de l'activité de toutes les communautés virales présentes. Contrairement à ces couches superficielles, les communautés biologiques du monimolimnion sont réduites aux seuls virus et procaryotes évoluant dans un environnement physico chimique contraignant (anoxie, absence de lumière) mais plus stable. Dans cette zone, la lyse virale est clairement et intimement liée à la productivité bactérienne (PB), même si, contrairement à la PB, les valeurs les plus élevées de la FVIC n'ont, paradoxalement, pas été enregistrées en profondeur. Les virus seraient donc le seul facteur de régulation des communautés bactériennes dans la zone anoxique monimolimnique du lac Pavin. Cette étude permet donc

de dire que le monimolimnion anoxique du lac Pavin, où le temps de résidence des eaux est de plusieurs dizaines d'années, présente un fonctionnement trophique essentiellement régit par la boucle virale (Matière organique dissoute (MOD) > procaryotes > virus > MOD), où les nutriments et substrats seraient séquestrés et recyclés plusieurs fois avant incorporation dans le sédiment ou retour dans les couches supérieures du lac. Cela expliquerait l'existence de virus typiques, endémiques, dans les couches profondes du lac Pavin.

3. Rôle de la lysogénie dans la colonne d'eau

Cette étude nous a permis de montrer, pour la première fois, qu'une fraction importante du bactérioplancton (jusqu'à 16 % de l'abondance totale) contient des génomes viraux viables, insérés dans les génomes bactériens. Les variations saisonnières de l'abondance relative des cellules procaryotes lysogènes semblent être liées à la disponibilité des hôtes potentiels (abondance et statut métabolique des procaryotes). Par ailleurs, la fréquence des cellules lysogéniquement infectées est plus faible et fluctue de façon antagoniste à la fréquence des cellules lytiquement infectées. La mise en place d'un 'mode de vie' lysogène ne semble être effective qu'en dessous d'un seuil de l'abondance des hôtes procaryotes. Cela indique que le 'mode de vie' lysogène serait, pour la communauté virale, plus un mécanisme de survie que de prolifération, pouvant, par ailleurs, avoir une incidence sur l'évolution adaptative des communautés d'hôtes. Dans la colonne d'eau du lac Pavin, les cellules lysogènes sont plus abondantes dans les couches mixolimniques, excluant l'hypothèse que la lysogénie serait un réservoir de virus dans les couches profondes du lac, et indiquent que l'évolution écologique des communautés d'hôtes dans ces couches n'est pas liée au mode d'expression virale qu'est la lysogénie.

4. Perspectives

Les recherches menées au cours de ce travail de thèse soulignent l'importance des hôtes potentiels dans les processus de diversification de la communauté virale aquatique, et dans la dynamique des abondances et des activités lytique et lysogénique saisonnières du virioplancton. Ces résultats proviennent d'observations empiriques, qui nécessitent, pour être confirmés, d'être testés par des approches expérimentales. Des expériences visant à répondre

en partie aux questions écologiques liées à la qualité des hôtes (statut métabolique, intégrité membranaire, résistance, diversité clonale…) ont été menées, mais nous n'avons pas eu le temps, avant la mise en page de ce document, de traiter les résultats acquis. Par ailleurs, l'isolation et la culture de couples virus-hôtes à partir d'échantillons naturels seraient nécessaires pour étudier les interactions entre virus, hôtes et environnement abiotique. D'autre part, dans les études de la littérature (y compris nos propres études), l'importance quantitative des activités lytique et lysogène des virus est exprimée en fonction de l'abondance totale du bactérioplancton. Cela ne rend sans doute pas bien compte de l'importance fonctionnelle des virus dans le fonctionnement des écosystèmes aquatiques, puisqu'une fraction significative du batérioplancton peut ne pas être active. Au cours des expériences dont il est question ci-dessus, nous avons estimé que, dans le lac Pavin, moins de 30 % des bactéries présentes étaient actives ou présentaient une intégrité membranaire. Enfin, l'activité virale de type pseudo lysogénique ou de type chronique reste, actuellement, totalement ignorée en écologie virale aquatique. Il en est de même pour l'intervention des virus dans les transferts horizontaux de gènes entre microorganismes, et l'impact de ces processus aléatoires dans la dynamique de la diversité microbienne. De manière générale, l'action des virus sur la diversification et sur la dynamique de la diversité microbienne, par le biais notamment de l'action des produits de la lyse virale (catalyse biogéochimique), ou de l'action plus ou moins directe des virus dans les échanges génétiques entre microorganismes, reste largement méconnue. Une attention particulière doit aussi être portée à la connaissance et à l'écologie des virus non-bactériophages, notamment des virus cyanophages et autres 'phytophages'.

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ANNEXES

Annexe 1: Dysbiosis in inflammatory bowel disease : a role for bacteriophages ?

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Dysbiosis in inflammatory bowel disease: a role for bacteriophages?

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LETTERS

Dysbiosis in inflammatory bowel disease: a role for bacteriophages?

Intestinal bacteria have been implicated in the initiation and amplification of inflammatory bowel disease (IBD). The dysbiosis theory, reviewed by Tamboli *et al* (*Gut* 2004;**53**:1), is that an imbalance between putative "harmful" versus "protective" bacterial species may promote chronic intestinal inflammation. Although several studies published so far support this hypothesis, the most vexing question posed by Tamboli *et al* remains "what is the origin of dysbiosis?". Bacteriophages outnumber bacteria by a factor of 10 in many natural ecosystems, exert a strong influence on bacterial diversity and population structure, and are probably involved in dysbiosis by destabilising bacterial communities.[1] They could be involved indirectly through gene transfer and genome reorganisation within the bacterial population or directly as immunomodulating agents[2] or by steric competition for microbe-associated molecular patterns on bacterial surfaces. However, bacteriophages are a neglected component of the gut microbiota. The first viral metagenomic study demonstrated a wide diversity (1200 genotypes) of uncultured bacteriophage species.[3] The present study aimed at measuring the total viral community associated with the gut mucosa and comparing viral abundance between healthy individuals and patients with Crohn's disease (CD), and also between the ulcerated and non-ulcerated mucosa of these patients.

Fourteen healthy individuals and 19 CD patients were recruited and gave their informed consent. Biopsies were obtained during colonoscopy and, for CD patients, from non-ulcerated and ulcerated tissues. Biopsies were disrupted by ultrasonication, filtered through 0.22 µm membranes and fixed in glutaraldehyde. For epifluorescence microscopy, biopsy supernatants were filtered through 0.02 µm membranes. Virus-like particles (VLPs) were stained with SYBR Green and counted on triplicate subsamples. VLPs were detected in every sample, and no contaminating bacteria were observed (fig 1A). Strikingly high numbers of VLPs were observed (fig 1B), with an average of 1.2×10^9 VLPs/biopsy (4.4×10^7–1.7×10^{10}). Transmission electron microscopy demonstrated that viral particles corresponded to bacteriophages, with morphotypes consistent with *Siphoviridae*, *Myoviridae* and *Podoviridae* being the dominant families (fig 2). Each individual seemed to be colonised by one dominant phage family. This result was confirmed by pulse-field gel electrophoresis of viral genomic DNA (data not shown). At the mucosal level and with specific reference to the potential role of bacteriophages in dysbiosis, CD patients harboured significantly more VLPs than healthy individuals (2.9×10^9 vs 1.2×10^8 VLPs/biopsy; Wilcoxon test $p=0.024$). Moreover, decreased amounts of VLPs were detected in CD ulcerated mucosa, with an average of 2.1×10^9 VLPs/biopsy compared with 4.1×10^9 VLPs/biopsy from non-ulcerated mucosa (fig 1). It can be hypothesised either that more viruses are produced or that they can survive longer in non-ulcerated areas. Whether bacterial composition and abundance differ when comparing non-ulcerated with ulcerated mucosa is still controversial although differing bacteriophage life cycles (lysis, lysogeny) could be responsible for some of these discrepancies.

This study shows for the first time a dense bacteriophage community specifically associated with the gut mucosa, reaching 10^{10}/mm^3 of tissue. Significantly more bacterio-

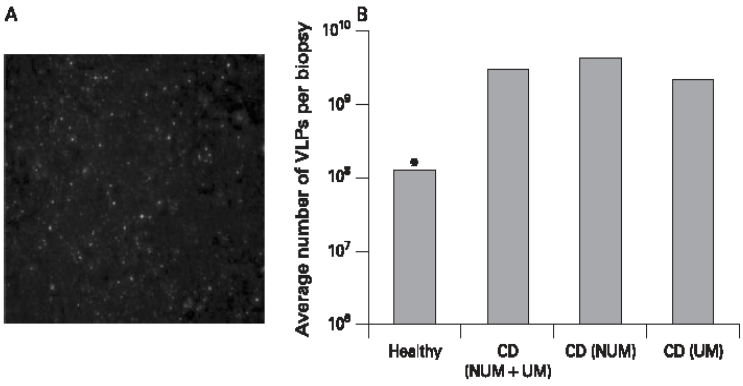


Figure 1 Concentrations of virus-like particles (VLPs) assessed by epifluorescence microscopy on gut mucosal samples from healthy individuals and Crohn's disease (CD) patients. (A) SYBR Green I-stained human mucosal viral concentrate from a healthy individual (concentrate diluted to 5×10^{-4}). (B) Average number of VLPs per biopsy in the different clinical groups. *$p = 0.024$ Wilcoxon text. NUM, non-ulcerated mucosa; UM, ulcerated mucosa.

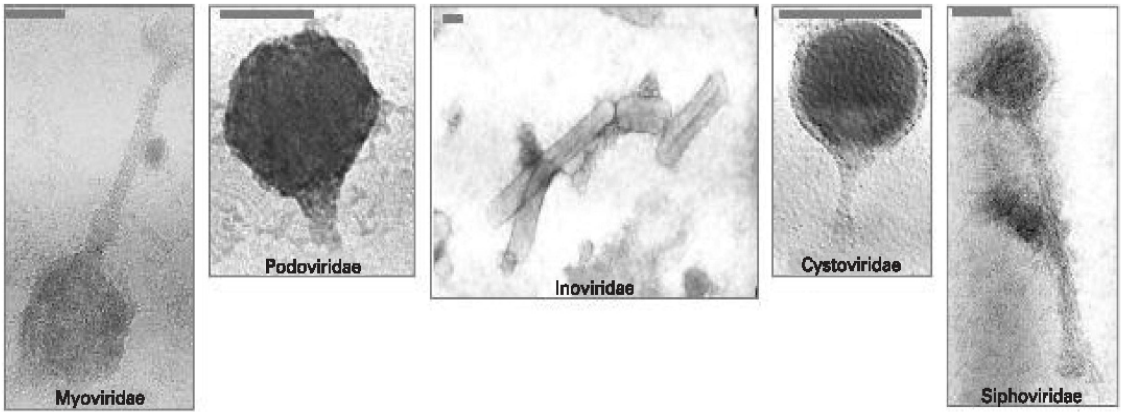


Figure 2 Transmission electron microscopy observation (80 kV, ×40 000 magnification) of bacteriophage morphotypes from mucosal samples. The dominant morphotypes are *Myoviridae*, *Podoviridae* and *Siphoviridae*. The grey bar represents 100 nm.

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phages were detected in the mucosa from CD patients than from healthy individuals. Tamboli *et al* discussed risk factors known to affect the gut microbial composition (host genetic background, method of birth delivery, early bacterial colonisation of neonates, diet and environment) which could be responsible for dysbiosis. Based on our results, we postulate that bacteriophages might also play a key role in the dysregulated immune response of IBD patients to the mucosal-associated bacterial population. More detailed studies that relate phage populations to disturbances in bacterial populations and the dysregulated host immune response on larger cohorts would help in establishing their role in the pathogenesis of IBD.

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Annexe 2: Short-term variations in abundances and potential activities of viruses, bacteria and nanoprotists in Lake Bourget

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ORIGINAL ARTICLE

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Short-term variations in abundances and potential activities of viruses, bacteria and nanoprotists in Lake Bourget

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Abstract Samples were collected at four depths every 6 h over a 42-h period during two contrasting seasons (June vs. December) from Lake Bourget, France, for evidence of circadian fluctuations in the concentrations and potential activities of viruses, prokaryotes and protists in relation to environmental conditions: temperature, chlorophyll *a* and dissolved organic carbon (DOC) concentrations. Considerable vertical and temporal fluctuations were observed for all variables. Circadian variations were noted for DOC and chlorophyll *a* concentrations. Despite the external abiotic forcing (light, water movements), the fluctuations of microbial variables (including viruses) in most cases were apparently linked to biotic factors and interactions. Standing stocks and activities, as well as the number and levels of correlations among the microbial components, were, surprisingly, higher in winter than in summer. We speculate that this was because trophic interactions prevailed over the seasonal forcing (i.e. temperature) in shaping the observed differences.

Keywords Lakes · Diurnal cycles · Viruses · Virioplankton · Bacteria · Protists

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Introduction

Because turnover rates are relatively high, species composition, cell counts, biomass and activities of pelagic microorganisms can vary substantially over short time scales (Sime-Ngando and Hartmann 1991; Amblard et al. 1994; Jugnia et al. 2000; Winter et al. 2004). Some microorganisms, such as ciliates, are motile, chemosensory and photosensitive, and others, such as copepods, can migrate vertically on a diel cycle (see Sime-Ngando and Hartmann 1991). In general, fluctuations in both biotic and abiotic parameters are discernible in pelagic ecosystems at the time scale of diurnal cycles.

Basically, this is because both autotrophic and heterotrophic processes depend on factors as obvious as light. Light can govern cycles of resources, such as phytoplankton exudates, and influence heterotrophic processes. Light can also influence the mortality of pelagic microbes, either directly through DNA damage (Herndl et al. 1993; Jeffrey et al. 1996) or indirectly, since it is a dominant mechanism of viral destruction and inactivation (Suttle and Chen 1992; Wommack et al. 1996; Noble and Fuhrman 1997; Weinbauer et al. 1997). Enhanced solar ultraviolet exposure has also been shown to be an inhibitory factor for nanoflagellate bacterivory (Ochs 1997; Ochs and Eddy 1998). Other studies have concluded that light may stimulate heterotrophic processes, such as photoenzymatic activity or photoreactivation (i.e. light-dependent DNA repair) in a bacterial community (Weinbauer et al. 1997) and may also facilitate digestion, grazing and growth rates of protozooplankton (Strom 2001). The direct impact of light on crustacean zooplankton and ichthyoplankton has also been partially tested (Browman et al. 2000).

However, it is usually difficult to differentiate the causes of short-term variability in natural communities. In addition to external abiotic forcing and day night cycles, a mixture of factors such as water movements, turbulence, population dynamics and interactions may be involved (Sime-Ngando and Hartmann 1991;

Sime-Ngando et al. 1991), including possibly chaotic fluctuations (Becks et al. 2005; Mandal et al. 2006). Microbial communities in a given sampling point within the water column can be imported or exported horizontally or vertically as a result of advection, thereby, changing the variance resulting from biotic interactions in relation to the diel cycle. Consequently, the effects of diel cycles on the biology of the plankton are usually difficult to isolate because they are obscured by the hydrology of the system. This is well known from lake studies (Sime-Ngando and Hartmann 1991; Sime-Ngando et al. 1991; Amblard et al. 1994; Jugnia et al. 1998, 2000; Tadonléké et al. 1998) compared to the oceans; where the use of free-floating buoy or drifter deployments is more practicable (Weinbauer et al. 1995; Graham et al. 2000; Winter et al. 2004).

The aim of the present study was to examine short-term variations in the abundances and potential activities of viruses, prokaryotes and nanoprotists in Lake Bourget, France, in relation to environmental conditions, i.e. temperature, chlorophyll *a* and dissolved organic carbon (DOC) concentrations. Samples were collected from one station at four different depths representative of the water column every 6 h over a 42-h period during two contrasting seasons (June vs. December) for evidence of circadian fluctuations. High-resolution profiles of temperature and of chlorophyll *a* concentration were determined in an effort to track the vertical structure (i.e. physical and biological) of the whole water column. This is the first attempt to study short-term variability in the plankton of Lake Bourget.

Materials and methods

Study site, sampling and physico-chemical parameters

Lake Bourget is located on the edge of the Alps (45°44′N, 05°51′E). It is an elongated (18 km and 3 km in length and width, respectively) north south orientated lake, with a surface area of 42×10^6 m^2, a total volume of 3.5×10^9 m^3, maximum and average depths of 145 m and 80 m, respectively and a water residence time of approximately 10 years. It has a catchment area of about 560 km^2, with maximum and average altitudes of 1,845 m and 700 m, respectively. Water quality restoration programmes started in the 1970s have significantly lowered the trophic status of the lake, from highly eutrophic to mesotrophic. In 2005, the water transparency varied between 2.4 m and 14.5 m, the total P concentration in winter was at about 23 µg l^{-1} and the maximum concentration of chlorophyll *a* was less than 13 µg l^{-1} (Jacquet et al. 2005a, b).

The sampling strategy was decided based on the available logistics and included two short-term sampling series carried out at a reference station known as point I in Gresine Bay (maximum depth Z_{max} = 35 m), located in the eastern part of the lake. For each series, four different depths were sampled (2, 10, 15 and 30 m) eight times, every 6 h, from 6:00 p.m. on 9th June to 12:00 noon on 11th June 2004 for the first series, and from 12:00 noon on 1st December to 6:00 a.m. on 3rd December 2004 for the second series. Before each sampling operation, high-resolution vertical profiles (i.e. continuous measurement in the whole water column) of temperature and chlorophyll *a* concentrations (Chl) were monitored using FluoroProbe® (BBE, Moldenke, Germany), a submersible spectrofluorometer configured for in situ measurements of chlorophyll *a* fluorescence, as described and detailed elsewhere (Leboulanger et al. 2002). For the June sampling series, DOC levels were determined in 15-ml aliquots of filtered (pre-combusted glass-fibre filters) samples collected in pre-combusted glass vials. The samples were then acidified to pH < 3 with 2 N HCl and the vials were flame-sealed and stored at 4°C in the dark until analysis. DOC concentrations were measured using a carbon analyser (Labtoc, UV promoted persulphate oxidation, IR detection), as previously described (Comte et al. 2006).

Virus and cell counts

The abundances of viruses, heterotrophic bacteria and picocyanobacteria were estimated immediately after sampling using flow cytometry. A 1-ml sample was analysed without adding any fixative or dye to analyse the autotrophic picoplankton community. In Lake Bourget, this community is typically dominated by Phycoerythrin-rich cyanobacteria (Humbert and Le Berre 2001; Briand et al. 2005; Jacquet et al. 2005a). Another 1-ml sample was fixed with glutaraldehyde (0.5%, v/v, final conc.) and bacterial and viral counts were performed as described elsewhere (Duhamel and Jacquet 2006; Duhamel et al. 2006). We used a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm with the standard filter set-up. Fluorescent microbeads (Molecular Probes) of diameter 1 µm were added to each sample as an internal standard. For heterotrophic bacteria, samples were diluted with 0.2-µm-filtered water from the lake, while for viruses, samples were diluted with 0.02-µm-filtered TE (Tris EDTA, pH = 8) buffer and heated for 10 min at 75°C. The samples were stained with SYBR Green I (1/10,000 final conc.) for 15 min in the dark and run at medium speed (ca 40 µl min^{-1}). Either a minimal number of 10,000 events were recorded in log mode for each sample or a minimal acquisition time of 4 min when the number of events was < 10,000. Plots of green fluorescence measured at 530 ± 30 nm versus 90° light scatter or forward-angle light scatter were used to discriminate and count the stained items. Bacteria with high DNA content (HDNA) were discriminated (i.e. from those cells with low DNA content) on the basis of their typical FCM signatures within the total community and were considered as the most

dynamic members of the bacterial assemblage (Gasol et al. 1999).

For the nanoflagellate counts, glutaraldehyde (1%, v/v, final conc.) fixed samples were filtered (pressure < 100 mmHg) over polycarbonate membranes (diameter: 25 mm, pore size: 0.8 µm), stained with primulin (Caron 1983), mounted between slides and glass cover slips with a non-fluorescent immersion oil and stored at −20°C until analysis. The slides were examined under UV light to count heterotrophic nanoflagellates and under blue light to count pigmented flagellates at 1,250× magnification. Green light was useful to display phycoerythrin-containing flagellates.

Bacterial production and enzyme activity

Bacterial production (BP) was determined from the incorporation of [methyl-^{3}H] thymidine (Amersham Biosciences, UK, 70 95 Ci/mmol of specific activity) into bacterial DNA (Bell 1993). For each sampling time and depth, three replicates and one formalin-killed control were inoculated with thymidine at a final concentration of 20 nM in glass vials and then incubated in-situ for 2 h. The live incubations were stopped by adding formalin and all samples were extracted in the laboratory using trichloroacetic acid (TCA, 10% final conc.) and kept on ice for 30 min. The samples were then filtered through a 25-mm-diameter, 0.22-µm-pore-size membrane (GTTP). The filters were then rinsed three times with 3 ml of TCA (5% final conc.). The filters were placed in scintillation vials, allowed to dry and solubilised with 1 ml of soluene. After adding 10 ml of scintillation cocktail (Hionic Fluor, Perkin Elmer), the radioactivity was counted with a 2100-TR (Packard Instruments) with counting efficiency corrected for quenching. Bacterial production, calculated in moles of ^{3}H-TdR incorporated into DNA, was converted into the number of cells produced by using a conversion factor (10^{18} cells mol^{-1}) recommended by Bell (1993). In all cases, the within-sample coefficients of variation (n = 3) were <15%.

For the December sampling series, the extracellular enzyme activity was determined as an additional proxy of bacterial activity in the winter. The cleavage of a model substrate leucine methyl-coumarin-amide (Leu-MCA; Sigma) was measured according to Somville and Billen (1983). Increasing amounts of the substrate Leu-MCA stock solution (40 mmol l^{-1}) were added to 2-ml bulk water subsamples collected at each depth (2, 10, 15 and 30 m), in order to obtain final concentrations ranging from 0 to 4,000 µmol l^{-1}. Incubation lasted one hour and was performed in the dark at 20°C and under slight movement with gentle stirring. The potential extracellular enzyme activity was then calculated for both unprocessed water and water filtered through the 2-µm filter, collected at each depth, by adding the fluorescent substrate to two replicate subsamples (2 ml) at the saturating concentration. The activities were similar in both fractions (Students t-test, $p > 0.05$) and were correlated ($r = 0.80$, $p < 0.01$). For this reason, only the activity in the unprocessed water is presented. A blank used in order to assess the non-enzymatic hydrolytic activity of the substrate was prepared by filtering a sufficient amount of water from each depth through a 0.2-µm-pore-size filter, by autoclaving it and by adding 230 µl of glycerine buffer to inhibit the enzyme activity before adding the substrate. After incubation, the fluorescence was determined using a fluorometric and luminometric reader (Fluoroskan Ascent FL, Thermo Labsystem), at excitation and emission wavelengths of 355 nm and 460 nm, respectively. A calibration curve was established by dissolving a 2 mmol l^{-1} stock solution of the standard MCA (Sigma) in Methyl Cellosolve and adding 0.22-µm-filtered and autoclaved water from the sampling site. The final results are expressed in µmol MCA l^{-1} h^{-1}. The variability among replicate incubations was typically small (CV < 5%).

Viral bacteriolysis

In formalin-fixed samples, the bacteria contained in 8-ml subsamples were harvested by centrifuging onto 400 mesh copper electron microscope grids with carbon-coated Formvar film using a Centrikon TST 41.14 swing-out rotor (Bettarel et al. 2004). Each grid was then stained for 30 s with uranyl acetate (2% w/w) and examined using a JEOL 1200EX transmission electron microscope operated at 80 kV at magnifications of 20,000 40,000×. At least 1,300 1,500 prokaryotic cells per sample were examined to determine the frequency of visibly infected cells (FVIC). Cells were scored as infected if they contained five or more intracellular viruses. For each sample, the mean burst size (viruses bacteria^{-1}) was estimated from the number of viruses in visibly infected cells. Because mature phages are visible only late in the infection cycle, FVIC counts were converted to the frequency of infected cells (FIC) using the equation $FIC = 9.524 \times FVIC - 3.256$ (Weinbauer et al. 2002). The FIC was then converted to viral-induced bacterial mortality (VBM, as a percentage per generation) according to Binder (1999) using the equation $VBM = (FIC + 0.6 \times FIC^2)/(1\ \ 1.2 \times FIC)$.

Statistical analyses

The data were subjected to one-way analysis of variance (absence of replicates for most variables prevents the use of two-way ANOVA) to test for effects of time or depth, and using correlation analysis to test for significant coupling between variables. In addition, a matrix of data was produced and analysed by means of principal component analysis (PCA) to identify combinations of variables that account for the largest amounts of the total variance observed. For all of the statistical analy-

ses, the data were normalised using logarithmic transformation.

Results

Temperature and chlorophyll *a* depth time profiles

In June, surface heating occurred in the late afternoon, increasing the relative thermal resistance to mixing. However, oscillations were apparent at the bottom boundary of the discontinuity layer, with a general cooling phase during the day-time and a deepening of the thermocline during the night (Fig. 1a). For all variables under study, the most significant depth-related difference was from temperatures recorded in June (Table 1). The water column was almost isothermal in December (7.8 8.1°C, Fig. 1b) but the temperatures fluctuated significantly over time during both seasons (Table 1). Chlorophyll *a* concentrations (Chl) were significantly (ANOVA, $p < 0.001$) higher in December (mean 6.9 µg l^{-1}) than in June (3.1 µg l^{-1}). Maxima were recorded in late afternoon and during the night-time in the thermocline in June and in the surface waters in December (Fig. 1b, d).

Short-term fluctuations in biological variables

Changes in concentration and rate measurements are shown in Figs. 2, 3, 4 and 5. For each sampling depth and campaign, the coefficients of variation for standing stocks over the study period ranged from 3% to 70%, apart for the abundance of autotrophic picoplankton recorded at 30 m depth in June, which exhibited a higher CV (211%) due to an exceptional peak recorded at 6:00 a.m., together with a deepening of the thermocline (Figs. 1a and 2c). When eliminating this peak, the CV drops to 35%. The highest CVs were calculated for bacterial production (range: 65 137% in June and 61 105% in December). Values for aminopeptidase activity measured in December ranged from 9% to 23%. Concentrations of the biological variables under study and the viral lytic activity were generally higher in December than in June (ANOVA, $p < 0.001$), similar to Chl. The few exceptions were for the abundance of autotrophic picoplankton and for bacterial production, which were significantly higher in June than in December (Figs. 1, 2, 3, 4 and 5).

ANOVA for the time effect yielded more significant differences in June than in December. Indeed, a significant effect of the sampling time was found for the esti-

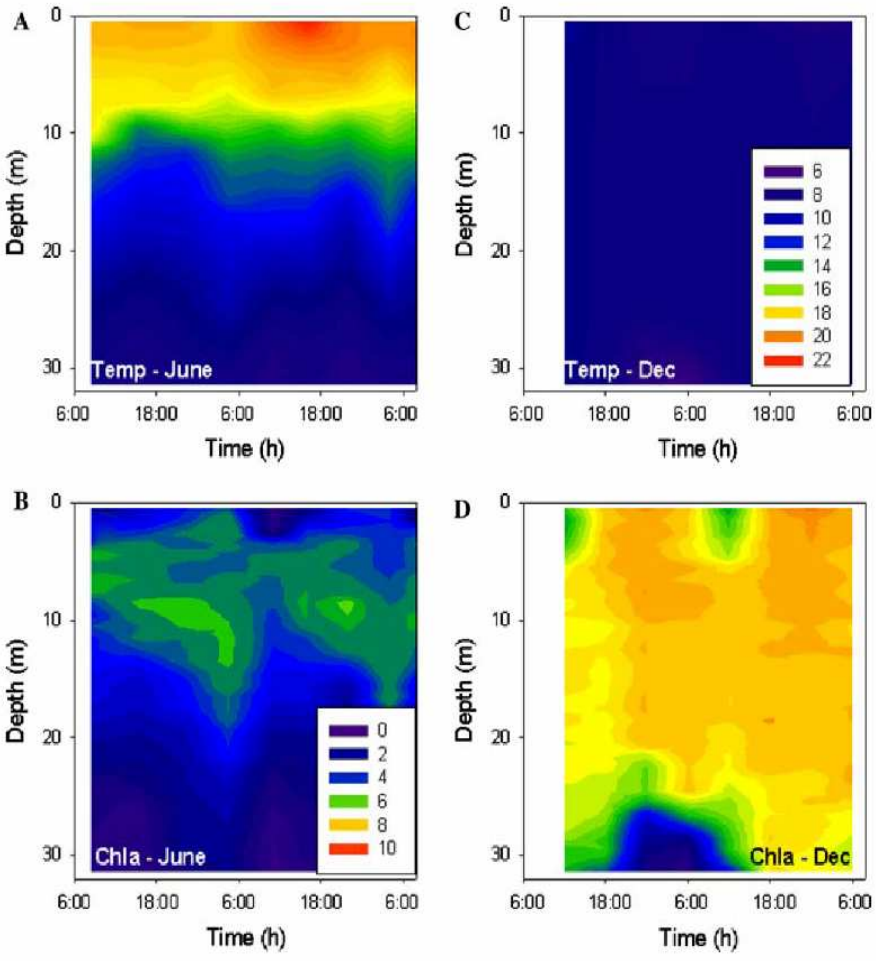


Fig. 1 Depth time diagrams of temperature and chlorophyll *a* concentrations in Lake Bourget, 9th to 11th June and 1st to 3rd December 2004, based on high-resolution vertical measurements (i.e. continuous measurements through the water column) carried out using a submersible spectrophotometer (FluoroProbe®, BBE, Moldenke, Germany)

mates of DOC, viral and HNF (heterotrophic nanoflagellates) abundances and bacterial production in June, and for temperature in December (Table 1). Significant effects of time, but also of sampling depth, were also recorded for temperature and Chl in June, and for viral abundance and bacterial production in December. The water column temperature, thus, varied significantly with the sampling time in December, whereas most of the biological variables varied more with depth than with time (Table 1).

During both campaigns, viral abundance increased with time at all depths and this was more marked in December than in June (Fig. 2a, d). Assuming an exponential growth model, viruses accumulated at 0.21 day^{-1} in June (r^2 = 0.58) and at 0.31 day^{-1} in December (r^2 = 0.83), corresponding to production rates of 0.6×10^7 and 2.4×10^7 viruses l^{-1} day^{-1}, respectively. These rates were two orders of magnitude lower that those (3.9 and 3.6×10^9 viruses l^{-1} day^{-1}, respectively) calculated from the viral-induced mortality (see below), bacterial production and burst sizes, which were substantially higher in December (range, mean: 18 81, 41 viruses $bacterium^{-1}$) than in June (10 48, 29 viruses $bacterium^{-1}$). A drop in viral and prokaryote abundances was noted during the first night in December at 30 m (Fig. 2d f), accounting for the significant variation noted with depth (Table 1). Similar to viruses, the abundance of HNF also generally increased with time. This was clearly related to the pattern during the last 24 h of sampling (Fig. 3a, c), with an associated accumulation rate of 0.28 day^{-1} (r^2 = 0.67). In contrast, the significant effect of time on bacterial production in June was related to a general decreasing trend at all depths from the second sampling point (Fig. 4a). Finally, a diel cycle was apparent with regard to DOC concentrations measured in June, with an increasing phase during the day-time at all sampling depths, reaching the highest value at 15 m (Fig. 4c).

Table 1 Results of analysis of variance (ANOVA) used to test for differences with sampling depth and with time

Variable	Depth		Time	
	F value	*p* value	*F* value	*p* value
June*				
Temperature (T °C)	**546**	**< 0.001**	**2.740**	**0.030**
DOC	2.120	0.120	**2.850**	**0.020**
Chlorophyll (Chl)	**44.020**	**< 0.001**	**4.200**	**0.004**
Viruses (VA)	1.960	0.150	**3.800**	**0.008**
Bacteria (BA)	**6.370**	**0.003**	0.770	0.610
HDNA	**7.400**	**0.001**	0.830	0.570
APP	**9.240**	**< 0.001**	0.060	0.990
HNF	2.010	0.140	**4.170**	**0.005**
PNF	2.220	0.110	1.500	0.220
FVIC	1.430	0.250	2.030	0.090
Bacterial production (BP)	0.100	0.950	**4.460**	**0.003**
December*				
Temperature	2.490	0.080	**3.590**	**0.010**
Chlorophyll	4.700	0.110	0.660	0.700
Viruses	**5.640**	**0.005**	**11.820**	**< 0.001**
Bacteria	**3.750**	**0.020**	0.800	0.590
HDNA	**3.150**	**0.040**	0.920	0.500
APP	**5.820**	**0.004**	0.930	0.500
HNF	**36.400**	**< 0.001**	1.890	0.120
PNF	**10.870**	**< 0.001**	1.700	0.162
FVIC	1.510	0.230	0.250	0.960
Bacterial production	**7.040**	**0.001**	**13.230**	**< 0.001**
Aminopeptidase (MCA)	**3.170**	**0.040**	0.580	0.750

All estimates were log-transformed. Temperature (T °C), concentrations of dissolved organic carbon (DOC), chlorophyll *a* (Chl), viruses (VA), total bacteria (BA), bacteria with high DNA content (HDNA), autotrophic picoplankton (APP), heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), rates of bacterial cell production and aminopeptidase extracellular enzyme activity (MCA)

* Differences between June and December were all significant ($p < 0.001$) for all variables. Significant values are in bold

Prokaryote production and mortality

Bacterial production ranged from 1 to 10×10^7 cells l^{-1} h^{-1} (mean 5×10^7 cells l^{-1} h^{-1}) in June and from 0.5 to 7×10^7 cells l^{-1} h^{-1} (mean 1×10^7 cells l^{-1} h^{-1}) in December (Fig. 4a, d). The frequency of visibly infected cells was less than 2.5% (mean = 1.3%) in June and less than 4% (2.3%) in December, corresponding to the destruction of 5 30% (mean 11%) and 10 58% (29%) of bacterial production by viruses, respectively (Fig. 5). We were not able to conduct grazing experiments during this study. However, a grazing study in the same lake carried out in 2002 (Comte et al. 2006) reported taxon-specific HNF clearance rates of 1.6 5 nl ind^{-1} h^{-1} (mean = 3.07 nl ind^{-1} h^{-1}). Relating this to our June campaign, this corresponds to a potential grazing impact ranging from 27% to 84% of bacterial production (mean = 52%). In December, the calculated grazing potential exceeded bacterial production, even when the minimum clearance rate given above was applied.

Relationships between variables

The number and the significant levels of empirical relationships between variables were higher in December than in June (Table 2). With a few exceptions in June, temperature, Chl and the abundances of viruses, of total bacteria and of bacteria with high DNA content were positively correlated to each other during both campaigns, as were the abundances of phototrophic and heterotrophic nanoflagellates. Data on bacteria with low DNA content (not shown) were not related to any other variables. In addition, viral abundance during both campaigns was positively correlated to HNF counts. The latter variable was negatively correlated to bacterial production in June, whereas bacterial production and Chl were positively correlated in December. The other correlations were positive and were detected in the December samples, including those between (1) viral abundance vs. bacterial production and enzyme activity, (2) FVIC versus HNF abundance, (3) the abundance of

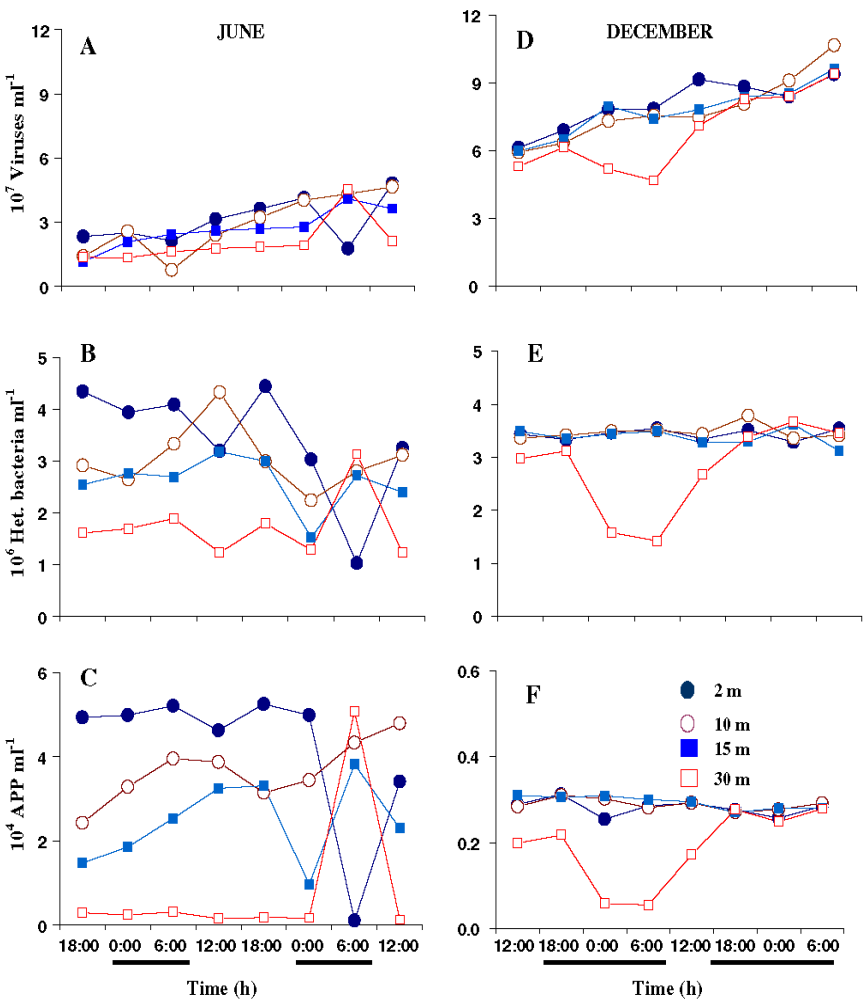


Fig. 2a–f Spatial and temporal changes in concentrations of virus-like-particles (**a**, **d**), heterotrophic bacteria (**b**, **e**), and autotrophic picoplankton (APP; **c**, **f**) in Lake Bourget between 9th and 11th June and 1st and 3rd December 2004. The *black bars* indicate the night-time period

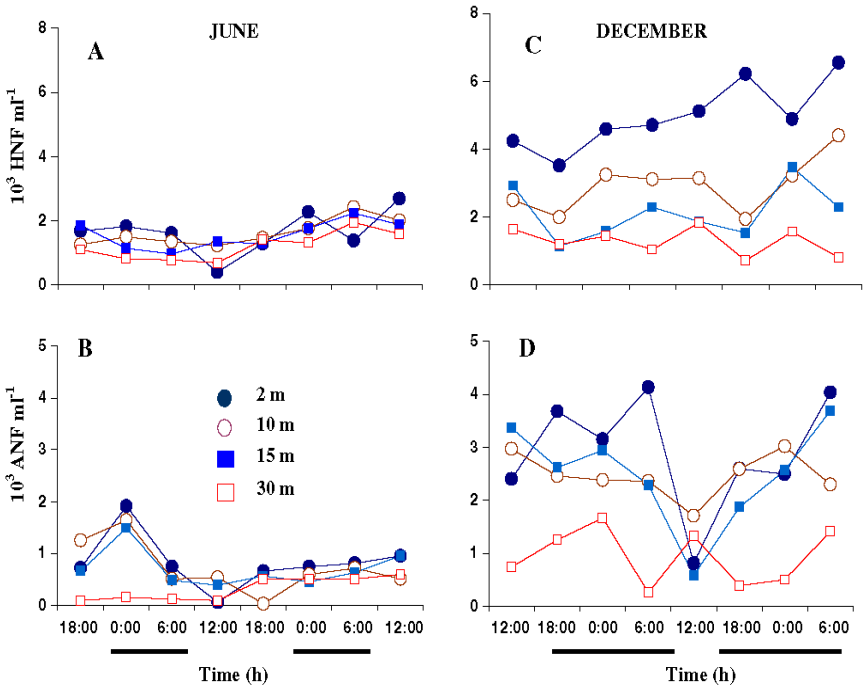


Fig. 3a–d Spatial and temporal changes in concentrations of heterotrophic (HNF; **a**, **c**) and autotrophic (ANF; **b**, **d**) nanoflagellates in Lake Bourget between 9th and 11th June and 1st and 3rd December 2004. The *black bars* indicate the night-time period

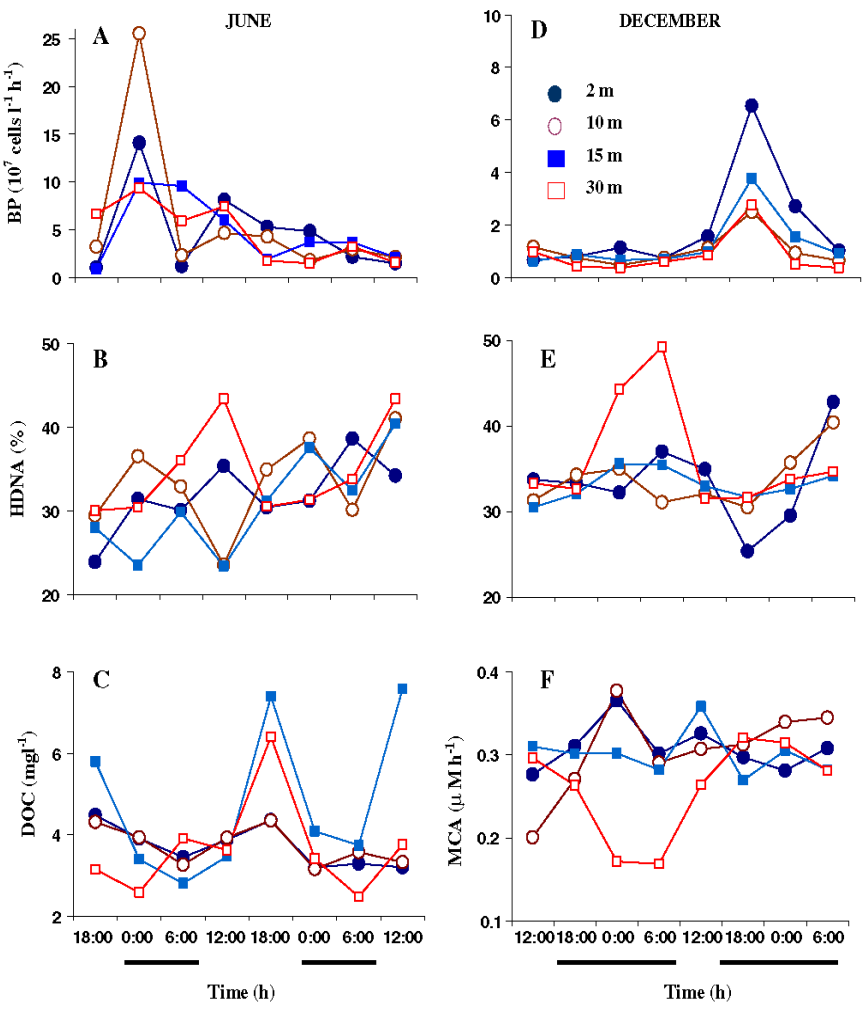


Fig. 4a–f Spatial and temporal changes in bacterial production (BP; **a**, **d**), percentage of heterotrophic bacteria with high DNA content (HDNA; **b**, **e** % of total abundance), dissolved organic carbon concentration (DOC (**c**, measured in June) and aminopeptidase extracellular activity (MCA; **f**, measured in December) in Lake Bourget between 9th and 11th June and 1st and 3rd December 2004. The *black bars* indicate the night-time period

total and high-DNA bacteria versus enzyme activity and (4) enzyme activity vs. temperature, Chl and the abundance of autotrophic picoplankton (Table 2).

For both sampling campaigns, the plots of the descriptors of the PCA grouped most of the variables along axis 1, the relationships between variables being apparently more numerous and closer in December than in June (Fig. 6a, c). In June, burst size (for which no correlation was noted) and the number of infected cells were isolated and opposed to the other variables. The majority of these variables were selected positively by the major principal component in June and negatively in December. The plots of the field observations showed that this selection was mainly related to the samples collected at 2, 10 and 15 m, which were opposed to those collected at 30 m (Fig. 6b, d).

Discussion

The present study is an attempt to analyse planktonic microorganisms on a time scale that is realistic in terms of their probable generation times. High-resolution temperature profiles recorded in the stratified water column in June showed internal oscillations with a period on the order of 12 18 h (Fig. 1a). Physical, chemical and the related biological isolines were shifting with the passage of these internal waves. For example, the abundances of viruses, bacteria and autotrophic picoplankton shifted markedly in the water column on June 11 at 6:00 a.m., coinciding with the deepening of the thermocline (Figs. 1a, 2a c). From these observations, we conclude that wind-forced internal waves have effects on the plankton dynamics in Lake Bourget (Vinçon-Leite et al. 1989), mainly as a result of the import/export advection of microbial stocks from the target sampling points.

Besides advection, the abundances of HNF in June were similar at all sampling depths, where they accumulated late in the sampling period at a rate (about 0.3 day^{-1}) that was typical of those of planktonic protists in temperate lakes during the spring growing season (Carrias et al. 2001). Similar patterns were also noted for viruses during the two seasons. Sharp increases in the abundances of bacteria and autotrophic picoplankton between 6:00 a.m. and 6:00 p.m. in the deepest water masses on 2nd December would require a

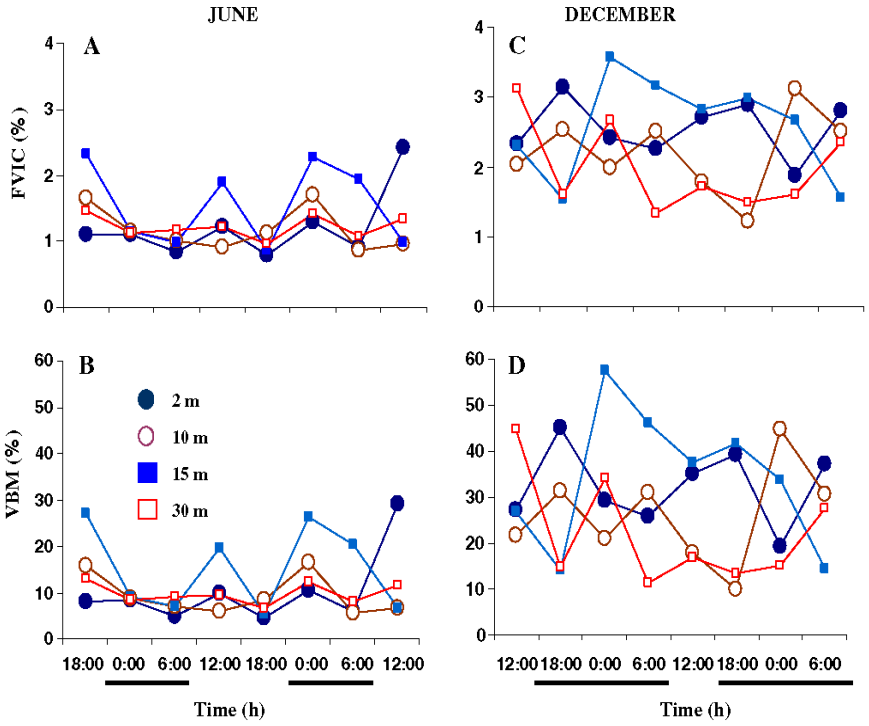


Fig. 5a–d Spatial and temporal changes in the frequency of visibly infected bacterial cells (FVIC; **a**, **c** % of total counts) and in the virus-mediated bacterial mortality (VBM; **b**, **d** % of bacterial production) in Lake Bourget between 9th and 11th June and 1st and 3rd December 2004. The *black bars* indicate the night-time period

Table 2 Results of Pearson's correlation analysis used to test for empiric correspondence among estimated variables

	VA	FVIC	BA	HDNA	BP	MCA	APP	HNF	PNF	Chl
June										
HDNA	**0.41***	−0.21	**0.91*****							
BP	−0.02	−0.15	0.04	0.02						
APP	**0.46****	−0.05	**0.92*****	**0.91*****	0.05	ND				
HNF	**0.75*****	0.23	0.22	0.25	**−0.45****	ND	**0.34***			
PNF	0.27	0.01	0.23	0.18	−0.14	ND	0.25	**0.57*****		
Chl	0.17	−0.08	**0.50****	**0.56*****	0.16	ND	**0.62*****	0.23	**0.34***	
T °C	0.24	−0.11	**0.62*****	**0.65*****	−0.08	ND	**0.66*****	0.27	0.28	**0.65*****
December										
BA	**0.61*****	0.18								
HDNA	**0.62*****	0.25	**0.80*****							
BP	**0.35**	0.02	0.29	−0.07						
MCA	**0.61*****	0.18	**0.74*****	**0.68*****	0.17					
APP	**0.58*****	0.23	**0.97*****	**0.79*****	0.26	**0.84*****				
HNF	**0.35***	**0.36***	**0.36***	**0.36***	0.27	**0.38***	**0.35***			
PNF	0.26	0.31	**0.45****	**0.41***	0.05	0.29	0.26	**0.58*****		
Chl	**0.64*****	0.22	**0.94*****	**0.73*****	**0.34***	**0.78*****	**0.64*****	**0.37**	**0.49****	
T °C	0.21	0.16	**0.80*****	**0.54****	0.16	**0.72*****	0.21	0.16	0.29	**0.69*****

See Table 1 for an explanation of the abbreviations. Significant correlations are in bold. Asterisks denote the levels of correlation as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The underlined coefficients denote common correlations for the two sampling periods

net community doubling time of 5 8 h. Moreover, bacterial production estimated via bottle incubations was higher and fluctuated more in June than in December, the values (10^7 10^8 cells l^{-1} h^{-1}) being typical of oligotrophic to mesotrophic lakes (Bettarel et al. 2004; Colombet et al. 2006). These observations suggest that part of the variations were intrinsic to microbial compartments during this study, despite noise from water movements.

Only a few of these variations were related to the day night cycles, perhaps because of the relatively low resolution in our sampling intervals. A diurnal cycle was noted for DOC measured in the water column in June, with maxima occurring during the day (Fig. 4c). In contrast to DOC, chlorophyll *a* concentrations in the surface waters peaked in the late afternoon and during the night, and were correlated to several variables during the two study seasons, as was the abundance of viruses

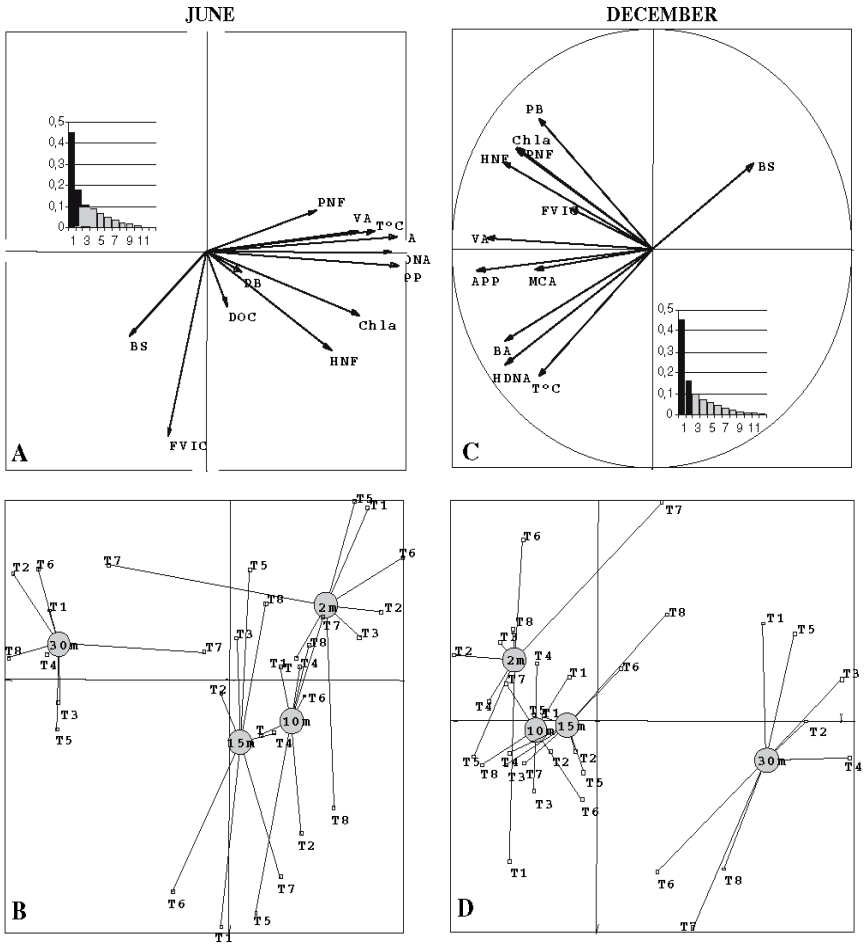


Fig. 6a–d Results of the principal component analysis (PCA) obtained from the main variables under study (**a**, **c**) and from the observations made for each sampling depth and time (**b**, **d**) in Lake Bourget between 9th and 11th June and 1st and 3rd December 2004. See Table 1 for an explanation of the previous abbreviations; BS = burst size, T1 T8 in panels **b** and **d** refer to the eight sampling hours for each of the four sampling depths. Horizontal *X*-axis = Axe 1, vertical *Y*-axis = Axe 2. *Inset* histograms in **a** and **c** are graduated from 0 to 0.5 and represent the fractions of variances (*Y*) explained by the different PCA axes (*X*, from Axe 1 onwards)

with that of autotrophic picoplankton (APP) (Table 2). The latter community was exclusively represented by Phycoerythrin-rich cyanobacteria, which usually appear to be easier to infect with viruses (Wilhelm et al. 2006). Phytoplankton communities were, thus, likely to be actively implicated in trophic interactions. From the temporal fluctuations of chlorophyll *a*, it is difficult to know whether phytoplankton cells were accumulating or possibly dividing in the late afternoon or during the night, as we have no data on diel patterns or light-related autoecology of phytoplankton populations, primarily of *Planktothrix rubescens*, the dominant cyanobacterium in Lake Bourget (Humbert and Le Berre 2001; Briand et al. 2005; Jacquet et al. 2005a). However, tentative explanations from the freshwater literature include (1) algal avoidance of exposure to high light, which is a common situation in aquatic systems (Tadonléké et al. 1998) and (2) increasing chlorophyll cell content with decreasing light intensity in some algal species, primarily cyanobacteria (Tadonléké et al. 1998).

Apart from two exceptions (i.e. bacterial production and autotrophic picoplankton abundance), all biological variables under study were significantly higher in the winter than in the summer (Table 2). In Lake Bourget, the phytoplankton biomass is typically dominated (>90% of the total) by the cyanobacterium *P. rubescens* for a significant part of the year (Jacquet et al. 2005a). Using both probe fluorescent measurements (i.e. the same fluoroprobe used in this study, calibrated with *P. rubescens*) and cell counts, it has recently been shown from depth-integrated seasonal data that this cyanobacterium typically peaks in early December (see Fig. 5 in Leboulanger et al. 2002). From our data and those from multi-year reports on Lake Bourget (Briand et al. 2005; Jacquet et al. 2005a), this is partly related to the fact that, in late autumn and winter, *P. rubescens* colonises the entire water column, whereas during the other seasons, its development is more restricted to specific zones in the water column. Multivariate analysis clustered most of the variables along axis 1 in June and December, isolating burst size and samples collected at 30 m from other variables collected in the upper water column. Correlations among these variables were higher in December than in June, in both their intensity and number, indicating that biological variables were trophically more dependent on each other in December than in June. This could be related to the grazing pressure. During our study, the abundance of zooplankton (dominant species *Daphnia hyalina* and *D. galatea*) was largely higher in spring/summer than in

winter (Laine and Anneville, unpublished) but no general diel pattern is available for metazooplankton in Lake Bourget.

We speculate that microbial interactions and the related cascading effects were more enhanced in winter than in summer. This was indicated by the couplings (1) between chlorophyll *a* and bacterial production (Ducklow and Carlson 1992), (2) between viral abundance and bacterial abundance production and enzymatic activity (Bettarel et al. 2003, 2004) and (3) between the abundances of viruses and bacteria and their potential HNF grazers (Gonzalez and Suttle 1993; Bettarel et al. 2005) in winter. It is tempting to attribute the higher abundance and potential bacterivory (that considerably exceeded bacterial production in all samples) of HNF in December (compared to June) to the reduction of grazing pressure from zooplankton, resulting in higher competition for food in HNF communities, a diversification of their diet and the occurrence of non-bacterivorous species. In contrast, higher zooplankton grazing pressure on HNF in June could have given a competitive advantage to the tiniest HNF cells, i.e. the dominant bacterivores in aquatic systems (Strom 2000), which are known to be strong stimulators of bacterial production (Posch et al. 1999; Simek et al. 2001; Sime-Ngando and Ram 2005). This may, thus, also help explain the two exceptions to the detected 'anomaly' (i.e. the fact that most biological variables under study were significantly higher in the winter than in the summer) noted for autotrophic picoplankton and bacterial production. We consider that the detected 'anomaly' was likely to be forced by the seasonal behaviour of the major phytoplankton population, in relation to the grazing environment and the related trophic interactions.

In conclusion, microbial parameters under study varied considerably over a short-term period in Lake Bourget. Circadian variations noted for DOC and chlorophyll *a* concentrations were found to match the day night cycle to some extent. However, despite the external abiotic forcing (light, water movements), the fluctuations in biological variables in most cases were apparently forced by trophic interactions. However, we think that a much tighter vertical and temporal sampling programme than ours would be required to better observe general patterns of diel changes in Lake Bourget. Clearly, our study could not distinguish all of the factors relating to short-term variations in the microbial components under study. It, therefore, left unresolved the question of the impact of micro- and mesozooplankton (such as ciliates and rotifers), the role of a variety of sources of food and predation, and possibly chaotic population fluctuations. Nevertheless, we believe that, short-term, in-situ models to study microbial components constitute a rather promising approach to understanding the overall functioning of the planktonic food web in natural lakes such as Lake Bourget.

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RESUME

Les objectifs de ce travail visaient à déterminer l'importance qualitative, quantitative et fonctionnelle des virus dans le Lac Pavin, en tenant compte des gradients liés à la profondeur. Il s'agissait, plus spécifiquement, d'examiner (i) la dynamique saisonnière et spatiale de la biodiversité des virus, (ii) l'importance des gradients liés à la profondeur dans les variations saisonnières de l'abondance et de l'activité lytique des virus, et (iii) l'importance du mode de vie lysogène. Grâce à la mise au point d'un protocole original de concentration, nous montrons que la dynamique de la diversité du virioplancton du lac est étroitement associée à celle des communautés microbiennes. Il en est de même pour les variations d'abondance et d'activité lytique. La prise en compte de l'ensemble de la colonne d'eau indique que la structure des réseaux trophiques microbiens et la place des virus dans ces réseaux dépendent de la profondeur, avec une simplification des modes trophiques, et des niveaux d'organisation biologique des communautés, et des interactions trophiques potentielles, avec la profondeur. Ainsi le fonctionnement trophique des couches profondes, réduites aux virus et procaryotes, serait essentiellement régi par la boucle virale. Finalement, jusqu'à 16 % du bacterioplancton serait lysogène. Ce mode de vie, antagoniste à celui lytique, dépend de la disponibilité de l'hôte et serait plus un mécanisme de survie que de prolifération virale, pouvant par ailleurs avoir une incidence sur l'évolution adaptative des hôtes. Les résultats acquis montrent que les virus seraient essentiels dans la diversification et l'écologie des communautés microbiennes, et dans les flux de matière et d'énergie circulant dans la colonne d'eau.

Mots clés : Lac Pavin - Virioplancton - Réseaux trophiques microbiens - Gradients verticaux - Lyse virale – Lysogénie

ABSTRACT

The main goal of this thesis was to assess qualitative, quantitative and functional significance of viruses in Lake Pavin, by taking into account the depth-related gradients in the water column. Specifically, we have examined the (i) spatio temporal dynamics of viral community diversity, (ii) significance of depth-related gradients in seasonal variations of viral abundance and lytic activity and (iii) relative significance of lysogenic 'life style'. From an original concentration protocol, we have shown that the dynamics of viral diversity in the lake was strongly linked to those of microbial communities. Similar for variations in viral seasonal abundance and lytic activity. Through the entire water column, microbial trophic network structure and the role of viruses in this network depended strongly on depth, with a simplification of trophic modes, of biological organization levels of communities, and of trophic interactions with depth. Biological limnology of the deep layers is reducte to viruses and prokaryotes, and is thus governed by the viral loop processes. Finally, up to 16 % of bacterioplankton could be lysogens. This way of life, antagonist with lytic one, depended on host availability and could be more a mechanism for the maintenance of viral traits, than can further have significant incidences on the evolutionary ecology of host communities. Overall, our results show that viruses are essential in the diversification and ecology of microbial communities, and in the matter and energy flows in the lake water column.

Key Words : Lake Pavin - Virioplankton - Microbial loop - Depth related gradients- Viral lysis- Lysogeny