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Emilie Lance

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PAR

Emilie LANCE

IMPACTS DES CYANOBACTERIES TOXIQUES SUR LES GASTEROPODES DULCICOLES ET SUR LEUR RÔLE DE VECTEUR DANS LE TRANSFERT DES MICROCYSTINES AU SEIN DU RÉSEAU TROPHIQUE

Thèse réalisée au sein de l'UMR CNRS 6553 ECOBIO (Université de Rennes 1), IFR CAREN Rennes, Equipes Rôle de la Biodiversité dans les Processus Ecologiques, et Forcage Anthropique et Biodiversité, de 2005 à 2008, dans le cadre de la formation de l'école doctorale Vie-Agro-Santé

Soutenance le 8 décembre 2008 devant le jury composé de :

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Introduction générale

Introduction générale

Les eaux douces continentales ne représentent que 3% du volume d'eau global terrestre mais elles jouent un rôle décisif dans le maintien de la vie sur terre (Wetzel, 1975). Or les écosystèmes dulcicoles, et en particulier les eaux stagnantes, sont parmi les plus menacés et globalement les moins bien protégés malgré les mesures d'aménagement et de conservation (Abromovitz, 1996; Moilanen et al., 2008). A leur évolution naturelle (comblement) s'ajoute une pression anthropique de plus en plus importante liée aux usages de l'eau (irrigation, transport, production d'énergie) et surtout à la pollution (rejets industriels, agricoles, domestiques…). Plus particulièrement, l'accroissement de la population mondiale a entraîné, depuis les 50 dernières années, une intensification et un changement des pratiques agricoles, associés à la destruction des végétations ripariennes et à une utilisation intensive de produits phytopharmaceutiques pour protéger les cultures et accroître les rendements de production (Wetzel, 1975; Reynolds, 1984). Outre les effets toxiques de l'introduction de ces molécules chimiques sur les réseaux trophiques aquatiques, les interventions humaines ont également conduit à un transfert important de nutriments (majoritairement phosphore et azote) depuis les bassins versants vers les eaux douces, engendrant une eutrophisation accrue des systèmes aquatiques et une augmentation de la production primaire du phytoplancton (Reynolds, 1984).

Les cyanobactéries, qui font partie du phytoplancton, sont parmi les plus anciens organismes apparus sur terre (précambrien) et les plus abondants et largement distribués (Paerl et al., 2001). Procaryotes autotrophes, elles sont présentes dans tous les types de milieux, terrestres ou aquatiques, et sous tous les climats. Dans les eaux douces, les cyanobactéries ont des capacités de prolifération importantes, donnant lieu à des « blooms » (épais tapis à la surface de l'eau ou dispersés dans toute la colonne) qui peuvent être observés dans toutes les régions du monde, y compris en Bretagne (Chorus et Bartram, 1999; Brient et al., 2004). Même si il s'agit d'un phénomène naturel, l'eutrophisation croissante associée au réchauffement climatique contribue à augmenter sérieusement la fréquence, la sévérité et la durée de ces blooms (Codd et al., 2005; Paerl & Huisman, 2008). D'un point de vue économique, les proliférations de cyanobactéries aggravent le problème actuel de gestion des eaux continentales en créant diverses nuisances (ex : coloration de l'eau, nuisances olfactives, perturbations des procédés de traitement des eaux d'alimentation…). D'un point de vue écologique, les fortes densités atteintes par les cyanobactéries lors des blooms (2 à 3

millions/ml) entraînent des perturbations du milieu, e.g. augmentation de la turbidité et mort des plantes aquatiques (habitat pour de nombreuses espèces), diminution de la teneur en oxygène, déséquilibre dans les communautés phytoplanctoniques (Chorus et Bartram, 1999). Mais leur plus sévère nuisance tient à leur propriétés toxiques, régulièrement observées en milieu naturel et qui constituent un problème majeur à la fois pour la santé des écosystèmes et pour la santé publique (mortalité massive de poissons, oiseaux, animaux domestiques; chez l'homme, nombreux malades sur tous les continents et parfois des morts) (pour revues : Codd et al., 2004, Zurawell et al., 2005).

Les cyanotoxines recouvrent une grande variété de structures chimiques (peptides cycliques, alcaloïdes et lipopolysaccharides) et de propriétés toxiques (neurotoxines, hépatotoxines, dermatotoxines, cytotoxines) (pour revues : Wiegand & Pflugmacher, 2005, Leflaive & Ten-Hage, 2007). Ces toxines sont intracellulaires et synthétisées par les populations de cyanobactéries en croissance, puis libérées dans le milieu à l'occasion de leur sénescence. Les hépatotoxines sont produites dans 40 à 75 % des blooms, les plus communes étant les microcystines (MCs), peptides cycliques dont 80 variants ont été isolés jusqu'à présent, différant principalement par 2 acides aminés en positions 2 et 4 de la molécule (ex : MC-LR pour leucine et arginine) (pour revue Zurawell et al., 2005). Les MCs sont produites par la plupart des genres de cyanobactéries et sont très fréquemment rencontrées en milieu naturel (Chorus et Bartram, 1999) [en Bretagne, plus de 76% des cyanobactéries prélevées en synthétisent (Vezie et al., 1997)]. L'attention spéciale qui leur est portée par la communauté scientifique et par les gestionnaires des ressources aquatiques est due à leur toxicité aiguë et aux dommages qu'elles induisent lors d'expositions chroniques à de faibles doses (pour revue : Dawson, 1998). Les MCs s'accumulent préférentiellement dans le foie des vertébrés et la glande digestive des invertébrés (pour revue : Zurawell et al., 2005). Elles interagissent spécifiquement avec les protéines phosphatases (Ppases), présentes chez tous les organismes et essentielles au maintien de l'intégrité cellulaire. On distingue 2 types de liaison : réversible, les MCs sont alors dites « libres » et peuvent être éliminées par les processus de détoxication impliquant les gluthations (Wiegand et al., 1999), ou covalente irréversible, elles sont alors dites « liées » et sont difficilement éliminées (Hastie et al., 2005). Dans les deux cas, les Ppases sont inhibées ce qui entraîne une désorganisation du cytosquelette de la cellule. Les interactions entre MCs et Ppases conduisent alors à des nécroses et à une destruction du tissu hépatique (potentiellement mortelle), mais aussi, et dans une moindre mesure, à des lésions du

tube digestif et des reins, et sont promotrices de tumeurs (Wiegand and Pflugmacher, 2005; Zurawell et al., 2005).

La problématique des MCs concerne à la fois l'écologie et la santé publique. L'homme peut être contaminé lors de baignades (voie orale ou nasale), par l'eau de boisson (libération de la toxine lors des procédés de traitement des eaux), par l'alimentation (coquillages, poissons, légumes arrosés avec de l'eau contaminée) et dans des circonstances plus exceptionnelles comme au Brésil, où 76 personnes ont trouvé la mort suite à une dialyse effectuée avec de l'eau contaminée (pour revues : Duy et al., 2000; Dietrich et Hoeger, 2005). Les organismes des réseaux trophiques dulcicoles et terrestres peuvent s'intoxiquer de 3 façons lors des proliférations de cyanobactéries : i) par les toxines solubilisées dans l'eau ou adsorbées sur les particules organiques et inorganiques (voie orale, nasale ou transcutanée), ii) en ingérant les cyanobactéries vivantes, iii) en consommant des proies ayant accumulé des toxines. La production de MCs lors des proliférations de cyanobactéries est donc susceptible d'affecter tout le réseau trophique et de perturber le fonctionnement des écosystèmes. Il s'avère nécessaire d'étudier l'impact des MCs sur les organismes afin de prédire les changements de composition des communautés aquatiques consécutives aux proliférations. Jusqu'à présent, beaucoup de recherches ont été menées sur des intoxications aiguës, essentiellement de vertébrés [mammifères (souris et rats), poissons], alors que les connaissances sur les conséquences d'une intoxication chronique par les MCs, en particulier sur les invertébrés aquatiques, restent très parcellaires (Zurawell et al., 2005; Wiegand et Plufgmacher, 2005).

Les mollusques gastéropodes sont présents dans tous les types d'écosystèmes dulcicoles (stagnants et courants) (Clarke, 1979; Dillon, 2000), où ils peuvent constituer une part importante de la biomasse des macroinvertébrés benthiques (jusqu'à 90%) (Hawkins & Furnish, 1987; Habdija et al., 1995; Strong et al., 2008; Balian et al., 2008). Les gastéropodes sont des bioindicateurs potentiels de différents types de pollution des eaux douces et considérés comme de bons modèles pour examiner les effets des polluants (pour revues : Salanki, 2000; Downs et al., 2001; Duft et al., 2007). Ce sont des organismes-cibles majeurs des cyanotoxines, dans la mesure où ils sont susceptibles de se contaminer par les toxines dissoutes dans l'eau (voie orale, branchiale/pulmonaire, transcutanée) ou adsorbées sur les particules organiques et minérales qu'ils ingèrent régulièrement, mais aussi d'ingérer des cyanobactéries toxiques vivantes présentes dans les communautés d'algues dont ils se nourrissent (Dillon, 2000). De plus, en tant que consommateurs primaires, les gastéropodes

occupent une place stratégique incontournable dans la chaîne trophique entre producteurs primaires et prédateurs, et sont consommés par un grand nombre d'espèces (sangsues, larves d'insectes, écrevisses, poissons, oiseaux, mammifères) (Dillon, 2000). En milieu naturel, des MCs ont déjà été détectées chez quelques espèces de gastéropodes pulmonés et prosobranches (Kotak et al., 1996 ; Zurawell et al., 1999; Chen et al., 2005; Gkelis et al., 2006; Gérard et al., 2008; Gérard et al., sous presse), d'où un risque de transfert de ces toxines dans les réseaux trophiques aquatiques et terrestres. Dans ces études, l'ingestion de cyanobactéries est soupçonnée d'être la cause majeure d'accumulation de MCs (les cyanotoxines dissoutes auraient un rôle mineur). Cependant, les cinétiques d'accumulation et d'élimination des MCs libres et liées, et leurs impacts sur les tissus des gastéropodes exposés aux cyanobactéries toxiques n'ont jamais été étudiés.

Depuis plusieurs années, des recherches sont menées en parallèle dans l'UMR Ecobio sur les cyanobactéries et sur les gastéropodes des eaux douces. Dans le cadre de l'axe thématique Écotoxicologie et Écodynamique des Contaminants (ECODYN 2003-2005) du programme national Ecosphère Continentale (ECCO), des travaux réalisés au laboratoire ont montré les effets pathogènes de l'exposition chronique à de faibles concentrations de MC purifiée (dissoute) sur les traits de vie (survie, croissance, fécondité) des gastéropodes, ainsi qu'une accumulation de la toxine dans leurs tissus (Gérard & Poullain, 2005; Gérard et al., 2005). Néanmoins, l'impact de l'ingestion de cyanobactéries toxiques sur les traits de vie des gastéropodes n'a jamais été étudié. D'autre part, lors d'un suivi à long terme en milieu naturel, Gérard et al. (2008) ont mis en évidence la relation entre le déclin d'une communauté lacustre de gastéropodes et les proliférations de cyanobactéries productrices de MCs.

Le but de cette thèse est de poursuivre ces recherches afin de déterminer l'impact des MCs sur les gastéropodes en fonction des voies de contaminations probables en milieu naturel (ingestion de cyanobactéries toxiques et exposition aux toxines dissoutes), et d'élargir l'étude à l'échelle du réseau trophique en termes de transfert de cyanotoxines aux prédateurs, comme l'illustre le schéma ci-après.

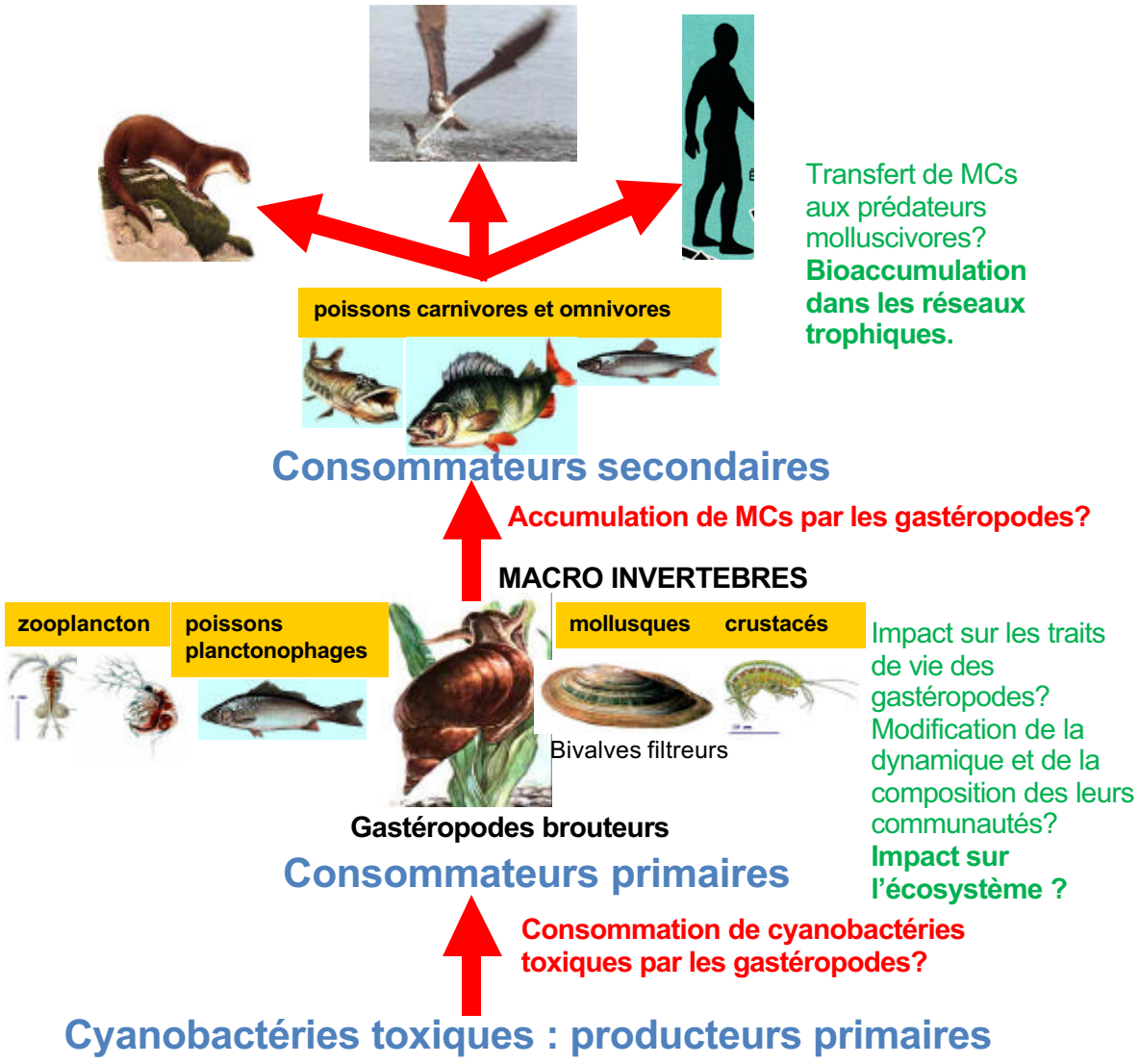


Schéma résumant les principaux objectifs de ce manuscrit : impact des cyanobactéries productrices de MCs sur les gastéropodes dulcicoles et rôle des gastéropodes dans le transfert des MCs au sein du réseau trophique aquatique (simplifié).

Ce document est divisé en 7 chapitres. Le premier chapitre est consacré à la description du contexte de notre étude et présente un état des connaissances d'une part sur les cyanobactéries : problématique des cyanobactéries toxiques, toxicité des MCs, voies de contamination des organismes, et enfin impact des cyanobactéries toxiques sur les organismes, et d'autre part sur les gastéropodes dulcicoles : présentation, biologie et écologie, interactions avec les cyanobactéries toxiques et conséquences. Les différents objectifs de la thèse sont présentés en conclusion de ce premier chapitre.

Le chapitre 2 présente l'impact des MCs sur les traits de vie de 2 espèces modèles de gastéropodes, le pulmoné *Lymnaea stagnalis* et le prosobranche *Potamopyrgus antipodarum*, ainsi que les conséquences en termes d'accumulation de MCs [travaux publiés sous forme de 3 articles (Lance et al., 2006, 2007, 2008)]. Au cours d'expériences, les 2 espèces de gastéropodes ont été exposées à deux âges différents (juvéniles et adultes) et pendant 5 semaines à une souche de cyanobactérie *Planktothrix agardhii,* productrice de MCs, puis placés pendant 3 semaines en conditions de dépuration. Plus précisément, cette partie vise à évaluer : 1) la consommation de cyanobactéries toxiques par les deux gastéropodes en présence ou absence de nourriture non toxique (salade), 2) les cinétiques d'accumulation et d'élimination de MCs libres dans leurs tissus, et 3) l'impact sur les traits de vie (survie, croissance, fécondité).

Compte tenu de la réduction de fécondité observée lorsque *L. stagnalis* est exposée à *P. agardhii* productrice de MCs (Lance et al., 2007, Chapitre 2) et à de la MC-LR dissoute (Gérard et Poullain, 2005), le chapitre 3 explore et quantifie l'impact de ces deux voies de contamination sur la fitness de la limnée en termes de taux d'éclosion des œufs, de durée du développement embryonnaire et de survie des néonates.

La nécessité d'associer à l'approche expérimentale une approche en milieu naturel pour comprendre l'impact réel des MCs sur les gastéropodes nous a incité à mener une étude dans 3 sites du Lac de Grand Lieu (Loire Atlantique) où prolifèrent des cyanobactéries toxiques de façon récurrente. Le chapitre 4 relate d'une part, les résultats obtenus en conditions semi-contrôlées (gastéropodes encagés) sur la réponse de nos 2 espèces modèles (*L. stagnalis* et *P. antipodarum*) en termes d'accumulation de MCs et d'impact sur la survie, et d'autre part, l'impact des MCs sur la structure des communautés de gastéropodes (abondance, richesse spécifique) à partir du suivi mensuel réalisé dans les 3 sites pendant 1 an.

Le chapitre 5 présente une étude expérimentale en partie réalisée dans le laboratoire de Toxicologie Environnementale du Pr. D. Dietrich (Université de Konstanz, Allemagne) et portant sur l'histopathologie (et sa réversibilité) et la distribution des MCs dans différents organes (glande digestive, glande génitale, pied, rein) du gastéropode pulmoné *L. stagnalis*, en fonction du mode de contamination (ingestion de *P. agardhii* vs MC-LR dissoute).

Le chapitre 6 prend en compte l'existence de MCs liées de manière covalente (irréversible) et le rôle potentiel des variants de MCs dans les processus d'accumulation. Cette étude a été réalisée en collaboration avec le laboratoire de Biochimie et Pharmacologie du Dr J. Meriluoto (Université de Turku, Finlande), où elle s'est déroulée de Septembre à Décembre 2007. Plus précisément, ce chapitre détaille 1) la cinétique d'accumulation et d'élimination des MCs totales (libres et liées) dans les tissus de *L. stagnalis* exposée à *P. agardhii* ou à de la MC-LR dissoute pendant 5 semaines puis placée en dépuration pendant 3 semaines, 2) la proportion relative des différents variants de MCs (dmMC-LR, dmMC-RR and MC-YR) accumulés dans le mollusque, ainsi que l'intensité de l'accumulation, en fonction de la proportion relative des variants dans les cyanobactéries.

Dans le chapitre 7, nous mesurons le transfert trophique de MCs (libres et liées) accumulées par *L. stagnalis* après ingestion de cyanobactéries toxiques, à un consommateur secondaire, l'épinoche *Gasterosteus aculeatus*. L'étude établit l'accumulation et l'élimination des MCs dans différents organes (foie, muscles, reins, branchies) du poisson, ainsi que l'impact de l'intoxication en termes d'histopathologie hépatique, de stress oxydant et de modifications comportementales (fréquence ventilatoire, comportement alimentaire et locomoteur).

Le chapitre 8 présente une synthèse et une discussion de tous les résultats, ainsi qu'une conclusion générale et des perspectives de recherche futures.

Chapitre 1 :

État des connaissances

Chapitre 1 : État des connaissances

1.1. Les cyanobactéries

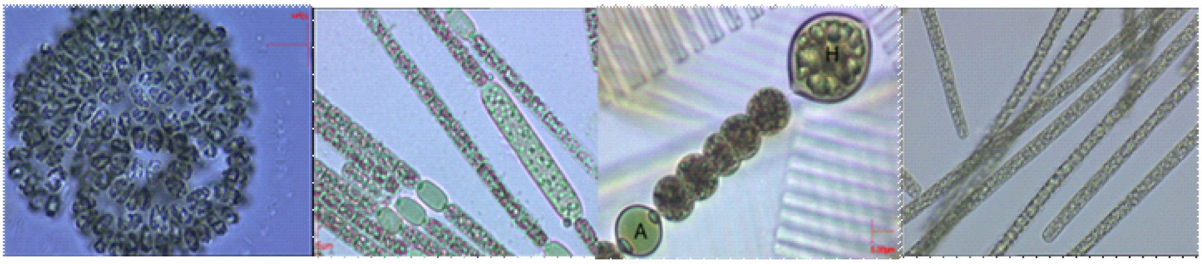
1.1.1. Position taxonomique

Les cyanobactéries sont des micro-organismes procaryotes photosynthétiques à Gram négatif. Elles sont aussi appelées Cyanophytes ou Cyanophycées, ou encore algues bleues, en raison de la présence d'une phycobiline bleue qui leur est propre. Elles sont classées du point de vue systématique dans le règne des Eubactéries, mais ont été longtemps rangées dans le règne végétal car elles présentent à la fois des propriétés spécifiques des bactéries et des caractéristiques propres aux algues. Comme les bactéries, elles sont dépourvues de membrane nucléaire et d'organites intracellulaires (mitochondrie, réticulum endoplasmique, dictyosome). Comme les algues, elles possèdent des pigments photosynthétiques (ex : chlorophylle a) et appartiennent à la communauté phytoplanctonique qui assure la production primaire par réalisation d'une photosynthèse productrice d'oxygène en utilisant l'eau comme donneur d'électron. Dans le code international de nomenclature botanique, elles appartiennent à la classe des cyanophycées comprenant différents ordres (ex : ordre des Nostocales) et familles et incluant 150 genres et 2000 espèces (pour revue : Duy et al., 2000).

1.1.2. Diversité morphologique et écologique des cyanobactéries

Les cyanobactéries font partie des plus anciens organismes apparus sur Terre (précambrien : 2.5 à 3.5 billions d'années) et des plus abondants et largement distribués (Paerl et al., 2001; Cox et al., 2005). Elles peuvent être unicellulaires, filamenteuses ou coloniales (Figure 1), et présentent des caractéristiques écologiques très variées qui leur ont permis de coloniser la plupart des habitats, aquatiques (dulcicoles et marins) ou terrestres. Un grand nombre d'entre elles sont adaptées à des environnements extrêmes (glaciers, sources chaudes, cendres volcaniques) (pour revue : Chorus et Bartram, 1999). En milieu aquatique, elles sont

planctoniques, c'est-à-dire en suspension dans la colonne d'eau, ou benthiques quand elles se développent sur les sédiments.



Cyanobactérie unicellulaire coloniale *Woronichinia naegelania*

Cyanobactérie organisée en trichomes *Aphanizomenon flos aquae*

Trichome de cyanobactérie *Anabaena smithii* avec un hétérocyste (H) et un akinète (A)

Cyanobactérie organisée en filament *Planktothrix agardhii*

Figure 1 : Différentes morphologies chez les cyanobactéries, d'après des photos de Luc Brient, université de Rennes 1.

Sous nos climats tempérés, certaines cyanobactéries filamenteuses (ex : *Anabaena sp, Aphanizomenon sp*) ou coloniales (ex : *Microcystis sp.)* se développent au printemps sous forme planctonique lorsque la température des eaux superficielles atteint 18-20°C, puis restent sous forme benthique de résistance (les akinètes) pendant la saison froide. D'autres, comme *Planktothrix sp*, peuvent se maintenir toute l'année sous forme planctonique dans des eaux froides thermiquement non stratifiées (t° uniforme > 5-6°C) (Briand et al., 2002).

Les cyanobactéries possèdent des caractéristiques morphologiques et physiologiques qui leur assurent un avantage compétitif sur les autres espèces du phytoplancton et leur permettent de dominer fréquemment les communautés phytoplanctoniques. Parmi ces caractéristiques, la présence de pseudo-vacuoles à gaz leur permet de moduler leur densité et de se déplacer verticalement afin d'optimiser leur position dans la colonne d'eau pour une exploitation optimale de l'intensité lumineuse (Walsby et al., 1994). De plus, les cyanobactéries possèdent des pigments surnuméraires (ex : phycocyanines et phycoérythrines), en plus de ceux que possèdent les autres micro-algues planctoniques (chlorophylles, carotènes et xanthophylles), qui permettent une utilisation plus large du spectre lumineux. Enfin, environ le tiers des cyanobactéries possèdent des cellules spécialisées, les hétérocytes, sièges d'une fixation d'azote moléculaire (Oberholster et al., 2004), à laquelle s'ajoute pour certaines espèces une meilleure affinité pour le phosphore et une capacité à le stocker, renforçant leur avantage compétitif (pour revue : Chorus et Bartram, 1999).

1.1.3. Proliférations ou « blooms » de cyanobactéries

Les cyanobactéries ont la capacité de proliférer lorsque les conditions leur sont favorables, typiquement dans les eaux de 20-30° C, de pH entre 6 et 9, à faible turbulence et à forte intensité lumineuse, et à teneur moyenne à élevée en phosphore et/ou azote (milieu eutrophe ou hypereutrophe) (Duy et al., 2000; Oberholster et al., 2004). Les blooms de surface (ou efflorescences) peuvent se former en 2 jours et se maintenir entre une et plusieurs semaines (Paerl et al., 2001). En région tempérée, ils ont généralement lieu pendant la période estivale [de mai à octobre en Bretagne, avec fréquemment 2 à 3 millions d'individus mL^{-1} d'eau (Brient et al., 2004)]. Ils disparaissent souvent lorsque les conditions changent (brassage des eaux, diminution de l'intensité lumineuse) en fin de période estivale. Parfois, des efflorescences apparaissent et disparaissent à plusieurs reprises selon les épisodes de refroidissements et de vents. Même si les blooms constituent un phénomène naturel, leur fréquence et leur sévérité sont augmentées par l'eutrophisation, souvent liée aux activités anthropiques (rejet d'intrants, d'adjuvants mousseux et autres) dans les bassins versants. De plus, le réchauffement climatique semble agir comme un catalyseur favorisant les proliférations de cyanobactéries partout dans le monde (Paerl & Huisman, 2008).

1.1.4. Effets indésirables des proliférations de cyanobactéries

a. Impact écologique lié aux fortes densités :

Les proliférations de cyanobactéries entraînent une augmentation de la turbidité des eaux et par suite, la mort des plantes aquatiques représentant un habitat pour de nombreuses espèces. Elles provoquent également une perturbation des réseaux trophiques aquatiques car elles prolifèrent au détriment des autres organismes photosynthétiques (pour revue : Zurawell et al., 2005). Lors de la dégénérescence des blooms, elles entraînent une diminution de la teneur en oxygène de l'eau menaçant la faune. De façon globale, les êtres vivants (plantes, animaux) sont toujours moins nombreux dans les endroits où prolifèrent des cyanobactéries (Wear & Gardner, 2001), posant un problème majeur pour la santé des écosystèmes.

b. Impact sur le cadre de vie et les usages anthropiques de l'eau:

La présence de cyanobactéries en forte densité peut gêner les activités nautiques en raison de colorations inhabituelles de l'eau (bleue, rouge ou verte) associées à des nuisances olfactives et des masses d'algues se déplaçant au gré des vents (Figures 2 et 3). Elle provoque également des perturbations du fonctionnement des procédés de traitement des eaux d'alimentation (colmatage des filtres, consommation accrue en réactifs de traitement) et une dégradation de la qualité des eaux de consommation insuffisamment traitées (présence de toxines et/ou de métabolites odorants) (Chorus et Bartram, 1999).

Figure 2 : Prolifération de cyanobactéries filamenteuses dans le lac de Grand Lieu (Loire Atlantique) en mai 2006.
Par Lance Emilie

Figure 3 : Prolifération de cyanobactéries coloniales dans le Frémur, en 2004.
Par Acou Anthony

c. Production de toxines

Les proliférations de cyanobactéries entraînent de plus en plus souvent des problèmes de santé publique en raison de la production de toxines par certaines souches. Chaque espèce présente à la fois des souches non toxiques et des souches toxiques capables de produire un ou plusieurs types de toxines. Une liste non exhaustive des espèces de cyanobactéries potentiellement toxiques et de leurs toxines est présentée en annexe 1. Les cyanotoxines recouvrent une grande variété de structures chimiques (peptides cycliques, alcaloïdes et lipopolysaccharides) et de propriétés toxiques (neurotoxines, hépatotoxines, dermatotoxines, cytotoxines et toxines irritantes) (pour revues : Wiegand & Pflugmacher, 2005, Leflaive & Ten-Hage, 2007). Les incidents les plus graves provoqués par les cyanobactéries sont le plus souvent attribués aux neurotoxines [anatoxines, les saxitoxines et leurs dérivés, et la β-N-méthyloamino-L-alanine (BMMA)] et aux hépatotoxines (Wiegand & Pflugmacher, 2005).

- Neurotoxines : les anatoxines et les saxitoxines sont des alcaloides qui affectent le système nerveux central et périphérique et ont pour cible la jonction neuro-musculaire. Elles entraînent une paralysie du corps et un arrêt respiratoire (Wiegand & Pflugmacher, 2005). Les saxitoxines inhibent la transmission nerveuse en bloquant les canaux sodiques. Elles sont aussi connues sous l'appellation de toxine paralysante des coquillages (PSPs). La BMMA provoque une excitation des neurones et agit comme un agoniste du récepteur glutamate dans le cerveau. Elle serait impliquée dans des maladies humaines neuro-dégénératives (détectée dans le cerveau des malades) lors d'expositions chroniques à faible dose (Cox et al., 2005).

- Hépatotoxines : ce sont les cyanotoxines les plus fréquemment rencontrées lors des proliférations de cyanobactéries (produites dans 40 à 75 % des blooms), et sont impliquées dans de nombreux épisodes d'intoxication (Chorus et Bartram, 1999). Elles comprennent les microcystines et les nodularines. Les microcystines, dont nous avons étudié l'accumulation et l'impact chez les mollusques gastéropodes lors de cette thèse, seront détaillées plus amplement dans la partie suivante (1.2.).

Depuis 1930, de nombreux cas d'intoxication humaine lors d'efflorescences de cyanobactéries ont été rapportés dans la littérature. Des études épidémiologiques ont montré une corrélation entre différents symptômes (diarrhée, vomissements, irruption cutanée, fièvre, infection des yeux et des oreilles…) et la densité des cyanobactéries (Dietrich et Hoeger, 2005). Les épisodes les moins graves consistent en des irritations du visage, des symptômes asthmatiques et des vomissements, comme reportés par des soldats ayant subi un entraînement dans des lacs contaminés par des blooms de *Microcystis aeruginosa* ou *Planktothrix agardhii* en Angleterre (Codd et al., 1999). L'exposition aux cyanotoxines qui a permis de démontrer leur potentiel toxique chez l'humain de manière spectaculaire est un incident survenu au Brésil en février 1996. Plus de 76 personnes sont décédées en 3 mois dans un centre d'hémodialyse par exposition intraveineuse à une eau contaminée par des microcystines (Azevedo et al., 2002). Par ailleurs, à partir d'études épidémiologiques menées en Chine, l'augmentation de l'incidence des carcinomes hépatiques dans certaines régions a été suggérée comme étant associée à l'ingestion régulière d'eau de surface contaminée par les cyanobactéries (Chorus, 2001). Enfin, outre les intoxications humaines, de très nombreux cas d'intoxications aiguës d'animaux domestiques (chiens, bovins, ovins, chevaux, porcs) ou sauvages (poissons, canards, oiseaux, rats musqués, visons, rhinocéros) dues aux cyanotoxines ont été décrits sur tous les continents (pour revues : Chorus et Bartram, 1999; Briand et al., 2003).

1.2. Les microcystines

1.2.1. Synthèse des microcystines

Les microcystines (MCs) sont les cyanotoxines le plus fréquemment rencontrées en milieu naturel (pour revue : Chorus et Bartram, 1999). Elles sont produites par de nombreux genres, dont *Microcystis, Planktothrix, Oscillatoria*, *Aphanizomenon, Nostoc, Anabaena*, et ont été détectées partout dans le monde, et dans des plans d'eau aux caractéristiques physico-chimiques variables. Cependant, dans les régions tempérées de l'hémisphère Nord c'est le genre *Planktothrix* qui domine le plus souvent (Scheffer *et al.*, 1997) et qui présente une forte production de MCs (Christiansen *et al.*, 2003). Chaque espèce de cyanobactérie présente des souches productrices ou non productrices de MCs qui coexistent dans les blooms (Briand et al., 2008). Une souche peut fabriquer plusieurs types de toxines ou plusieurs variants de MCs (Carmichael, 1992). Les MCs sont intracellulaires et synthétisées par les cellules en croissance, et la production dépend à la fois de : 1) la présence de gènes de biosynthèse dont l'expression peut être régulée par des facteurs environnementaux (e.g. lumière, température, azote, phosphore) (Schatz et al., 2007), 2) du taux de croissance des cellules et de la disponibilité des nutriments (Briand et al., 2008), 3) de la proportion entre souches toxiques et non toxiques (Briand et al., 2008), et 4) de l'état physiologique des clones toxiques (Chorus, 2001).

On ne sait toujours pas quel est le rôle des MCs, même si certains scientifiques supposent qu'il s'agit d'un système de défense contre l'herbivorie (Carmichael, 1992, Jang et al., 2003, 2004). Cependant, le fait que la capacité à produire des MCs par les cyanobactéries préfigure la lignée des Métazoaires conduirait à écarter cette hypothèse (Schatz et al., 2007). Deux hypothèses sont actuellement privilégiées : 1) les MCs seraient des métabolites secondaires des cyanobactéries, dont la production, régulée au niveau cellulaire, pourrait être une réponse à un stress abiotique (température, intensité lumineuse, salinité, nutriments) (Sivonen et Jones, 1999); et 2) les MCs seraient des métabolites primaires dont l'expression est constitutive, et la proportion relative de clones possédant ou non le gène de synthèse des toxines déterminerait le niveau global de toxicité de la population (Briand et al., 2005). Au vu des données actuelles, la question n'est pas encore élucidée.

1.2.2. Présence des microcystines en milieu naturel

Les concentrations de MCs intracellulaires enregistrées en milieu naturel à travers le monde varient de 1 à 8600 µg MC g^{-1} DW (pour revue Christoffersen, 1996). En Bretagne, plus de 76% des cyanobactéries prélevées synthétisent des MCs avec des concentrations intracellulaires allant de 70 à 4000 µg g^{-1} DW (Vezie et al., 1997). Cependant, dans une population de cyanobactéries il y a toujours quelques cellules sénescentes qui larguent leur contenu dans le milieu (en phase de croissance de 10 à 20% de la teneur totale en toxines est extracellulaire) (Jones et Orr, 1994). Lors de la décomposition de l'ensemble du bloom, l'autolyse des membranes permet la diffusion massive des endotoxines dans le milieu extérieur (Jones et Orr, 1994). Par ailleurs, les stress physiques (ex : irradiation aux UV) ou chimiques (emploi d'algicides ou de floculants lors du traitement des eaux de boisson) induisent un stress oxydant qui conduit à la mort des cyanobactéries et à la libération en masse des toxines dans le milieu (Ross et al., 2006).

Après libération dans l'eau, les MCs peuvent être dissoutes ou particulaires (adsorbées sur des particules et des sédiments). Les concentrations mesurées de MCs dissoutes dans le milieu sont variables : 37 µg MC L^{-1} dans un lac eutrophe finlandais (Lindholm et al., 1989), 141 µg MC L^{-1} dans un lac eutrophe danois (Hyenstrand et al., 2003) et de 1300 à 1800 µg MC L^{-1} pendant 9 jours en Australie après traitement aux algicides (Jones & Orr, 1994). Les MCs peuvent persister jusqu'à 6 mois dans le sédiment sec et 2 ou 3 mois dans l'eau (Rapala, 1994), mais sont le plus souvent éliminées dans les 2 semaines qui suivent la sénescence des proliférations de cyanobactéries grâce à deux processus : la photolyse et la biodégradation microbienne. La photolyse comprend à la fois la décomposition et l'isomérisation des MCs en des isomères géométriques non toxiques, et est amplifiée par la présence des pigments cyanobactériens dissous ou d'acides humiques (Tsuji *et al.,* 1994). La biodégradation microbienne, dont le produit final est souvent du dioxyde de carbone, peut s'effectuer rapidement dans les lacs qui subissent régulièrement des blooms cyanobactériens et où les communautés bactériennes sont mieux adaptées et plus efficaces (e.g. en 8 jours la concentration de MC passe de 50 µg L^{-1} à un niveau indétectable avec 24% piégée dans les sédiments et le reste dégradé) (Christoffersen et al., 2002 ; Hyenstrand et al., 2003). En résumé et de manière générale, la concentration en MCs dissoutes est forte (> 1 µg MC L^{-1}) pendant la phase de sénescence des populations de cyanobactéries toxiques (et dans les jours qui suivent), et plus faible (< 1 µg MC L^{-1}) lors de la prolifération.

1.2.3. Structures et propriétés physico-chimiques

Les MCs sont des petits heptapeptides monocycliques, de poids moléculaire compris entre 900 et 1100 Daltons, et de structure générale cyclo(-D-Ala-**X**-D-MeAsp-**Y**-Adda-D-Glu-Mdha), où D-Ala et D-Glu sont deux D-acides aminés (respectivement l'alanine et l'acide glutamique), le D-MeAsp est l'acide D-erythro-β-methylaspactic et le Mdha correspond au N-methyldehydroalanine (Dawson, 1998). Un nouvel acide aminé C-20, de nom abrégé Adda, est propre aux cyanobactéries et est de structure acide (2S,3S,8S,9S)-3-amino-9-methoxy-2-6,8-trimethyl-10-phenyldeca-4,6-dienoic. Adda, très flexible, correspond à une queue hydrophobe s'étendant dans la partie postérieure de la MC (Figure 4).

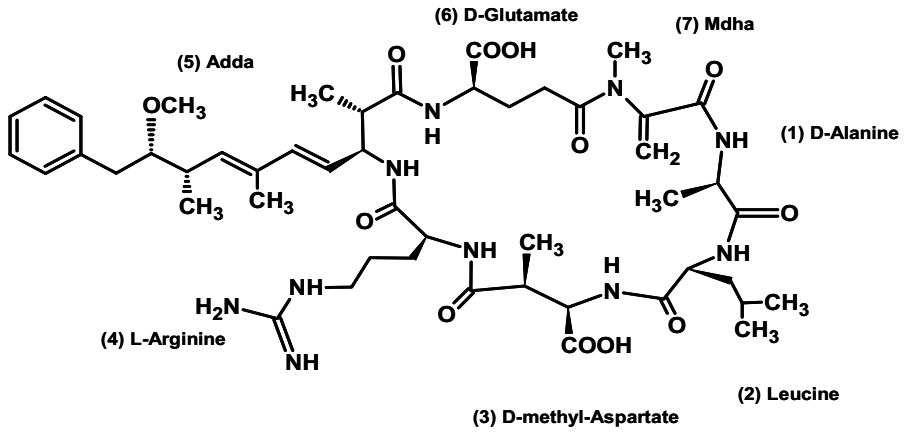


Figure 4 : Structure de l'hépatotoxine MC-LR en tant qu'exemple de MC

Presque 80 variants structuraux de MC ont été isolés jusqu'à présent, différant principalement par deux acides aminés variables en position 2 et 4, représentés par X et Y dans la formule chimique ci-dessus (Sivonen et Jones, 1999 ; Dietrich & Hoeger, 2005). Le plus étudié des variants, et probablement le plus répandu, est la MC-LR pour laquelle les deux acides aminés variables sont la leucine (L) et l'arginine (R) (Figure 4). Des modifications (isomérisation géométrique, substitution de groupements, démethylation…) dans la constitution des autres acides aminés engendrent également des analogues de MCs (Dawson, 1998).

Les MCs sont extrêmement stables dans l'eau et peuvent tolérer des changements physico-chimiques radicaux, incluant ceux du pH et de la température (jusqu'à 300°C) (Harada et al., 1998). Les acides aminés variables et invariables les constituant incluent des résidus polaires et apolaires mais les MCs sont globalement solubles dans l'eau, avec différents degrés de solubilité entre les variants (par exemple la MC-LR est plus hydrophile que les MC-LF et –LW) (Harada, 1996; Sivonen & Jones, 1999; Codd et al., 2005).

1.2.4. Toxicité aiguë et chronique des microcytines

a. Toxicité aiguë et doses létales

La toxicité aiguë est généralement exprimée par la dose létale 50% (DL50[1]). Les tests de toxicité aiguës (ou chroniques) ont très souvent été établis sur des vertébrés (rat ou souris) et aucune donnée n'est disponible concernant les mollusques. De plus, la toxicité de tous les variants n'a pas été étudiée et ceux pour lesquels on dispose d'informations sont principalement ceux disponibles en tant que standards. Selon Skulberg et al. (1984), les DL50 des MCs sont intermédiaires entre celles de l'amanite (muscarine) et du cobra (neurotoxine). Les MCs sont très toxiques en administration intra péritonéale (ip) chez la souris, et un peu moins par voie orale (annexe 2) (Sivonen et Jones, 1999). Le niveau de toxicité peut varier entre les variants et par exemple, la DL50 des MC-LR, -LA, -YR et –YM sont de même ordre de grandeur, tandis que celle de la MC-RR est 10 fois plus élevée. De même, la DL50 des MCs peut varier entre les espèces : inférieure à 1.7 mg kg^{-1} par voie orale pour la carpe commune mais de 1.7 à 6.6 mg kg^{-1} pour la truite (pour revue : Fischer and Dietrich, 2005).

b. Toxicité sub-chronique et chronique

Les études sur la toxicité sub-chronique[2] et chronique[3] sont essentielles pour connaître et prévenir les effets néfastes d'une exposition à moyen et long termes à de faibles doses de toxines. La dose sans effet nocif observé (DSENO) mesurée chez la souris par gavage avec de la MC-LR pure pendant 13 semaines est de 40 µg. kg^{-1}. j^{-1} (annexe 2). La DSENO est de 333 µg. kg^{-1}. j^{-1} lorsque l'intoxication a lieu par ingestion pendant 43 jours de cyanobactéries produisant de la MC-LR. En terme de toxicité chronique chez le rat, des nécroses rénales sont observées après 8 mois d'intoxication par voie intra-péritonéale à très faibles doses de MC-LR ou de MC-YR (10 µg. kg^{-1}) tous les deux jours. Ces effets toxiques posent le problème de santé publique qui consiste à savoir quelle est la dose maximale journalière tolérable à long terme pour l'Homme (pour revue : Dietrich et Hoeger, 2005).

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[1] dose en µg de toxine purifiée/kg nécessaire pour entraîner la mort de 50% des individus testé en 24h

[2] Les études de toxicité subchronique consistent en l'administration de doses répétées pendant au moins 90 jours

[3] Les études de toxicité chronique consistent en une exposition répétée pendant une grande partie de la vie de l'animal (18 mois pour la souris et 2 ans pour le rat).

1.2.5. Distribution des microcystines dans les cellules des organes cibles

Lorsque les MCs pénètrent oralement dans l'organisme, elles résistent à la digestion dans le tractus gastro-intestinal et passent dans la circulation sanguine via l'iléon (Msagati et al., 2006), puis sont préférentiellement transportées vers le foie qui constitue le principal organe cible (Tencalla and Dietrich, 1997; Fischer et Dietrich, 2000). Pour entrer dans les hépatocytes, les MCs ont besoin de transporteurs car elles ne traversent pas les membranes en raison de leur hydrosolubilité. Chez les vertébrés, le transport actif des MCs dans les cellules est assuré par les transporteurs des acides biliaires qui induisent un organotropisme vers les organes où ils sont très nombreux (le foie en particulier, mais aussi l'intestin et le cerveau au niveau de la barrière hémato-encéphalique) (Eriksson et al. 1990; Fischer et al., 2005; Dietrich et Hoeger, 2005). La cinétique de distribution des MCs dans les organes cibles peut varier en fonction des différentes MCs car des changements dans la structure de la molécule sont susceptibles de modifier son affinité avec les transporteurs des acides biliaires[1] (Dietrich et Hoeger, 2005).

Chez certains invertébrés (malacostracés, mollusques gastéropodes et bivalves), la glande digestive est l'organe cible majeur des MCs, et dans une moindre mesure le tractus digestif et la glande génitale (Zurawell et al., 2001; Chen et al., 2005; Chen & Xie, 2005; Xie et al., 2007; Zhang et al., 2007). Jusqu'à présent, aucun transporteur d'acides biliaires n'a été mis en évidence chez les gastéropodes, ce qui suggère une pénétration des MCs dans les cellules via d'autres transporteurs.

1.2.6. Mécanismes de toxicité des microcystines et impact dans l'organisme

Après pénétration dans une cellule, les MCs peuvent établir 2 types de liaison avec des protéines phosphatases (Ppases) à sérine/thréonine (Ppase1, Ppase2A, Ppase4 et Ppase5) généralement situées dans le cytosol. Le premier type de liaison intervient dans un délai de quelques minutes à quelques heures et correspond à une interaction non covalente, donc

[1] Il est intéressant de noter que la MC présente une concordance de structure avec la phalloïdine, une hépatotoxine produite par le champignon *Amanita phalloides* (amanite phalloïde) aussi transportée dans les hépatocytes via les transporteurs d'acides biliaires.

réversible, entre le groupement Adda (hydrophobe) de la MC et une boucle hydrophobe située près de la sous-unité catalytique de la Ppase. Dans cette configuration, la partie cyclique de la MC bloque l'accès au site actif de l'enzyme, inhibant son fonctionnement (Goldberg et al., 1995; Maynes et al., 2006). La seconde interaction intervient dans un délai de quelques heures, et correspond à la fixation covalente et irréversible du groupement carbonyle du résidu Mdha des MCs, sur les groupements cystéine 273 et 226 des sous-unités catalytiques des Ppases (Hastie et al., 2005; Maynes et al., 2006).

Les Ppases catalysent la déphosphorylation des protéines, un processus de base dans le fonctionnement des cellules, et participent ainsi au contrôle d'un grand nombre de processus cellulaires (synthèse des protéines, transcription, division cellulaire, neurotransmission…). L'inhibition rapide de ces enzymes par les MCs entraîne une perte de l'intégrité du cytosquelette (microfilaments, filaments intermédiaires et des microtubules) par modification de l'état de phosphorylation de ses protéines (Eriksson et al., 1989). Ceci engendre une déformation de la cellule, une perte d'adhésion des cellules entre elles, des nécroses étendues et une destruction de l'architecture hépatique (Kondo et al., 1992), entraînant secondairement une hémorragie intrahépatique sévère pouvant causer la mort (Dawson, 1998). Bien que les MCs atteignent prioritairement le foie, des dommages similaires mais moins sévères ont été observés dans le tube digestif (déformation des entérocytes et hémorragies) et dans les reins (lésions cellulaires dans le glomérule et le tube rénal proximal) des vertébrés (pour revues : Dietrich et Hoeger, 2005; Zurawell et al., 2005).

D'autres paramètres physiologiques peuvent être perturbés par les MCs, notamment les concentrations en enzymes plasmatiques. Par exemple, chez les poissons, Gupta & Guha (2006) décrivent une augmentation des concentrations en bilirubine (issue de la destruction des globules rouges), cholestérol et triglycérides, et une modification des taux d'aspartate- et d'alanine-aminotransférases. La balance ionique est aussi affectée en raison de l'inhibition des pompes ioniques Na^+, K^+, $HCO3^-$, Ca^{2-}-ATPases dans les cellules épithéliales des branchies (pour revue : Malbrouck et Kestemont, 2006). La figure 5 résume un exemple de toxicité des MCs chez les poissons.

Par ailleurs, la pénétration des MCs dans les cellules entraîne la production de radicaux libres et initie une cascade oxydante avec formation et accumulation d'espèces réactives à l'oxygène (ROS : Reactive Oxygen Species). Les ROS sont extrêmement toxiques pour les cellules car ils entraînent une inactivation enzymatique, une peroxydation des lipides, et des dommages sur l'ADN (e.g. mutations ponctuelles, cassures, réarrangement

chromosomiques…) (Kankaanpää et al., 2007). En général, l'augmentation de la quantité de ROS active les mécanismes de défense des cellules qui réduisent le stress oxydant[1]. Des enzymes antioxydantes, la glutathion peroxydase (GPx) et la superoxyde dismutase (SOD), participant à l'élimination des ROS et à la lutte contre les processus de lipoperoxydation, sont activées en présence de MCs (Wiegand et al., 1999; Cazenave et al., 2006). L'activité promotrice de tumeur des MCs a été démontrée chez la souris : apparition de nodules dans le foie après administration intrapéritonéale de MC-LR à très faible dose (10 µg kg^{-1}), ainsi que sur la peau et dans le colon après absorption d'extraits de cyanobactéries administrés avec l'eau de boisson (Duy et al., 2000; Humpage et al, 2000).

Enfin, l'effet toxique des MCs sur la reproduction et l'embryogenèse des poissons a été démontré (réduction des taux de survie et d'éclosion des embryons, effets tératogènes, diminution de la quantité de lipides dans les réserves vitellines) (Oberemm et al., 1999; Liu et al 2002; Jacquet et al., 2004; Huynh-Delerme et al., 2005; Cazenave et al., 2006; Lecoz et al., 2008). Chez la souris, une dose journalière de MC-LR (2000 µg. kg^{-1}) entre le 6ème et le 15ème jour de la gestation provoque un retard de poids et d'ossification du squelette des embryons (pour revue Dietrich et Hoeger, 2005).

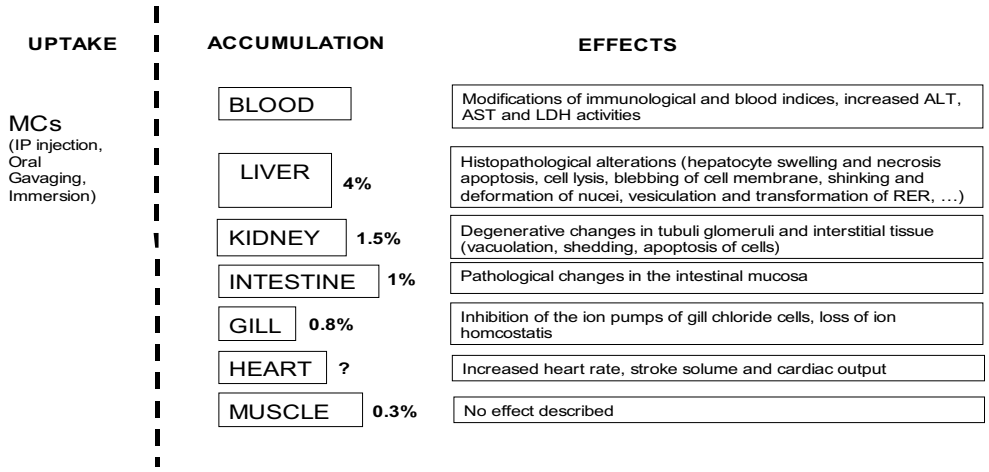

Figure 5 : Mécanismes de toxicité des MCs che les poissons. D'aprés Malbrouck et Kestemont, 2006. ALT = alanine aminotransferase, AST = aspartate aminotransferase, LDH = lactate deshydogénase, RER = rough endoplasmic reticulum, GSH = glutathione, GST = glutathione-S-transferase.

[1] stress oxydant : c'est un déséquilibre entre les systèmes antioxydants et les systèmes pro-oxydants en faveur de ces derniers.

1.2.7. Modalités d'accumulation des microcystines dans les organismes

Après pénétration dans les organismes, les MCs s'accumulent sous deux formes dans les tissus en fonction de leur interaction avec les protéines phophatases (Ppases) (cf partie 1.2.5.). Lorsque la liaison covalente MC-Ppase n'a pas eu lieu, la MC est dite « libre » dans les tissus, et partiellement métabolisée puis excrétée par des systèmes de détoxication décrits ci après. Lorsque la liaison covalente a eu lieu, la MC est dite « liée» de manière irréversible dans les tissus et son élimination, qui n'a pas été démontrée, a probablement lieu lors du renouvellement des Ppases ou des cellules endommagées.

De façon générale, les MCs libres peuvent être métabolisées via les enzymes de détoxication, les glutathions, principalement dans les cellules du foie des vertébrés et de la glande digestive des invertébrés (cytosol et microsome). La conjugaison des MCs avec le glutathion (GSH) est réalisée par une enzyme de biotransformation de phase II, la glutathion-S-transférase (GST). La GST catalyse la réaction d'addition entre les MCs et le groupement thiol du glutathion générant ainsi des composés plus hydrosolubles et moins toxiques, qui n'induisent pas d'inhibition des Ppases et qui peuvent être éliminés dans la bile ou dans les urines. Cette étape majeure de la détoxication a été décrite pour des organismes très différents : végétaux (*Ceratophyllum demersum*), des invertébrés (zooplancton : *Daphnia sp., Artemia salina* et bivalve *: Dreissena sp.*), poisson (*Dano rerio*), et des mammifères (souris et rat) (Kondo et al., 1992; Pflugmacher, 1998; Wiegand et al., 1999; Metcalf et al., 2000 Pietsch et al., 2001; Takenaka, 2001; Cazenave et al., 2006). L'élimination de la MC peut également se faire par conjugaison à la cysteine, démontrée *in vitro* (Kondo et al., 1992) et *in vivo* (Plufgmacher et al., 2001). L'élimination fécale et l'excrétion urinaire de la MC non conjuguée ont été observées mais sont faibles (respectivement 1% et 9% de la dose) car la fixation des MCs dans le foie diminue la quantité disponible dans le plasma.

Les MCs sont rarement totalement éliminées par les processus de détoxication et l'accumulation varie selon les organismes. Outre les impacts induits au niveau cellulaire, histologique et physiologique décrits dans la partie 1.2.5., l'accumulation de MCs s'accompagne souvent d'impact négatif sur les traits de vie (survie, croissance et fécondité), décrits dans les parties qui suivent (pour certains organismes aquatiques). Ces impacts sont généralement attribués à la demande énergétique accrue pour la métabolisation des toxines et la réparation des dommages induits, entrainant une réallocation d'énergie en défaveur des traits de vie (Wiegand et al., 1999, Wiegand and Pflugmacher, 2005).

1.3. Contamination des réseaux trophiques par les microcystines

1.3.1. Principales voies de contamination des organismes

Les organismes vivant dans/près des eaux où proliférèrent des cyanobactéries productrices de MCs peuvent se contaminer de manière directe par les MCs dissoutes dans l'eau (ou adsorbées sur les sédiments) et par les MCs intracellulaires (en consommant des cyanobactéries toxiques), et de manière indirecte par les MCs accumulées dans leurs proies (transfert trophique). L'importance relative des différentes sources de contaminations, variant selon les espèces, est résumée dans le schéma ci après (Figure 6).

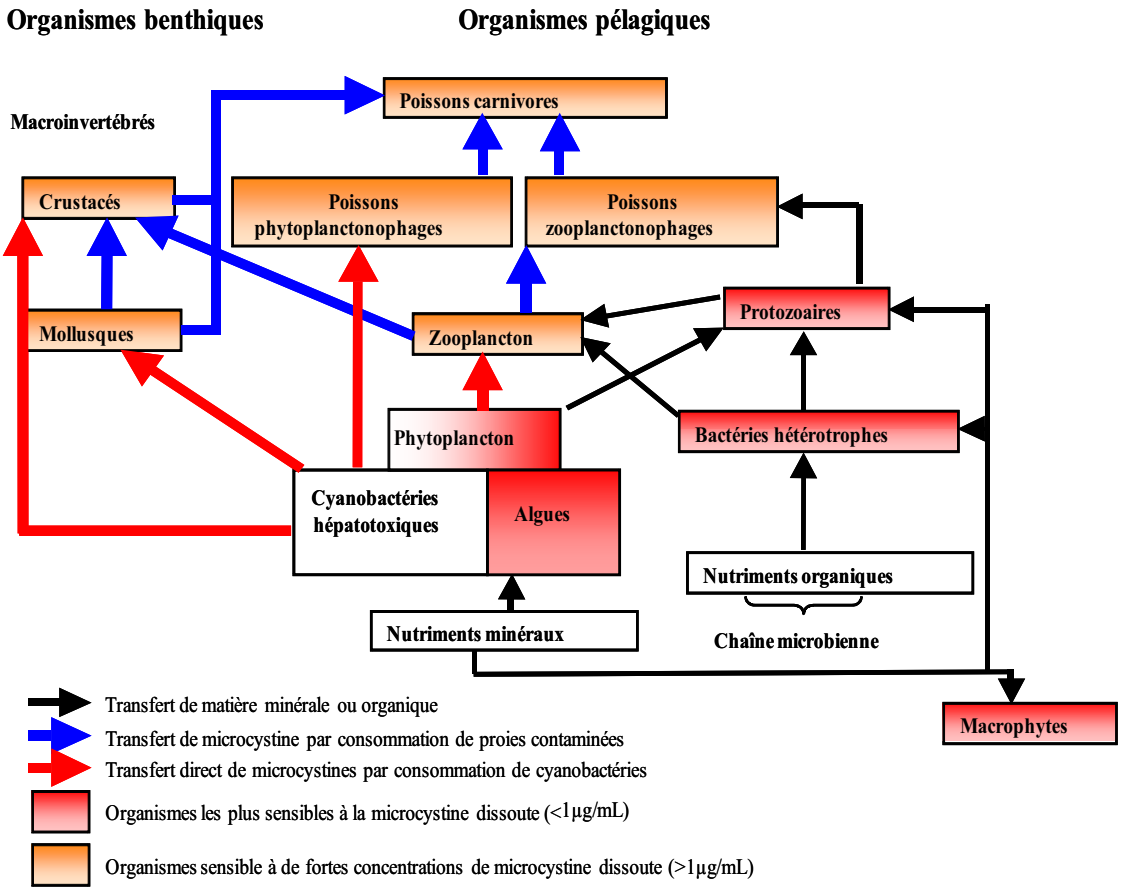

Figure 6 : Schéma simplifié des biocénoses dulcicoles et voies de contamination par les microcystines. Par soucis de simplification, les larves d'insectes et les adultes aquatiques ne sont pas représentés car les informations sur leur interaction avec les cyanobactéries et les MCs sont rares. Le réseau trophique aquatique est, en réalité, bien plus complexe car de nombreux organismes changent de régimes alimentaires plusieurs fois au cours de leur vie.

1.3.2. Contamination directe par les microcystines présentes dans le milieu

Sous forme dissoute [abondante lors de la sénescence du bloom mais à de faibles concentrations quelques semaines après et tout au long de la saison de prolifération] ou adsorbées sur les sédiment ou des particules alimentaires, les MCs pénètrent dans les organismes via l'eau de boisson, la peau ou les branchies (Hyestrand et al., 2003). De manière générale, il existe une relation allométrique pour la concentration de toxine dissoute tolérée (effets sublétaux et létaux), et les petites espèces (e.g., algues, bactéries, protozoaires) ainsi que les macrophytes semblent plus sensibles que les espèces de moyenne (zooplancton) ou de grande taille (macroinvertébrés, poissons) (Christoffersen, 1996). Les MCs inhibent la croissance et la photosynthèse de certaines espèces du phytoplancton. Chez les macrophytes, l'absorption de MCs dissoutes engendre une réduction des taux de croissance et de germination, induit des changements morphologiques (réduction du nombre de feuilles) et physiologiques (changement dans les ratios de chlorophylles a et b), et entraîne un stress oxydant (e.g. *Ceratophyllum demersum, Lemna minor*) (pour revues : Wiegand et Plufgmacher, 2005; Zurawell et al., 2005). De même, la toxicité des MCs dissoutes a été démontrée sur les protozoaires (ex : réduction du taux de croissance de flagellés, inhibition du taux de respiration) (pour revue : Christoffersen, 1996). Par contre, les poissons se montrent globalement peu sensibles à des expositions de MCs dissoutes de concentration proche de celle des milieux naturels (Tencalla et al., 1994; Landsberg, 2002). De manière générale, le zooplancton, les macroinvertébrés et les poissons se contaminent principalement par ingestion de cyanobactéries toxiques (pour revues : Ibelings et Chorus, 2007; Wiegand et Pflugmacher, 2005; Malbrouck et Kestemont, 2006). Chez l'homme, selon Dawson (1998), une consommation régulière d'eau contaminée pourrait favoriser des cancers du foie et du tube digestif. L'OMS, en 1999, a fixé une concentration maximale d'hépatotoxines de 1µg MC-LR L^{-1} d'eau potable.

1.3.3. Contamination directe par ingestion de cyanobactéries toxiques

De nombreux travaux mettent en évidence l'ingestion de cyanobactéries toxiques par le zooplancton (Rotifères, Copépodes et Cladocères), consommateur primaire au sein du réseau trophique (Lampert, 1987; Rothhaupt, 1991; DeMott, 1999; Thostrup et Christoffersen,

1999; Rohrlack et al., 2001). Cependant, certaines espèces de Copépodes et de Rotifères présentent des adaptations comportementales (i.e. tri des souches non toxiques de cyanobactéries) qui augmentent leurs capacités à coexister avec les cyanobactéries toxiques (ex. Copépodes) (Fulton & Paerl, 1987, Paerl et al., 2001). La sélection des particules alimentaires par filtrage actif est courante chez les Copépodes (Fulton et Pearl, 1987), alors que la plupart des Cladocères (Daphnies) font un filtrage passif et sont incapables de trier les particules alimentaires (Rohrlack et al., 1999). En présence de cyanobactéries toxiques, les Daphnies se protègent en inhibant leur filtration et en augmentant les rejets post-abdominaux, et diminuent fortement leur alimentation. Ainsi, en milieu naturel, les blooms de cyanobactéries sont souvent associés au déclin des populations de grands Cladocères et au développement des populations de Copépodes et Rotifères (Paerl et al., 2001). Néanmoins, les études sur le zooplancton sont nombreuses et parfois controversées du fait de l'hétérogénéité des souches de cyanobactéries utilisées, et des larges différences entre les espèces (Yanuso et Sugaya, 1998). Par exemple, certaines Daphnies conservent un fort taux de filtration en présence d'espèces de cyanobactéries coloniales ou de grande taille dont la structure rend les cellules incapturables (Lampert, 1987; De Bernardi & Giussani, 1990).

Au sein des macroinvertébrés aquatiques (crustacés, mollusques et insectes) les données disponibles (concernant quelques taxons : écrevisses, bivalves), suggèrent que l'ingestion de cyanobactéries serait la voie prédominante d'intoxication (pour revue : Wiegand et Pflugmacher, 2005). Par exemple, dans une pièce d'eau contaminée par des blooms, 99% des écrevisses (*Pacifastacus lemusculus*) présentent des cyanobactéries dans leur estomac (Liras et al., 1998). Comme pour le zooplancton, les cyanobactéries toxiques ont un impact écologique sur les populations de bivalves car elles peuvent favoriser les espèces capables de trier les souches toxiques des non toxiques. Certains bivalves ont la possibilité de trier les particules ingérées et parfois de rejeter sélectivement les cyanobactéries toxiques [i.e. *Planktothrix agardhii* est prélevée par *Anodonta anatina* mais son taux d'ingestion est faible car elle est fortement rejetée dans les pseudofaeces] (Bontes et al., 2007). Les dreissenes sont également capables de détecter la toxicité des cyanobactéries et ainsi d'éviter leur ingestion par réduction des taux de filtration et augmentation du taux de rejet (Juhel et al., 2006). Cependant, comme chez le zooplancton, l'ingestion de cyanobactéries va dépendre de la taille du bivalve ainsi que de la cyanobactérie (filamenteuse ou colonies) (Naddafi et al., 2007).

Les cyanobactéries peuvent constituer une source de nourriture pour certaines espèces de poissons herbivores (e.g. *Aphanizomenon* pour le gardon) (Kamjunke et al., 2002). Carbis

et al. (1997) ont montré qu'elles sont la principale source de nourriture d'une population de carpe exposée à des blooms de *M. aeruginosa*. Cependant, certaines espèces de poissons semblent coexister avec les cyanobactéries toxiques par des adaptations similaires à celles rencontrées chez le zooplancton et les bivalves (e.g. adaptations comportementales chez certains poissons phytoplanctonophages). Le tilapia (*Oreochromis sp*) et la carpe argentée (*Hypophtalmichthys molitrix*), par exemple, diminuent leur activité de broutage quand la proportion de *Microcystis* dans le phytoplancton augmente. La reconnaissance des cellules toxiques est effectuée via des composants membranaires des cyanobactéries (Beveridge et al., 1993 ; Keshavanta et al., 1994). D'après l'étude de Xie et al. (2007) sur plusieurs espèces de poissons d'un lac tempéré (dont *Carassius auratus* et *Cyprinus carpio*), le taux de broutage diminue progressivement au fur et à mesure que le pourcentage de cyanobactéries toxiques augmente. De plus, certains poissons zooplanctophages comme les épinoches se nourrissent moins lors de la prolifération de cyanobactéries, en raison d'un accroissement de la turbidité de l'eau (vision amoindrie) et d'un encombrement des branchies (Engström-Öst et al., 2006).

1.3.4. Accumulation de MCs et impact dans le réseau trophique aquatique

Chez le zooplancton (Rotifères, Copépodes et Cladocères), l'accumulation de MCs est souvent conséquente (chez les espèces qui consomment des cyanobactéries toxiques), et varie de 0,3 à 3900 µg MCs g^{-1} DW (Watanabe et al., 1992; Ferrao-Filho et al., 2002). Dans un lac eutrophe, environ 80% du zooplancton contient des MCs, avec une moyenne estivale de 63 à 211 µg MCs g^{-1} DW et un maximum de 1352 µg MCs g^{-1} DW (Ibelings et al., 2005). De plus, les MCs ont un impact négatif sur les taux de survie, la croissance et la reproduction du zooplancton (Lampert, 1987; Rothhaupt, 1991; DeMott et al., 1991; Thostrup et Christoffersen, 1999; Rohrlack et al., 2001). Par exemple, chez les Cladocères, la croissance et la reproduction sont affectées et le temps de survie est inversement proportionnel au taux d'ingestion de cyanobactéries toxiques (Rohrlack et al. , 2001).

Concernant les invertébrés, des mortalités de Chironomidae, d'Oligochaetes, d'écrevisses (*Orconectes limosus*) et de bivalves (*Anodonta piscinalis* et *Unio tumidus*) ont été observées en Pologne durant un épisode intense de prolifération de cyanobactéries toxiques en 1992 (Codd et al., 2004). En France (Bretagne), une étude révèle que les mollusques bivalves sont très affectés par la présence de cyanobactéries productrices de MCs (Gérard et al., 2008). Certaines espèces semblent plus résistantes aux MCs, comme *Anodonta cygnea* qui accumule de fortes quantités de MCs sans impact (Eriksson et al., 1989).

Cependant, l'impact des MCs sur les bivalves a été peu étudié et la plupart des études reportent uniquement l'accumulation et la détoxication. Watanabe et al. (1997) ont mis en évidence une accumulation de MCs (plus de 3 µg par individu) chez des moules (*Anodonta woodonia*, *Unio douglasiae*) originaires d'un lac dans lequel prolifèrent chaque année *Microcystis*. D'après Ibelings et al. (2005), 89% des *Dressena polymorpha* d'un lac eutrophe néerlandais contiennent des MCs dans leurs tissus à une concentration maximale de 27 µg g^{-1} DW. Enfin, pendant les blooms algaux dans un estuaire australien soumis à l'eutrophisation, *Mytilus edulis* peut accumuler jusqu'à 90 mg g^{-1} DW (dose largement létale pour les souris), les rendant particulièrement impropres à la consommation (Falconer et al., 1992). Au laboratoire, *Anodonta cygnea* exposée aux cyanobactéries *Planktothrix agardhii* (Eriksson et al., 1989) ou *Aphanizomenon issatschenksi* (Pereira et al., 2004) accumulent des MCs, essentiellement dans la glande digestive. La détoxication n'est pas toujours totale et dépend de la quantité de MCs accumulée ainsi que de l'espèce de bivalve considérée. Par exemple, chez les anodontes, l'élimination de la toxine est rapide (69 à 88%) au cours des 6 premiers jours en eau saine (Prepas et al., 1997) mais se stabilise ensuite. D'après Eriksson et al., (1989), les MCs sont toujours détectées dans des anodontes après un séjour de 2 mois en eau saine. Cependant, toutes ces études ont probablement sous estimé la quantité réelle de MCs dans les tissus des bivalves, car n'ont pas dosé les MCs liées. Ces MCs liées ont été mises en évidence par Williams et al. (1997a) chez *M. edulis* et par Dionisio Pires et al. (2004) chez *D. polymorpha*, ou elles représentent 1% des MCs totales la première semaine et 38% la deuxième semaine d'ingestion de *M. aeruginosa*.

Les crustacés accumulent des MCs mais semblent moins sensibles à leurs effets toxiques que les autres macroinvertébrés. Vasconcelos et al. (2001) montre que l'écrevisse américaine *Procambarus clarkii* ingère des cyanobactéries *Microcystis sp.* sans impact sur la croissance, mais avec une accumulation de MCs (jusqu'à 2.9 µg de MC g^{-1} DW) dans les intestins et la glande digestive. Pendant la période de dépuration, les MCs sont pratiquement toutes éliminées en 23 jours. D'autre part, les écrevisses nourries avec des souches toxiques ont une meilleure croissance que les écrevisses à jeun, et les tests de toxicité aiguë montrent une faible mortalité des larves exposées (au maximum de 35% en 48h). De même, 50% des écrevisses (*Pacifastacus lemusculus*) accumulent des toxines dans leur glande digestive après 14 jours d'ingestion de cellules toxiques de *P. agardhii*, sans qu'aucune mortalité ou effets négatifs ne soient observés (Liras et al., 1998). Cependant, un impact physiologique des MCs (inhibition des ATPases, augmentation de l'activité des GST) sur les branchies du crabe *Chasmagnatus granulatus* a été obervé par Vinagre et al. (2002). L'accumulation de MCs a

aussi été démontrée chez des crevettes (*Palaemon modestus* et *Macrobrachium nipponensis*) d'un lac eutrophe soumis à des blooms intenses et réguliers (Chen & Xie, 2005), avec comme sites principaux d'accumulation, la glande digestive et la gonade, mais aussi les œufs (8 à 29% des MCs dans les œufs).

Chez les poissons, de nombreuses études reportent l'accumulation de MCs (annexes 3 et 4), accompagnée d'impact physiologiques et histologiques (Ernst et al., 2005; 2006; 2007). La concentration de MCs est souvent plus importante dans le foie et le tube digestif, modérée dans les reins et les gonades, et plus faible dans les muscles, mais il existe de grandes variations entre les espèces (pour revue : Malbrouck et Kestemont, 2006). L'accumulation dépend des modalités d'exposition (intensité et durée), à court et à long terme, et des processus d'élimination. Une étude menée sur des stades juvéniles de carpe (*Cyprinus carpo*) exposés deux années consécutives à des MCs dissoutes (simulation de libérations naturelles annuelles lors des blooms) a montré une augmentation de l'activité enzymatique de détoxication lors de la deuxième exposition. Il y aurait alors une meilleure tolérance chez les organismes régulièrement exposés (Palikova *et al.*, 2004). Les impacts sur les traits de vie des poissons ont été peu étudiés. En milieu naturel, des mortalités massives ont été associées à l'occurrence de blooms toxiques (Rodger et al., 1994), et une étude à laquelle nous avons participé révèle une contamination par les MCs de 50% des anguilles prélevées dans un site en Bretagne (Frémur) affectant leur condition (quantité de réserves lipidiques) avant la migration (Acou et al., 2008).

1.3.5. Contamination indirecte par ingestion de proies contaminées

Les MCs, dont nous venons de décrire l'accumulation chez un grand nombre d'organismes, peuvent être transmises le long des chaînes trophiques aquatiques et terrestres. Un transfert de MCs a été supposé suite à une importante mortalité de saumons d'élevage liée à l'ingestion de copépodes et de larves de crabe ayant accumulé des MCs (Williams et al., 1997b). En milieu naturel, l'hypothèse d'une contamination des poissons omnivores ou carnivores par transfert trophique a déjà été relatée chez plusieurs espèces (Ibelings *et al.*, 2005 ; Gkelis *et al.*, 2006 ; Xie *et al*, 2005 ; 2007) mais peu d'études l'ont clairement établi. Ibelings et Chorus (2007) distinguent 3 types de devenir des MCs dans les réseaux trophiques: 1) la bioaccumulation, lorsque la concentration de MCs chez un organisme excède celle dans les cyanobactéries, 2) la biomagnification, lorsque la concentration de MCs augmente en progressant dans le niveau trophique, et 3) la biodilution, lorsque la concentration de MCs

diminue en progressant dans le niveau trophique. Ibelings et al. (2005) ont mis en évidence une présence de MCs à tous les niveaux trophiques (cyanobactéries, zooplancton, bivalves, poissons zooplanctophages, malacophages et piscivores), mais aucune biomagnification n'a été observée. Selon ces auteurs, les MCs auraient plutôt tendance à subir une biodilution en raison des processus de détoxication ayant lieu à chaque niveau trophique. En effet, en laboratoire, Lauren-Määttä et al. (1995) n'ont pas détecté de MC-LR chez des larves de Diptère (*Chaoborus sp.*) nourries avec du zooplancton (*Daphnia pulex*) contaminé. Les auteurs supposent que la MC-LR (au moins 96,5%) a été métabolisée (ou excrétée) par les Diptères. Une autre explication à l'absence de biomagnification entre les poissons planctonophages et carnivores est proposée par Fisher et Dietrich (2000). Selon ces auteurs, l'accumulation de MC devrait être moins importante chez les espèces carnivores comparé aux planctonophages en raison de variations dans les capacités d'absorption intestinale : un poisson herbivore accumulerait plus de MCs qu'un carnivore car il prélève l'intégralité de la toxine du bol alimentaire grâce à un iléon long et de large surface (les carnivores ont un iléon court donc absorberaient moins de toxine lors de la consommation de proies contaminées). Cependant, Xie et al. (2007) montrent que l'accumulation de MC est relativement faible dans les organes de la carpe argentée planctonivore alors qu'elle est importante chez les poissons omnivores et prédateurs. Néanmoins, les MCs liées de manière covalente ont très rarement été prises en compte et pourraient engendrer un transfert non négligeable qui passerait inaperçu lors des dosages avec les techniques classiques utilisées.

Le transfert des MCs à l'homme est possible via la consommation de fruits de mer ou de poissons contaminés (annexe 5) (Ibelings and Chorus, 2007). Ainsi, dans la baie de Sepetiba au Brésil, un suivi de 11 mois des quantités de MCs dans les crevettes, crabes et muscles de poissons, a permis de montrer une accumulation supérieure à la limite autorisée par l'OMS (Magalhaes et al., 2003). Les MCs peuvent également se fixer sur, et s 'accumuler dans, des salades arrosées avec de l'eau contaminée (Codd et al., 1999). Le riz est aussi source d'intoxication lorsque des blooms de cyanobactéries se développent dans les rizières. De même, la consommation de suppléments alimentaires à base de cyanobactéries (dont le genre *Spirulina*.) présente une source potentielle d'exposition et est soupçonnée d'être à l'origine d'un cas de décès aux Etats Unis (pour revue : Dietrich et Hoeger, 2005).

1.4. Les interactions cyanobactéries-gastéropodes dulcicoles

1.4.1. Présentation des Gastéropodes dulcicoles

Les gastéropodes d'eau douce (environ 4000 espèces validées) sont présents sur tous les continents, à l'exception de l'Antarctique, et peuplent à peu près tous les types d'habitat dulcicole (Strong et al., 2008). Ils appartiennent principalement aux Caenogastropoda et aux Heterobranchia, dont font partie respectivement la plupart des Prosobranches (groupe polyphylétique) et des Pulmonés Basommatophores (Strong et al., 2008).

Les Prosobranches d'eau douce ont une origine marine et leur histoire évolutive est purement aquatique. Ils ont une respiration majoritairement branchiale (Aldridge, 1983) et dans une moindre mesure tégumentaire, et sont munis d'un opercule calcaire ou corné porté par le pied qui obture l'ouverture de la coquille lorsque l'animal est rétracté. La figure 7 illustre un exemple de Prosobranches Valvatidae et Bithyniidae.

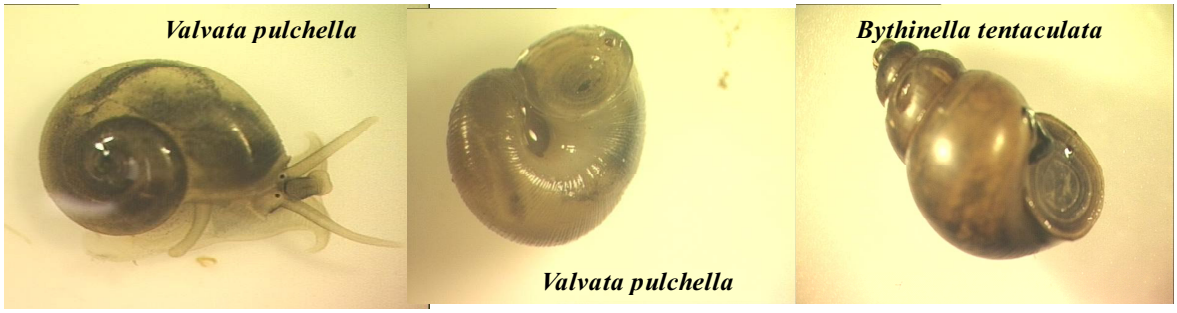

Figure 7 : Photo des Prosobranches Valvatidae (*Valvata pulchella)* et Bithyniidae *(Bythinella tentaculata)* (taille réelle respectivement environ 3.5 et 10 mm) individus prélevés dans le lac de Grand Lieu (44).

Les Pulmonés Basommatophores, dépourvus d'opercule, dérivent des Pulmonés terrestres et se sont adaptés secondairement à la vie en eau douce. Ils possèdent un poumon richement vascularisé qui peut être rempli d'air ou d'eau selon la disponibilité en dioxygène dissous, et muni d'un orifice unique, le pneumostome (MacMahon, 1983). Le degré d'adaptation à la vie aquatique diffère selon les familles (dans l'ordre croissant : Lymnaeidae < Physidae < Planorbidae < Ancylidae). La plupart ont une respiration pulmonaire (les échanges gazeux se font avec l'atmosphère lors de remontées) et cutanée [le tégument constitue un échangeur gazeux non spécifique mais efficace lorsque la teneur en O_2 dissous

est forte (Dillon, 2000)]. Chez les Pulmonés les plus aquatiques (Planorbidae, Ancylidae), la cavité pulmonaire s'atrophie, des branchies secondaires se différencient, et la respiration devient essentiellement cutanée et branchiale. Les Planorbidae possèdent également un pigment respiratoire, l'hémocyanine, et peuvent vivre complètement immergées dans des eaux plus profondes (MacMahon, 1983; Russel-Hunter, 1978). Les photos ci-dessous (Figure 8) illustrent des exemples de Pulmonés Planorbidae, Lymnaeidae et Physidae.

Figure 8. Photos des Pulmonés Planorbidae *Bathyomphalus contortus*, Lymnaeidae *Radix ovata* et Physidae *Physa acuta* (taille réelle respectivement environ 4, 12 et 9 mm), individus prélevés dans le lac de Grand Lieu (44).

a. Biologie des gastéropodes

Chez tous les gastéropodes d'eau douce, la reproduction est uniquement sexuée. Chez les Prosobranches les sexes sont séparés (sauf chez *Valvata sp.*) alors que chez les Pulmonés il y a toujours hermaphrodisme (Dillon, 2000). L'autofécondation est possible mais en général il y a une préférence pour la fécondation croisée. Les femelles ou les individus matures (Pulmonés) collent leurs pontes gélatineuses sur le substrat (à l'exception des Neritidae chez qui les œufs sont pondus isolément). Certains Prosobranches (ex : *Potamopyrgus antipodarum*) sont vivipares et parthénogénétiques (Dillon, 2000).

Les Pulmonés sont plutôt annuels et ont globalement évolué vers la semelparité (mortalité après la reproduction), probablement en relation avec les fortes contraintes de l'habitat dulcicole (variations soudaines des conditions physicochimiques) (Calow, 1978). Les Prosobranches tendent à être pérennes (2 à 5 ans) et itéropares, comme les formes marines dont ils dérivent. En région tempérée, il existe généralement une seule saison de reproduction, le printemps (monovoltinisme). Cependant, chez certaines espèces [comme *Planorbis planorbis* en Bretagne (Costil & Daguzan, 1995)] on peut observer un bivoltinisme avec une génération de printemps et une de fin d'été, ou plus exceptionnellement une reproduction toute l'année pour *P. antipodarum*. L'histoire de vie d'un organisme est le résultat à la fois

des forces évolutives à long terme et des réponses immédiates à son environnement. Le type d'habitat conditionne l'évolution de chaque population vers l'une ou l'autre des stratégies. Chez les adultes, la mortalité est principalement liée à l'effort de reproduction bien que quelques individus survivent parfois une deuxième année. Chez les juvéniles, la mortalité est très élevée et est principalement due à la prédation (Dillon, 2000).

b. Ecologie des gastéropodes

Les gastéropodes dulcicoles sont particulièrement abondants dans les zones littorales des lacs ou rivières à faible courant, et sont essentiellement benthiques ou concentrés sur les macrophytes immergés ou émergés (Dillon, 2000). Du fait de leur faible mobilité, les communautés de gastéropodes sont très influencées pas la qualité de leur milieu et sont de bons indicateurs de l'état de santé de l'écosystème (Elder & Collins, 1991; Salanki, 2000; Downs et al., 2001; Lefcort et al., 2002). En général, les Pulmonés aquatiques préfèrent les habitats peu profonds (petits hydrosystèmes) donc instables sur le long terme (s'assèchent et disparaissent), alors que les Prosobranches sont plus typiques des grandes pièces d'eau stables. D'après Russel-Hunter (1961), le succès des populations de mollusques dépend de leurs capacités à subir des variations physiologiques considérables. Les populations de Pulmonés sont plutôt euryèces et tolèrent bien les variations de conditions du milieu en raison de leur plasticité élevée. Ils présentent une grande diversité intra-spécifique, éco-phénotypique et génétique. Les Prosobranches, exclusivement aquatiques (donc dépendants de la quantité d'oxygène dissous et de la qualité de l'eau) et très liés au substrat dont ils extraient la nourriture, ont une moindre tolérance aux variations du milieu que les Pulmonés (Russel-Hunter, 1978; Aldridge 1983; MacMahon 1983; Dillon 2000).

De nombreux facteurs abiotiques conditionnent la présence des gastéropodes, mais certains comme la température, le niveau d'eau ou la disponibilité de nourriture sont plus déterminants (à partir du moment où la teneur en calcium dissous dépasse 5 mg L^{-1}). En région tempérée, la température influence la survie, la croissance, la reproduction et la taille à la maturité sexuelle (Costil, 1997). Beaucoup de Pulmonés hivernent et migrent vers les eaux plus profondes quand la température devient trop basse (Aldridge, 1983). A forte température, la teneur en O_2 diminue dans l'eau, obligeant les Pulmonés à rester près de la surface en zone littorale, même en l'absence de nourriture. Les Pulmonés vivant près du littoral sont plus tolérants aux variations de température (et donc aux variations du taux d'oxygène dissous)

que les Prosobranches vivant à de plus grandes profondeurs (Aldridge, 1983). Un autre facteur influençant l'abondance et la richesse spécifique des communautés de gastéropodes est la productivité du milieu (niveau trophique). Par exemple, les mollusques de 45 plans d'eau de Bretagne présentent une distribution le long d'un gradient trophique, avec certaines espèces (e.g. *Lymnaea glabra*) caractéristiques des milieux oligotrophes et d'autres (*Hippeutis complanatus, P. planorbis, Planorbis corneus, Lymnaea stagnalis*) caractéristiques des milieux plus eutrophes. (Legendre et al., 2004 ; Costil et Clément, 1996). La concentration en phosphore semble être un paramètre significatif, en raison de son rôle dans l'augmentation de la densité de périphyton consommé par les gastéropodes (Dillon, 2000).

c. Place des gastéropodes dans le réseau trophique aquatique

Les gastéropodes jouent un rôle dominant dans les réseaux alimentaires benthiques des eaux courantes et stagnantes (Brendelberger, 1997) car ils constituent une part importante (jusqu'à 90%) de la biomasse de macroinvertébrés benthiques (Hawkins & Furnish, 1987; Habdija et al., 1995; Strong et al., 2008; Balian et al., 2008). Ce sont des herbivores-détritivores et brouteurs, qui consomment des détritus et le périphyton algal se développant sur les macrophytes, les supports rocheux des zones peu profondes et autres substrats (e.g., coquille de mollusques) (Reavell, 1980 ; Abbott & Bergey, 2007). Même si le régime alimentaire des gastéropodes dulcicoles est qualifié de généraliste, un choix alimentaire par broutage sélectif est démontré au laboratoire pour plusieurs espèces (Lodge, 1986 ; Underwood & Thomas, 1990). Les gastéropodes influencent ainsi les communautés au sein du périphyton [ex : réduction de la biomasse, modification de la structure taxonomique des communautés, stimulation de la production primaire par relarguage de nutriments dissous) (Mac Collum, 1998; James et al., 2000; Aberle et al., 2005)], mais sont aussi influencés en retour par son abondance (réduction de la densité et de la croissance des gastéropodes) (Liess & Haglung, 2007). De plus, la présence de gastéropodes favorise indirectement les macrophytes, à la fois par le relarguage de nutriments, et par le retrait du périphyton présent sur les feuilles et permettant aux macrophytes de mieux capter la lumière et les nutriments (modification de la compétition périphyton-macrophytes) (Brönmark, 1990; Steinman et al., 1987). Les gastéropodes brouteurs sont considérés comme des espèces ingénieurs des écosystèmes d'eau douce en raison de leur implication dans le contrôle de la productivité des zones littorales (Abbott & Bergey, 2007).

Par ailleurs, les gastéropodes sont les proies de nombreux prédateurs, le plus souvent omnivores : oiseaux d'eau, poissons (Salmonidés, Cyprinidés, Siluriformes, Perciformes, Anguilliformes), amphibiens (Urodèles et Anoures), sangsues (Glossiphoniidae), écrevisses et insectes (coléoptères hydrophiles et dytiques, larves de tabanidae et d'odonates, hémiptères) (Michelson, 1957, Turner & Chislock, 2007). Ils ont donc un rôle clé dans la structuration du réseau trophique en tant que lien important entre les producteurs primaires et les consommateurs secondaires (Kerans et al., 2005), et peuvent être à l'origine de 30% du transfert total d'énergie au sein des communautés dans les ruisseaux (Habdija et al., 1995).

1.4.2. Interactions cyanobactéries-gastéropodes en milieu naturel : voies de contamination par les microcystines

a. Contamination par les MCs dissoutes ou adsorbées sur des particules

Les possibles voies de pénétration des MCs dissoutes chez les gastéropodes Pulmonés et Prosobranches sont : i) l'ingestion d'eau contaminée [de l'ordre de 8 à 12 µl h^{-1} g^{-1} poids frais chez *Lymnaea stagnalis* selon De Witt (1996)], ii) le remplacement à partir de l'eau environnante de l'hémolymphe émise par les gastéropodes par le pore hémal (réduction de 35%) lorsqu'ils se rétractent dans leur coquille [phénomène fréquent chez les Pulmonés lors d'un stress, comme face à un prédateur (Rigby & Jokela, 2000)], iii) la perméabilité de l'épiderme [l'entrée de matière particulaire à travers l'épiderme par pinocytose a été démontrée chez *L. stagnalis* (Zylstra, 1972)], et en particulier des surfaces respiratoires, pulmonaire ou branchiale [branchie des Prosobranches et pseudobranchies de certains Pulmonés (Planorbidae, Ancylidae)]. La contamination par les MCs particulaires peut avoir lieu chez les Pulmonés lors de l'ingestion de particules minérales (nécessaires à la trituration de leurs aliments dans l'estomac), et chez les Prosobranches lors de la filtration de leur nourriture en suspension dans l'eau (Aldridge, 1983) ou lors de l'ingestion de sédiments dont ils extraient les nutriments, soit dans la cavité buccale, soit dans le tube digestif (Aldridge, 1983 ; Dorgelo & Leonards, 2001).

b. Contamination par ingestion de cyanobactéries toxiques

La plupart des gastéropodes qui vivent dans les zones littorales sont conditionnés par les abondances relatives des ressources alimentaires (Pinel-Alloul & Magnin, 1978, Reavell, 1980, Brendelberger, 1997). Ainsi, en période estivale sous nos latitudes, ils peuvent être amenés à consommer de grandes quantités de cyanobactéries qui envahissent l'eau et les supports rocheux et macrophytiques du littoral, accumulées par effet du vent (Scheffer *et al.*, 1997). La consommation de cyanobactéries (*Planktothrix sp., Anabaena sp.* et *Merismopedia sp.*) a été vérifiée chez *Lymnaea catascopium* pendant la période de prolifération dans divers lacs eutrophes (Pinel-Alloul et Magnin, 1979), et démontrée en laboratoire chez *Pseudosuccinea collumella* et *Physa vernalis* et *Physella heterostropha* (Kesler et al., 1986; Mac Collum et al., 1998). Une ingestion journalière et constante de cyanobactéries productrice de MCs a été démontrée chez le Prosobranche (Viviparidae) japonais *Sinotaia histrica*, exposé pendant 15 jours à *Microcystis* (Ozawa et al., 2003). Cette ingestion est suivie d'une accumulation de MCs dans la glande digestive, maximale après 10 jours (436 µg g^{-1} DW), et d'une détoxication lente et incomplète (la concentration en MC s'élève encore à 127 µg g^{-1} DW après 15 jours). Zurawell et al. (2001) ont également montré la présence de cellules de *Microcystis aeruginosa* contenant des MCs dans le tube digestif de *L. stagnalis* prélevée en milieu naturel.

c. Importance relative des voies de contamination par les microcystines

La part relative des voies de contamination des gastéropodes Pulmonés et Prosobranches par les MCs n'a pas été mise en évidence. Ces gastéropodes, différant par leurs caractéristiques écophysiologiques, leur cycle de vie et leurs exigences écologiques (cf paragraphe 1.4.1.) peuvent en effet présenter des réponses variables à l'exposition aux MCs intracellulaires ou dissoutes. Au laboratoire, le Pulmoné *L. stagnalis* accumule 100 fois moins de MCs libres que le Prosobranche *P. antipodarum* (0.4 µg g^{-1} de poids frais pour le Prosobranche) lors d'une exposition à de la MC-LR dissoute (Gérard et al., 2005; Gérard et Poullain, 2005), suggérant une contamination importante des Prosobranches par les toxines dissoutes. En milieu naturel, l'accumulation de MC dans les tissus de (jusqu'à 140 µg MC g^{-1} DW pour *Lymnaea stagnalis,* 130 µg MC g^{-1} pour *Physa gyrina,* 40 µg MC g^{-1} pour *Helisoma trivolvis*) prélevés dans des lacs canadiens est proportionnelle à la quantité de toxine présente dans les cyanobactéries (jusqu'à 1526 µg g^{-1} DW), mais n'est pas corrélée à celle de la toxine dissoute dans l'eau (jusqu'à 1.2 µg L^{-1}) (Kotak et al., 1996; Zurawell et al., 1999). Ce serait

donc essentiellement par ingestion de cyanobactéries toxiques que les Pulmonés accumuleraient des toxines.

1.4.3. Devenir des microcystines ingérées chez les gastéropodes

Une proportion parfois non négligeable (supérieure à 60%) de micro-algues peut survivre au passage dans le tube digestif des gastéropodes (Cuker, 1983 ; Underwood & Thomas, 1990, Brendelberger, 1997). Les micro-algues partiellement digérées sont souvent celles dont la structure rend la trituration difficile, comme les diatomées entourées d'une squelette siliceux, ou certaines cyanobactéries formées de colonies encapsulées dans une enveloppe mucilagineuse (Zurawell et al., 2001). Ainsi, Zurawell et al. (2001) ont élucidé certains aspects du devenir de *Microcystis aeruginosa* et de ses MCs chez le Pulmoné *L. stagnalis*, et montrent que la majorité des MCs libres détectées dans les limnées provient des cyanobactéries intactes présentes dans le tractus alimentaire (protégées de la digestion par le mucus entourant les colonies). Les cyanobactéries non digérées sont éliminées dans les fèces dans les 8 heures après ingestion, représentant 57% de la concentration initiale de MCs mesurée chez le mollusque. Cependant, en cas d'ingestion de cyanobactérie filamenteuse (sans mucus) par les gastéropodes, il est probable qu'une plus grande proportion des MCs soit libérée après lyse mécanique et enzymatique des cellules dans le gésier et dans l'estomac. En effet, l'estomac des Prosobranches contient un stylet cristallin, le protostyle, dont le rôle dans la digestion est à la fois chimique par les enzymes qu'il contient, et mécanique par sa rotation rapide. Chez les Pulmonés, il n'existe pas de stylet cristallin, mais leur gésier est composé de muscles et de tendons puissant et est rempli de particules minérales (sable) permettant de la trituration des aliments (digestion mécanique complétée par une digestion chimique grâce à diverses enzymes déversées par la glande digestive) (Carriker, 1946; Dillon, 2000). Le tableau présenté en annexe 6 récapitule l'ensemble des organes impliqués dans la digestion de *L. stagnalis* ainsi que leurs fonctions. Les MCs, résistantes à la digestion dans le tractus gastro-intestinal des eucaryotes (Msagati et al., 2006), sont ensuite dirigées et accumulées dans la glande digestive, site majeur de digestion intracellulaire, sécrétion, assimilation, excrétion, et du métabolisme chez les gastéropodes (Carriker, 1946 ; Zurawell, 2001). D'après Chen et al. (2005), la glande digestive est l'organe qui contient la plus grande teneur de MCs libres (4.4 µg MC g DW^{-1}), suivi par le tractus digestif (1.69 µg MC g DW^{-1}) et la gonade (0.72 µg MC g DW^{-1}) chez le Prosobranche Viviparidae *Bellamya aeruginosa* prélevé dans un lac chinois.

Etant donné que les Ppases sont présentes dans toutes les cellules eucaryotes, il est fortement probable que les MCs puissent interagir avec ces enzymes après pénétration dans les cellules de la glande digestive des gastéropodes, et induire des dommages similaires (histopathologie) à ceux observés chez les vertébrés. De plus, aucune de ces études ne reporte le dosage de toxines liées de manière covalente aux Ppases, malgré leur probable forte implication dans les phénomènes de transfert au sein du réseau trophique.

1.4.4. Impact des microcystines sur les gastéropodes

L'impact des cyanobactéries et des MCs sur les traits de vie des gastéropodes a été très peu étudié (Gérard & Poullain, 2005, Gérard et al., 2005) à la fois lorsque nous avons commencé cette thèse et encore aujourd'hui (excepté nos travaux). L'exposition pendant 6 semaines à de la MC-LR dissoute (33 µg L^{-1}) induit une diminution des traits de vie (survie, croissance et fécondité) chez le Prosobranche *P. antipodarum* quel que soit son âge (Gérard & Poullain, 2005), alors que seule la fécondité est affectée chez *L. stagnalis* (Gérard *et al.*, 2005). Selon ces études, l'activité locomotrice des deux espèces n'est pas modifiée par l'exposition à la MC solubilisée. D'autre part, selon l'étude préliminaire de Gevrey *et al.* (1972), la limnée *Radix auricularia* survit à l'ingestion de cyanobactéries toxiques (complexe *Microcystis farlowania - Pseudoanabaena franqueti*), sans qu'il y ait de pathologie apparente. En milieu naturel, la toxicité des cyanobactéries sur les gastéropodes a été suggérée par Armitage & Fong (2004) qui ont constaté une forte augmentation de la biomasse des cyanobactéries due à l'enrichissement en nutriments (P et N) d'un milieu estuarien ayant eu pour conséquence d'accroître la mortalité du Pulmoné *Cerithidea californica*, résultat probable d'une ingestion massive de cyanobactéries toxiques.

1.4. Conclusion et objectifs de la thèse :

Cette synthèse des connaissances montre qu'en dehors des travaux de Gérard et Poullain (2005) et Gérard et al., (2005), et malgré la réalité de la confrontation des cyanobactéries et des gastéropodes au sein de leur habitat, en particulier dans les eaux eutrophes, l'impact des cyanobactéries productrices de MCs sur les gastéropodes dulcicoles a été rarement étudié. Les quelques études répertoriées reportent l'accumulation de MCs libres chez des Prosobranches et des Pulmonés, le plus souvent en milieu naturel (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005). Ainsi, à travers une approche à la fois écotoxicologique, écophysiologique et écologique, la méthodologie adoptée associe des expériences de laboratoire à une étude *in situ*. Les recherches sont menées à différents niveaux d'organisation biologique : tissus, organisme, population, communauté.

Les deux espèces modèles de gastéropodes choisies pour les expériences en milieux contrôlé et semi contrôlé, le Pulmoné *Lymnaea stagnalis* (Lymnaeidae) et le Prosobranche *Potamopyrgus antipodarum* (Hydrobiidae), sont représentatives des communautés bretonnes et diffèrent par leurs caractéristiques écophysiologiques, leur cycle de vie et leurs exigences écologiques, ce qui devrait permettre d'avoir une vision plus globale des modalités de réponses des gastéropodes à l'exposition aux cyanobactéries toxiques. *L. stagnalis*, très étudiée en écotoxicologie, est un Pulmoné ovipare de grande taille (≤ 5 cm) (Ökland, 1990), qui se reproduit principalement au printemps. *P. antipodarum* est une espèce de petite taille (≤ 5 mm) (Strzelec & Serafinski, 1996), ovovivipare et parthénogénétique, dont les femelles portent des embryons toute l'année avec un pic d'abondance au printemps. Les cyanobactéries utilisées sont des souches toxiques de *Planktothrix agardhii*, l'une des espèces les plus communes dans les lacs eutrophes peu profonds d'Europe (Scheffer *et al.*, 1997) et présentant une production de MCs plus élevée que les espèces unicellulaires ou coloniales (Christiansen *et al.*, 2003). Afin de rester dans une situation concrète, nous avons utilisé des concentrations de cyanobactéries et de toxines représentatives de la majorité des situations rencontrées en milieu naturel (Christoffersen, 1996, Chorus, 2001). Par ailleurs, l'UMR Ecobio affiche des compétences reconnues au plan international sur l'utilisation de sites ateliers dans l'étude du fonctionnement des écosystèmes. Parmi ces sites, le Lac de Grand Lieu (Loire Atlantique), réserve naturelle suivie depuis plus de 20 ans, connaît des blooms récurrents de cyanobactéries, dont *P. agardhii*, et a été choisi comme site d'étude privilégiés de ce programme de recherche. Enfin, le modèle de poisson choisi pour les expériences de transfert des MCs est l'épinoche, *Gasterosteus aculeatus* (Gasterosteidae), petit poisson

carnassier euryhalin des zones tempérées de l'hémisphère Nord, utilisé comme espèce bioindicatrice de par sa large répartition et sa sédentarité (Sanchez et al., 2007). Son régime alimentaire est considéré comme omnivore et peut inclure des mollusques selon leur disponibilité dans le milieu (Bruslé et Guignard, 2001).

Ainsi, dans un premier temps l'objectif de cette thèse était de :

- En conditions contrôlées, évaluer l'ingestion de cyanobactéries productrice de MCs par les 2 modèles de gastéropodes, y compris en présence d'une autre ressource alimentaire non toxique.
- Connaître la réponse des 2 modèles à cette ingestion, en termes d'impact sur les traits de vie et de processus d'accumulation versus détoxication.
- Approfondir les impacts observés sur la fécondité du Pulmoné via l'étude des conséquences d'une exposition aux MCs dissoutes dans le milieu ou à des cyanobactéries toxiques sur la descendance (éclosion, développement embryonnaire, survie des néonates).
- Connaître la réponse des 2 modèles de gastéropodes en conditions semi-contrôlées (encagés en milieu naturel), et évaluer l'impact des cyanobactéries toxiques par un suivi de la structure des populations naturelles de gastéropodes (différents sites contaminés).

Ces études, comparées à celle entreprises avec les 2 mêmes espèces exposées aux MCs dissoutes (Gérard & Poullain, 2005; Gérard et al., 2005) permettront de déterminer les principales voies de contamination chez les Prosobranches et chez les Pulmonés, ainsi que leurs conséquences. Elles permettront également de déterminer le modèle accumulant le plus de toxines et ainsi, dans un deuxième temps, d'approfondir notre démarche et de :

- Étudier et comparer les impacts histopathologiques suite à l'exposition du modèle de gastéropode choisi aux MCs dissoutes ou à des cyanobactéries toxiques. Associée à une étude sur la localisation des MCs liées dans les tissus, ces expériences auront pour but de différencier le devenir des toxines dans le mollusque selon le mode de contamination.
- Adapter une technique pour le dosage des MCs liées dans les tissus, jamais mises en évidence chez les gastéropodes, et jouant un rôle prépondérant dans la contamination des réseaux trophiques car très difficilement éliminées par les organismes.
- Mettre en évidence le transfert des MCs dans la chaine trophique depuis les gastéropodes à leurs prédateurs (modèle poisson), afin d'évaluer le rôle vecteur des gastéropodes et les risques de contamination du réseau trophique.

Chapitre 2 :
Exposition de 2 gastéropodes dulcicoles, *Lymnaea stagnalis* (Pulmoné) et *Potamopyrgus antipodarum* (Prosobranche), à une cyanobactérie productrice de microcystines

Chapitre 2 : Exposition de 2 gastéropodes dulcicoles, *Lymnaea stagnalis* (Pulmoné) et *Potamopyrgus antipodarum* (Prosobranche), à une cyanobactérie productrice de microcystines

Au sein de leur habitat, les mollusques gastéropodes sont confrontés aux proliférations de cyanobactéries toxiques, particulièrement dans les eaux eutrophes. Les interactions entre cyanobactéries toxiques et gastéropodes ont rarement été étudiées, et les quelques études disponibles rapportent l'accumulation de microcystines mais jamais leur impact. En milieu naturel, l'accumulation de MCs semble être plus importante chez les Pulmonés que chez les Prosobranches, et il a été suggéré qu'elle était une conséquence directe de l'ingestion de cyanobactéries toxiques (Kotak et al., 1996; Zurawell et al., 1999; Chen et al., 2005 ; Chen et Xie, 2005). Cependant, en laboratoire, le pulmoné *Lymnaea stagnalis* accumule 100 fois moins de MCs que le prosobranche *Potamopyrgus antipodarum* lorsque tout deux sont exposés à de la MC-LR dissoute dans le milieu (Gérard et al., 2005; Gérard et Poullain, 2005). Les pulmonés et les prosobranches, très différents sur le plan des caractéristiques écophysiologiques, sont susceptibles de présenter des voies de contamination différentes et/ou des réponses variables (en termes de stratégie d'allocation des ressources et de processus de bioaccumulation versus détoxification) aux toxines cyanobactériennes. C'est pourquoi, il nous a paru essentiel d'établir les principales voies de contamination par les MCs des pulmonés et prosobranches et de comparer leur accumulation de MCs ainsi que ses impacts.

Ce chapitre comporte 3 études distinctes portant sur 2 espèces de gastéropodes, le pulmoné *Lymnaea stagnalis* et le prosobranche *Potamopyrgus antipodarum*, exposées à deux âges différents (juvéniles et adultes) à des souches de *P. agardhii* productrice de MCs (faibles doses représentatives de la majorité des situations rencontrées en milieu naturel*)* pendant 5 semaines, et vise à évaluer : 1) la consommation de cyanobactéries toxiques des deux gastéropodes en présence ou absence de nourriture non toxique (salade), 2) les cinétiques d'accumulation et d' élimination de MCs dans leurs tissus, et 3) l'impact sur leurs traits de vie

(survie, croissance, fécondité). L'intoxication est suivie par 3 semaines de dépuration pendant lesquelles les mollusques ne reçoivent que de la salade.

Les résultats montrent que *L. stagnalis* et *P. antipodarum* ingèrent des souches toxiques de *P. agardhii* même en présence de salade. Après 5 semaines d'intoxication, 61% des toxines ingérées sont accumulées par *L. stagnalis* (Lance *et al*., 2006), et seulement 2.6% par *P. antipodarum*, (Lance et al., 2008). Après la période de dépuration, la détoxification est rapide et totale chez *P. antipodarum* alors qu'elle est partielle (90%) chez *L. stagnalis*. Les traits de vie (croissance et la fécondité) de *L. stagnalis* et *P. antipodarum* sont affectés par l'ingestion de *P. agardhii*. On observe un effet age-spécifique caractérisé par une baisse des taux de croissance chez les juvéniles et une baisse de fécondité chez les adultes. Ces deux traits de vie sont diminués pendant la contamination pour les deux espèces, mais l'impact est réversible durant la dépuration chez le prosobranche. Dans le cas du pulmoné l'impact est plus sévère et irréversible (Lance et al., 2007).

Alors que de précédents travaux (Gérard & Poullain, 2005; Gérard et al., 2005) montraient que le prosobranche était plus sensible que le pulmoné (i.e. diminution de la survie, croissance et fécondité versus diminution de la fécondité) aux toxines dissoutes dans le milieu et accumulait plus de MCs, nous observons l'inverse lorsque les gastéropodes ingèrent des cyanobactéries toxiques. Ainsi, ces résultats ont permis de mettre en évidence :

- les MCs présentes dans le milieu ont toujours un impact négatif sur les gastéropodes, qu'elles soient dissoutes dans l'eau ou dans les cyanobactéries vivantes ;

- il existe une variabilité dans les voies de contamination : le prosobranche *P. antipodarum* se contamine par les deux voies mais semble plus sensible aux toxines dissoutes, alors que le pulmoné *L. stagnalis* est peu sensible et très faiblement contaminé par les toxines dissoutes mais fortement contaminé (accumulation de MCs 1300 fois supérieure) et affecté par l'ingestion de cyanobactéries toxiques;

- il existe une variabilité dans l'efficacité des processus de détoxification selon l'espèce de gastéropode (apparemment plus efficace chez le prosobranche) et l'âge (les juvéniles accumulent plus de MCs que les adultes).

2.1. Interactions between Cyanobacteria and Gastropods. I. Ingestion of toxic *Planktothrix agardhii* by *Lymnaea stagnalis* and the kinetics of microcystin bioaccumulation and detoxification[1]

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Abstract

The last two decades have been marked by an increasing occurrence of toxic cyanobacterial blooms in aquatic ecosystems. These pose an expanding threat to the environment and to human health. Among the intracellular toxins produced by cyanobacteria, microcystins (hepatotoxins) are the most frequent and widely studied. As an ubiquitous herbivore living in eutrophic freshwaters, the freshwater snail *Lymnaea stagnalis* (Gastropoda: Pulmonata) is particularly exposed to cyanobacteria. The toxic filamentous *Planktothrix agardhii* is common in temperate lakes and is therefore, a potential food resource for gastropods. We have studied the consumption of *P. agardhii* by *L. stagnalis* juveniles and adults in the presence or absence of non-toxic food (lettuce) over a 5-weeks period. Intoxication was followed by a 3-weeks detoxification period when snails were fed only on lettuce. The kinetics of microcystin accumulation and detoxification in the gastropods were established using the ELISA analytical method. The results showed an ingestion of toxic *P. agardhii* by *L. stagnalis*, even in the presence of lettuce, and the absence of food selection regardless of the age of the snails. Juveniles and adults consumed the same number of cells per ml and consumption was proportional to food availability. On average, 63% of cyanobacteria available were taken up during the first 24 hours. After 5 weeks of intoxication, 61% of the toxins present in the ingested cyanobacterial cells had accumulated in snail tissues (95% in the digestive-genital gland complex) with a concentration up to 80.4 ± 4.9 µg g DW^{-1}. Toxin accumulation was greater in the gastropods fed on *P. agardhii* alone than those fed on the mixed diet, and was also greater in juveniles than in adults. After the removal of toxic cyanobacteria, detoxification was rapid: 64% of the toxins disappeared from snail tissues during the first week, but microcystins were still detected after 3 weeks (on average, 3.5 ± 0.9 µg g DW^{-1}). These results are discussed in terms of potential contamination to the food web.

1. Introduction

Cyanobacteria can form massive blooms in freshwater bodies and produce a wide range of toxins such as hepatotoxins, neurotoxins and lipopolysaccharides. Hepatotoxins have a more widespread occurrence and are found in 40 to 75% of cyanobacterial blooms (Chorus and Bartram, 1999). The most studied hepatotoxins are the microcystins, cyclic heptapeptides of which 80 variants have been identified (Dietrich and Hoeger, 2005). Contamination of organisms can occur by exposure to soluble toxins, direct consumption of cyanobacterial cells and by consumption of contaminated prey. Microcystins have been recognized to accumulate and induce extensive damage in several organisms including zooplankton, bivalves and fish, after ingestion of cyanobacterial cells (for review: Zurawell et al., 2005).

Freshwater gastropods have rarely been considered in toxic cyanobacteria studies. However, these organisms represent an important part of freshwater macroinvertebrate biomass. They are important links between primary producers and higher consumers, and they often play key roles in structuring aquatic communities (Habdija et al., 1995). Recently, pathogenic effects of dissolved microcystin-LR on life-traits have been demonstrated in laboratory experiments on two gastropod species: the prosobranch *Potamopyrgus antipodarum* has shown a decrease in survival, growth and fecundity (Gérard & Poullain, 2005) while the pulmonate *Lymnaea stagnalis* has shown a decrease in fecundity (Gérard et al., 2005).

Freshwater pulmonates like lymnaeids inhabit shallow littoral zones and are predominantly herbivores. They are considered as indiscriminate grazers searching for food in the entire water column and on various substrates of the littoral and adapt their diet to the relative abundance of available resources (Bovbjerg, 1968; Reavell, 1980; Brendelberger, 1997). As cyanobacteria can dominate phytoplankton community and colonize littoral waters in bloom periods, it is therefore relevant to ask whether these grazers would consume large quantities of toxic cyanobacteria and whether they would be affected by this consumption. The few field studies which have included gastropods (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005) have hypothesized a consumption of toxic cyanobacteria following a positive relationship found between toxin concentration in phytoplankton and microcystin accumulation in gastropod tissues.

L. stagnalis is characteristic of eutrophic aquatic systems (Clarke, 1979) which are more prone to cyanobacterial blooms. *Planktothrix agardhii*, a filamentous planktonic species is the most common cyanobacterium in eutrophic lakes of temperate areas (Scheffer et al., 1997; Brient et al., 2004) and has a greater cellular toxin production than other colonial or unicellular species (Christiansen et al., 2003). Although this species is planktonic, wind events have been shown to concentrate filaments in littoral zones. Indeed, Webster and Hutchinson (1994) predict that a blue-green population should be more strongly concentrated towards the downwind end of a lake. This prediction is in accord with measured distributions in lakes. Densities of toxic cyanobacteria can thus become very high in shallow waters and with the filaments trapped and accumulating in dense macrophytes, on rocks and littoral sediments, increasing the probability of grazing by snails.

This study is the first part of a research program on freshwater cyanobacteria-gastropod interactions and examines the potential of *L. stagnalis* to ingest *P. agardhii* and to accumulate microcystins. The second part of our work focuses on the negative impact of toxic cyanobacteria ingestion on the life traits of gastropods (survival, growth, fecundity), and the plasticity of the response according to their development stage (juveniles or adults). Results will be presented in a separate publication. Consumption of cyanobacterial cells by gastropods in the presence or not of non-toxic food was monitored during 5 weeks to study the feeding behaviour of *L. stagnalis*. Toxin production by *P. agardhii* was evaluated to assess the quantity of microcystins ingested by snails. Additionally, we investigated the potential of *L. stagnalis* to accumulate microcystins by establishing the kinetics of accumulation and detoxification of microcystins in gastropod tissues during a 5-weeks intoxification period followed by a 3-weeks detoxification period, and by calculating the ratio of accumulated/ingested toxins.

We focused the discussion on the potential accumulation of cyanotoxins by lymnaeid snails in the field and contamination risk for the food web, since these gastropods are consumed daily by numerous invertebrates (crayfish, leeches, aquatic insects as adult coleopterans or larval tabanids) and vertebrates (fish, waterfowl) (for review: Michelson, 1957), which in turn are consumed by aquatic or terrestrial predators like fish, amphibians, musk rats and birds.

2. Material and methods

2.1. Biological material

Prior to the experiments, juvenile and adult *L. stagnalis* (respectively 14 ± 1 and 25 ± 1 mm shell length) were acclimatized at constant temperature (20 ± 1°C) and photoperiod (12-h/12-h, light/dark cycle) and fed on dried lettuce ad libitum during 7 days. The filamentous cyanobacterium *P. agardhii*, originating from the recreational watersport site at Viry (Essone, France), was maintained in a modified medium (20 mL of liquid BG11 per litre of dechlorinated water). Cyanobacteria were placed in an incubation room at constant temperature (25 ± 2°C) and photoperiod (12-h/12-h, light/dark cycle) at an irradiance of 40 $\mu E\ m^{-2}\ s^{-1}$. The algal concentrations of 200 000 cells mL^{-1} were provided twice a week to the gastropods. These algal suspensions produced dmMC-LR and MC-RR (detected by HPLC-MS), with a total concentration of 5 $\mu g\ L^{-1}$ expressed as microcystins-LR equivalents (MC-LReq) and measured by high-performance liquid chromatography (HPLC, section 2.3.b.). The density of cyanobacteria used in this study is similar to that commonly found, and often exceeded in natural systems worldwide (Chorus and Bartram, 1999).

2.2. Experimental set up

Following the period of acclimatization, snails were divided into 4 groups according to age and diet for a 5-weeks intoxication experiment: (1) cyanobacterial suspension as the only food source for juveniles (juv cyano) and (2) for adults (ad cyano), (3) a mixture of cyanobacterial suspension and lettuce ad libitum for juveniles (juv cyano+let) and (4) for adults (ad cyano+let). Each group consisted of 35 replicates i.e. 35 isolated individuals, in glass containers of 15 mL for each juvenile and 40 mL for each adult. Preliminary observations showed that gastropods consumed the *P. agardhii* suspension of 200 000 cell mL^{-1} in 3 days, hence cyanobacteria suspension was renewed twice a week. Control glass containers of 15 and 40 mL were filled in the absence of gastropods, with cyanobacteria suspension with and without lettuce during 5-weeks. Preliminary experiments performed over 20 days on 30 replicates with and without lettuce showed that the *P. agardhii* growth was similar and constant. It was assumed that 2 replicates per treatment were sufficient to measure cyanobacterial growth, and the influence of the presence of lettuce on the development of

cyanobacteria, as the conditions for growth (light, temperature, nutrient) were identical. After the intoxication period, all gastropods were fed solely on lettuce ad libitum and maintained in dechlorinated non-toxic water during a 3-weeks detoxification period.

2.3. Cyanobacterial cells and microcystin ingestion estimates

2.3.a. Growth and ingestion model

To estimate the number of cells ingested by L. stagnalis, cyanobacterial densities were determined daily on microscope with a Nageotte chamber. The total length of all *P. agardhii* filaments in a 50 µL volume was measured and expressed in cells mL^{-1} assuming an average length of one cell of *P. agardhii* was 3.12 µm, based on 50 measurements. These cell counts were made in all groups and controls for the 5-weeks intoxication period (10 renewals of suspension). Cyanobacterial growth rate (µ) was determined from the rate of change in the cyanobacterial biomass (B) over time in the controls, as follows:

$$dB/dt = \mu B \quad (1)$$

The time interval used in this study was one day and we used the discrete time form of its precedent transition equation to estimate the daily growth rate (µ) as follows:

$$B_{t+1} = B_t (1+\mu) \quad (2)$$

where B_t and B_{t+1} are respectively the biomass of cyanobacteria at time t and t_{+1} (in cells mL^{-1}). An assumption was made that µ was not influenced by the presence or absence of a gastropod. Hence, the daily gastropod ingestion rate of cyanobacteria in the treatments without lettuce was obtained by including the ingestion as a loss term (S) in equation (2) as follows:

$$B_{(S)\,t+1} = B_{(S)\,t} \times (1 + \mu - S) \quad (3)$$

where $B_{(S)\,t}$ and $B_{(S)\,t+1}$ are respectively the biomass of cyanobacteria at time t and t_{+1} (in cells mL^{-1}) in the treatments with snails and without lettuce. The total number of cells per ml ingested by snail per day was obtained by multiplying the cyanobacterial biomass $B_{(S)\,t}$ by the ingestion rate (S).

As preliminary tests revealed that the addition of lettuce introduced and enhanced the development of microorganisms that led to a cyanobacterial loss, we also estimated the

quantity of cells ingested by each snail in the presence of lettuce in their diet. Several assumptions were required to quantify this: (1) that cyanobacteria have the same growth rate (μ) in the presence of lettuce or not, (2) the snails do not consume microorganisms and (3) the daily loss rate of cyanobacteria due to the presence of lettuce (L) is independent of the presence of a snail. We first estimated L as follows:

$$B_{(L)\,t+1} = B_{(L)\,t} \times (1 + \mu - L) \qquad (4)$$

where $B_{(L)\,t+1}$ and $B_{(L)\,t}$ are respectively the biomass of cyanobacteria at time t and t_{+1} (in cell mL^{-1}) in the controls with lettuce and no snails. The daily snail ingestion rate of cyanobacteria in the presence of lettuce (SL) was obtained by considering the presence of microorganisms, as follows:

$$B_{(SL)\,t+1} = B_{(SL)\,t} \times (1 + \mu - L - SL) \qquad (5)$$

where $B_{(SL)\,t+1}$ and $B_{(SL)\,t}$ are respectively the density of cyanobacteria at time t and t_{+1} (in cell mL^{-1}) in the treatments with lettuce and snails. The total number of cells per mL ingested daily by a snail in presence of lettuce was estimated by multiplying $B_{(SL)\,t}$ by SL.

The percentage of cells ingested by snails compared to those available (%conso) in the absence of lettuce was calculated as follows:

$$\%conso = 100 \times [S / (1+\mu)] \qquad (6)$$

The %conso in the presence of lettuce was calculated with SL instead of S in equation (6). Ingestion of cyanobacteria by snails was estimated for each group and expressed in several ways: as the average total number of cells ingested per snail and per renewal of suspension (twice a week), as the average number of cells ingested per snail and per ml of available suspension twice a week, and as the average total number of cells ingested per dry weight of snail and per renewal of suspension, in %conso per day or in average %conso per 3 days.

2.3.b. Ingestion of microcystins: HPLC analysis

Total microcystin content ingested per snail per week was estimated from the cyanobacterial cell toxin concentration and the average number of cyanobacterial cells ingested. Toxin concentration in cyanobacterial cells was determined each week with a HPLC with diode array detection (HPLC-DAD) and a variable-wavelength UV detector operating at 238 nm. Prior to HPLC analysis, cells harvested by filtration (nylon cloth, 2 μm pore size)

were suspended in 0.5 mL of 85% methanol in water and centrifuged at 7000 G for 7 min. The volume injected was 20 µl with a flow rate of 1 mL min^{-1}. The separation was performed on a microspher C18 reverse-phase column (3 µm) under isocratic conditions with a mobile phase of 10 mm ammonium acetate and acetonitrile (7.4:2.6) for 10 min. Concentration was expressed as microgram cellular MC-LReq per litre of suspension. Microcystin contents estimated were combined with cell counts to derive a relation between algal density and microcystin production to obtain the amount of toxins produced by one cell of *P. agardhii*.

2.4. Quantitative analysis of MC-LReq in exposed snail tissues and quality control of microcystin measurement

Every week, 2 snails were randomly chosen from each group and were starved for ca. 24h to empty their gut contents (Carriker, 1946) to ensure that the microcystin measurement reflected only assimilated toxins and did not include microcystins in the undigested filaments of *P. agardhii* or microcystins in the gut. Snails were removed from their shells, freeze-dried and weighed prior to microcystin analysis. This analysis was performed by immuno-assay with an ELISA Microcystin Plate Kit (Envirologix INC), which detects all of the 6 purified hepatotoxins of common bloom-forming cyanobacteria, especially MC-LR and MC-RR, from 0.05 µg L^{-1} threshold and to the nearest 0.01 µg L^{-1} (Codd et al., 1997, Gilroy et al., 2000). All microcystins of the *P. agardhii* strain used were thus detected and expressed in MC-LReq using MC-LR, given by the supplier as standard. Microcystins were extracted with 2 mL of 100% methanol (Codd et al., 1997). Each snail was crushed in 1 mL of 100% MeOH and then crushed again after 12h at 4°C with 1 mL MeOH added. For the immuno-assay analysis the extract was diluted with water to less than 5% MeOH (Beattie et al, 1998). At the end of both the intoxication and detoxification periods, microcystin analysis was performed separately on the cephalopedial zone and the digestive-genital gland complex. The aim was to determine the relative importance of visceral mass and foot muscle tissue in the accumulation. Microcystin contents in snail tissues are expressed in µg g FW^{-1} (fresh weight) and in µg g DW^{-1} (dry weight) according to the relation previously established: FW = 7.47 * DW (n = 30, R Pearson = 0.94, $\underline{P} < 0.05$). The values were calculated by taking into account extraction recovery and possible matrix-induced signal enhancement or suppression with the ELISA test because of unspecific binding to and/or denaturing of the antibodies. Control snails, free of microcystins, were freeze-dried and homogenized in a mortar, spiked with MC-LR standard (5 µg g^{-1})

(Dionisio Pires et al., 2004). The extraction was performed as described previously and the recovery for the extraction was calculated. The matrix effect (i. e. effect of snail tissue) was checked by spiking control snails with MC-LR standard (5 µg g^{-1}) and the response was compared to 100% methanol spiked with the same amount (Dionisio Pires et al., 2004). The average recovery was 72 ± 5.3% and matrix effect was negligible (from 0.05 to 4.8% of differences between matrix and methanol results with an average of 1.7 ± 0.4%). Similar results about the matrix effect with the ELISA test were observed by Ernst et al. (2005). The percentage of bioaccumulation (%acc) was calculated each week based on the ratio between the average quantity of MC-LReq accumulated in snail tissues between weeks 1 and n (in µg) and the estimated average quantity of MC-LReq ingested between weeks 1 and n (in µg). During the detoxification period, the percentage of toxin elimination from snail tissues (%detox) was calculated between weeks by the expression:

$$\% \text{ détox} = 100 \times [\text{MC-LReq}_{n} - \text{MC-LReq}_{n+1}] / \text{MC-LReq}_{n} \quad (7)$$

2.5. Statistical analysis

Pearson's coefficient (R Pearson) was calculated to assess the existence of a correlation between: (1) the quantity of microcystins produced and the density of cyanobacterial suspensions (n = 90), (2) the fresh and dry weights of snails (n = 30). One analysis of covariance (ANCOVA) was performed on the cyanobacteria counts in the controls to compare their growth rates (µ) between weeks and volumes of culture medium (n=120). Two-way analyses of variance (ANOVA) with repeated measures and the Tukey-HSD multiple comparison test were performed to compare: (1) the total number of cyanobacterial cells and the number of cyanobacterial cells per mL ingested per snail, (2) the average %conso of snails over a 3 days period, (3) the total number of cyanobacterial cells ingested per dry weight of snails, (4) the quantity of microcystins bioaccumulated in µg g DW^{-1}, (5) the %acc, (6) the %detox. Differences were considered to be statistically significant at $P < 0.05$. Data are reported as means ± standard errors (± S.E.).

3. Results

3.1. Ingestion of *P. agardhii* cells and microcystins

The daily growth rate μ of *P. agardhii* was similar in the 15 or 40 mL containers and between weeks in the controls (cyanobacteria alone) (ANCOVA, n = 120, $P = 0.53$), and was 0.19 ± 0.02 d^{-1} (Fig. 1). When lettuce was present, cell density of *P. agardhii* was lower (Fig. 1), showing a loss of cyanobacteria to the introduced microorganisms which almost compensated for the growth rate. When snails were present, with or without lettuce, cyanobacteria density was lower than in their absence (Fig. 1), demonstrating the consumption of *P. agardhii* by *L. stagnalis*.

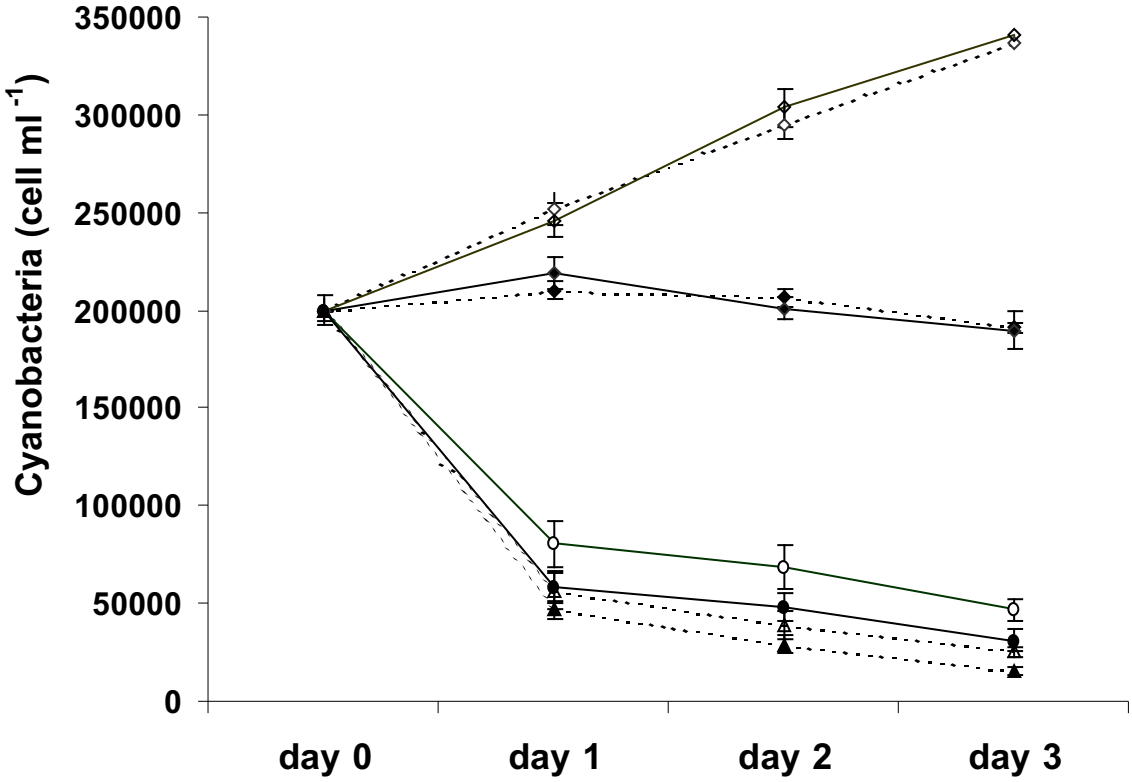

Figure 1. Dynamics of *P. agardhii* populations during 3 days: alone (open diamond) and with lettuce (closed diamond), in the presence of snails as juveniles (triangle) and adults (circle) without (open) and with lettuce (closed) (dotted line for 15 mL and continuous line for 40 mL glass containers).

In the 40 mL algal suspension, adults consumed 2.5 times more cells than juveniles in a 15 mL algal suspension over the 3 days, with and without lettuce (ANOVA $F_{3,20} = 434.3$, $P < 0.05$ and Tukey HSD, $P < 0.05$) (Table 1). When expressed in cell number per mL,

consumption was similar in adults and juveniles (Tukey HSD, $P > 0.05$) but different according to the diet (ANOVA $F_{3,20} = 23.5$, $P < 0.05$). Snails with lettuce consumed less cells per ml than snails without lettuce, regardless of age (Tukey HSD, $P < 0.05$) (Table 1). This difference reflects the diminution of the availability of cyanobacteria due to the presence of lettuce.

Table 1. Mean (± S.E.) ingestion of *P. agardhii* cells by *L. stagnalis* juveniles and adults over 3 days during the 5-weeks intoxication period in the presence or absence of lettuce.

	cells per snail	cells per snail per mL
juv cyano (juveniles fed on cyanobacteria)	3 376 000 ± 18 000	225 000 ± 1 200
juv cyano+let (juveniles fed on cyanobacteria and lettuce)	2 882 000 ± 124 000	192 000 ± 8 000
ad cyano (adults fed on cyanobacteria)	8 428 000 ± 160 000	211 000 ± 4 000
ad cyano+let (adults fed on cyanobacteria and lettuce)	7 214 000 ± 358 000	180 000 ± 9 000

The mean %conso over 3 days, which takes account of the decline in available cyanobacterial cells for snails when microorganisms were present, was not significantly different between groups and weeks (ANOVA, respectively $F_{3,20} = 2.3$ and $F_{4,20} = 1.3$, all $P > 0.05$). In each age class, it was similar in the presence or not of lettuce (Tukey HSD, all $P > 0.05$), suggesting an absence of non-toxic food selection in juveniles and adults. In addition, the daily %conso changed over the 3 days during the intoxication period (ANOVA, $F_{2,108} = 91.4$, $P < 0.05$). It was significantly higher the first day (Tukey HSD, $P < 0.05$), 62.8 ± 1.7% for all groups, whereas it did not significantly differ during the remaining 2 days (Tukey HSD, $P > 0.05$), mean of 33.1 ± 1.8%.

When normalised against dry weight of snails, total number of cells ingested differed according to age (ANOVA, $F_{3,20} = 97.6$, $P < 0.05$). Adults ingested in total 1.4 times more cells per g of dry weight than juveniles (Tukey HSD, $P < 0.05$). Such differences are relevant for comparisons of the differences in bioaccumulation of toxins per g of weight.

The total quantity of microcystins ingested was estimated from the production of MC-LReq by one *P. agardhii* cell (3.10^{-8} µg, n = 45, R Pearson = 0.9, $P < 0.05$). It was about 2.5 times higher for adults than for juveniles, in relation with the total number of cells ingested.

At the end of the 5 weeks-intoxication period, the total content of microcystins consumed was estimated for adults at 2.2 ± 0.0 µg ind^{-1} without lettuce and 1.9 ± 0.1 µg ind^{-1} with lettuce, and for juveniles at 0.9 ± 0.0 µg ind^{-1} without lettuce and 0.7 ± 0.0 µg ind^{-1} with lettuce.

3.2. Bioaccumulation and detoxification

Microcystin contents in snails increased steadily during the intoxication and reached a maximum of 80.4 ± 4.9 µg g DW^{-1} (10.8 ± 0.7 µg g FW^{-1}) after 5 weeks in the case of juveniles without lettuce (Fig. 2).

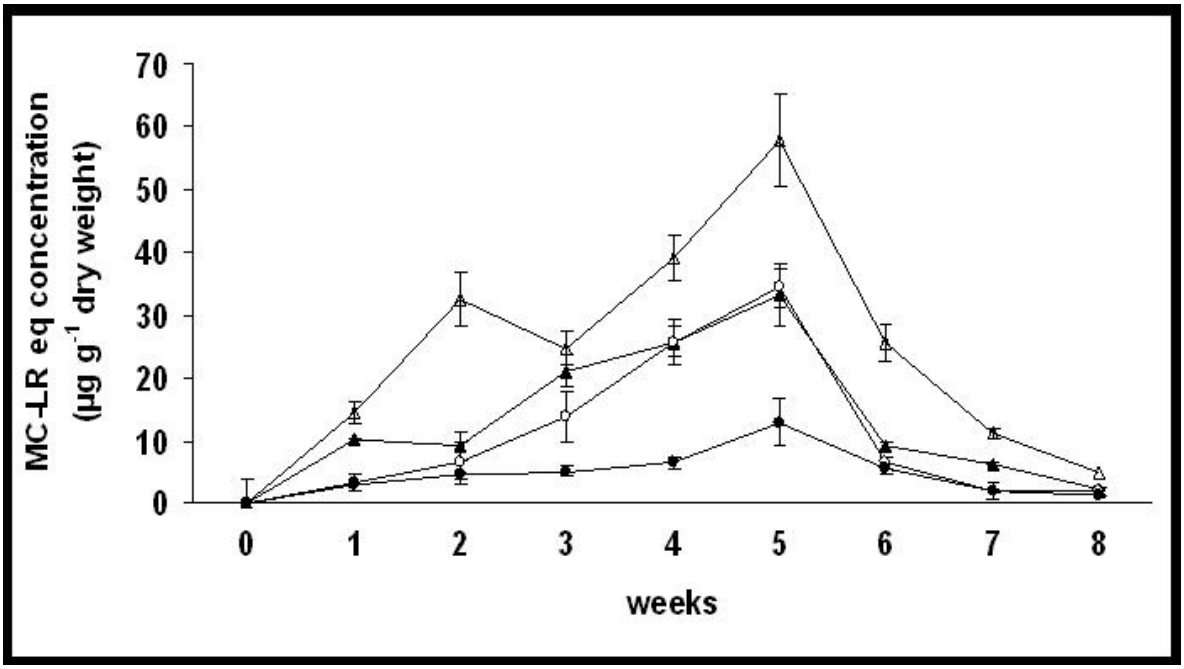

Figure 2. Kinetics of MC-LReq in *L. stagnalis* tissues (µg g DW^{-1}) of juveniles (triangle) and adults (circle) fed on cyanobacteria without (open) and with lettuce (closed) during 5 weeks of intoxication, then fed on lettuce during 3 weeks of detoxification.

Differences in MC-LReq contents per g were significant between groups (ANOVA, $F_{3,20}$ = 24.8, $P < 0.05$). Juveniles accumulated 2 times more toxins per g than adults (Tukey HSD, $P < 0.05$), in spite of having consumed 1.4 times less cells per g of dry weight (and 2.5 times less cells in total). Moreover, individuals fed on cyanobacteria alone accumulated more toxins per weight than those fed on the mixed diet (Tukey HSD, $P < 0.05$). The %acc, which takes into account differences in cell consumptions, varied between groups (ANOVA, $F_{3,20}$ = 7.1, $P < 0.05$) and showed similar trends. In general, juveniles accumulated greater

proportions of ingested microcystins than adults, and for both ages, snails fed on *P. agardhii* alone had a greater %accu than snails fed on mixed diet (Fig. 3). However, only the differences between adults fed on *P. agardhii* alone and the other groups were significant (Tukey HSD, $P < 0.05$). An average of 61% of total ingested toxins was accumulated by all gastropods after the intoxication.

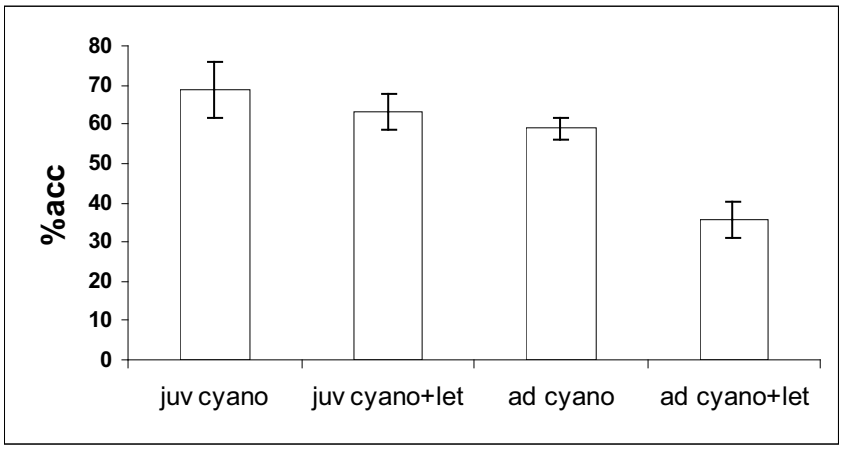

Figure 3. Mean (± S.E.) percentage of ingested MC-LReq accumulated in *L. stagnalis* tissues (% acc) of juveniles (juv) and adults (ad) fed on cyanobacteria without (cyano) and with lettuce (cyano+let) during 5 weeks of intoxication.

In addition, more than 95% of accumulated toxins were located in digestive-genital gland complex (less than 5% in cephalopedial zone) (Table 2).

Table 2. Mean (± S.E.) concentration of MC-LReq (µg g DW^{-1}) in *L. stagnalis* digestive-genital gland complex at the end of 5-weeks intoxication and 3-weeks detoxification periods according to the age and the presence of lettuce (see abbreviations in table 1).

	juv cyano	juv cyano+let	ad cyano	ad cyano+let
End of intoxication	194.8 ± 13.5	170.5 ± 12.9	135.8 ± 1.4	35.8 ± 4.0
End of detoxification	22.1 ± 2.1	20.4 ± 4.9	9.4 ± 0.9	4.6 ± 0.5

During the 3 weeks-detoxification period, mean microcystin contents declined in all groups from 48.0 ± 7.9 µg g DW^{-1} to 3.5 ± 0.9 µg g DW^{-} (or from 6.4 ± 0.9 µg g FW^{-1} to 0.4 ± 0.1 µg g FW^{-1}). Adult snails fed on *P. agardhii* alone, which had accumulated the highest

total quantity of toxins at week 5 (in the whole body and not per g of weight), eliminated every week a greater proportion of the toxin contents in their tissues than other groups (ANOVA, $F_{3,156} = 11.5$, $P < 0.05$ and Tukey HSD, $P < 0.05$), which showed similar percentages of detoxification (Tukey HSD, $P > 0.05$) (Table 3). Moreover, the percentage of detoxification was significantly higher the first week: 63.6 ± 2.1% for all groups (ANOVA, $F_{2,156} = 19.7$, $P < 0.05$ and Tukey HSD, $P < 0.05$), whereas elimination of toxins did not significantly differ during the last two weeks, respectively 54.5 ± 3.0% and 50.2 ± 2.9% (Tukey HSD, $P > 0.05$). At the end of the experiments, snails had eliminated on average 91.9 ± 0.5% of the accumulated toxins.

Table 3. Mean (± S.E.) percentage of MC-LReq eliminated every week in *L. stagnalis* tissues (% detox) during the 3-weeks detoxification period, according to the age and the presence of lettuce (see abbreviations in table 1).

Weeks	juv cyano	juv cyano+let	ad cyano	ad cyano+let
6	42.1 ± 2.4	76.9 ± 1.1	81. 0± 0.9	54.4 ± 3.9
7	55.9 ± 2.3	31.0 ± 2.4	67.8 ± 5.6	63.3 ± 1.9
8	55.2 ± 2.1	63.9 ± 0.9	43.7 ± 5.0	37.9 ± 3.6

4. Discussion

The results show that *L. stagnalis* ingested hepatotoxic strains of *P. agardhii*, even in the presence of another non-toxic food source (lettuce). Therefore no food selection by *L. stagnalis* occurred in contrast to some copepod and fish species which have been shown to select and ingest only the non-toxic food in laboratory experiments (for review: Zurawell et al., 2005). On average 63% of cyanobacterial cells were consumed during the first 24 hours and 33% during the remaining 2 days. We observed that cyanobacterial filaments sank quickly to the bottom of containers after the renewals of suspension, forming a dense layer of food for snails. Sedimentation was probably due to the absence a buoyancy control necessary to access light since the light climate was homogenous in glass containers. Search for food by *L. stagnalis* is done by random movements (Bovbjerg, 1968) and the decrease of cyanobacteria consumption after 24h may be a consequence of the decrease in encounter rates between snail and cyanobacterial cells. The search for food is accomplished by waving the head from side to side, and the tentacles are sensitive to food contact. As cyanobacteria were consumed, their concentrations decreased and the probability of detection by the snail was reduced.

In the presence of lettuce, cyanobacteria were consumed by snails, but *P. agardhii* availability was also decreased due to microorganisms. Snails consumed the same proportion of available cells as when fed only on cyanobacteria, but ingested a smaller total number of cells. As found by Sheerboom and Geldof (1978), the amount of food ingested is always related to the amount available and *L. stagnalis* fed almost continuously day and night without reaching a satiety threshold. Comparison between adults and juveniles showed that they consumed the same number of cells per mL. The feeding behaviour of *L stagnalis* was similar irrespective of age and of alternative non-toxic food source, the determining factor was the availability of cyanobacteria in the medium.

Following consumption of toxic cyanobacteria, lymnaeid snails rapidly accumulated microcystins readily detectable at the end of the first week of intoxication period (a maximum of 619 ng of MC-LReq in total body) and increasing with time (a maximum of 3.2 µg in total body after 5 weeks). Juvenile snails fed on cyanobacteria had the greatest microcystin concentration per g at week 5: on average 80.4 ± 4.9 µg g DW^{-1} (Fig. 2). A similar bioaccumulation was observed in the gastropod *Sinotaia histrica* after consumption of a toxic *Microcystis* strain (Ozawa et al., 2003). In Canadian lakes, Zurawell et al. (1999) reported

similar or higher values of microcystin accumulation in three gastropod species (up to 140 µg g DW^{-1} for *L. stagnalis*, 130 µg g DW^{-1} for *Physa gyrina* and 40 µg g DW^{-1} for *Helisoma trivolis*). They considered that the concentration of MC-LReq in tissues was correlated with toxins found in phytoplankton (up to 1526 µg g DW^{-1} in hypereutrophic lakes), and not with dissolved toxins (up to 1.2 µg L^{-1}). In laboratory experiments, *L. stagnalis* exposed for 6 weeks to 33 µg L^{-1} dissolved MC-LR accumulated only a maximum of 0.06 µg g DW^{-1} (Gérard et al., 2005). For comparison, *L. stagnalis* in this study, exposed to 5 µg L^{-1} of intracellular MC-LReq, accumulated almost 1300 times more toxins after 5 weeks. It appears from these results that gastropods accumulated microcystins mainly by grazing toxic phytoplankton, and to a lesser extent, via uptake of dissolved toxins.

Based on the percentage of accumulation, an average of 61% of total ingested toxins was accumulated by all gastropods after the intoxication. However, microcystin concentrations found in *L. stagnalis* were probably underestimated in the present experiment, due to the limitation in microcystin extraction from snail tissues. Indeed, an undetermined part of microcystins, i.e. those covalently bound to protein phosphatase, are not extractable by methanol and not detectable by the ELISA test. Thus, the microcystin concentrations we reported in tissue samples correspond to free and metabolised microcystins (i. e. conjugated with glutathione and cysteine, with which microcystin antibodies of the ELISA test crossreact by immunoaffinity (Metcalf, 2000)). The existence of non-extractable microcystins has been demonstrated in some bivalves by several authors (Williams et al., 1997; Dionisio Pires et al., 2004; Dietrich and Hoeger, 2005). Less than 0.1% of the total microcystins was extractable with methanol in saltwater mussels (Williams et al., 1997). In zebra mussels, covalently bound MC-LR was generally lower than free unbound MC-LR but could reach 62% of free MC-LR (Dionisio Pires et al., 2004). Further investigations are required to estimate the percentage of covalently bound and free microcystins in snail tissues.

The 39% of total ingested microcystins that were not measured in *L. stagnalis* are thought to have been partly: (i) eliminated in the gizzard and cecal string fraction of the faeces (undigested cells) during the first hours post ingestion. According to Zurawell et al. (2006), 57% of the initial microcystin concentration is found in this faeces fraction of *L. stagnalis* within 8h after removal from microcystin-containing cyanobacteria exposure. Other possible mechanisms by which ingested microcystins escaped measurement include: (ii) entering the digestive gland, where intracellular digestion of cyanobacteria occurred, followed by excretion in the digestive gland fraction of the faeces (Carriker, 1946; Zurawell et al., 2006);

(iii) entering in the digestive gland and accumulating through binding covalently to protein phosphatase enzymes and thus being undetectable (Dietrich and Hoeger, 2005).

The major accumulation site of microcystins in both experimentally exposed and wild invertebrates and vertebrates is the digestive gland (or liver) (Vasconcelos, 1995; Cazenave et al., 2005). According to Chen et al. (2005), the mean distribution of microcystins in the gastropod *Bellamya aeruginosa* is as follows: 64.5% in the digestive gland (4.4 µg g DW^{-1} MC-LReq), 24.8% in the digestive tractus (1.7 µg g DW^{-1} MC-LReq), 10.6% in the genital gland (0.7 µg g DW^{-1} MC-LReq) and 0.2% in the foot (0.05µg g DW^{-1}). In this study, more than 95% of accumulated MC-LReq are detected in the digestive-genital gland complex of *L. stagnalis*, with an average concentration of 135 µg g DW^{-1} at the end of intoxication period.

Bioaccumulation capacity showed two trends according to age and diet: (1) juveniles accumulated a greater proportion of ingested toxins than adults (66 vs 47%), (2) lymnaeids which received only cyanobacteria had a greater accumulation than those fed on cyanobacteria with lettuce (64 vs 50%). Moreover, despite a larger intake of toxic food per body weight, adult snails had less amounts of toxins per g, indicating they were more efficient in detoxifying and/or excreting the toxins than juveniles. Detoxification processes have been shown to occur in various organisms (e.g., plants, invertebrates and vertebrates) and allow organisms to survive under cyanobacterial stress (for review Cazenave et al., 2006). Accumulated microcystins can be metabolized into less harmful compounds after conjugation with gluthatione via glutathione-S-transferase or glutathione-peroxidase, resulting in microcystin excretion or physiological degradation. Previous studies, reported in Cazenave et al. (2006), have demonstrated that toxic cyanobacteria induce the production of reactive oxygen species (ROS) in relation with the immunological system. This oxidative stress is known to be reduced by the activity of antioxidant enzymes, such as glutathione-peroxidase. The higher accumulation of cyanotoxins in juvenile (versus adult) *L. stagnalis* may be due to the less well developed, and therefore less competent, immune system (Dikkeboom et al., 1985), with a consequently less efficient detoxification system. Moreover, the resource allocated to detoxification processes, which has a high energy cost, should be derived from a common pool of limited resources used by all fitness-associated traits (Rigby and Jokela, 2000). Thus snails which had received lettuce with cyanobacteria had a lower accumulation probably due to the enhanced energy uptake allowing a more efficient detoxification. The consequences of toxic cyanobacteria consumption in terms of impact on life traits and energy

allocation according to the age and the food diet in *L. stagnalis* will be reported in a separate publication (in preparation).

As a consequence of detoxification processes in organisms exposed to toxic cyanobacteria, the potential contamination of the food web may be thought to be limited. At present, few toxicological studies (Lauren-Määttä et al., 1995; Engström-Öst et al., 2002; Ibelings et al., 2005) have investigated the transfer of microcystins in aquatic food web, and zooplankton and bivalves have mainly been considered as toxin vectors, not freshwater gastropods. Ibelings et al. (2005) showed a transfer of microcystins without biomagnification from zooplankton and zebra mussel to fish in a lake in the Netherlands. In the laboratory, accumulation of hepatotoxic nodularin was demonstrated in shrimps and three-spined sticklebacks fed on cyanobacteria-fed copepods (Engström-Öst et al., 2002). In this study, due to the elimination of 64% of the MC-LReq content in *L. stagnalis* tissues during the first week free of cyanobacteria, and of 92% after 3 weeks, the risk of toxin transfer to gastropod predators in the field is probably small outside bloom events. However, this risk remains as microcystins were still detectable in snail tissues after 3 weeks of detoxification (on average, 3.5 ± 0.9 µg g DW^{-1}). A similar detoxification efficiency is also demonstrated by Zurawell et al. (2006): the cumulative microcystin loss from *L. stagnalis* was 95% at 22°C (80% at 10°C) after 6 days following the removal from toxic cyanobacteria exposure, and 99,5% at 22°C (97,5% at 10°C) after 30 days.

However, as gastropods are generally common and abundant in fresh waters (Habdija et al., 1995) and are consumed by various invertebrate and vertebrate predators (for review: Michelson, 1957), the accumulation of microcystins in their body could lead to a significant contamination of aquatic and terrestrial food webs. Moreover, the risk of toxin transfer to higher trophic levels is probably much higher in natural conditions for two reasons. Firstly, the accumulation reported in this experiment is underestimated due to the absence of detection of covalently bound microcystins in snails. Secondly, the concentration of cyanobacterial suspensions in this study was 200 000 cell mL^{-1}, which is similar if not less than those regularly observed in lakes, particularly in eutrophic waters (Chorus and Bartram, 1999). According to Brient et al. (2004), 70% of monitored lakes in Brittany (France) reach a cyanobacterial density between 100 000 and 5 millions cell mL^{-1} during the summer period. *P. agardhii* is a common microcystin producer in the Northern hemisphere (Scheffer et al., 1997) and may have an extended proliferation period, from April to October (Chorus and Bartram, 1999), or even persist perennially for many years by maintaining minimal density

during winter (Briant et al., 2002). Hence, when cyanobacteria dominate the phytoplankton community for an extended period in eutrophic lakes, the quantity of toxic cells ingested by gastropods is probably far higher than in this experiment, resulting in a more important toxin accumulation in the field as suggested by Zurawell et al. (1999), especially in bloom periods. Consequently, the increased accumulation may delay the detoxification period and lengthen its duration.

To evaluate the risk of cyanotoxin transfer in the field, based on the age-dependant differences in microcystin accumulation by snails demonstrated here, we need to consider the life cycle of freshwater gastropods and the structure of their populations and communities during the blooms. Despite the high infraspecific interpopulation plasticity of freshwater gastropods and the numerous life cycle patterns described in temperate regions (Calow, 1978), pulmonates are generally annual, univoltine and semelpare (even if *L. stagnalis* is among the few pulmonate species able to live for two years), whereas prosobranches tend to be perennial and iteroparous. The main breeding season takes place in late spring or early summer, and coincides with the beginning of the cyanobacteria population maxima. Most gastropods are consequently exposed to cyanobacteria from birth, when they are the most vulnerable to the predation pressure which focuses on neonates and juveniles, whereas the mortality of adults is mostly related to the reproductive effort (Calow, 1978). Moreover, juveniles, that accumulate more toxins per weight than adults, are probably more susceptible to the toxicity of cyanobacteria, with consequences for their life traits. Results on the impact of toxic cyanobacteria ingestion by *L. stagnalis* on survival, growth, fecundity and locomotion will soon be published in a separate publication. To complete this experimental research program, long term investigations are required in the field to demonstrate the cyanotoxin vector role of freshwater gastropods and the transfer patterns through the food web.

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2.2. Interactions between cyanobacteria and gastropods. II. Impact of toxic *Planktothrix agardhii* on the life-history traits of *Lymnaea stagnalis* [2]

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Abstract:

Hepatotoxins are frequently produced by many cyanobacterial species. Microcystins (MCs) are the most frequent and widely studied hepatotoxins, with potentially hazardous repercussions on aquatic organisms. As a ubiquitous herbivore living in eutrophic freshwaters, the snail *Lymnaea stagnalis* (Gastropoda: Pulmonata) is particularly exposed to cyanobacteria. The toxic filamentous *Planktothrix agardhii* is common in temperate lakes and is therefore, a potential food resource for gastropods. In the first part of this study, we demonstrated the ingestion of toxic *P. agardhii* by *L. stagnalis* during a 5 weeks exposure, with concomitant accumulation of, on average, 60% of total MCs ingested. After 3 weeks of non-toxic food (lettuce), approximately 90% of MCs were eliminated from tissues. Here, we investigate the impact of toxic *P. agardhii* consumption on the life-history traits (survival, growth and fecundity), locomotion and the structure of digestive and genital glands of juvenile and adult *L. stagnalis*. We observed a decrease of growth regardless of age, although this was more marked in juveniles, and a reduction of fecundity in adults. Survival and locomotion were not affected. Reduction of growth and fecundity continued to be observed even after feeding of non-toxic food for 3 weeks. The structure of the digestive gland was altered during the intoxication period but not irreversibly as cells tended to recover a normal status after the 3 weeks detoxification period. No histopathological changes occurred in the genital gland and oocytes, and spermatozoids were present in the gonadic acini. The density of cyanobacterial suspensions used in this study was comparable to those regularly observed in lakes, particularly in eutrophic waters. These results are discussed in terms of the negative impact of toxic cyanobacteria on natural communities of freshwater gastropods, and potential cascading effects on the equilibrium and functioning of the ecosystem.

1. Introduction

Contamination of freshwater bodies by toxic cyanobacteria is a subject of serious international concern since the toxins produced are known to cause a range of sublethal and lethal effects to organisms, and enter the food chain by accumulating in tissues (for review: Zurawell et al., 2005). Cyanobacteria produce many types of toxins, the most studied and widespread are the hepatotoxic microcystins (MCs) of which 80 variants have been identified (Dietrich and Hoeger, 2005). Although the organ specificity of MCs is primarily the liver, similar but less severe damage can occur within the gastrointestinal tract and kidney, and MCs are potential tumour promoters (for review: Zurawell et al., 2005) also. Contamination of organisms can occur by exposure to extracellular toxins (released after cell lysis into the surrounding water), intracellular toxins (direct ingestion of cyanobacteria) or toxins incorporated in organisms (consumption of contaminated prey). Exposure to toxic cyanobacterial cells has been shown to induce accumulation of MCs in freshwater organisms (for review: Zurawell et al., 2005).

Gastropods inhabit shallow littoral zones of temperate lakes and ponds and are important primary consumers (Dillon, 2000). The few studies involving gastropods focused on the bioaccumulation and detoxification (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005; Gkelis et al., 2006; Lance et al., 2006). Exploration of toxin impact on gastropod life traits has been limited so far (Gérard & Poullain, 2005; Gérard et al., 2005), although it is necessary to evaluating the impact of toxic cyanobacterial blooms on freshwater populations and communities at the scale of the individuals. Ingestion of toxic cyanobacteria by gastropods has been suggested by several authors following a positive relationship found between toxin concentration in phytoplankton and in snail tissues (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005). In the initial report of this research (Lance et al., 2006), the consumption and consequent MC accumulation by the pulmonate *Lymnaea stagnalis* fed on toxic strains of *Planktothrix agardhii* was demonstrated. Given that the pathogenic effects of dissolved MC-LR (one variant of MCs) on life-history traits have been demonstrated for *L. stagnalis* via a decrease in fecundity (Gérard et al., 2005), and for the prosobranch *Potamopyrgus antipodarum* via decreases in survival, growth and fecundity (Gérard & Poullain, 2005), it is relevant to ask whether toxic *P. agardhii* ingestion would have a negative impact on the life-history traits of *L. stagnalis.*

L. stagnalis is a characteristic species of eutrophic aquatic systems (Clarke, 1979), which are more prone to cyanobacterial blooms. This gastropod has been used as an indicator

species of various pollutants such as heavy metals and pesticides (reviewed in Gomot, 1997). *P. agardhii* is the most common cyanobacterium in eutrophic lakes of temperate areas (Scheffer et al., 1997; Brient et al., 2004), with a greater cellular toxin production than other cyanobacterial species (Christiansen et al., 2003). Here, in the second part of the study, we investigate the effect of the consumption of *P. agardhii* on the life-history traits (survival, growth and fecundity), locomotion and structure of the digestive and genital glands of *L. stagnalis*. Experiments were conducted during a 5 weeks intoxication period when snails consumed toxic cyanobacteria with or without lettuce, followed by 3 weeks of detoxification when snails consumed only lettuce.

Several studies on gastropods have shown that survival, growth and fecundity were predominantly affected by various abiotic and biotic stresses (e.g. natural or anthropogenic pollution, parasitism) (Abd Allah et al., 1997; Gérard & Théron, 1997; Bacchetta et al., 2002; Duft et al., 2003; Gérard & Poullain, 2005). Therefore, we hypothesize that both accumulation and detoxification of MCs by *L. stagnalis* will affect its life-history traits and physiology. In natural systems, chronic exposures of populations, such as those occurring under repeated and prolonged proliferation of toxic cyanobacteria, which covers the breeding seasons of gastropods (Calow, 1978), may interfere with the reproductive process and the development of juveniles, and consequently may have a demographic impact. As crawling of aquatic gastropods constitutes the most costly form of locomotion in the animal kingdom, due to the need for mucus secretion (Denny, 1980, 1984) which is highly energy demanding in *L. stagnalis* according to Calow (1974) locomotory activity was measured. We hypothesise that a decrease in locomotory activity may allow a release of energy which can be used for the detoxification processes. Finally, the structure of both digestive and genital glands of *L. stagnalis* was studied to highlight a possible irreversible degradation usually caused by MCs after entrance into cells (for review: Zurawell et al., 2005).

Results are discussed in terms of the direct poisoning risk toxic cyanobacteria pose for gastropods in the field in relation to the age structure of populations, with potential repercussions on the whole ecosystem (e.g. microalgal assemblages, food web).

2. Material and methods

2.1. Biological material

Adult and juvenile *L. stagnalis* from an established laboratory population, producing 8000 individuals per year, were used in this study. The *L. stagnalis* massive stock has been maintained in the Experimental Unit of the Institut National de Recherche en Agronomie (INRA, Rennes, France) under laboratory conditions (14/10 L/D, 20 ± 1°C) for 8 years, with 100 individuals per 30L aquarium. Prior to the experiments, juveniles and adults *L. stagnalis* (14 ± 1 and 25 ± 1 mm shell length respectively) were acclimatized at constant temperature (20 ± 1°C) and photoperiod (14/10 L/D) in dechlorinated tap water and fed on dried, pesticide-free, lettuce *ad libitum* during 7 days. The filamentous cyanobacterium *P. agardhii*, originating from the recreational watersport site at Viry (Essone, France), was maintained in a modified medium (20 mL of liquid BG11 per litre of dechlorinated water). Cyanobacteria were placed in an incubation room at constant temperature (25 ± 2°C) and photoperiod (12-h/12-h, light/dark cycle) at an irradiance of 40 µE m^{-2} s^{-1}. The algae at densities of 200 000 cells mL^{-1} were provided twice a week to the gastropods. These algal suspensions produced toxic dmMC-LR and MC-RR (detected by HPLC-MS), with a total concentration of 5 µg L^{-1} expressed as MC-LR equivalents (MC-LReq), corresponding to a concentration of 280 µg g^{-1} dry weight of *P. agardhii*, and measured by high-performance liquid chromatography (Lance et al., 2006). Cyanobacterial densities were determined using a microscope with a Nageotte chamber. The total length of all *P. agardhii* filaments in a 50 µL volume was measured and expressed in cells mL^{-1} assuming an average length per cell of 3.12 µm, based on 50 measurements.

2.2. Exposure of snails to food items including cyanobacteria

Following the period of acclimatization, snails were divided, for a 5 weeks intoxication experiment, into four groups according to diet: (1) snails fed on lettuce *ad libitum* (let) or (2) starved (starv), (3) snails exposed to a density of toxic cyanobacteria of 200 000 cells mL^{-1} (cyano) and (4) snails exposed to the same density of cyanobacteria and fed on lettuce *ad libitum* (cyano+let). Each group consisted of 40 replicates i.e. 40 isolated individuals (20 juveniles and 20 adults), in glass containers of 15 mL for each juvenile and 40

mL for each adult. Preliminary observations showed that gastropods consumed the *P. agardhii* suspension of 200 000 cell mL^{-1} in 3 days, hence the cyanobacteria suspension was renewed twice a week. After the intoxication period, all gastropods were fed solely on lettuce *ad libitum* and maintained in dechlorinated non-toxic water during a 3 weeks detoxification period.

2.3. Life-history trait and locomotion measurements

Shell sizes were assessed each week by measuring the maximum shell length to the nearest 0.1 mm. Weekly growth rates refer to the percentage of growth per week, calculated from the ratio between the increase (in mm) in shell size (S) between weeks n and n+1 and the shell size at week n, by the expression:

$$\text{Growth rate} = 100 \times [S_{n+1} - S_n] / S_n \quad (1)$$

The reproduction of each snail was monitored at weekly intervals by recovering the egg masses laid per individual and counting the number of embryos they contained. After 5 and 8 weeks, locomotory activity was estimated from the distance travelled by the snails over a fixed period. Each snail was placed for 15 min in a container filled with 15 mL water. After this period and following the removal of the snails, 10 mg of carmine were added to each box to adhere to the mucus tracks (Calow, 1974). The length of the mucus trails produced, revealed as red bands, was then measured with the help of a digital curvimeter to the nearest 1 mm (distance moved, D, measured in centimetres per 15 min).

2.4. Histology of digestive and genital glands

Histological analyses were made of 10 specimens from each treatment on weeks 5 and 8, at the end of intoxication and detoxification periods respectively. Snail bodies were removed from their shells, then fixed in Bouin's fluid, cut into serial 6-µm-thick sections and stained with Heidenhain blue (Martoja and Martoja-Pierson, 1967). Longitudinal sections of the digestive and genital glands were observed using an optical microscope to detect morphological differences in the structure of digestive and gonadic lobules and connective tissue, attributable to the different diets of the snails.

2.5. Statistical analysis

Two-way analyses of variance (ANOVA) with repeated measures and the Tukey-HSD multiple comparison test were performed to compare the life history parameters (shell growth, number of eggs) between diets. Analyses of covariance (ANCOVA) were performed on the data of locomotory activity. Differences were considered to be statistically significant at $P < 0.05$. Data are reported as means ± standard errors (± S.E.).

3. Results

3.1. Effects on the life-history traits: survival, growth and fecundity

Survival : no mortality occurred during the 8 weeks study in any of the treatments irrespective of age and diet.

Growth : Juvenile, and adult, weekly growth rates differed significantly between diets during both the intoxication and detoxification periods (Table 1).

Differences in shell size of juveniles fed different diets increased during the intoxication period (Fig. 1). There were no statistically significant differences between weekly growth rates of starved juveniles and juveniles exposed to cyanobacteria alone, but growth rates of both groups were significantly lower than those of juveniles fed on lettuce. Moreover, the weekly growth rates of juveniles fed on lettuce with cyanobacteria were significantly reduced compared to the controls fed only with lettuce. Differences in adult shell sizes between diets were less marked (Fig. 2). Starvation and ingestion of toxic cyanobacteria, either with or without lettuce, led to similar growth rates, that were significantly lower than those of the control snails (Table 1).

Table 1: Statistical comparisons (repeated-measure ANOVA with F and P values) for the weekly growth rate and the fecundity (egg masses and eggs) of *Lymnaea stagnalis* varying with diets during the intoxication and detoxification periods.

	Intoxication	Detoxification
Juveniles weekly growth rate	$F_{6,710} = 20.6$ *	$F_{6,422} = 9.8$ *
Adults weekly growth rate	$F_{6,710} = 15.8$ *	$F_{6,422} = 2.8$ *
Number of mass egg by adults	$F_{6,758} = 8.5$ *	$F_{6,406} = 5.8$ *
Number of eggs by adults	$F_{6,758} = 8.3$ *	$F_{6,406} = 11.8$*

* significant at $P < 0.05$

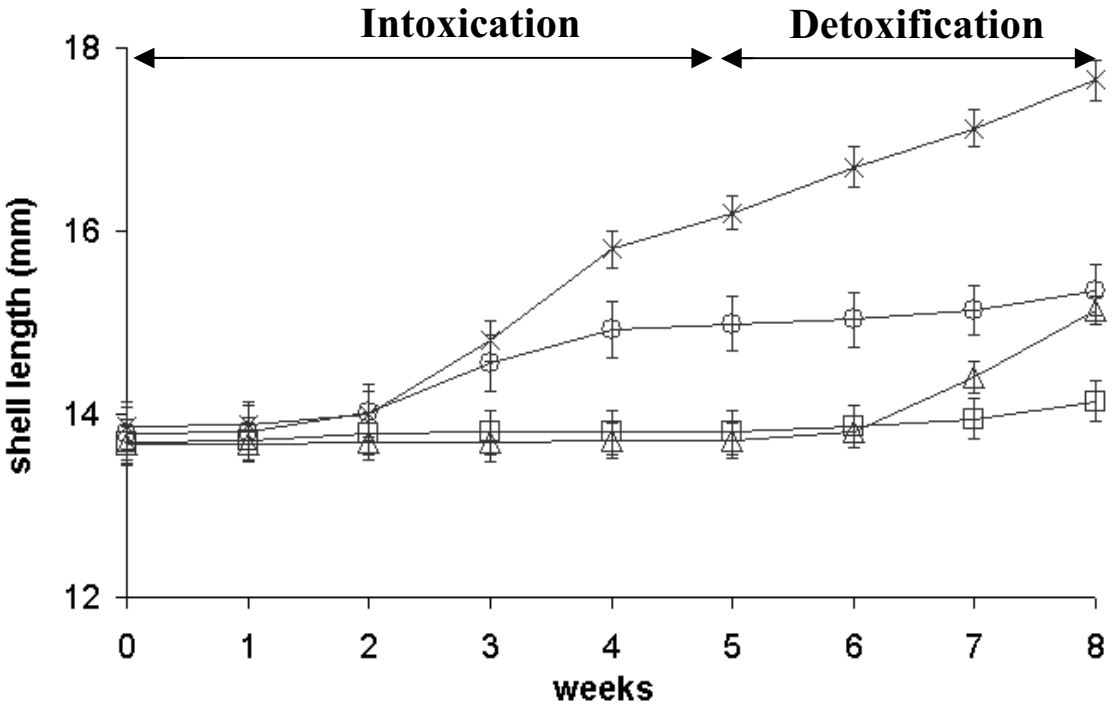

Figure 1. Growth rate of juvenile *Lymnaea stagnalis* (shell length in mm ± SE) fed on lettuce (cross), on cyanobacteria without (square) and with lettuce (circle), and starved (triangle) during a 5-week intoxication period, followed by a 3-week detoxification period.

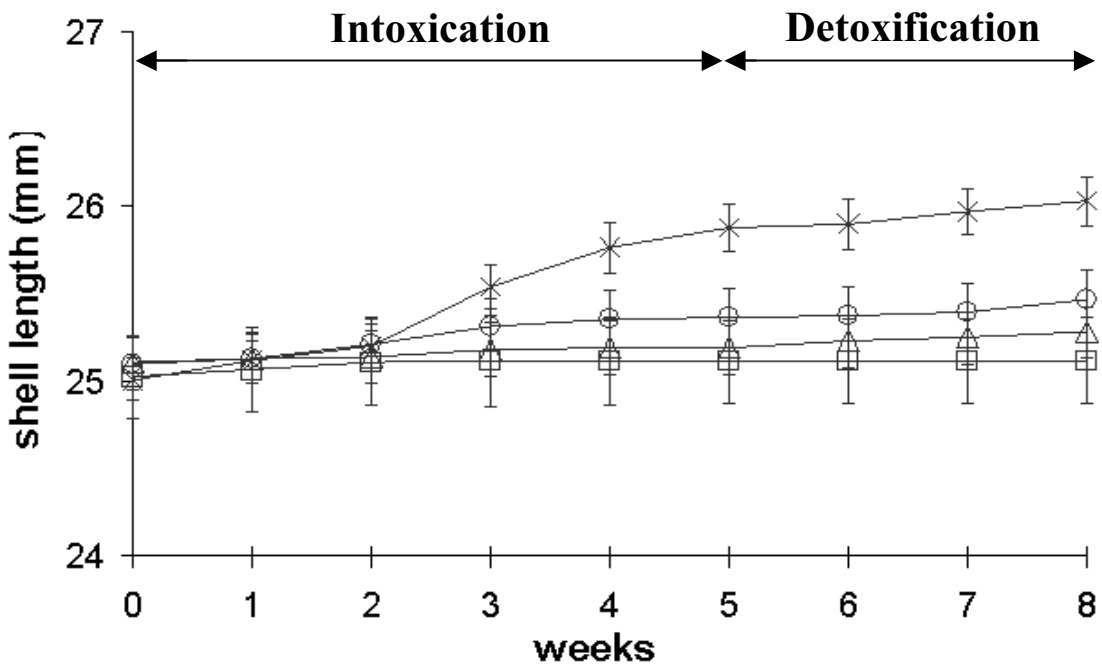

Figure 2. Growth rate of adult *Lymnaea stagnalis* (shell length in mm ± SE) fed on lettuce (cross), on cyanobacteria without (square) and with lettuce (circle), and starved (triangle) during a 5-week intoxication period, followed by a 3-week detoxification period.

During the detoxification period, the weekly growth rates of all juveniles previously exposed to toxic cyanobacteria, with or without lettuce, were significantly lower than those of juveniles never exposed, starved and controls. For adult snails, only those previously fed on cyanobacteria alone showed growth rates significantly lower than other treatments, for which there was no significant differences. The average weekly growth rates during each period are presented in Table 2.

Table 2: Average weekly growth rates of juvenile and adult *L. stagnalis* for each group during the intoxication and detoxification periods by toxic *P. agardhii* (see abbreviations in section 2.2).

	Period	Groups			
		let	starv	cyano	cyano+let
Adults	Intoxication	0.68 ± 0.08	0.06 ± 0.02	0.07 ± 0.02	0.21 ± 0.04
	Détoxification	0.19 ± 0.04	0.12 ± 0.03	0.00 ± 0.00	0.14 ± 0.04
Juveniles	Intoxication	3.28 ± 0.39	0.05 ± 0.02	0.17 ± 0.05	1.70 ± 0.24
	Détoxification	2.86 ± 0.29	3.42 ± 0.48	0.83 ± 0.19	0.82 ± 0.17

Fecundity: egg laying was not observed for juveniles that were not sexually mature. For adults, egg laying was drastically reduced in all groups during the first week of experiment (Fig. 3), probably due to the stress of isolation in individual glass containers. Fecundity varied significantly between diets during the entire experiment (Table 1), and control snails had significantly greater egg mass and egg numbers than other treatments (Fig. 3 and Fig. 4). From the second week of the intoxication period, a decrease in reproductive effort was observed for snails fed on cyanobacteria with lettuce, and a complete cessation for snails starved or fed on cyanobacteria alone (Fig. 3). During the detoxification period, 16% of snails previously starved started to lay again. Snails previously fed on cyanobacteria with lettuce started to lay again at a similar rate. Snails previously fed on cyanobacteria alone remained non-reproductive, except for 5% of snails (1 individual) that laid 1 egg mass (Fig. 3 and Fig. 4).

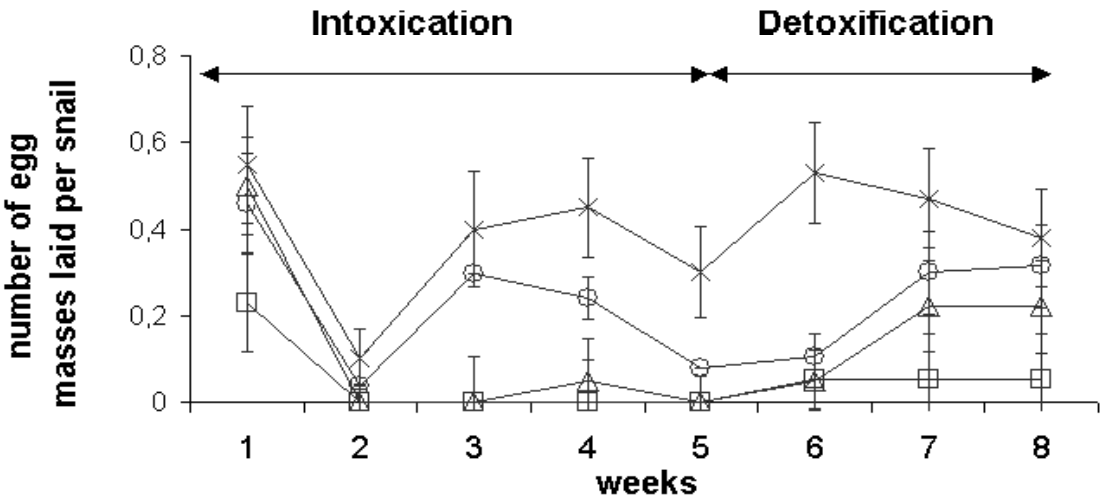

Figure 3. Egg mass laying (± SE) per *Lymnaea stagnalis* fed on lettuce (cross), on cyanobacteria without (square) and with lettuce (circle), and starved (triangle) during a 5-week intoxication period, followed by a 3-week detoxification period.

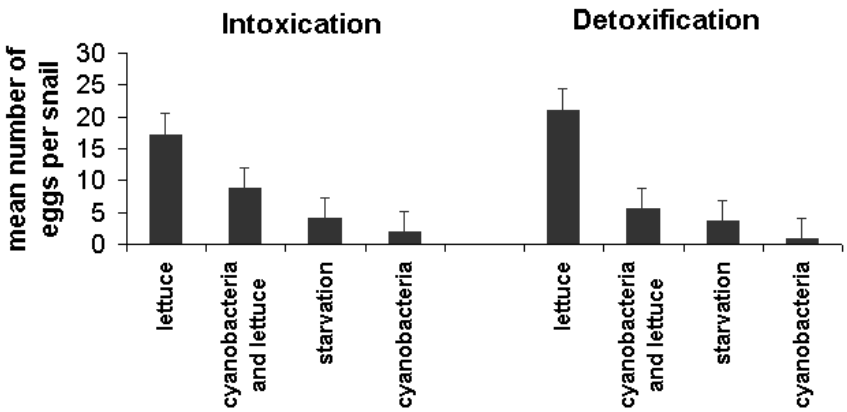

Figure 4. Mean number of eggs (± SE) laid per *Lymnaea stagnalis* during a 5-week intoxication period, followed by a 3-week detoxification period.

3.2. Effects on locomotion

There was a great variability between snails in the locomotory activity measured at the end of the intoxication and detoxification periods. Locomotion was not influenced by the diet (ANCOVA, $P > 0.05$), but was different according to age for both intoxication and detoxification periods with a distance covered by juveniles significantly longer than that of adults (ANCOVA, all $P < 0.05$).

3.3. Histology of the digestive and genital glands

The digestive gland of gastropods comprises three basic cell types: large digestive cells, small columnar basophilic cells and large pyramidal basophilic cells. The digestive cells with a basal nucleus and various apical vacuoles are the most abundant (Ünlü et al., 2005). At the end of the intoxication period, the structure of the digestive gland of stressed snails (starved or fed with cyanobacteria with, or without, lettuce) was different from that of control snails (fed on lettuce): with flattened epithelial cells compared to high and larger lumen of the digestive lobules, and greater vacuolisation of cells. After 3 weeks of detoxification, the digestive gland tended to recover to a structure similar to that of the controls. No change was observed in the structure of genital gland regardless of the diet (stressed or control snails) and the age (juveniles or adults). Oocytes and spermatozoids were always present in the gonadic acini in spite of the significant decreased fecundity in stressed adults.

4. Discussion

4.1. Impact on life-history traits

There is dearth information regarding the lethal effect of microcystins on gastropods, regardless of the uptake routes and of the origin of cyanotoxins (intracellular or dissolved). Despite the ingestion of toxic cyanobacteria, survival of *L. stagnalis* was unchanged during our experiments. No change in the survival of the lymneid *Radix auricularia* was found after ingestion of a complex of two neurotoxic cyanobacteria species: *Microcystis farlowiana* and *Pseudanaboena franqueti* (Gevrey et al., 1972) also. In the same way, no change occurred in the survival of *L. stagnalis* exposed during 6 weeks to 33 µg L^{-1} purified soluble MC-LR (Gérard et al., 2005). The locomotory activity of *L. stagnalis* remained unchanged irrespective of diet during our experiment, and the locomotion of juveniles was significantly greater than that of adults regardless of dietary group. Gérard et al. (2005) observed the same effect in *L. stagnalis* during a 6 weeks exposure to 33 µg L^{-1} purified soluble MC-LR. The egg laying behaviour of adults (immobile snails) may explain their lesser activity compared to juveniles (Gérard, 1996). In this study, during the stress imposed on *L. stagnalis* by toxic cyanobacteria or starvation, energy allocated to locomotion was maintained despite the highly calorific cost of crawling (Calow, 1974), possibly due to its vital role in searching for food (Bovbjerg, 1968).

In contrast, the consumption of toxic *P. agardhii* led to clearly demonstrated negative effects on the growth and the fecundity of *L. stagnalis*, with an age-specific effect characterized by a severe decrease of growth rate when snails were intoxicated as juveniles, and a reduction of reproductive effort when intoxicated as adults. These two life-history traits were inhibited during contamination and remained negatively affected during the depuration. Juveniles fed on *P. agardhii* showed a lower growth rate than controls, even for snails that had ingested lettuce with toxic cyanobacteria. Starvation induced the same effects but juvenile snails previously starved returned to a growth rate similar to the controls when they were fed once again. Thus toxic cyanobacteria consumption induced in juveniles a slowing down of the growth that persisted after the return to a non-toxic diet. In contrast, exposure to purified MC-LR (33µg L^{-1}) during 6 weeks did not induce a decrease of growth of juvenile *L. stagnalis* (Gérard et al., 2005) and resulted in MCs accumulation in tissues 1300 times less than in our study with an intracellular MC-LReq concentration of 5 µg L^{-1} (Lance et al., 2006). It appears

from these results that pulmonates accumulated MCs mainly by grazing toxic phytoplankton and the impacts on life-history traits are less severe when accumulation is lower.

In adult *L. stagnalis*, the ingestion of toxic *P. agardhii* led to a reduced growth but to a lesser extent compared to juveniles, and the main negative effect was a severe decrease of fecundity. Adults fed only on toxic cyanobacteria stopped egg laying completely, not only during the intoxication period but also during the 3 weeks of detoxification. A similar cessation of reproduction was also observed in starvation conditions, but with the great difference that starved snails returned to lay when they were fed once again. Moreover, fecundity of snails fed on lettuce with toxic cyanobacteria was significantly lower than the fecundity of the controls, including the detoxification period where the egg number was 4 times smaller. Decrease of fecundity is a classic response of adult *L. stagnalis* to various chemicals shown both during and after experimental exposure (Jumel et al., 2002; and Coutellec & Lagadic, 2006). Shifts in resource allocation may be engaged as anticipatory safety measures, even before absolute shortages arise rather than reflecting direct constraints. In general, abiotic and biotic stresses (e.g. natural or anthropogenic pollution, parasitism) are important factors known to affect growth and fecundity and to influence gastropod life histories, distribution and abundance (Streit & Peter, 1978; Gérard & Théron, 1997; Bacchetta et al., 2002; Lefcort et al., 2002; Duft et al., 2003; Gérard & Poullain, 2005). The age-specific response in growth and fecundity to the intoxication is basically an energy problem, as stated by Gérard & Théron (1997) for *Biomphalaria glabrata* infected by *Schistosoma mansoni*. Resource allocation patterns differ between individuals at different stages of their development, and the energy of juvenile gastropods is mainly allocated towards somatic production and differentiation, whereas the energy of adults is mainly channelled to egg production.

During the 5 weeks of toxic cyanobacteria consumption, gastropods accumulated 61% of MCs present in the ingested cells (Lance et al., 2006), and eliminated 92% of these toxins during the 3 weeks with non toxic diet. Accumulation and detoxification processes have been shown to occur in various organisms (e.g., plants, invertebrates and vertebrates) and to allow the organism to survive under cyanobacterial stress (for review: Cazenave et al., 2006). Resources allocated to the detoxification processes, which have a high energy cost, should be derived from a common pool of limited resource, inducing trade-offs that probably affect life-history traits and physiology. In their study of MC detoxification processes, Wiegand et al. (1999) interpreted the decrease of a life-trait as the result of an increased energy demand

required to eliminate toxins. Here, *L. stagnalis* fed on cyanobacteria with lettuce had a lower accumulation of MC than those which received only cyanobacteria (50 vs 64%) (Lance et al., 2006), associated with less severe effects on life-history traits. As an additional energy resource, lettuce would allow limited growth and reproduction with a concomitant detoxification.

4.2. Histological studies

Despite a decrease of fecundity in adult *L. stagnalis*, no visible pathology was detected in the genital gland, and spermatozoids and oocytes were present in the gonadic acini of both juvenile and adult snails. It suggests that organogenesis of the genital gland continued in stressed juveniles that begin to produce gametes before the sexual maturity, and that the decreased fecundity of stressed adults was not due to the absence of gametogenesis in the genital gland. According to Chen et al. (2005), the mean distribution of MCs in the prosobranch *Bellamya aeruginosa* was 64.5% in the digestive gland, 24.8% in the digestive tractus and 10.6% in the genital gland. These results suggest that the genital gland is a site of minor accumulation but could be impaired by toxins. Despite of the production of sperm and oocytes within the ovotestis, egg laying is functionally impossible when accessory sexual organs (e.g. albumen, nidamental and prostate glands, spermatheca) are not developed or when their growth is altered as demonstrated in *B. glabrata* infected by *S. mansoni* (Théron & Gérard, 1994). In this study, negative effects on fecundity in spite of the presence of gametes in the genital gland may be due to an impairment of accessory sexual organs and egg laying process via a direct action of MCs (hormonal perturbation for instance) and/or a reallocation of resource toward MCs detoxification.

The digestive gland of gastropods is the primary site of intracellular digestion, accumulation, detoxification and metabolism, and can be influenced by MCs (Zurawell, 2001). This work showed that histopathological alterations of the digestive gland of *L. stagnalis* occurred after a single oral injection of dissolved MC-LR into the oesophagus in a dose range of 0.0002 to 0.02 µg per snail. Bleb formations, vacuolisation, separation of the basal lamina from the cell, cell lysis, and massive necrosis at the highest dose, were observed. The cellular changes in the digestive gland were consistent with those observed in liver tissue of both mammals and fish during oral or intraperitoneal exposure to MCs (Zurawell, 2001). MCs are potent inhibitor of protein serine/threonine phosphatases in the PPP family (PP1,

PP2A, PPP4 and PPP5) (Hastie et al., 2005), which are essential for growth and maintenance of the cell structure, and can cause severe and irreversible degradation of hepatic tissues depending on the dose (for review: Zurawell et al., 2005). Surprisingly, pathologic effects on the digestive gland seem to be more severe with 0.02 µg of purified MC-LR orally injected (Zurawell, 2001), than in our experiment with 1.41 µg of MCs ingested with cyanobacterial cells (average total MC content of all ingested cells at week 5). Indeed, our histological analysis revealed only limited changes in the digestive gland of *L. stagnalis* due to the consumption of toxic *P. agardhii*, and that the digestive lobules tended to recover a healthy aspect during the detoxification period. This difference of effect may be due to the uptake routes and/or the type of MCs: impact of purified MC-LR on tissues may be more severe than that of MCs synthesized by *P. agardhii* (dmMC-LR and MC-RR). Alternatively, purified MC-LR may penetrate cells more easily than metabolised MCs resulting from the digestion of cyanobacteria. These aspects need further investigation in future experiments.

The changes we observed (e.g. thinning of digestive epithelium, vacuolisation of cells) were comparable with those in starved *L. stagnalis* or described for parasitized gastropods (Moore et al., 1973). As demonstrated in lymnaeids, physiological responses to many stresses induce a mobilization of glycogen, an indicator of energy reserve, and may result in a modified expression of life-history traits as a consequence of energy reallocation (Baturo et al., 1995; Jumel et al., 2002; Coutellec and Lagadic, 2006). The energy balance can thus be altered by the stressful effect of MCs, as shown by Juhel et al. (2006) for the zebra mussel exposed to toxic strains of *Microcystis aeruginosa*. These authors suggested that the depression in the energy balance was due to the production of pseudofaecal material rich in mucus ("pseudodiarrhoea"), which is energetically expensive to produce, and to the enzymatic degradation of MCs via Glutathione S-transferase during the detoxification process. In the present study, there is insufficient evidence to decide whether the MCs are responsible for the diminution of energy reserve and fecundity by direct interference in the energy balance (perturbation of the glycogen metabolism) and in the laying process, or operate by indirect effects on the reallocation of resources toward detoxification.

4.3. Implications of these results for natural gastropod communities

From our experiments and the reported pathogenic effects of soluble MC-LR exposure on gastropods (Gérard et al., 2005, Gérard & Poullain, 2005), one can envisage a serious

hazard for gastropod species living in eutrophic waters, subject to recurrent proliferations of toxic cyanobacteria. The absence of a mortality effect found in this study may be due to the low cyanobacterial density and low MCs production (200 000 cells mL^{-1} producing 5µg L^{-1} of MC-LReq) and the 5 weeks exposure duration. However: i) many monitored lakes in Brittany (France) reach *P. agardhii* densities between 100 000 and 2 millions cell mL^{-1} during the summer period (Brient et al., 2004); ii) MC-LReq concentrations often exceed 280 µg g^{-1} dry weight (Sivonen and Jones, 1999); iii) *P. agardhii*, the common MC producer in the Northern hemisphere (Scheffer et al., 1997), may have an extended proliferation period, from April to October, or even persist perennially for many years by maintaining minimal density during winter (Briant et al., 2002). In a similar way, *L. stagnalis* did not appear to be adversely affected by the presence of cyanobacteria containing MCs at a concentration of 72 µg g^{-1} dry weight (Zurawell et al., 2006).

Moreover, as we monitored the fecundity but did not consider the viability of embryos in the group laid after ingestion of cyanobacteria, the real impact of toxic cyanobacteria on the populations of gastropods was probably underestimated. Gomot (1997) showed that embryo development was the most sensitive period during an exposure of *L. stagnalis* to cadmium concentrations. The probability of survival of *L. stagnalis* in an environment polluted with toxic cyanobacteria is thus likely to be even less than predicted by our results. Gastropods are known to be potential bioindicators of freshwater pollution from various sources, and good models to assess the effects of pollutants, due to their sensitivity, low mobility and contact with polluted sediments (Elder & Collins, 1991; Gomot, 1997; Salanki, 2000; Downs et al., 2001; Lefcort et al., 2002). Thus, in natural systems, there are probably additive or synergistic effects due to the presence of more than one stressor, leading to more severe impacts on life-history traits and/or to a reduced detoxification capacity.

Cyanotoxins are mainly retained within cyanobacterial cells during bloom development, but are released into the surrounding medium by senescence and lysis of the bloom at the end of proliferation period (autumn in temperate regions) (for review: Zurawell et al., 2005). For freshwater gastropods, chronic exposure to cyanobacterial blooms leads to the ingestion of potentially toxic cyanobacteria during the proliferation period and to intense exposure to soluble microcystins at the end of this period. To evaluate the risk of such impacts we need to consider the life cycle of freshwater gastropods and the structure of their populations during blooms. The main breeding season takes place in late spring or early summer (Calow, 1978), and coincides with the beginning of the cyanobacteria population maxima. Consequently,

most gastropods are exposed to cyanobacteria from birth, and thus, would have a decreased growth rate whereas the reproductive effort of adults would be altered. These two modes of MC exposure with negative impacts on populations of gastropods may also have potential cascading effects on the equilibrium and functioning of the whole aquatic ecosystem.

White et al. (2005) proposed a theoretical model to help managing toxic blooms and minimize their toxic effects in relation to both human and ecological risks. The predictive model for cyanotoxin bioaccumulation is based on the monitoring changes in toxin availability (temporal, spatial and different uptakes routes) throughout the progression of a toxic bloom, and on the internal tissue concentrations of aquatic organisms. However, the authors noted that there was, unfortunately, no currently available data on tissue toxin levels and sublethal or lethal effects of toxic cyanobacteria. Our study on the interactions between cyanobacteria and gastropods defines, in part, 3 of the 4 principal elements required for the application of the management model (White et al., 2005) to *L. stagnalis*. These known elements are: the likely uptake route seems to be the ingestion of cyanobacteria (Lance et al., 2006) while dissolved toxins produce only minor tissue contamination (Gerard et al., 2005); - the minimum exposure time required for the occurrence of bioaccumulation is 1 week at 5 µg L^{-1} intracellular MC-LReq (Lance et al., 2006). We emphasise that our measurements are of toxin concentration in tissues (Lance et al., 2006), and the demonstration of sublethal effects i.e reduction of growth and fecundity occurred over the same time span.

4.4. Conclusion

In the future, measuring and predicting the impact of toxic cyanobacteria on gastropod populations in fresh waters threatened by recurrent blooms is essential due to their key role in the ecosystem as herbivorous grazers (Dillon, 2000) and strong linkages they provide between primary producers and higher consumers in the food web (for reviews: Michelson, 1957; Habdija et al., 1995). A diminution of density of gastropods will have indirect negative consequences on the populations of the predator organisms i.e crayfish, leeches, aquatic insects, fish, waterfowl (for review: Michelson, 1957), and indirect positive consequences on the proliferation of toxic cyanobacteria. It is known that gastropods may largely control algal biomass and productivity by grazing, and influence the periphyton and macrophyte

assemblages (for review Liess & Hillebrand, 2004). It is becoming increasingly clear that invasion by toxic cyanobacteria poses a serious threat to biodiversity. This problem may increase dramatically if the ecological functions of gastropods as grazers are impaired by the sublethal effects of cyanotoxins.

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2.3. Consumption of toxic cyanobacteria by *Potamopyrgus antipodarum* (Gastropoda, Prosobranchia) and consequences on life traits and microcystin accumulation [3]

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Abstract

Among the wide range of toxins produced by cyanobacterial blooms, microcystins (MCs) are the most common and are known to accumulate in aquatic organisms. Freshwater gastropods are grazers and likely to ingest toxic cyanobacteria, particularly *Planktothrix agardhii*, one of the most common species in the northern hemisphere. The study examines i) the ingestion of toxic *P. agardhii* by the prosobranch *Potamopyrgus antipodarum*, ii) the kinetics of MC accumulation and depuration in snail tissues during and post-exposure, and iii) the impact of MCs on their life traits (survival, growth and fecundity). We showed that *P. antipodarum* ingested 71% of cyanobacteria available during the first 24h in the presence or not of non-toxic food, and accumulated 1.3% of ingested MCs during the 5-week intoxication period. Elimination of MCs was total after 3 weeks of depuration. A decrease of growth and fecundity was observed during the intoxication period, but it was reversible after the end of exposure. Results are discussed in terms of variation of the response between prosobranch and pulmonate gastropod to toxic cyanobacteria exposure, and the negative impact of toxic cyanobacteria on natural communities of freshwater gastropods.

1. Introduction

During the past decades, the significant increase of cyanobacteria proliferation in eutrophic waters worldwide has become a serious threat to human health and aquatic biota (Chorus and Bartram, 1999). Indeed, almost 50 cyanobacterial species are known to produce toxins (e.g., hepatotoxins, dermatotoxins, and neurotoxins) and approximately 75% of blooms have been shown to contain often more than one variant of toxins released in the water essentially during senescence (Chorus and Bratram, 1999). The most studied and widespread toxins are the potent hepatotoxic microcystins (MCs), cyclic heptapeptides of which 80 variants have been identified (Dietrich and Hoeger, 2005). MCs are tumour promoters and have been found to accumulate in vertebrates and invertebrates with potential damages to several organs (for review: Zurawell et al., 2005). Freshwater gastropods (i.e., Prosobranchia and Pulmonata) are mostly indiscriminate grazers inhabiting the littoral area where cyanobacteria frequently form scums (Chorus and Bartram, 1999). They are essential in food web as direct consumers of phytoplankton and as preys of numerous invertebrates and vertebrates (Dillon, 2000). Contamination of gastropods may occur via ingestion of toxic cyanobacteria and extracellular cyanotoxins dissolved or adsorbed on various particles. Due to the ecological and physiological differences between prosobranchs and pulmonates (e.g., respiration, feeding habits, phenotypic plasticity), one may expect that uptake route of cyanotoxins and contamination level will be different. Consumption of toxic cyanobacteria has been suggested in the field for pulmonates (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003) and prosobranchs (Chen et al., 2005; Xie et al., 2007; Zhang et al., 2007). In the laboratory, ingestion of toxic *Planktothrix agardhii*, one of the most common cyanobacterial species in the northern hemisphere, by the pulmonate *Lymnaea stagnalis* was demonstrated with consequent MC accumulation and negative impact on the life traits (Lance et al., 2006, 2007). Negative effects were also observed following exposure to purified dissolved MC-LR in *L. stagnalis* and in the prosobranch *Potamopyrgus antipodarum* but with minor MC accumulation (Gérard et al., 2005; Gérard & Poullain, 2005; Gérard C., personal communication). As *P. antipodarum* seemed to be more sensitive to dissolved MC-LR exposure than *L. stagnalis* according to these authors, it was worthy i) to assess the potential ingestion of toxic cyanobacteria by *P. antipodarum,* ii) to investigate the consequences of such ingestion on MC accumulation and life traits (i.e., survival, growth, and reproductive effort), iii) to compare these effects for the prosobranch and the pulmonate in relation with the

uptake route of cyanotoxins. In this study, *P. agardhii* containing MCs at an environmental relevant concentration were fed to *P. antipodarum* during 5 weeks, i.e., potential duration of a cyanobacterial bloom in eutrophic lake (e.g., Zurawell et al., 1999). The intoxication period was followed by a 3-week depuration period to determine the decrease of MCs from tissues and the impact on life traits when toxic cyanobacteria were removed. The discussion focused on the comparison between the prosobranch *P. antipodarum* and the pulmonate *L. stagnalis,* in terms of the negative impact of toxic cyanobacteria on natural communities of freshwater gastropods, and the potential cascading effects on the equilibrium and functioning of the ecosystem.

2. Material and methods

2.1. Biological material

The prosobranch snail *P. antipodarum,* native to a population of parthenogenetic females in a wetland stream (Pleine-Fougères, France), were mass-reared under laboratory conditions (20 ± 1°C, LD: 12-12, in dechlorinated tap water) and fed on dried lettuce *ad libitum*. The filamentous cyanobacterium *P. agardhii*, strain 75-02, originating from the recreational watersport site at Viry (Essone, France), was isolated and provided by the National Museum of Natural History (Paris, France). Cyanobacterial cultures were grown in 2L glass flasks with BG 11 medium in an incubation room under continuous agitation (25 ± 2°C, LD: 12-12, irradiance of 40 µE m^{-2} s^{-1}), and were replenished with a new medium every two weeks to ensure exponential growth. The algal densities provided to snails twice a week were taken from these cultures and were of 100 000 cells mL^{-1} producing dmMC-LR and MC-RR, with a total concentration of 2.6 µg L^{-1} expressed as MC-LR equivalents (MC-LReq), i.e. 146 µg g-1 dry weight (DW) of *P. agardhii*, and measured by high-performance liquid chromatography (Lance et al., 2006).

2.2. Experimental set up

Prior to the experiments, juvenile (sexually immature) and adult snails (respectively 2 ± 0.2 and 4 ± 0.2 mm shell length) were acclimatized for 7 days (20 ± 1°C, LD: 12-12, and fed on dried lettuce *ad libitum*) as 5 individuals with 15 mL of dechlorinated water per 50 mL

container. Twenty one replicates of 5 snails were assigned to each of the 8 groups according to diet and age of the snails: (1) snails fed on lettuce *ad libitum* (let) as juveniles and (2) as adults, (3) snails starved (starv) as juveniles and (4) as adults, (5) snails exposed to a density of toxic cyanobacteria of 100 000 cells mL^{-1} (cyano) as juveniles and (6) as adults, (7) snails exposed to the same density of toxic cyanobacteria and fed on lettuce *ad libitum* (cyano+let) as juveniles and (8) as adults. Among these 21 replicates of 5 snails per group, 5 replicates were used for the life traits study during the entire experiment and 16 for the mc accumulation and depuration study (2 different replicates each week, which added to 10 sacrificed snails per week and per group). A total of 420 juvenile and 420 adult snails were used in this experiment. Preliminary observations showed that gastropods consumed the *P. agardhii* suspension in 3 days; hence cyanobacteria suspension was renewed twice a week. Control containers were filled in the absence of gastropods, with 15 mL of cyanobacteria suspension with and without lettuce during 5 weeks, and randomly placed near containers with gastropods. After the intoxication period, all gastropods were fed solely on lettuce *ad libitum* and maintained in dechlorinated tap water during a 3-week depuration period.

2.3. Ingestion estimates of cyanobacterial cells and MCs.

2.3.a. Ingestion estimate of cyanobacterial cells

The ingestion of cyanobacteria by *P. antipodarum* was estimated for each group from the disappearance of cyanobacterial cells in containers with snails compared with controls without snails. Densities of cyanobacteria in containers were determined daily during the 5-week intoxication experiment on microscope with a Nageotte chamber (Lance et al., 2006). These cell counts were made in all the groups including controls for every new cyanobacterial suspension (10 renewals). Cyanobacterial growth rate (μ) was determined from the rate of change in the cyanobacterial biomass (B) over time in the controls, as follows:

$$dB/dt = \mu B \quad (1)$$

The time interval used was one day and the discrete time form of the precedent transition equation allowed estimating the daily growth rate (μ) as follows:

$$B_{t+1} = B_t (1+\mu) \quad (2)$$

where B_t and B_{t+1} are respectively the biomass of cyanobacteria at time t and t+1 (in cells mL^{-1}). An assumption was made that μ was not influenced by the presence or absence of a gastropod. Hence, the daily gastropod ingestion rate of cyanobacteria in the treatments without lettuce was obtained by including the ingestion as a loss term (S) in equation (2) as follows:

$$B_{(S)\,t+1} = B_{(S)\,t} \times (1 + \mu - S) \quad (3)$$

where $B_{(S)\,t}$ and $B_{(S)\,t+1}$ are respectively the biomass of cyanobacteria at time t and t+1 (in cells mL^{-1}) in the treatments with snails and without lettuce.

The total number of cells per ml ingested by the 5 snails per day was obtained by multiplying the cyanobacterial biomass $B_{(S)\,t}$ by the ingestion rate (S). Ingestion was expressed in four ways as (i) the %consumption per day for the 5 snails in glass containers, (ii) the average % consumption per 3 days for the 5 snails in glass containers, (iii) the average total number of cells ingested per snail and per renewal of suspension (twice a week), and (iv) the average number of cells ingested per snail and per ml of available suspension twice a week.

2.3.b. Ingestion estimate of MCs

Total MC ingested per snail per week was estimated from the MC concentration in cyanobacterial cells and the average number of cells ingested. MC concentration in cyanobacterial cells was determined twice a week during the preparation of the suspension provided to snails, that reached a concentration of 2.6 μg L^{-1} MC-LReq measured with a HPLC with diode array detection (HPLC-DAD) and a variable-wavelength UV detector operating at 238 nm, as described in Lance et al. (2006). These measured MC contents were combined with cell counts to derive a relation between algal density and MC concentration to obtain the amount of MC contained in one cell of *P. agardhii*.

2.4. Quantitative analysis of MC-LReq in exposed snail tissues and quality control of MC measurement

Every week, 10 snails (2 replicates) were randomly chosen from each group and were starved for ca. 24h to empty their gut contents (Carriker, 1946) to ensure that the MC measurement reflected only assimilated MCs, and did not include MCs in the undigested

filaments of *P. agardhii* nor in the gut. Snails were removed from their shell, freeze-dried and weighed prior to MC analysis. This was performed by immuno-assay with an ELISA MC Plate Kit (Envirologix INC) as described in Lance et al. (2006). MC contents in *P. antipodarum* tissues were expressed in µg g FW^{-1} (fresh weight) and in µg g DW^{-1} according to the relation previously established: FW = 5.55 * DW (n = 30, R Pearson = 0.97, $\underline{P} < 0.05$). The values were calculated by taking into account extraction recovery and possible matrix-induced signal enhancement or suppression with the ELISA test because of unspecific binding to and/or denaturing of the antibodies. Control snails, free of MCs, were freeze-dried and homogenized in a mortar, spiked with MC-LR standard (5 µg g^{-1}) (Dionisio Pires et al., 2004). The extraction was performed and the recovery for the extraction was calculated. The matrix effect (i.e. effect of snail tissue) was checked by spiking control snails with MC-LR standard (5 µg g^{-1}) and the response was compared to 100% methanol spiked with the same amount (Dionisio Pires et al., 2004). The average recovery was surprisingly 99.8 ± 1.2% and matrix effect was negligible (average of 2.8 ± 0.6% of differences between matrix and methanol results). The small amount of *P. antipodarum* tissue used (2 mg) could explain the 100% recovery of extraction with 2 mL methanol.

The percentage of accumulation (%acc) was calculated each week based on the ratio between the average quantities of MC-LReq accumulated in snail tissues between weeks 1 and n (in µg) and the estimated average quantities of MC-LReq ingested between weeks 1 and n (in µg). During the depuration period, the percentage of MC elimination from snail tissues (% detox) was calculated between weeks n and n+1 by the expression:

$$\% \text{ detox} = 100 \times [\text{MC-LReq}_{n} - \text{MC-LReq}_{n+1}] / \text{MC-LReq}_{n} \quad (4)$$

2.5. Life trait measurements

For all the snails, shell size was measured to the nearest 0.1 mm every week. The weekly growth % refers to the percentage of growth per week, calculated as the ratio between the increase of the shell size (S in mm) between week n and n+1 and that at week n:

$$\text{Weekly growth } \% = 100 \times [S_{n+1} - S_{n}] / S_{n} \quad (5)$$

The reproduction was monitored at the end of intoxication and depuration periods by counting the number of embryos harboured in the oviduct pouch of 5 randomly dissected individuals in each group.

2.6. Statistical analysis

Pearson's coefficient (R Pearson) was calculated to assess the existence of a correlation between: (1) the quantity of MCs produced and the density of cyanobacterial suspensions (n = 50), (2) the fresh weight (FW) and dry weight (DW) of the snails (n = 30). Two-way analyses of variance (ANOVA) with repeated measures and the Tukey-HSD multiple comparison test were performed to compare: (1) the number of cyanobacterial cells per mL ingested per snail, (2) the average %consumption of snails over a 3-day period, (3) the daily %consumption of snails, (4) the %acc, (5) the growth rate, between groups and weeks. Test t was used to compare the number of embryos per group. Differences were considered to be statistically significant at $P < 0.05$. Data are reported as mean ± standard error (S.E.).

3. Results

3.1. Ingestion of *P. agardhii* cells and MCs

The average 3-day dynamics of *P. agardhii*'s suspensions used during the 5-week intoxication experiment was lower when snails were present, with or without lettuce, than in their absence (Fig. 1), demonstrating the consumption of toxic cyanobacteria by *P. antipodarum*. Moreover, the average daily growth rate of *P. agardhii* although negative was higher in the controls without snails than when snails were present, with or without lettuce ($\mu = -0.14 \pm 0.06\ d^{-1}$ vs $-0.47 \pm 0.09\ d^{-1}$ respectively without and with snails) (Fig. 1). Whatever the age of the snails and whether toxic cyanobacteria were the only source of food or not, there was no significant differences in cell numbers consumed per snail and per mL (ANOVA $F_{3,39} = 1.02$, $P > 0.05$) (Table 1) and in the mean %consumption over 3 days (ANOVA, $F_{3,39} = 0.34$, $P > 0.05$). The daily %consumption was similar between groups (ANOVA, $F_{3,\ 119} = 1.73$, $P > 0.05$) but changed over the 3 days during the intoxication period (ANOVA, $F_{2,119} = 20.32$, $P < 0.05$). Consumption was significantly higher on the first day (71.3 ± 4.3% of cyanobacteria consumed by all the five snails) (Tukey HSD, $P < 0.05$), whereas it did not significantly differ during the remaining 2 days (45.8 ± 4.4% of the remaining cells) (Tukey HSD, $P > 0.05$).

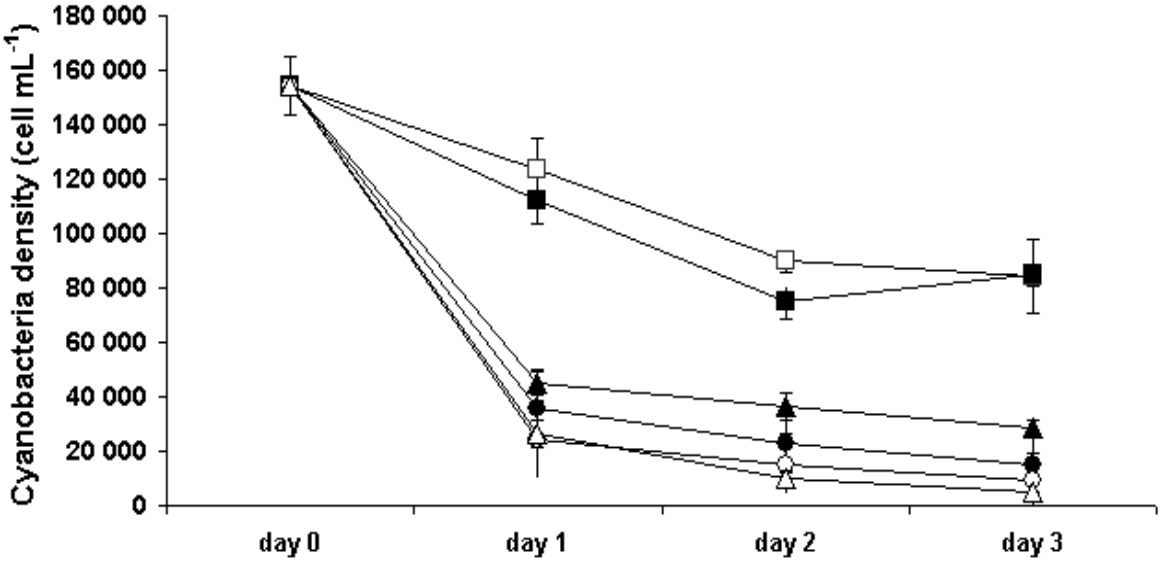

Figure 1. Mean (± S.E.) 3-day dynamics of *Planktothrix agardhii* during the 5-week intoxication experiment: alone (open square) and with lettuce (closed square), in the presence of snails as juveniles (triangle) and adults (circle) without (open) and with lettuce (closed).

Table 1. Mean (± S.E.) ingestion of *Planktothrix agardhii* cells by *Potamopyrgus antipodarum* juveniles and adults over 3 days during the 5-week intoxication period in the presence or absence of lettuce (cells per snail, cells mL^{-1} per snail).

	cells per snail	cells mL^{-1} per snail
juveniles fed on cyanobacteria (juv cyano)	$283.10^3 \pm 55.10^3$	$19.10^3 \pm 4.10^3$
juveniles fed on cyanobacteria and lettuce (juv cyano+let)	$326.10^3 \pm 37.10^3$	$28.10^3 \pm 2.10^3$
adults fed on cyanobacteria (ad cyano)	$303.10^3 \pm 80.10^3$	$20.10^3 \pm 5.10^3$
adults fed on cyanobacteria and lettuce (ad cyano+let)	$312.10^3 \pm 66.10^3$	$21.10^3 \pm 4.10^3$

The total quantity of MCs ingested was estimated from the number of ingested cells and from the concentration of MC-LReq in one *P. agardhii* cell measured twice a week during 5 weeks ($2.6.10^{-8} \pm 0.2.10^{-8}$ µg, n = 50, R Pearson = 0.92, $P < 0.05$) (Fig. 2). At the end of the 5-week intoxication period, the total content of MCs consumed was estimated for each adult at 73.6 ± 7.8 ng ind^{-1} without lettuce and 84.6 ± 5.6 ng ind^{-1} with lettuce, and for each juvenile at 78.8 ± 4.6 ng ind^{-1} without lettuce and 81.1 ± 6.9 ng ind^{-1} with lettuce.

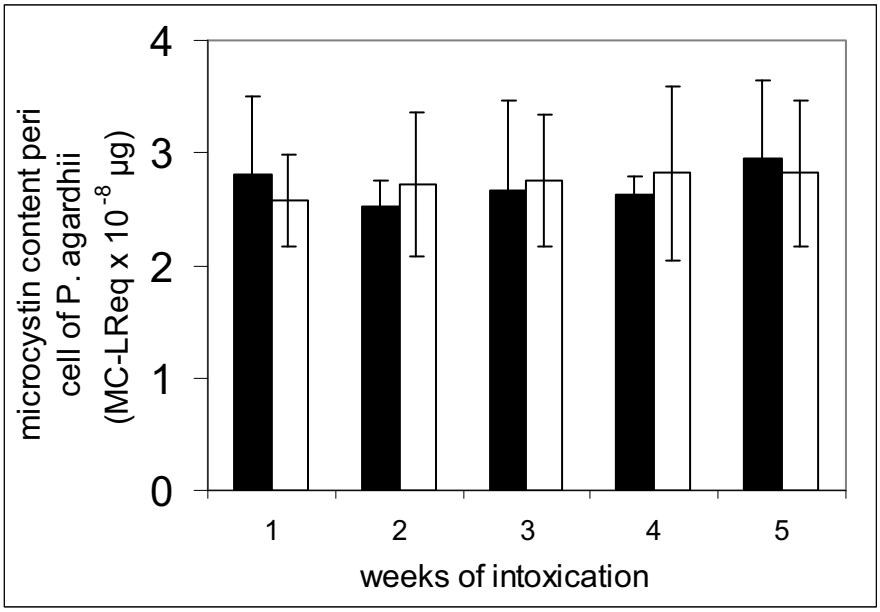

Figure 2. Mean (± S.E.) concentration of MC-LReq (µg) per cell of *Planktothrix agardhii* during the 5-week intoxication period, two cyanobacterial suspensions per week: first in black, second in white

3.2. Accumulation and depuration

After 5 weeks of intoxication, MC contents in snail tissues reached a maximum of 4.9 ± 0.5 µg g DW^{-1} (0.9 ± 0.0 µg g FW^{-1}) in case of juveniles without lettuce (Fig. 3). The %acc varied significantly between groups and weeks (ANOVA, $F_{3,39}$ = 17.4 and $F_{4,39}$ = 9.2, P < 0.05). It was similar for juveniles and adults in each diet (Tukey HSD, P > 0.05), but whatever the age, snails fed on toxic cyanobacteria alone showed a greater %acc than snails fed on mixed food (Tukey HSD, P < 0.05) (Fig. 4). Moreover, %acc was significantly higher during the two first weeks of intoxication than during the 3 following weeks (Tukey HSD, P < 0.05). An average of 1.29 ± 0.2 % of total ingested MCs were accumulated by all gastropods during the intoxication period, with 1.65 ± 0.1 % during the two first weeks and 1.0 ± 0.0 % during the 3 remaining weeks. During the depuration period, MCs decreased rapidly in all groups from 2.5 ± 0.2 µg g DW^{-1} to 0.0 ± 0.0 µg g DW^{-} (or from 0.5 ± 0.0 µg g FW^{-1} to 0.0 ± 0.0 µg g FW^{-1}) (Fig. 3). The percentage of detoxification was 75.7 ± 3.1% for all groups during the first week and MCs were completely eliminated after 3 weeks (Table 2).

Table 2. Mean (± S.E.) percentage of MC-LReq eliminated every week in *Potamopyrgus antipodarum* tissues (% detox) during the 3-week depuration period, calculated between weeks n and n+1, according to the age and the presence of lettuce (see abbreviations in table 1 or in section 2.2).

Weeks	juv cyano	juv cyano+let	ad cyano	ad cyano+let
6	70.1 ± 5.2	81.4 ± 2.1	71.8 ± 2.4	79.4 ± 1.5
7	83.0 ± 3.5	87.6 ± 1.8	86.4 ± 3.3	85.7 ± 2.7
8	100.0 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

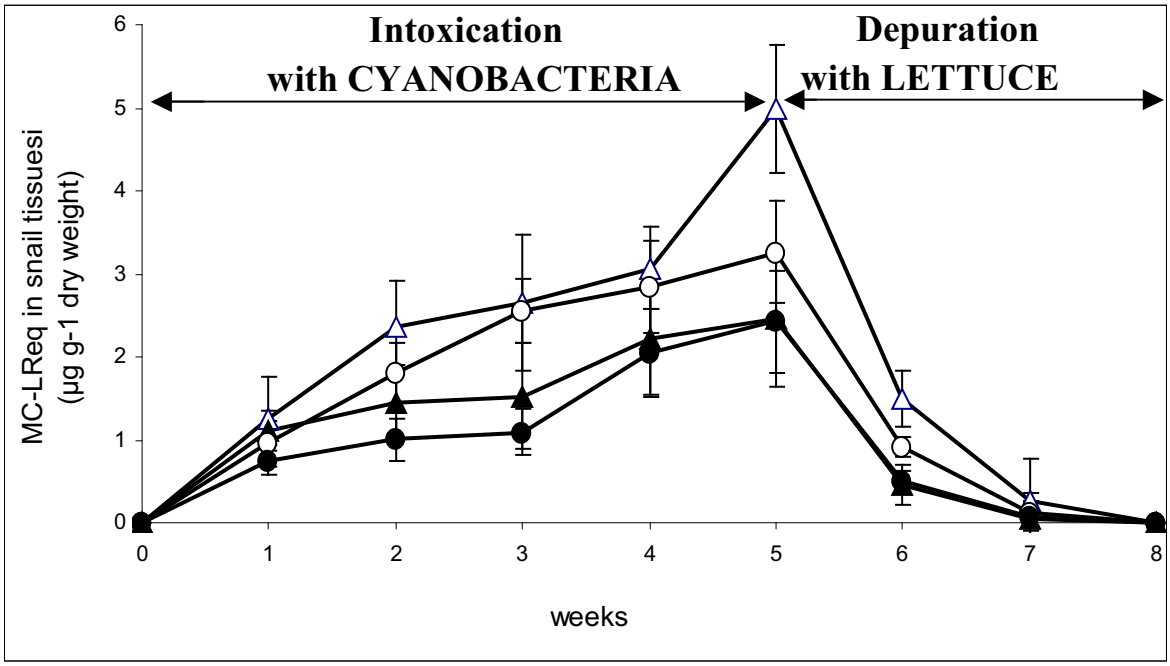

Figure 3. Kinetics of MC-LReq in *Potamopyrgus antipodarum* tissues (µg g DW-1) (± S.E.) of juveniles (triangle) and adults (circle) fed on cyanobacteria without (open) and with lettuce (closed) during 5 weeks of intoxication, then fed on lettuce during 3 weeks of depuration (n = 80).

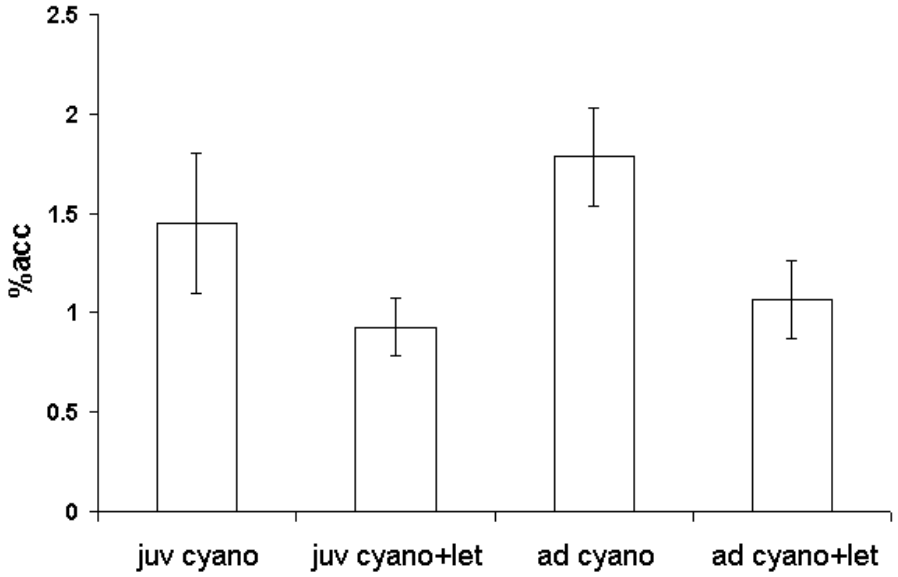

Figure 4. Mean (± S.E.) percentage of ingested MC-LReq accumulated in *Potamopyrgus antipodarum* tissues (% acc) of juveniles (juv) and adults (ad) fed on cyanobacteria without (cyano) and with lettuce (cyano+let) during 5 weeks of intoxication (n = 50).

3.3. Effects on the life traits: survival, growth and fecundity

No mortality occurred during the experiment whatever the age and the diet.

The weekly percentage of growth of juveniles differed significantly between groups during the whole experiment (ANOVA, for intoxication and depuration periods respectively, $F_{3,319} = 44.5$ and $F_{2,239} = 9.4$, $P < 0.05$). Differences in juvenile sizes increased during the intoxication period (Fig. 5).

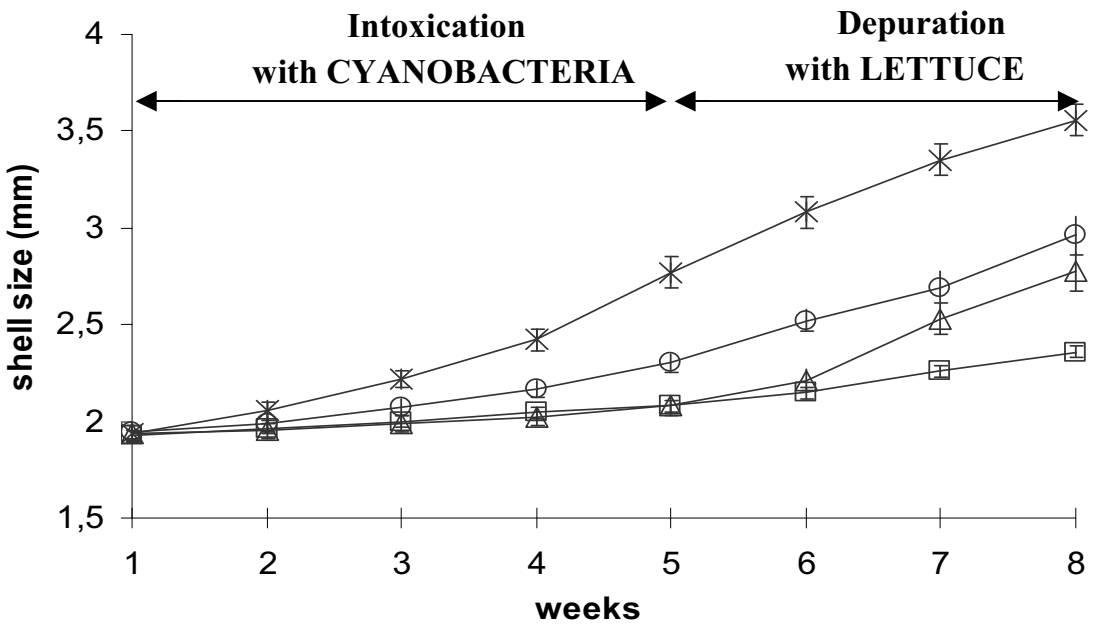

Figure 5. Growth of juvenile *Potamopyrgus antipodarum* (shell size in mm ± SE) fed on lettuce (cross), on cyanobacteria without (triangle) and with lettuce (circle), and starved (square) during a 5-week intoxication period, and a 3-week depuration period (n = 100).

The weekly percentage of growth of starved juveniles and those fed on cyanobacteria alone were similar (Tukey HSD, $P > 0.05$), but were significantly lower than that of juveniles fed on lettuce, with or without toxic cyanobacteria (Tukey HSD, all $P < 0.05$) (Table 3). Moreover, the weekly percentage of growth of juveniles fed on lettuce with cyanobacteria was decreased compared to the controls fed only on lettuce (Tukey HSD, $P < 0.05$) (Table 3). During the depuration period, negative effect induced by toxic cyanobacteria disappeared since the weekly percentage of growth of all juveniles previously exposed to toxic cyanobacteria, with or without lettuce, were similar to that of the controls (Tukey HSD, $P > 0.05$). Only snails previously starved showed significantly lower growth than other snails (Tukey HSD, all $P < 0.05$) (Table 3).

Table 3: Average weekly percentage of growth of juvenile and adult *Potamopyrgus antipodarum* for each group during the 5-week intoxication and the 3-week depuration periods (see abbreviations in table 1).

	Period	Groups			
		let	starv	cyano	cyano+let
Juveniles	Intoxication	9.5 ± 0.8	1.9 ± 0.3	1.7 ± 0.2	4.4 ± 0.6
	Depuration	9.0 ± 0.9	4.2 ± 0.4	10.1 ± 1.0	8.8 ± 0.8
Adults	Intoxication	1.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
	Depuration	1.4 ± 0.3	0.8 ± 0.1	1.2 ± 0.3	1.0 ± 0.1

Differences in adult shell sizes between diets were significant during the intoxication period (ANOVA, $F_{3,319} = 10.1$, $P < 0.05$). Percentages of growth were similar in case of starvation and ingestion of toxic cyanobacteria, with or without lettuce, (Tukey HSD, all $P > 0.05$), and were significantly lower than those of control snails (Table 1, Tukey HSD, all $P < 0.05$). These effects were reversible during the depuration period (ANOVA, $F_{2,239} = 1.1$, $P = 0.37$), where all groups presented a similar weekly percentage of growth (Table 3).

Juvenile snails being sexually immature during the whole experiment harboured no embryo. At the end of intoxication, fecundity of adult snails starved or fed on toxic cyanobacteria alone was similar (t test, T = 0, $P = 1$, DL = 6), but was significantly lower than that of control snails (respectively, t test, T = 2.7, $P = 0.03$ and T = -2.6, $P = 0.04$, DL = 6) (Fig. 6). Only a few embryos were found in the oviduct pouch of females fed on toxic cyanobacteria with or without lettuce (4.5 ± 2.2 and 0.75 ± 0.3 embryos per female respectively). However, the number of embryos in females fed on cyanobacteria with lettuce was not significantly lower (t test, T = -1.08, $P = 0.3$, DL = 6) than that of the controls (8.5 ± 4.2 embryos per female). After the depuration period, only snails previously starved presented significantly lower embryos than controls (t test, T = 2.7, $P = 0.03$, DL = 8) (Fig. 6).

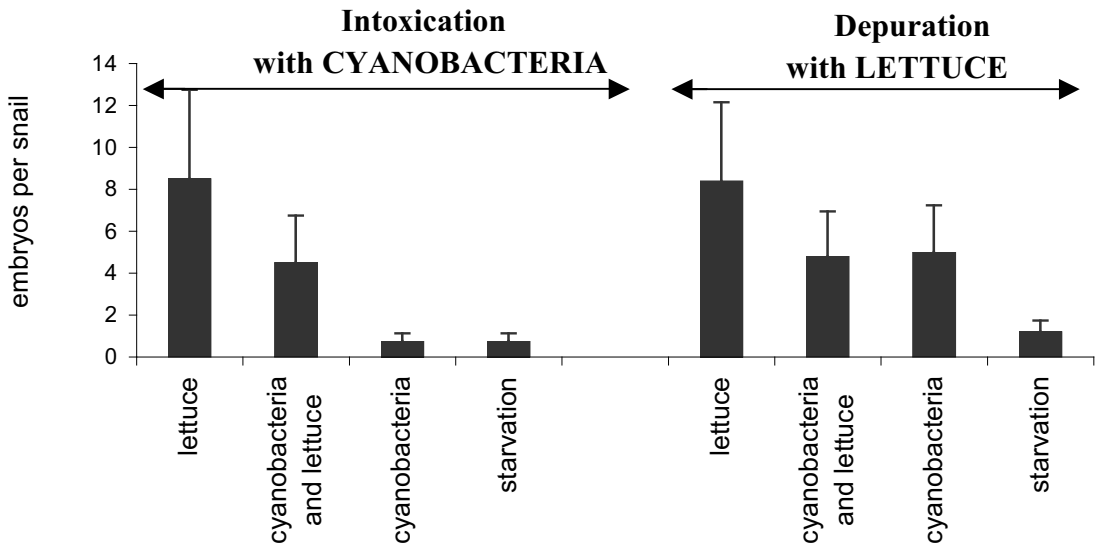

Figure 6. Mean number of embryos (± SE) per *Potamopyrgus antipodarum* female after the 5-week intoxication period, and after the 3-week depuration period (n = 40).

4. Discussion

4.1. Ingestion of toxic cyanobacteria and MC accumulation

The prosobranch *P. antipodarum* is considered as a generalist feeder, i.e. both grazer and detritivore, which is attracted to food by chemical stimuli in water and will eat a wide range of substances (Haynes and Taylor, 1984). Our study demonstrated that *P. antipodarum* ingested hepatotoxic *P. agardhii* regardless of age and of presence or absence of non-toxic food, as previously shown for the pulmonate *L. stagnalis* in a similar experiment (Lance et al., 2006). *P. antipodarum* appeared also unable to discriminate between toxic and non-toxic food, in contrast to copepods and fish species (for review, Zurawell et al., 2005), suggesting that it could ingest toxic cyanobacteria in the field. Around 70% of cyanobacterial cells present in suspension were consumed by 5 individuals during the first day and 46% of the remaining cells during the next 2 days. These ingestion rates were of the same order for a single individual of *L. stagnalis* exposed to *P. agardhii* with respectively 63% during the first day and 33% during the next 2 days (Lance et al., 2006).

The ingestion of toxic cyanobacteria led to MC accumulation in *P. antipodarum* tissues. MC concentration increased linearly to an average of 3.3 ± 0.4 µg g DW^{-1} in all snails after 5 weeks of intoxication. Following the removal of toxic cyanobacteria, MCs decreased strongly in tissues and were completely eliminated after 3 weeks of depuration. In field studies, MC accumulation was of the same order in the prosobranch *Viviparus contectus* from a European lake (from 2.9 to 3.3 µg g DW^{-1}) (Gkelis et al., 2006), as well as in the digestive gland of the prosobranchs *Bellamya aeruginosa* (4.14 and 2.33 µg g DW^{-1}) (respectively Chen et al., 2005 and Zhang et al., 2007) and *Sinotaia histrica* (5.38 µg g DW^{-1}) (Xie et al., 2007), in Asian lakes. On the other hand, Zurawell et al., (1999) reported much higher values of MC accumulation in 3 species of pulmonates from Canadian lakes (up to 140 µg g DW^{-1} for *L. stagnalis*, 130 µg g DW^{-1} for *Physa gyrina* and 40 µg g DW^{-1} for *Helisoma trivolvis*). This is consistent with the laboratory experiments of Lance et al. (2006) on *L. stagnalis* that accumulated 48.0 ± 7.9 µg MC g DW^{-1} after 5 weeks of feeding on toxic *P. agardhii* at 5 µg L^{-1} MC-LReq. Many authors of field studies found that MC accumulation in gastropods was depended on MC level in the phytoplankton and was a result of direct ingestion (Zurawell et al. 1999, Yokoyama and Park, 2002; Chen et al., 2005; Xie et al., 2007; Zhang et al., 2007). However, according to Zhang et al. (2007), MC content in the gastropod tissues was also

correlated with the extracellular MCs dissolved in water. Nevertheless, in our studies, for both *L. stagnalis* and *P. antipodarum*, MC accumulation was greater by grazing toxic cyanobacteria at a maximum concentration of 5 µg. L^{-1} than by exposure to dissolved MC-LR at 33 µg. L^{-1} (Lance et al., 2006, Gérard et al., 2005, Gérard C., personal communication). These results are in accordance with those of White et al. (2006) on the prosobranch *Melanoides tuberculata* exposed to intracellular and dissolved cylindrospermopsins. However, the difference in MC accumulation between the two contamination pathways is far greater for *L. stagnalis* (i.e., 1300 times) than for *P. antipodarum* (i.e., 1.5 times). Thus it appears from our results and these other studies that prosobranchs may accumulate less MCs from the ingestion of toxic cyanobacteria and that transfer of MCs from intoxicated prosobranchs through the food web is probably negligible compared to pulmonates.

The differences in the percentage of accumulation in *P. antipodarum* (1.3% of total ingested MCs) and *L. stagnalis* (61.0%) suggest differences in the capacity of MC assimilation and/or metabolization in the digestive system. Ingested MCs that were not quantified in snail tissues of both species are thought to have been partly: (i) eliminated in the gizzard and caecal string fraction of the faeces (undigested cells) during the first hours post ingestion; (ii) entering the digestive gland and digested, followed by excretion in the digestive gland fraction of the faeces (Dillon, 2000; Zurawell et al., 2006); (iii) entering the digestive gland and accumulating through binding covalently to protein phosphatase enzymes and thus being undetectable (Dietrich and Hoeger, 2005). Indeed, particles ingested by gastropods are grinded in the gizzard and only particles ≤ 4µm pass into the digestive gland for intracellular and/or extracellular digestion, the others passing into the prointestine to be compacted in faeces (Dillon, 2000). As Zurawell et al. (2006) suggested, MCs are intracellular and failure of gizzard to mechanically or enzymatically disrupt cells may limit their uptake by the gastropod digestive system. Moreover, freshwater gastropods are able to adapt their enzymes to optimally suit the digestion of the most abundant food source (Brendelberger, 1997). During the digestion processes, elimination of toxic cells in the gizzard and caecal string fraction could account for a greater part of ingested MCs in *P. antipodarum*, whereas MCs might be mainly transported in the digestive gland in *L. stagnalis*. A second hypothesis to explain differences in MC assimilation between the two species is a possible better detoxification system for *P. antipodarum*, which allow a greater excretion in the digestive gland fraction of the faeces and limited toxin accumulation. Indeed, in vertebrates and invertebrates, the toxicity of MCs can be reduced and their elimination can be enhanced by a

conjugation reaction to glutathione, catalysed by glutathione-S-transferase (GSTs) or with cysteine (for review: Cazenave et al., 2006). Efficient elimination of MCs has been demonstrated in some molluscs both in the laboratory, i.e., 100% of the accumulated MCs were successfully eliminated by *P. antipodarum* after 3 weeks of depuration in our study, and by mussels in less than 15 days (Vasconcelos 1995, Yokoyama and Park 2003, Dionisio Pires et al., 2004), and in the field, i.e., *S. histrica* was able to depurate MC-LR efficiently after the period of cyanobacteria proliferations (Xie et al., 2007). However, contrary to these species, MCs were still detectable in *L. stagnalis* (on average, 3.5 ± 0.9 µg g DW^{-1}) after a 3-week depuration (Lance et al., 2006), and in *B. aeruginosa* in a Chineese lake after the end of bloom period in November (almost 3 µg g DW^{-1} in the digestive gland) (Chen et al., 2005). According to our results, *P. antipodarum* accumulated a significantly lower percentage of ingested MCs during the last three weeks of intoxication than during the first two weeks. This result suggests that two weeks could be the time required for the establishment of the detoxification processes that appear earlier and more efficient than that of *L. stagnalis*. However, concentrations reported in tissue samples correspond to free MCs only and an unknown amount of MCs probably also accumulated in *P. antipodarum* tissues in a covalent form, not detected by the ELISA test. We are unaware of a study that measures covalently bound MCs in gastropod tissues but this will soon be investigated.

4.2. Impact on life traits

This study demonstrated that the ingestion of toxic cyanobacteria by *P. antipodarum* did not affect survival but induced a decrease of growth and fecundity, as shown for *L. stagnalis* (Lance et al., 2007). MCs are inhibitors of protein phosphatases (PP1, 2A, 4, and 5) that play a crucial role in homeostasis and are potent liver tumour initiators and promoters (for reviews: Hastie et al., 2005; Zurawell et al., 2005). Moreover, their second most important target organ in freshwater invertebrates is the reproductive system (Chen and Xie, 2005). Resources allocated to the costly detoxification processes must imply trade-offs that probably affect life traits (for review: Cazenave et al., 2006). The elimination of MCs might explain why both species showed impact on life traits during the ingestion of toxic cyanobacteria. As for *L. stagnalis* (Lance et al., 2007), pathogen effects were less severe for *P. antipodarum* when snails were fed with toxic cyanobacteria in combination with non-toxic food. The availability of a non-toxic food probably interferes with MC accumulation rate, as

found by Soares et al. (2004) in fish, and allows relative growth and reproduction with a concomitant detoxification. The impact on the life traits of the prosobranch following toxic cyanobacteria ingestion appeared less severe than for the pulmonate since negative effects were reversible for *P. antipodarum* during the depuration period whereas the life traits remained negatively affected for *L. stagnalis*. The better recovery of *P. antipodarum* may partly be explained by its greater ability to detoxify MCs during the ingestion of toxic cyanobacteria. Thus it required less energy for the MC elimination during the depuration period compared to the pulmonate with consequences on the life traits.

Surprisingly, *P. antipodarum* seems to be more sensitive than *L. stagnalis* to dissolved MC-LR exposure (vs ingestion of toxic cyanobacteria). Indeed, a 6-week exposure to 33 µg. L^{-1} dissolved MC-LR induced a decrease in survival, growth and fecundity of *P. antipodarum* (Gérard and Poullain, 2005), whereas only a decrease in fecundity of *L. stagnalis* (Gérard et al., 2005). On the contrary to the pulmonate, the impact on the life traits of the prosobranch is more severe due to dissolved MC exposure than due to toxic cyanobacteria ingestion, despite an almost equivalent MC accumulation with both contamination pathways. After ingestion of toxic cyanobacterial cells, MCs are partly accumulated in the digestive gland or excreted, significantly reducing the MC transport in other organs (Zurawell et al., 2006). In contrast, when prosobranchs are exposed to dissolved toxins, MCs may penetrate through the cell membranes of the gill epithelia as suggested by White et al. (2006) for cylindrospermopsin. Then, they may be distributed in various organs, possibly impairing the homeostasis of the snails as shown for fish species (e.g., perturbation of the metabolism, induction of physiological stresses, histopathological and vascular damages) (for review: Malbrouck and Kestemont, 2006). Moreover, the gill of prosobranchs being involved in osmoregulation (Little, 1981), MCs could inhibit the ion transport in the gill leading to unbalanced homeostatis and sometimes to death as shown for some freshwater fish (for review: Malbrouck and Kestemont, 2006). On the other hand, the limited contamination of *L. stagnalis* by dissolved MCs may be due to limiting factors that prevent from a massive penetration of dissolved toxins in the internal medium (e.g., the aerial respiration). Nevertheless, the mechanisms involved in the higher sensibility of *P. antipodarum* to dissolved MC-LR compared to the ingestion of cyanobacteria containing MCs need to be clarified by further investigations.

4.3. Implications for natural gastropod communities

Interactions between toxic cyanobacteria and gastropods are likely to be frequent and prolonged in fresh waters of temperate regions (Zurawell et al., 1999; Xie et al., 2007; Zhang et al., 2007). In natural conditions, the prosobranch *P. antipodarum* can be contaminated by MCs via grazing of toxic cyanobacteria during proliferations, as demonstrated by our results, and via absorption of MCs released in the water (dissolved or adsorbed on sediment particles) when blooms collapse (Chorus and Bartram, 1999), as demonstrated by Gérard and Poullain (2005). Depending on environmental conditions, MC concentrations can vary from 1 to 8600 $\mu g\ L^{-1}$ (Christoffersen, 1996 for review), suggesting that gastropods can be exposed to high amount of MCs. Our investigations on gastropods-cyanobacteria interactions suggest that chronic exposures to toxic cyanobacteria and cyanotoxins lead to MC accumulation and have a negative impact on the gastropod life traits. Moreover, a negative impact may also occur on developing embryos, as demonstrated for the prosobranch *Melanoides tuberculata* exposed to cylindrospermopsin (Kinnear et al., 2007). Recently, Zhang et al. (2007) first reported MC accumulation in embryos harboured in *B. aeruginosa* (prosobranch) females collected in a lake exposed to dense toxic *Microcystis* blooms, indicating MC transfer from females to their offspring. Developing embryos are probably not protected in the same way against contamination by MC in the brood pouch of oviduct (e.g., *B. aeruginosa* or *P. antipodarum*) or in the gelatinous capsule surrounding egg masses (e.g., pulmonates). Whereas *P. antipodarum* tend to be perennial (up to 2.5 years), ovoviviparous and able to breed all year long, most pulmonates are annual and oviparous with breeding generally in late spring in temperate regions (Calow, 1978). To be annual or perennial implies great differences in the exposure to toxic cyanobacteria in terms of intensity, timing, and frequency. According to our present knowledge, one can suppose that impact would be more severe on pulmonates vs prosobranchs during the ingestion of cyanobacteria in the bloom period, whereas it would be more severe on prosobranchs vs pulmonates during the senescence when MCs are released in water. Moreover, *P. antipodarum* is a special prosobranch since it can be invasive in some areas where it was introduced (e.g., Australia, North America) and may impact on native macroinvertebrates (Schreiber et al., 2002, Richards et al., 2004, Kerans et al., 2005). Differences in contamination pathways of gastropods by toxic cyanobacteria could influence competitive interactions with consequences on population dynamics. The occurrence of toxic blooms in fresh waters may provide a possible explanation for the observed decrease in some

gastropod populations and changes in the structure of the communities (Gérard et al., in press).

4.4. Implications for food web dynamics

Gastropods have a key role in the ecosystem as herbivorous grazers and they provide strong linkages between primary producers and higher consumers in the food web (for reviews: Michelson, 1957; Dillon, 2000). Despite its small size (< 6 mm), *P. antipodarum* can significantly reduce algal biomass by grazing (Biggs and Lowe, 1994; Suren, 2005). However, rapid and total MC-detoxification by *P. antipodarum* may limit the risk of MC transfer in the food web, while this risk is greater with *L. stagnalis* (Lance et al., 2006). A decrease of the gastropod densities will have indirect negative consequences on the populations of predators, i.e., crayfish, leeches, aquatic insects, fish, waterfowl (for review: Michelson, 1957), and indirect positive consequences on the proliferation of toxic cyanobacteria. It is known that gastropods may largely control algal biomass by grazing and that primary producers can increase in abundance if grazers are selectively eliminated by toxic cyanobacteria (Biggs and Lowe, 1994; Liess & Hillebrand, 2004; Suren, 2005). The MC contamination of gastropods in the field, probably resulting in negative impacts on the populations, may also have potential cascading effects on the equilibrium and functioning of the whole aquatic ecosystem.

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Chapitre 3 : Impact des microcystines sur la fitness du pulmoné *L. stagnalis* exposé à une cyanobactérie toxique ou à de la microcystine-LR dissoute

Chapitre 3 : Impact des microcystines sur la fitness du pulmoné *L. stagnalis* exposé à une cyanobactérie toxique ou à de la microcystine-LR dissoute[1]

Les précédents travaux ont montré que l'accumulation de microcystines (MCs) dans les tissus du gastéropode pulmoné *L. stagnalis* et l'impact sur ses traits de vie (survie, croissance, fécondité) varient selon son âge (juvéniles, adultes), la voie de contamination (exposition à une cyanobactérie productrice de MCs ou à de la MC-LR dissoute) et la présence ou non d'une autre source de nourriture non toxique (Gérard et al., 2005; Lance et al., 2007). Compte tenu de la réduction de fécondité observée chez les adultes, et ceci, quelles que soient les modalités de l'expérience, nous avons voulu mesurer l'impact des deux sources de contamination sur la fitness de *L. stagnalis* en termes de taux d'éclosion des œufs, de durée du développement embryonnaire et de survie des néonates.

Les résultats de l'étude confirment la réduction de fécondité des limnées intoxiquées par les MCs, quelles que soient les modalités d'expérience. D'autre part, le taux d'éclosion des œufs issus de parents intoxiqués (exposés à 33 µg MC-LR L^{-1} ou à *Planktothrix agardhii* produisant 10 µg MCs L^{-1}) est inchangé par rapport aux témoins y compris lorsque les pontes restent en présence de cyanobactéries toxiques après l'oviposition. Cependant, ce taux d'éclosion est réduit lorsque les pontes restent en présence de MC-LR dissoute après l'oviposition. Ces résultats suggèrent une perméabilité des pontes à la MC-LR dissoute, et donc une contamination possible des embryons avant éclosion en milieu naturel, en particulier lors de la lyse des blooms. Par ailleurs, quel que soit le mode de contamination de la limnée, la durée du développement embryonnaire est raccourcie par rapport aux témoins et la survie des néonates est réduite (mesurée à 5, 10 et 15 jours après la naissance). Enfin, la présence de MCs n'est pas détectée dans les pontes des limnées intoxiquées, ni chez les néonates nés en eau non contaminée ou en présence de MC-LR dissoute. Cependant, les néonates présentent des MCs dans leurs tissus dans les 24h qui suivent leur naissance et dans les jours qui suivent lorsqu'ils coexistent avec des cyanobactéries toxiques, suggérant qu'ils sont capables d'ingérer des cyanobactéries très précocement. Tous ces résultats suggèrent un impact négatif des MCs sur la fitness des gastéropodes en milieu naturel, à la fois lors des proliférations de cyanobactéries toxiques et lors de la lyse des blooms.

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[1] Cette partie a été réalisée avec Marion Tanguy, stagiaire de Master 1 à l'université de Rennes 1 d'avril à juin 2007

Impact of microcystins on the fitness of *Lymnaea stagnalis* (Gastropoda, Pulmonata) exposed to toxic cyanobacteria or dissolved microcystin-LR

Introduction

Biotic and abiotic stresses (e.g. parasitism, pesticides, heavy metals) are known to frequently affect the fecundity of gastropods and their progeny (e.g., hatching, survival), as demonstrated for example for Lymnaeidae (Singh & Agarwal, 1986; Gomot, 1998; Russo & Lagadic, 2004; Leung et al., 2007; Coutellec et al., 2008; Pietrock et al., 2008). Previous studies on the pulmonate *Lymnaea stagnalis* showed that fecundity (number of eggs and egg-masses) is significantly decreased when exposed to microcystin (MC)-producing cyanobacteria (Lance et al., 2007) and dissolved MC-LR (Gérard et al., 2005). The few studies on the impact of MCs on hatching percentage, development time or survival of progeny always involve fish (Oberemm et al., 1999; Jaquet et al., 2004; Liu et al. 2002; Huynh-Delerme et al., 2005; Lecoz et al., 2008). Due to MC-accumulation in *L. stagnalis* (Zurawell et al, 1999; Gérard, et al., 2005; Lance et al., 2006), one can expect a transfer of MCs to the offspring during parental contamination with potent deleterious effects. MC-transfer from females to their developing embryos in the oviduct pouch has been recently demonstrated for the first time in the field for the ovoviviparous prosobranch *Bellamya aeruginosa* (Viviparidae) (Zhang et al., 2007). For another ovoviviparous prosobranch *Melanoides tuberculata* (Thiaridae), Kinnear et al. (2007) demonstrated that the exposure to extracellular cylindrospermopsin (CYN), a cyanobacterial hepatotoxin, induces an increase in the number of hatchlings released from parents, but a decrease when exposed to the CYN-producing cyanobacteria *Cylindrospermopsis raciborskii*. These authors therefore suggest that consumption of toxic cyanobacteria may be more deleterious for the gastropod progeny than exposure to dissolved cyanotoxin. However, developing embryos are probably not protected in the same way against MC contamination in the brood pouch of oviduct in the case of ovoviviparous species (e.g., *M. tuberculata, B. aeruginosa*) or in the gelatinous capsule surrounding egg masses in case of oviparous gastropods as Lymnaeidae. Moreover, it is important to determine whether a further MC-exposure of egg masses and neonates originated from MC-intoxicated parents can increase the toxic effect since they stay in the parental

environment in field conditions. Zurawell et al. (2001) did not demonstrate any impact on the survival of *L. stagnalis* embryos following egg exposure to dissolved MC-LR (up to 10 µg L^{-1}), but he suggested that higher MC concentrations could induce a penetration of the toxin in the egg mass. Exposure of fish eggs and embryos to MC-contaminated water revealed that the chorion (i.e., outer membrane which surrounds the embryo) is resistant to MC penetration (Oberemm et al., 1999) except for high concentrations (> 1 mg L^{-1}) (Wiegand et al., 1999) resulting in deleterious effects. Contamination of embryos may occur via MC-transfer from MC-exposed females to the vitellus during vitellogenesis and oogenesis.

Here, we investigate the impact of MC-contamination on the fecundity (numbers of eggs and egg masses), egg hatching (delay and percentage), and neonate survival when *L. stagnalis* adults are exposed to the MC-producing (10 µg L^{-1}) cyanobacteria *Planktothrix agardhii* (PMC 75-02) or dissolved MC-LR (33 µg L^{-1}). Adult contamination was followed or not by a further MC-exposure of egg masses, and also of neonates after hatching, in order to investigate potential additional effects of intracellular or dissolved MCs. MC accumulation was measured in adults, in their faeces and egg masses, and in neonates aged of 24h, 10 and 15 days. The discussion focuses on several hypotheses according to the contamination pathway (intracellular and extracellular MCs): i) can the impact on the progeny during parental contamination be attributed to indirect negative effects of MCs (e.g. shift in allocation resources towards detoxification) or to a direct MC-contamination of egg masses before oviposition, and ii) can it be a penetration of MCs in the egg masses after oviposition and/or a MC uptake by the neonates.

3.1. Material et Methods

3.1.1. Biological material

Adults of *L. stagnalis* were obtained from a laboratory population in the Experimental Unit of the Institut National de Recherche en Agronomie (U3E INRA, Rennes). Prior to the experiment, individuals (25 ± 3 mm shell length) were isolated in glass containers of 35 mL, acclimated to the experimental conditions (12/12 L/D, 20 ± 1°C) and fed on pesticide-free lettuce for 7 days. The filamentous cyanobacterium *Planktothrix agardhii* (strain PMC 75-02) was cultured as described in Lance et al. (2006). The *P. agardhii* suspension contained a total concentration of 10 µg MC-LR equivalents (MC-LReq) per litre, measured by HPLC as described in Lance et al. (2006) (see chapter 2). For dissolved MC-exposure, MC-LR was obtained from Alexis Corporation (USA) and solubilized with 0.1% MeOH in dechlorinated water for final MC-LR concentrations of 33 µg L^{-1}.

3.1.2. Experimental set up

During 3 weeks, *L. stagnalis* individuals (160) were maintained in glass containers of 35 mL. They were divided in 4 groups of 40 individuals varying in diet and composition of the medium: «controls» (dechlorinated water and lettuce), «cyano» (cyanobacterial suspension at 10 µg MC-LReq L^{-1}), «cyanolet» (cyanobacterial suspension with lettuce), «D33let» (dechlorinated water with dissolved MC-LR at 33 µg L^{-1} and lettuce). These groups were divided in two sub-groups according to further treatments on egg masses and neonates. Egg masses were sampled every day, and maintained without adult either in the adult contaminated medium ("cyano/cyano", "cyanolet/cyanolet", "D33let/D33let") or in dechlorinated water ("cyano/water", "cyanolet/water", "D33let/water"). Cyanobacterial suspensions as well as the control medium and the dissolved MC-LR medium were renewed twice a week.

3.1.3. MC accumulation in adult and neonate snails, in egg masses and faeces

MC content was measured every week in 2 individuals per treatment, and in their egg masses and faeces. For each group, MC content in all neonates hatched from 2 egg masses

was assessed at 24h, 10 and 15 days after birth. MC extraction from snail tissues, egg masses and faeces, and analysis by immuno-assay were performed as described in Lance et al. (2006) with an ELISA Microcystin Plate Kit (Envirologix INC) with detection threshold of 0.05 µg L^{-1} and to the nearest 0.01 µg L^{-1} (Gilroy et al., 2000).

3.1.4. Evaluation of the impact on the fitness of *L. stagnalis*

Number of egg masses and egg number per mass: We did not sample egg masses laid during the first week (week 0) because ovum and spermatozoid maturation and fecundation probably occurred before the beginning of the intoxication. After the first week, egg masses were sampled every day and eggs per mass and per individual were quantified. The index d_0 represents the day of laying.

Hatching percentage at day n was calculated each day from d_0 (day of laying) to d_f (last day of hatching = hatching of the maximal number of neonates), and expressed as follows:

Hatching percentage at d_n = 100 x (Number of individual hatched at d_n/ Number of eggs at d_0)

Hatching delay corresponds to the number of days between the laying d_0 and the hatching of the maximum number of neonates d_f.

The percentage of neonate survival was assessed at 5, 10 and 15 days after the hatching of the maximal number of neonates, and was expressed as follows:

%survival d_{f+n} = 100 x (Number of neonates surviving at d_{f+n}/ Number of neonates hatched at d_f)

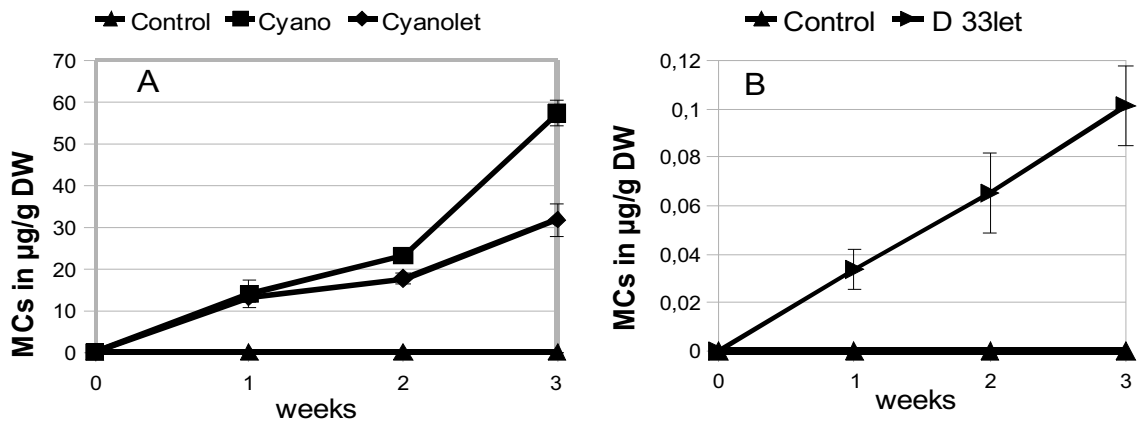
3.1.5. Statistical analysis

Data did not follow a normal distribution (according to the Kolmogornov-Smirnov test) and were thus analysed for differences between treatment groups using the Kruskall-Wallis (KW) test and 2 by 2 treatment groups using 1) the Mann-Whitney U-test for the numbers of egg masses and of eggs per mass per adult and the hatching delay, 2) the Chi2 test for the hatching percentage. Significant differences were determined at $p < 0.05$ for all statistical analyses. No statistics have been performed on the percentage of neonate survival due to a lack of data (only 4 egg masses were available for snails exposed to cyanobacteria). Data are reported as mean ± standard error (± SE).

3.2. Results

3.2.1. MC accumulation in *L. stagnalis* adults and egg masses

Snails exposed to intracellular (10 µg L^{-1}) and dissolved (33 µg L^{-1}) MCs accumulate these toxins from the first week of exposure. The accumulation is higher after ingestion of toxic *P. agardhii* vs MC-LR exposure (57.34 ± 0.20 µg g^{-1} DW after 3 weeks for the "cyano" group and 31.81 ± 0.89 µg g^{-1} DW for the "cyanolet" group vs 0.10 ± 0.03 µg g^{-1} DW for the "D33let" group) (Fig. 1). Moreover, from the second week, ingestion of toxic *P. agardhii* induces a significant higher MC-accumulation in absence ("cyano") vs presence ("cyanolet") of lettuce (Fig. 1). MCs are also detected in the faeces of intoxicated snails, in a higher concentration for the "cyano" group (1.39 ± 0.07 µg MC g^{-1} DW) versus "D33let" (0.16 ± 10^{-3} µg MC g^{-1} DW) and "cyanolet" (0.20 ± 0.01 µg MC g^{-1} DW). However, whatever the



treatment, MCs are never detected in egg masses.

Fig. 1: MC accumulation (µg MC g^{-1} DW ± SE) in adult *L. stagnalis* exposed during 3 weeks to A) toxic *P. agardhii* at 10 µg MC-LReq L^{-1} B) 33 µg dissolved MC-LR L^{-1} [Control = dechlorinated water and lettuce, Cyano = cyanobacterial suspension at 10 µg MC-LReq L^{-1}, Cyanolet = cyanobacterial suspension with lettuce, D33let = dechlorinated water with dissolved MC-LR at 33 µg L^{-1} and lettuce].

3.2.2. Impact of extracellular and intracellular MCs on the fecundity of adult *L. stagnalis*

The percentage of laying adults during the 3 weeks of exposure significantly varies among treatment groups (Test χ^2, ddl=3, $p<0.05$). Controls and snails exposed to toxic *P.*

agardhii with lettuce show a similar number of individuals laying (Test χ^2, ddl=1, p>0.05), that is significantly higher than for snails exposed to toxic *P. agardhii* alone (Test χ^2: p<0.05) or to 33 µg MC-LR L^{-1} with lettuce (Test χ^2, ddl=1, p<0.05). On average, 21.67 ± 6.32% of control snails are laying, 24.17 ± 4.95% for "cyanolet" group, 5.83 ± 2.74% for "cyano" and 5.83 ± 3.66% for "D33let" (Fig. 2 A). However, the egg number per mass is similar among all treatment groups during the 3 week-exposure (KW: ddl=3, H=0.34, p>0.05) (Fig. 2 B), with an average of 20.95 ± 2.65 eggs per mass. Consequently, the average number of eggs per individual is significantly lower in “cyano” (3.06 ± 0.90) and “D33let” (4.21 ± 1.11) groups compared to "cyanolet" (17.15 ± 2.13) and control (14.47 ± 1.84) groups (Fig. 2 B).

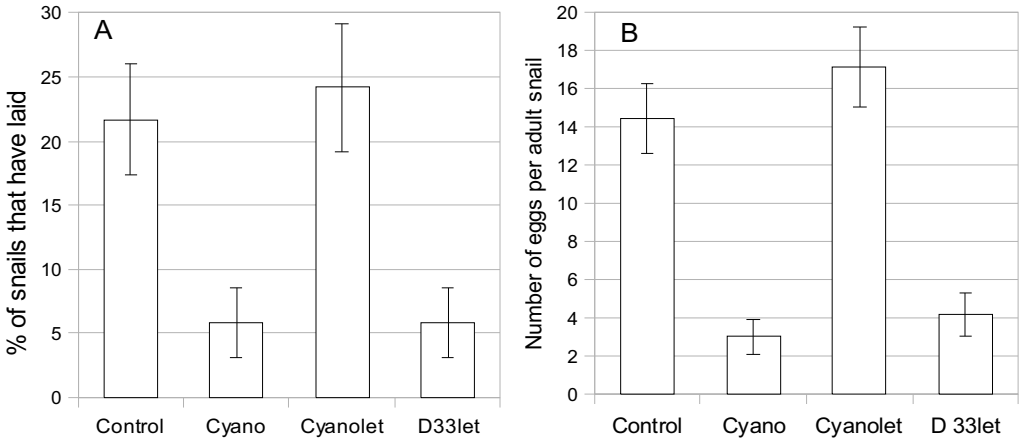


Fig. 2: Impact of MC-intoxication (exposure to *P. agardhii* at 10 µg MC-LReq L^{-1} or dissolved MC-LR at 33 µg L^{-1}) on *L. stagnalis* fecundity at the end of the 3 weeks of intoxication: A) percentage of adults laying, B) mean number of eggs per adult snail (±SE). See legend in the title of Table 1 or in part 3.1.2.

3.2.3. Impact of extracellular and intracellular MCs exposure on egg hatching

The MC-impact on hatching implies to compare on one hand control snails with treatments involving adult intoxication alone (egg masses incubated in free MC medium after oviposition), and on the other hand treatments involving adult intoxication alone with those involving intoxication of both adults and egg masses.

3.2.3.a. Impact on the hatching kinetics and percentage

Adults exposed to MCs but not egg masses after laying: The time required to reach the maximum percentage of hatching is significantly different between treatment groups (KW: ddl=3, H=51.62, p<0.05) (Fig. 3) and most eggs of all MC-exposed groups hatch significantly earlier than those of control snails (25 days). Compared to the controls, the maximal hatching

percentage is reached earlier for “cyano/water” group (16 days) (MW: W=105, $p<0.05$), followed by “D33let/water” group (18 days) (MW: W=106, $p<0.05$), and then “cyanolet/water” (20 days) (MW: W=11011; $p<0.05$).

The hatching percentage at day 15 after the egg laying is significantly different between treatment groups (KW: ddl=3, H=29.9, $p<0.05$). For control snails, only 4.18 ± 2.93% of eggs are already hatched at the 15th day, which is significantly lower than for intoxicated snails: “cyano/water”: 80.46 ± 7.45%, “cyanolet/water”: 61.25 ± 6.84%, “d33let/water”: 63.23 ± 17.75% [MW: W(controls, “cyano/water”)=82, W(controls,”cyanolet/water”)=450, W(controls, “D33let/water”)=80; $p<0.05$] (Fig. 3).

Regardless of the duration between oviposition and hatching, the maximal hatching percentage is similar between all groups (KW: ddl=3, H=1.95, $p>0.05$), with a mean of 84.18 ± 2.02% of eggs (Fig. 3).

***Adults and egg masses both exposed to MCs*:** The time required to reach the maximum percentage of hatching is significantly different between treatment groups (KW: ddl=5, H=64.47, $p<0.05$). Eggs of the "d33let/d33let" group are hatching significantly later (day 25) than those of "d33let/water" (day 18) (W=120, $p<0.05$). A similar pattern is observed for eggs of "cyano/cyano" (day 21) vs "cyano/water" (day 16) and of "cyanolet/cyanolet" (day 24) vs "cyanolet/water" (day 24) [MW: W(cyano/water,cyano/cyano)=246; W(cyanolet/water,cyanolet/cyanolet)=5951, $p<0.05$)] (Fig. 3).

The maximal hatching percentage varies between groups (KW: ddl=5, H=14.51, $p<0.05$). Eggs exposed to dissolved MC-LR have a significant lower maximal hatching percentage than eggs in free MC water (64.09 ± 6.19% vs 90.00 ± 2.46%) (MW: W=190, $p<0.05$) (Fig. 3). Exposure of eggs to toxic cyanobacteria have no effect on their hatching percentage and differences between "cyano/cyano" vs "cyano/water" groups and between "cyanolet/cyanolet" vs "cyanolet/water" are not significant (MW, W=179 and W=127, $p>0.05$) (Fig. 3).

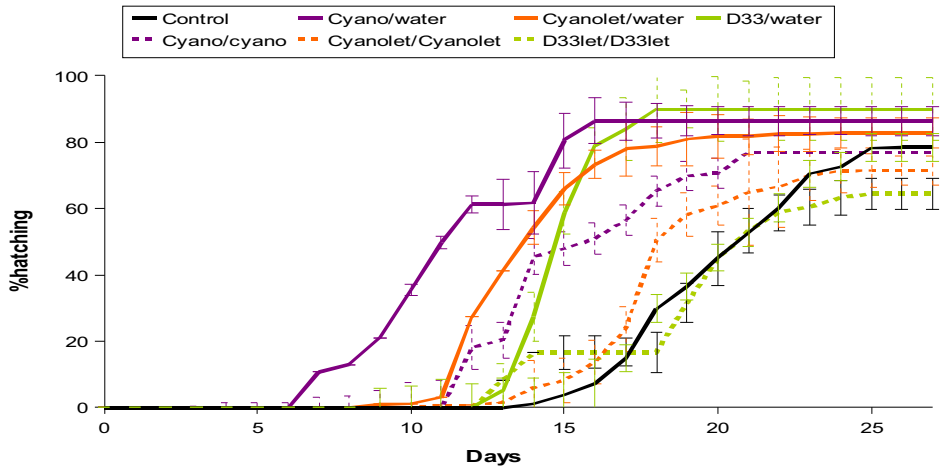


Fig. 3: Kinetics of hatching percentage (± SE) for eggs laid by intoxicated *L. stagnalis* adults (exposed during 3 weeks to *P. agardhii* at 10 µg MC-LReq L^{-1} or dissolved MC-LR at 33 µg L^{-1}) and exposed or not to MCs after laying, as a fonction of days after laying. See legend in the title of Table 1 or in part 3.1.2.

3.2.3.b. Impact on the total number of neonates born during the experiment

***Adults exposed to MCs but not egg masses after laying*:** The total number of neonates born per adult in 3 weeks is similar for controls (12.6 ± 3.71.) and snails exposed to toxic *P. agardhii* with lettuce (10.3 ± 2.99). However, snails exposed to *P. agardhii* alone or to 33 µg MC-LR L^{-1} with lettuce showed far less descendants per adult than controls (respectively 3.45 ± 0.09 and 3.70 ± 0.11 for "cyano/water" and "d33let/water").

***Adults and egg masses both exposed to MCs*:** The total number of neonates born per adult in 3 weeks is similar when egg masses are previously exposed to toxic cyanobacteria or incubated in free MC water: respectively 10.10 ± 1.47 and 10.30 ± 2.99 for "cyanolet/cyanolet" and "cyanolet/water"; and respectively 3.45 ± 0.09 and 2.60 ± 0.07 for "cyano/cyano" and "cyano/water". However, the neonate number is lower when egg masses are previously exposed to dissolved MC-LR (2.45 ± 0.07 for "d33let/d33let") vs incubated in free MC water (3.70 ± 0.11 for "d33let/water").

3.2.4. Impact of extracellular and intracellular MCs on neonates

3.2.4.a. Impact on the neonate survival

***Adults exposed to MCs but not egg masses after laying*:** The percentage of survival at 5, 10 and 15 days of neonates born from adults exposed to 33 µg MC-LR L^{-1} is similar to that of

neonates from control snails (Fig. 5A). However, survival of neonates from snails exposed to toxic cyanobacteria, with or without lettuce, is lower compared to controls (Fig. 4A). The survival rate of 15 day-neonates is 88.15% ± 1.15% for "controls" and 87.67% ± 1.24% for "d33let/water", against 82.90% ± 1.07% for "cyanolet/water" and 70.59% ± 1.41% for "cyano/water" groups.

***Adults and egg masses both exposed to MCs*:** The continuous presence of extra- or intra-cellular MCs in the medium of egg masses, and then neonates, tends to reduce neonate survival. The neonate survival at 5, 10 and 15 days for adult snails and egg masses both exposed to 33 µg MC-LR L^{-1} ("d33let/d33let") is lower than when egg masses are incubated in free MC water ("d33let/water") (Fig 5B). A similar pattern is observed for the "cyanolet/cyanolet" versus "cyanolet/water" groups (Fig 5B). The mean percentage of survival of juveniles from the "cyano/cyano" groups could not have been calculated because only 2 egg masses were laid (Fig 4B). The survival rate of 15 day-neonates is respectively 77.78% ± 2.94% for "d33let/d33let" and 60.42% ± 1.68% for "cyanolet/cyanolet" groups.

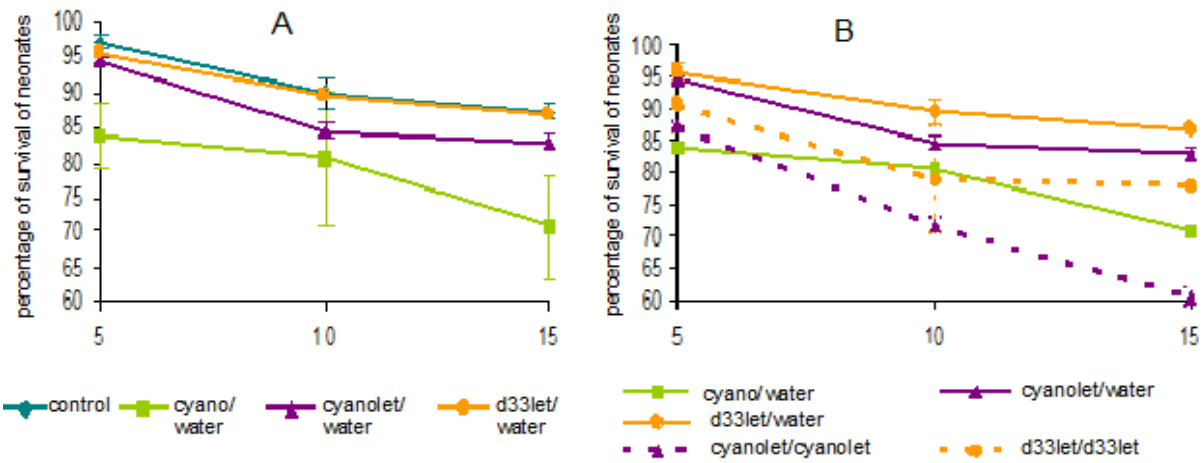


Fig 4 : Percentage of survival (± SE) of neonates at 5, 10 and 15 days according to A) treatments of parent snails (exposed during 3 weeks to *P. agardhii* at 10 µg MC-LReq L^{-1} or dissolved MC-LR at 33 µg L^{-1}), and B) the further treatment after laying of egg masses and neonates. See legend in Table 1 and part 3.1.2.

3.2.4.b. MC accumulation in *L. stagnalis* neonates

When egg masses produced by MC-intoxicated adults are placed in free MC water after laying ("cyanolet/water", "d33let/water"), no MC is detected in the tissues of neonates (table 1). No MC accumulation is also found in neonates exposed continuously to dissolved

MC-LR ("d33let/d33let"). But neonates accumulate MCs from the first day after their birth when they are exposed continuously to toxic cyanobacteria ("cyanolet/cyanolet").

	24h	10 days	15 days		24h	10 days	15 days
Controls	0	0	0	cyano/cyano	0.33 ± 0.08	0.32 ± 0.10	0.50 ± 0.15
cyanolet/water	0	0	0	d33let/water	0	0	0
cyanolet/cyanolet	0.18 ± 0.05	0	0.07 ± 0.01	d33let/d33let	0	0	0
cyano/water	0	0	0				

Table 1: MC accumulation (± SE) (µg g^{-1} DW) in *L. stagnalis* neonates 24 hours, 10 and 15 days after their birth, according to treatments. See legend in the title of Table 1 or in part 3.1.2.

3.2.5. Impact of extra-and intra-cellular MC-exposure on the overall fitness

***Adults exposed to MCs but not egg masses after laying*:** At the end of the experiment, the total number of surviving 15-days old descendents per *L. stagnalis* adult tends to be lower for snails exposed to toxic cyanobacteria alone (2.43 ± 0.49) and to dissolved 33 µg MC-LR L^{-1} (3.20 ± 0.63) vs control snails (10.90 ± 1.83) (Fig. 5).

***Adults and egg masses both exposed to MCs*:** The number of surviving 15-days old neonates per adult snail tends to be lower when egg masses are previously exposed to intra- (no juvenile surviving) or extra-cellular MCs (1.90 ± 0.41) compared to those placed in free MC water (Fig. 5).

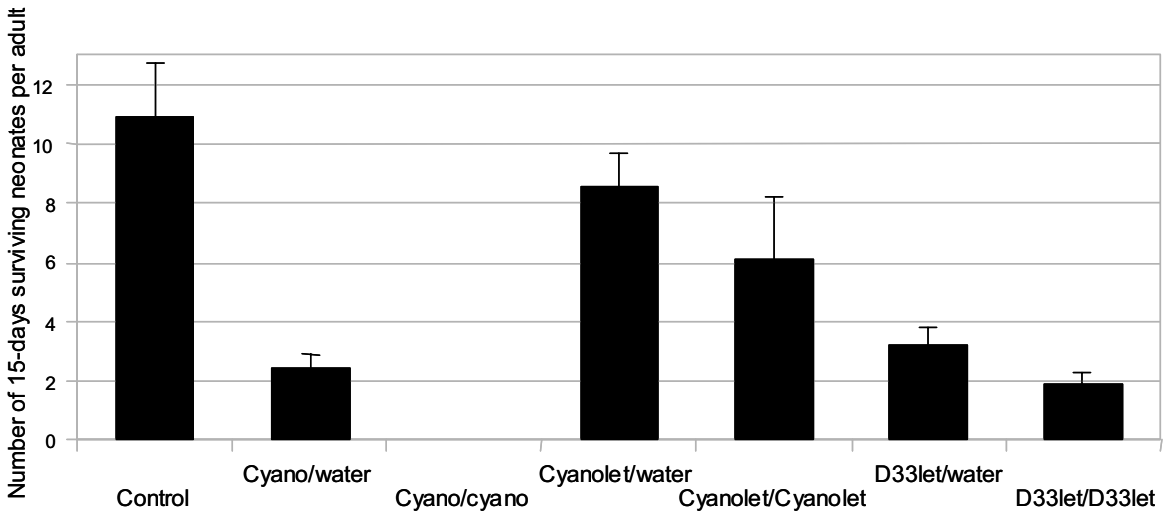


Fig 5 : Number of 15-days old neonates surviving per *L. stagnalis* adult according to A) treatments of parents, B) further treatment of egg masses and neonates. See legend in the title of Table 1 or in part 3.1.2.

3.3. Discussion

Contamination of L. stagnalis adults by toxic cyanobacteria or dissolved MC-LR exposure

L. stagnalis adults accumulated MCs in their tissues after 3 weeks of ingestion of toxic cyanobacteria (10 µg MC-LReq L^{-1}) or exposure to dissolved MC-LR (33 µg L^{-1}), but in a far less extent for this latter contamination pathway (i.e. 570 times less). These results are in accordance with previous studies (Gérard & Poullain, 2005; Lance et al., 2006), demonstrating a ratio of 1300 between MC accumulation after a 5-week ingestion of toxic cyanobacteria (5 µg MC-LReq L^{-1}) and a 6-week exposure to dissolved MC-LR (33 µg L^{-1}). Field studies also found that MC accumulation in gastropods is generally depending on MC level in the phytoplankton (and not often correlated to dissolved MCs), thus mostly due to ingestion of toxic cyanobacteria (Zurawell et al. 1999, Yokoyama and Park, 2002; Chen et al., 2005; Xie et al., 2007).

Impact of extra- and intra-cellular MCs on the fecundity of adult L. stagnalis

The fecundity of *L. stagnalis* was significantly decreased when exposed to toxic cyanobacteria or to dissolved MC-LR, as already demonstrated respectively by Lance et al. (2007) and Gérard et al. (2005). According to these authors, ingestion of toxic cyanobacteria also induced a decrease of growth, not observed with dissolved MC exposure. Lance et al. (2007) interpreted this difference as a weak penetration of the dissolved MC-LR in the snails. However, this study reveals that dissolved MCs, resulting in a far less accumulation than intracellular ones in exposed gastropod tissues, induced a similar negative impact on egg numbers laid by intoxicated *L. stagnalis* over 3 weeks. Even if both MC-contamination routes lead to a decreased fecundity of the snails, processes implied in this side-effect are different. Indeed, after ingestion of toxic cyanobacteria by gastropods, a disruption of cells is expected to occur in their gizzard (Carriker, 1946). A fraction of the released MCs is then eliminated in the gizzard string fraction of the faeces, and the other fraction enters the digestive gland and is accumulated in the digestive cells or excreted in the digestive gland string fraction of the faeces (Carriker, 1946; Zurawell et al., 2006). Here, we demonstrate the presence of MCs in body tissues (up to 57.34 ± 0.20 µg g^{-1} DW) and faeces (up to 1.39 ± 7.10^{-2} µg g^{-1} DW) of *L.*

stagnalis after 3 weeks of intoxication. MC accumulation mainly occurs in the digestive gland (Lance et al., 2006), and is possibly followed by its metabolization that may limit MC-transport towards other organs. Accumulated MCs can be metabolized into less harmful compounds after conjugation with glutathione via glutathione-S-transferase (GST), resulting in MC excretion or physiological degradation. Such detoxification processes have been shown to occur in various organisms (e.g., plants, invertebrates and vertebrates) (Pflugmacher et al., 1998; Wiegand et al., 1999; Pietsch et al., 2001). Resources allocated to such detoxification processes, which have a high energy cost, should be derived from a common pool of limited resources, inducing trade-offs that probably affect the fecundity. The fact that snails exposed to toxic cyanobacteria with lettuce accumulated less MCs and showed a minor decrease in fecundity seems to confirm this hypothesis. As an additional energy resource, lettuce would allow both concomitant reproduction and detoxification. Contamination by dissolved MCs can occur through oral water intake [8-12 µl/g/h for *L. stagnalis* (De Witt, 1996)] or penetration via epidermal cells, particularly where tegument is thinner as in the pulmonary cavity, highly vascularized (Dillon, 2000). MCs may thus be distributed in the entire body via haemolymph, before being accumulated in the digestive gland. Therefore, the strong decrease in the number of egg-masses laid by *L. stagnalis* exposed to dissolved MC-LR may be a consequence of a direct dysfunction in genital gland (gametogenesis) and/or accessory sexual organs (albumen, nidamental and prostate glands, spermatheca) due to the occurrence of MC-LR in the haemolymph, and not the result of a trade-off as suggested with intracellular MC contamination.

Impact of extracellular and intracellular MC exposure of L. stagnalis adults on egg hatching and neonate survival

Despite the fecundity of *L. stagnalis* was decreased due to intracellular or extracellular MC-exposure, there was no impact on the hatching percentage as demonstrated by Coutellec and Lagadic (2006) and Leung et al. (2007) for *L. stagnalis* exposed to xenobiotics. In our study, the development duration of the embryos was significantly reduced with both MC-intoxication pathways in contrast to exposure to pesticide and heavy metals, also reducing the gastropod fecundity (Singh et Agarwal, 1986; Gomot, 1998; Ellis-Tabanor, 2005; Coutellec et al., 2008). In the case of the ovoviparous *M. tuberculata* exposed to extracellular CYN, the number of hatchlings released from parent snails increased, suggesting that stress due to this

cyanotoxin induced abortion (Kinnear et al., 2007). The toxicity of MC-LR has been demonstrated for fish embryos, i.e., decreased hatching rate, growth and survival, increased frequency of abnormal embryos (Oberemm et al., 1999; Wiegand et al., 1999; Huynh-Delerme et al., 2005; Liu et al 2002). Hatching of medaka (*Oryzia latipes*) embryos was dose-dependent advanced after injection of MC-LR into the vitellus (Jacquet et al., 2004). A similar but less severe effect was also recorded after injection of the cyanobacterial strain we used here (*P. agardhii* PMC 75-02) (Lecoz et al., 2008). In contrast, we showed that the hatching occurred earlier when *L. stagnalis* parents were exposed to toxic *P. agardhii* vs dissolved MC-LR, and only the former contamination pathway induced a negative impact on the progeny survival. The decrease of both development duration of *L. stagnalis* embryos and neonate survival may be attributed to a parental effect (changes in energy allocation that may alter offspring development) and/or to a direct impairment of egg viability induced by MCs. Consequently, despite MC accumulation mainly in the digestive gland and excretion in faeces occurring after toxic cyanobacteria ingestion, and in a far less extent after dissolved MC-LR exposure, it is not excluded that a part of MCs can reach the genital gland and/or the accessory organs via haemolymph transport with potent deleterious effects. Indeed, Zhang et al. (2007) demonstrated a high positive correlation between MC content of the offspring and the gonad of ovoviparous *B. aeruginosa* females, indicating that MCs could be transferred from adult females to their young with physiological connection. In oviparous gastropod species, gametes may be contaminated by MCs in the genital gland during gametogenesis, and also vitellogenesis for oocytes. Later, MC-contamination of eggs may occur during the construction of the egg masses by accessory sexual organs in the parental organism. Indeed, after internal fertilization, eggs are enclosed in perivitelline fluid (containing proteins and calcium) from the albumen gland and coated by a perivitelline membrane, then they are further encapsulated in the oviduct with mucopolysaccharides and mucoproteins (with which MCs might be bound) secreted by various tissues before oviposition (Dillon, 2000). Even if the occurrence of MCs was demonstrated in spermatozoa and oocytes in the genital gland (Chapter 5), MCs were not detected in eggs laid by intoxicated *L. stagnalis* in the present study. Further investigations are needed in order to assess how MCs can affect the functioning of genital gland and accessory sexual organs as well as gametogenesis, vitellogenesis and embryogenesis of freshwater gastropods.

Egg masses laid by intoxicated parents and exposed to extracellular and intracellular MCs: impact on hatching, survival and MC accumulation in neonates

Egg masses exposed to dissolved 33 µg MC-LR L^{-1} after laying (MC-LR intoxicated parents) showed a decreased hatching percentage and an increased hatching delay compared to those incubated in non toxic water, suggesting the MC-LR entrance in egg masses. The MC-uptake by eggs has been demonstrated for the zebrafish *Danio rerio* exposed to 2.5 mg ^{14}C-labelled MC-LR L^{-1}, followed by an activation of two metabolization/excretion enzymes (GST and glutathione peroxidase) (Wiegand et al., 1999). In gastropods, no impact was observed by Zurawell (2001) on embryo survival of *L. stagnalis* after egg exposure to lower MC-LR concentrations (up to 10 µg L^{-1}). But, the author suggests that at higher concentration, MC-LR may penetrate in the egg mass via the tunica capsule, potentially damaged in the parent and/or in the external medium. The tunica capsule imbibes water over time in order to soften the egg mass and to allow developed snails to escape into their environment (Dillon, 2000). MC-penetration in egg masses can impair the common matrix surrounding the egg cells or the lamellar membranes enveloping each egg within the mass. The exposure of egg masses to toxic cyanobacteria only induced a delayed hatching, and was less severe than dissolved MC-LR exposure. Since toxic cyanobacteria cannot penetrate in egg masses, the negative effect recorded can be explained by the presence of few MCs released by cyanobacteria due to the senescence of some cells (Chorus et Bartram, 1999) and potentially penetrating in egg masses. This would require further investigations.

Continuous exposure to dissolved MC-LR after oviposition did not induce MC accumulation in neonates, but neonates exposed to toxic cyanobacteria accumulated MCs from the first day after hatching, suggesting that they can ingest toxic cyanobacteria very early in their life.

At the end of the experiment, both the proportion of successfully developed surviving 15-day old snails and the neonate survival were reduced when parents have been exposed to toxic cyanobacteria and dissolved MC-LR (vs controls). Moreover, synergistic effect occurred when egg masses stay in the toxic parental medium. Previous experiment on 2-month old juveniles of *L. stagnalis* showed no effect on growth and survival when exposed to dissolved MC-LR (Gérard et al., 2005) but a decrease in growth when exposed to toxic cyanobacteria (Lance et al., 2007). The 15-day old snails have probably less physiological capacities to detoxify and survive under cyanobacterial stress than 2-month old snails. These results

confirm the differential effect of MCs on gastropods according to the age and the contamination route, and suggest a potentially strong demographic impact on natural gastropod populations. There would need to be strengthened by deeper investigations in the field.

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Chapitre 4 :
Impact des cyanobactéries toxiques sur la communauté de gastéropodes du lac de Grand Lieu (44)

Impact of toxic cyanobacteria on gastropod communities in a eutrophic lake (Grand-Lieu, France)

Projet d'article

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Introduction

Gastropods, including Pulmonata and Prosobranchia, are generally common in fresh waters where they can be abundant and represent up to 90% of invertebrate biomass (Hawkins & Furnish, 1987; Habdija et al., 1995; Strong et al., 2008; Balian et al., 2008). As primary consumers, they constitute an important link between primary producers (e.g., potentially toxic cyanobacteria) and higher consumers (e.g., crayfish, fish, and waterfowl) (for reviews: Michelson, 1957; Dillon, 2000). The contamination of gastropods by MCs can occur mainly via feeding activity on toxic cyanobacteria and via absorption of cyanotoxins that are dissolved or adsorbed on particles and food items. Gastropods are also expected to be contaminated during gill or pulmonary breathing. In the field, positive relations between the MC concentrations in the phytoplankton and accumulation in gastropods suggest that they are primarily contaminated by consumption of toxic cyanobacteria (Kotak *et al.*, 1996; Zurawell *et al*, 1999; Ozawa *et al*., 2003; Chen *et al*., 2005). However, Zhang *et al* (2007) recently demonstrated that MC accumulation in the prosobranch *Bellamya aeruginosa* was correlated with both intracellular and extracellular MCs in the field. In the laboratory, consumption of toxic cyanobacteria (intracellular MCs) seems to be the major contamination pathway for the pulmonate *Lymnaea stagnalis* leading to a great accumulation compared to that induced by absorption of dissolved MC, whereas both intracellular or extracellular MC exposures seem equally contaminate the prosobranch *Potamopyrgus antipodarum* (Gérard & Poullain, 2005; Gérard, et al., 2005; Lance *et al*., 2006; 2007; 2008). According to these authors, the prosobranch is more susceptible to dissolved MC-LR exposure (decreased survival, growth and fecundity) than to ingestion of toxic cyanobacteria (decreased growth and fecundity), whereas it is the contrary for the pulmonate (decreased growth and fecundity with toxic cyanobacteria ingestion vs only decreased fecundity with dissolved MC-LR exposure).

Pulmonates show fundamental broader physiological and ecological tolerances compared to freshwater prosobranchs, and greater genetic and phenotypic plasticity (for reviews: Russel-Hunter, 1978; Aldridge 1983; MacMahon 1983; Dillon 2000). According to their differences in feeding habits, ecology and physiology, one can expect that natural populations of gastropods will be affected in waters where toxic cyanobacteria often proliferate, but probably not to the same extent between pulmonates and prosobranchs. In a long term field study, Gérard et al. (2008) related the decline of a gastropod community to

recurrent toxic cyanobacterial proliferations. Moreover, changes in the structure of several molluscan communities differently exposed to toxic cyanobacteria were recently demonstrated by Gérard et al. (2009): pulmonates (vs prosobranchs and bivalves) constituted the dominant taxa in waters exposed to high densities of cyanobacteria. Moreover, MC accumulation was found to be higher in pulmonates (vs prosobranchs), in the laboratory (Gérard et al., 2005; Lance et al., 2006, 2008) and in the field (Zurawell et al., 1999, Ozawa et al., 2003; Chen et al., 2005; Gkelis et al., 2006; Zhang et al., 2007; Gérard et al., 2009).

Our study-site is the protected Grand Lieu Lake, inhabiting by several hundred animal species (approximately 250 bird and 50 mammal species). This freshwater ecosystem is one of the richest in France. However, the lake has become increasingly eutrophic since 1960s, mainly because of agricultural pollution, and since 1980's, submerged macrophytes almost completely disappeared while turbidity dramatically increased due to yearly cyanobacterial blooms (Marion and Brient, 2000; Vezie et al., 1998; Paillisson and Marion, 2002). Three stations were chosen according to their differences in cyanobacteria and MC occurrence (low, medium and high contamination) in the past few years.

To quantify the impact of cyanobacteria on gastropod communities, we first assessed monthly inter-station variation in cyanobacterial proliferations (density and composition of communities) and measured MC concentrations in the three stations, during one year. We also sampled gastropods in these stations to investigate how the cyanobacteria occurrence may influence the community structure. We predicted that gastropod abundance and richness would be lower at highest cyanobacteria densities with differences depending on the taxonomic group considered (prosobranchs vs pulmonates). Secondly, to investigate MC accumulation in the gastropod community related to cyanobacterial density and toxicity and to the taxonomic group considered, we measured MC accumulation in snails sampled monthly during the year in the three stations. We expected that MCs would be detected at higher concentrations in snails inhabiting sites subjected to the highest densities of cyanobacteria, and in pulmonates vs prosobranchs. Thirdly, we encaged in the three stations the two gastropod species previously studied in the laboratory, *L. stagnalis and P. antipodarum* (Gérard & Poullain, 2005; Gérard et al., 2005; Lance et al., 2006, 2008), in order to confirm differences in MC accumulation in the field. Results are discussed in terms of both negative impact of toxic cyanobacteria on the dynamic and composition of gastropod communities, and MC accumulation among prosobranchs and pulmonates.

4.1. Material and Methods

4.1.1. Study-site

Grand-Lieu Lake is a large shallow eutrophic natural floodplain system located in Western France (Eastern Brittany, 47°05'N,1°39'W), which covers 4000 ha during summer and 6300 ha during winter, by flooding adjacent peaty marsh grasslands. During the summer, half of the area is composed of a peat fen with *Phragmites*, *Salix* and *Alnus*, which becomes progressively exposed in early summer, and provides a large amount of potential habitats for gastropods. Most of the permanently flooded area of the lake is covered from April to October by extensive beds of floating-leaved macrophytes (about 1000 ha of *Nymphae alba*, *Nymphae lutea*, *Trapa natans*) (Paillisson and Marion, 2002). Water level fluctuations of the lake follow the seasonal cycle of rainfall in the catchment and the water depth reaches up to 2.2 m in the floating macrophyte area from November to May. From June to September, the water level of the lake generally falls by 0.40 m in the shores and by 0.70 m in the floating macrophyte area (Marion and Brient, 1998). Three shoreline stations where cyanobacteria have already been recorded and varying in abundance were defined for both gastropod caging experiment and field study (Fig. 1): Malgogne (drainage channel with slow running water) where cyanobacteria rarely occurred during the past 10 years, Senaigerie (stagnant water) that was contaminated yearly from the 2000s by recurrent blooms of toxic cyanobacteria but at lower densities (up to 250 000 cell/ml) and with lower intracellular MC concentration (up to 2.9 µg MC/l) than Capitaine (stagnant water), that was contaminated yearly from the 2000s by recurrent blooms of toxic cyanobacteria (up to 1 000 000 cell/ml and 8 µg MC/l) (L. Brient, unpublished data).

4.1.2. Water and phytoplankton samplings and MC analysis

Water and phytoplankton were sampled monthly from March 2006 to March 2007 at all three stations, but fortnightly in May, June, August, September and October 2006 (17 samplings per site, total of 51 samplings in all sites). The samples were collected with a 1 meter column without concentration, stored in the dark and used for species identification and enumeration of cyanobacteria and other phytoplankton (Chlorophyceae, Zygophyceae, Chrysophyceae, Dinophyceae, Cryptophyceae, Euglenophyceae, Diatoms). When possible,

cyanobacteria were identified to the species level, and their densities (cells.mL^{-1}) were determined using a microscope with a Nageotte chamber (50 µL). Frequencies of occurrence (FO) (% of samplings a species is collected in the 17 samplings per station or 51 samplings for all stations) were calculated for each cyanobacteria species with density > 20 000 cell.mL^{-1}, and whatever the density. The concentrations of intracellular MCs in cyanobacteria and dissolved MCs in water samples were determined using a HPLC with diode array detection (HPLC-DAD) and a variable-wavelength UV detector operating at 238 nm. Prior to HPLC analysis, cells harvested by filtration (nylon cloth, 2 µm pore size) were suspended in 0.5 mL of 85% methanol in water and centrifuged at 7000 G for 7 min. The volume injected was 20 µl with a flow rate of 1 mL.min^{-1}. The separation was performed on a microsphere C18 reverse-phase column (3 µm) under isocratic conditions with a mobile phase of 10 mm ammonium acetate and acetonitrile (7.4:2.6) for 10 min. As MC-LR was the standard used, concentration was expressed as microgram cellular MC-LR equivalents (MC-LReq) per litre.

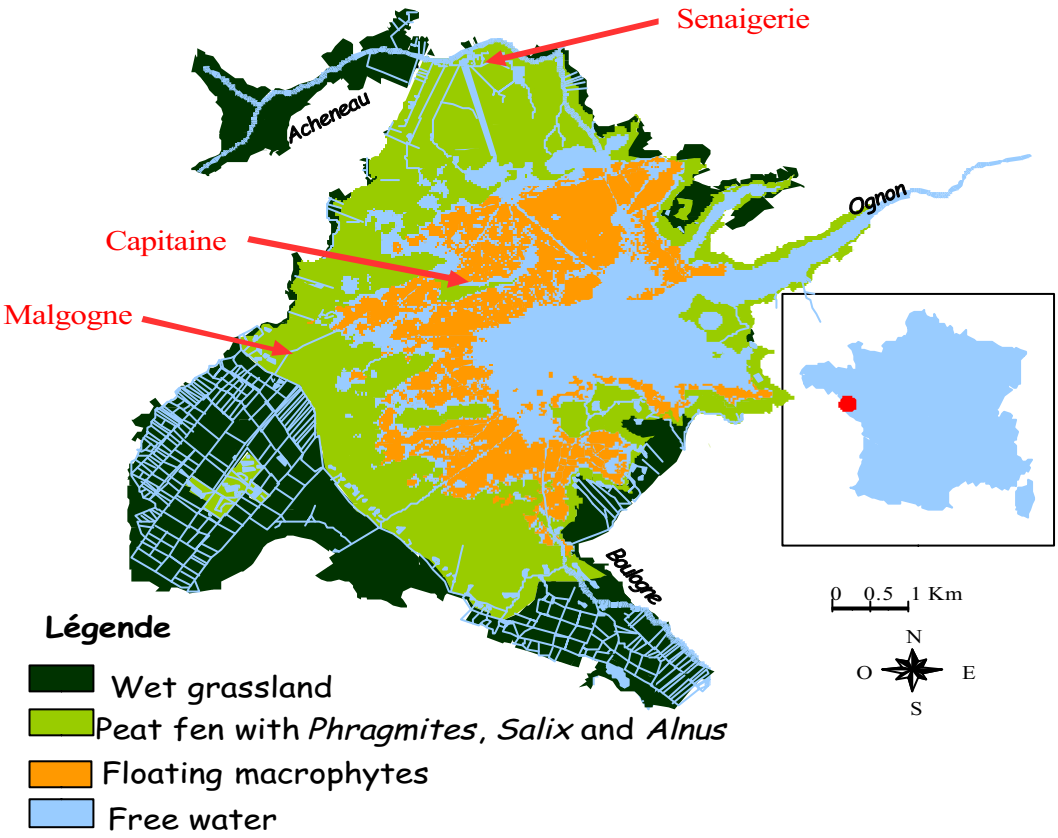


Figure 1: Ecological regions of the Grand Lieu Lake (France) and study-stations (Capitaine, Sénaigerie, Malgogne), by J.M. Paillisson.

4.1.3. Gastropod samplings and caging experiment

Gastropods were collected every month from March 2006 to March 2007 (except in December 2006) at the 3 stations (Malgogne, Senaigerie, Capitaine) using a pond-net (nylon mesh: 1 mm, square aperture: 0.5 x 0.5 m) and sweeping the column of water in a littoral area of 10 m length, 2 m width and 2 m maximum depth, during 3 minutes. Samplings were examined in the laboratory and all gastropods were systematically identified according to Gloër & Meier-Brook (1994), and measured (height for conic shells, diameter for discoid shells) with Pegase Pro Software (precision: 0.1 mm). The variables used to characterize the gastropod communities were: species richness, abundance, percentage of abundance (relative abundance of a species), and frequencies of occurrence (percentage of months a species is collected in the 11 sampling months per station and 33 for all stations). The rarest snail species, i.e., with a frequency of occurrence less than 5% (i.e., *Planorbis carinatus, Lymnea palustris, Physa heterostropha, Ferissia wautieri, Anisus leucostoma and Viviparus lacustris*) were excluded from statistical analysis. MCs were quantified monthly in the tissues of almost all gastropods sampled, using one or several individuals, depending on their body fresh weight (FW), excepted for few species for which the amount of fresh body tissues was not sufficient. A total of 113 MC analyses were performed corresponding to 22 gastropod species.

The two gastropod species used for the caging experiment, *Potamopyrgus antipodarum* (Prosobranchia) and *Lymnaea stagnalis* (Pulmonata), were originated respectively from an established laboratory population [Experimental Unit of the Institut National de Recherche en Agronomie, Rennes, France] and from a field population of parthenogenetic females in a wetland stream (Pleine-Fougères, France). All gastropods were mass-reared under laboratory conditions (20 ± 1°C, LD: 12-12, in dechlorinated tap water) and fed on dried lettuce *ad libitum*. From May to August 2006, 200 *P. antipodarum* and 54 *L. stagnalis* individuals (respectively 3.5 ± 0.5 and 30 ± 3 mm in shell length) were enclosed at each station in cages (40 cm length, 40 cm large and 6 cm depth for *P. antipodarum* and 40 cm length, 40 cm large and 50 cm depth for *L. stagnalis*) respectively of 1 and 10 mm monofilament nylon mesh with wood support frames. These size meshes allowed water and phytoplankton to move freely into the cages, creating an environment similar to the water lake. The cages with pulmonates were half maintained submerged and half emerged, and those with prosobranchs were completely submerged and maintained closer to the sediments,

both attached to a 5-meter length brass stake. This design allowed prosobranchs to use sediment for nutrition and pulmonates to aerial breathe at the water surface. Survival of snails was assessed 2 times per month during the experiment. MC measurement in gastropod tissues was made for 30 individuals of *P. antipodarum* and 6 of *L. stagnalis* at 20 (10th June), 50 (10th July) and 80 (11th August) days after their introduction in the lake. A replicate consisted of 10 *P. antipodarum* or 2 *L. stagnalis* pooled for analyses (i.e. 3 replicates per species).

4.1.4. MC analyses in gastropods

For the caging experiment or field study, snails were first removed from their shell, freeze-dried, and weighted. The MC analysis was performed by a method of immuno-assays with an ELISA Microcystin Plate Kit (Envirologix INC) which detects all of the 6 purified hepatotoxins of common bloom-forming cyanobacteria, especially MC-LR and MC-RR, from 0.05 μgL^{-1} threshold and to the nearest 0.01 μgL^{-1} (Gilroy *et al*., 2000). MCs were extracted with 2 mL of 100% methanol, as described in Lance et al. (2006), and MC concentration in the gastropods was expressed in MC-LReq $\mu g\ g^{-1}$ DW.

4.1.5. Data analyses

We examined pairwise relationships between intracellular MCs in cyanobacteria, abundance of cyanobacteria potentially producing MCs and total cyanobacteria abundance, using linear (proportionality) or exponential (threshold effect) correlations. The retained correlation model was that for which the r value was the highest. One-way ANOVAs were performed to test for differences in abundance and species richness of pulmonates, prosobranchs and the whole gastropod community according to stations, and also, in MC concentration in *Physella acuta* (the only gastropod species occurring in the 3 stations) according to stations. All data were log-transformed to meet assumptions of normality and variance homogeneity. Differences were regarded as significant when $P = 0.05$. Data are reported as mean ± standard error (SE).

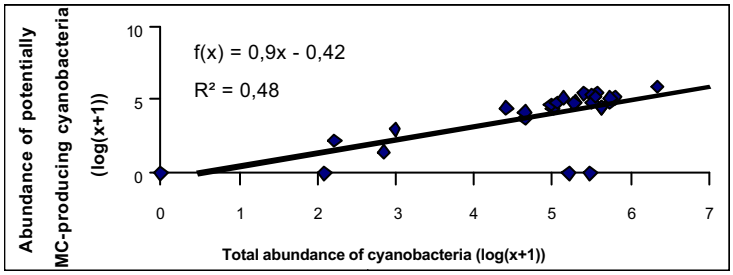
4.2. Results

4.2.1. Cyanobacteria contribution to phytoplankton and MC concentrations

4.2.1.a. ***Among station variation in cyanobacteria proliferation***

From March to November 2006 (i.e. proliferation period), cyanobacteria represented from 0 to 99.8% (mean 59.8 ± 9.1%) of phytoplankton community at Malgogne, against respectively from 57.2 to 97.4% (mean 75.9 ± 5.1%) at Senaigerie and from 58.9 to 99% (mean 78.6 ± 4.6%) at Capitaine (Fig. 2). Malgogne was the lower cyanobacteria-contaminated station (an average 68 400 ± 23 600 cell mL all over the year vs 200 900 ± 45 000 at Senaigerie and 485 500 ± 153 400 at Capitaine) (Fig. 3). At Malgogne, cyanobacterial densities were inferior to 15 000 cells mL^{-1} during the study, except from July to October 2006 where they were comprised between 51 000 and 318 000 cells mL^{-1}. At Capitaine and Senaigerie, cyanobacteria densities were superior to 45 000 cells mL^{-1} (excepted from November 2006 to February 2007). Cyanobacteria were particularly abundant during the spring 2006 in Capitaine (> 2 000 000 cells mL^{-1}) and Senaigerie (up to 616 000 cells mL^{-1}).

A total of 19 cyanobacteria species were identified during the study, among them 7 species potentially producing MCs (*Anabaena flos-aquae, Microcystis aeruginosa, Microcystis flos aquae, Microcystis wesenbergii, Oscillatoria sp, Plankthotrix agardhi*) (Table 1). In the 3 stations, the potentially producing MC-species were less abundant than non MC-producing species (Fig. 3), but their abundances correlated well (see below).



The potentially producing MC-species the most frequently sampled was *Planktothrix agardhii* (FO 35.3%), followed by *Oscillatoria* sp. (13.7%), and *Microcystis floas-aquae* (11.7%) (Table 1). At Malgogne, 5 potentially producing MC-species were present in June, July, and October (FO 17.6%) among them 3 with densities superior to 20 000 cells mL^{-1} (Table 1). At Malgogne, the mean density of potentially producing MC-species was 17 900 ± 10 600 all over the year (Fig. 3). At Senaigerie and Capitaine, 7 potentially producing MC-species occurred in 70.6% of samplings, among them 5 at Senaigerie and 6 at Capitaine with

densities > 20 000 cells mL^{-1} (Table 1). At Senaigerie and Capitaine, the mean densities of potentially producing MC-species were respectively 60 900 ± 12 100 and 124 400 ± 32 300.

Table 1: Cyanobacteria frequencies of occurrence (FO, %) at density > 20 000 cell/mL and whatever the density in brackets of species sampled in the 3 study-stations (Capitaine, Sénaigerie, Malgogne) of the Grand Lieu Lake from March 2006 to March 2007. The cyanobacteria potentially producing MCs (MC-prod) (Chorus et Bartram, 1999) are indicated in red.

Species	FO of species at density > 20 000 cell/mL and whatever the density in brackets, during the 17 samplings in each site			**FO of species at density > 20 000 cell/mL and whatever the density in brackets, during the 51 samplings in all sites**
	Malgogne	**Senaigerie**	**Capitaine**	
Anabaena flos aquae	0 (11,7)%	5.8 (11.7)%	5.8 (11.7)%	**3.9 (7.8)%**
Anabaena planktonica	0 (0)%	5.8 (23.5)%	5.8 (11.7)%	**1.9 (9.8)%**
Anabaena spiroides	5.8 (11.6)%	11.7 (47)%	11.7 (76.4)%	**9.8 (45)%**
Aphanizomenon flos aquae	0 (5.8)%	0 (11.7)%	5.8 (11.7)%	**1.9 (9.8)%**
Aphanizomenon gracile	5.8 (11.6)%	11.7 (23.5)%	35.2 (47)%	**17.6 (27.4)%**
Aphanocapsa sp. (picoplankton)	35.3(35.3)%	6.5 (6.5)%	17.6 (41.1)%	**39.2 (47)%**
Coelomoron sp.	0 (0)%	5.8 (11.7)%	0 (5.8)%	**1.9 (5.9)%**
Aphanothece sp.	23.5 (23.5)%	5.8 (11.7)%	0 (5.8)%	**9.8 (13.7)%**
Limnothrix redekeii	0(23.5)%	17.6 (47)%	35.3 (47)%	**17.6 (39.2)%**
Merismopedia sp	0(0)%	11.7 (23.5)%	11.7 (29.4)%	**7.8 (17.6)%**
Microcystis aeruginosa	0(0)%	0 (23.5)%	5.8 (23.5)%	**1.9 (15.6)%**
Microcystis flos aquae	0 (0)%	11.7(11.7)%	23.5 (29.4)%	**11.7 (13.7)%**
Microcystis wesenbergii	0 (17.6)%	0 (5.8)%	0 (5.8)%	**0 (9.8)%**
Oscillatoria sp	5.8 (17.6)%	23.5 (41.1)%	11.7 (29.4)%	**13.7 (29.4)%**
Plankthotrix agardhii	5.8 (29.4)%	44(76.4)%	52.9(70.5)%	**35.3 (58.8)%**
Pseudanabaena catenata	0 (0)%	0 (23.5)%	17.6(23.5)%	**17.6 (47)%**
Pseudanabaena limnetica	0 (0)%	0 (5.8)%	5.8 (11.6)%	**5.8 (17.6)%**
Synechoccochus sp. (picoplankton)	17.6 (17.6)%	0 (0)%	0(0)%	**17.6 (17.6)%**
Number of species sampled	**6 (11)**	**12 (18)**	**13 (17)**	**16 (19)**
Number of MC-prod species	**3 (5)**	**5 (7)**	**6 (7)**	**6 (7)**

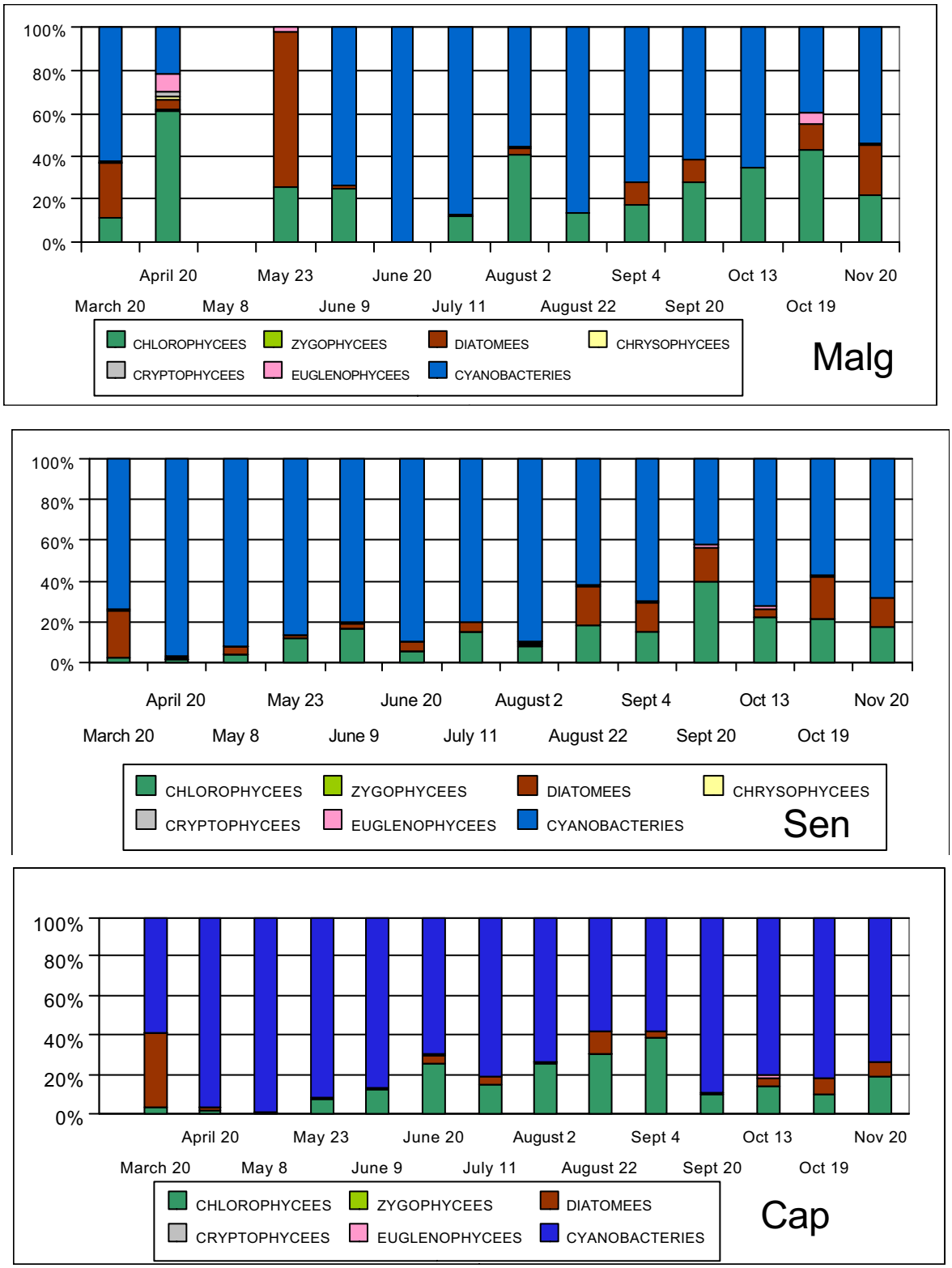


Figure 2: Temporal fluctuations in the percentage of different phytoplankton taxa from March 2006 to Novembre 2007 (proliferation period) in the 3 stations [Malgogne (Malg), Senaigerie (Sen), Capitaine (Cap)] of the Grand Lieu Lake.

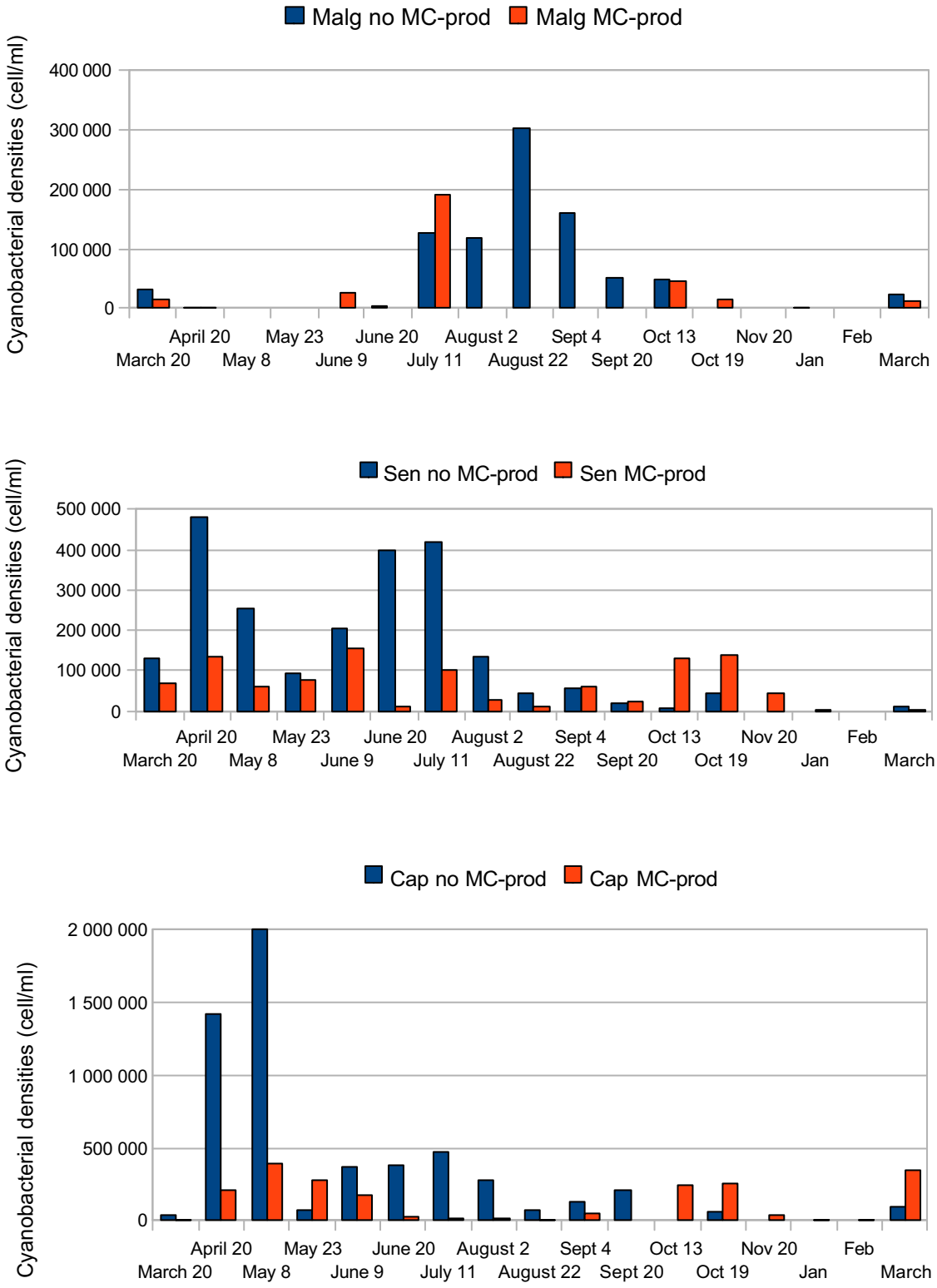
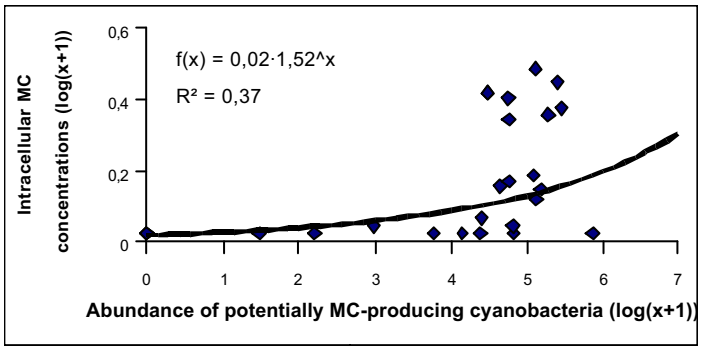


Figure 3: Densities of cyanobacteria (cell/mL) producing MCs (MC prod) or not (no MC prod) from March 2006 to March 2007 in the 3 stations [Malgogne (Malg), Senaigerie (Sen), Capitaine (Cap)] of the Grand Lieu Lake.

4.2.1.b. ***Extra- and intra-cellular MC concentrations***

Extracellular dissolved MCs were never detected in Malgogne water samples. In other stations, concentrations always remained below the HPLC-detection limit, except in May, August and November 2006 in Capitaine (3.96 ± 0.35 µg L^{-1}, and up to 5.60 µg L^{-1}) and in May and August 2006 in Senaigerie (3.46 ± 0.41 µg L^{-1}, and up to 4.94 µg L^{-1}).

Intracellular MCs in phytoplankton samples were detected at all the stations, with concentrations comprised between 0.07 and 4.50 µg L^{-1}, but were not correlated with the total abundance of potentially MC-producing cyanobacteria (see below).



At Malgogne, intracellular MCs were detected 3 times during the year: 0.16 µg L^{-1} in June 2006 and 0.44 µg L^{-1} in October 2006 coinciding with the presence of *P. agardhii* and in a lesser extent *Oscillatoria sp*. (Fig. 4), and 1.28 µg L^{-1} in July 2006 associated with *P. agardhii* occurrence (Fig. 4). At Senaigerie, intracellular MC concentration was inferior to 0.6 µg L^{-1} from May to last June 2006, and then increased from July (1.54 µg L^{-1}) to November (2.95 µg L^{-1}), coinciding with the presence of *P. agardhii* and *Oscillatoria sp.* (Fig. 4). At Capitaine, intracellular MC concentration up to 1.63 µg L^{-1} from May to June 2006 (Fig. 4) was essentially due to the presence of *P. agardhii*, and in a lower extent of *M. aeruginosa* (the highest density in May with 56 000 cells mL^{-1}) (Fig. 4). No MC was found in July and August 2006 (Fig. 4), coinciding with the total disappearance of *P. agardhii,* and in spite of the presence of *M. aeruginosa* and *Oscillatoria sp.*. From September to November 2006, *P. agardhii* and *Oscillatoria sp* proliferated and we observed a maximum intracellular MC concentration of 7.16 µg L^{-1} (Fig. 4). During the year, mean intracellular MC concentration occurring at Malgogne was 0.13 ± 0.08 µg L^{-1}, 0.72 ± 0.22 µg L^{-1} at Senaigerie and 1.34 ± 0.50 µg L^{-1} at Capitaine. The contamination intensity of the study-stations by cyanobacteria and intracellular MCs was thus in increasing order: Malgogne, Senaigerie and Capitaine.

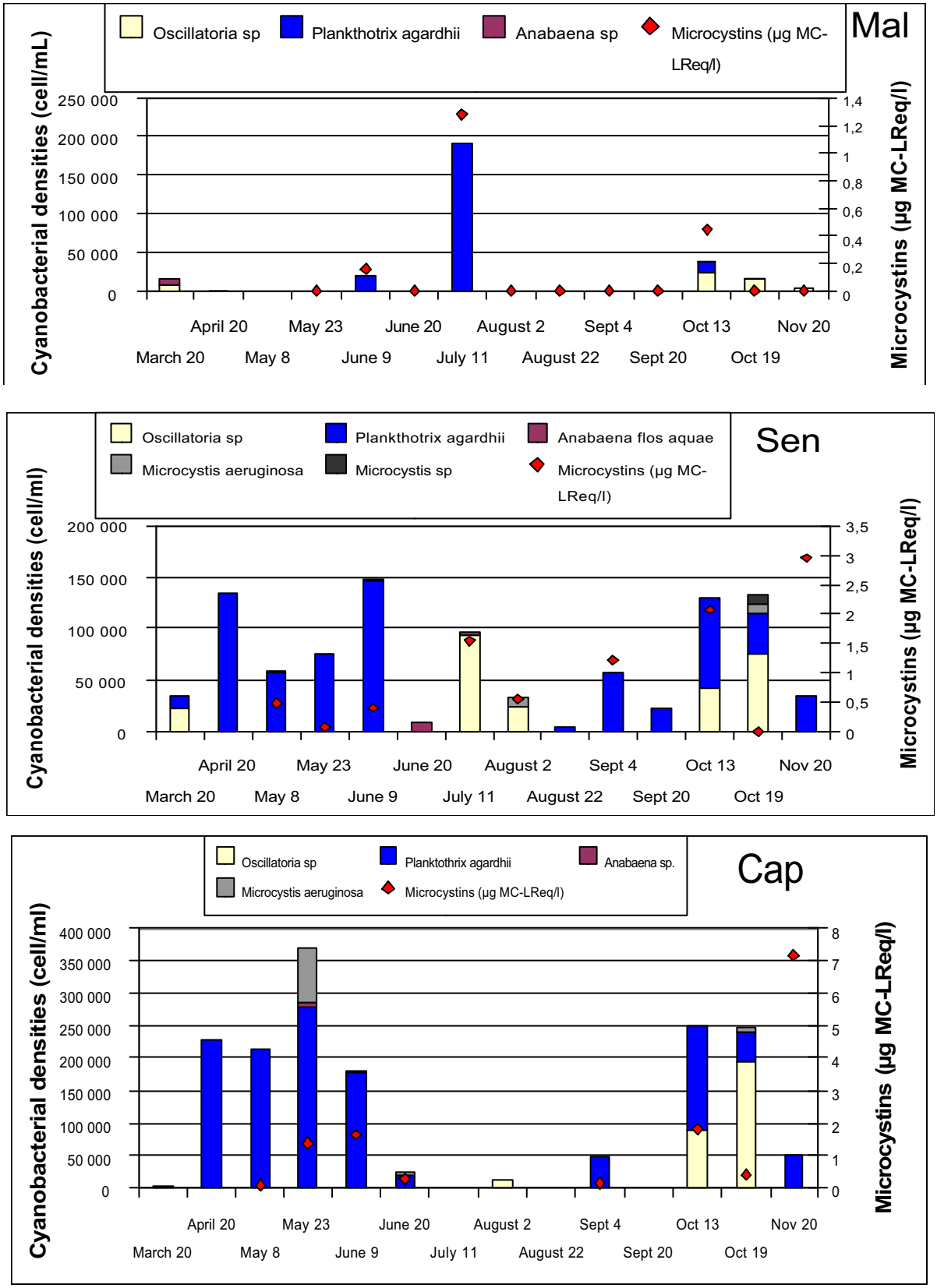


Figure 4: Intracellular MC concentrations (µg/L) and densities of potentially MC-producing cyanobacteria from March 2006 to November 2006 (proliferation period) at the 3 stations [Malgogne (Malg), Senaigerie (Sen), Capitaine (Cap)] of Grand Lieu Lake.

4.2.2. Impact on gastropod communities

During the one-year study, a total of 2599 gastropods belonging to 23 species were collected in the 3 stations among them 16 pulmonates (Lymnaeidae, Physidae, Planorbidae, Ancylidae) and 7 prosobranchs (Viviparidae, Bithyniidae, Hydrobiidae, Valvatidae) (Table 2). The total abundance frequency of pulmonate species (82.3%) was higher than that of prosobranchs (17.6%). Among pulmonate families, Planorbidae was the most frequently sampled, followed by Physidae. The most occurring species was the physid *Physella acuta* (84.85%, present in the 3 sites), followed by the two prosobranchs *Valvata cristata* and *Valvata piscinalis* (respectively 39.39% and 33.33%). The occurrence frequency of other species was inferior to 30.30%, among them 6 rare species (i.e., *Planorbis carinatus, Stagnicola palustris, Ferissia wautieri, Anisus spirorbis* and *Viviparus lacustris*) occurring only one time (3.03%) (Table 2).

The species richness and abundance of gastropods differed significantly between stations (Anova, respectively $F_{2,30} = 21.03$ and $F_{2,30} = 8.11$; $p < 0.05$). At Malgogne, the mean gastropod abundance was 146.0 ± 33.1 during the one-year study, vs 84.4 ± 27.1 at Senaigerie and 5.6 ± 0.9 at Capitaine; only Malgogne and Capitaine were significantly different (Tuckey HSD, $p < 0.05$). During the study, 61.8% of the total gastropod abundance was sampled at Malgogne, vs 35.5% at Senaigerie and 2.4% at Capitaine (Table 2). The mean gastropod species richness was significantly higher at Malgogne (4.6 ± 0.8) during the study, than at Senaigerie (1.6 ± 0.5) (Tuckey HSD, $p < 0.05$), and than at Capitaine (0.4 ± 0.1) (Tuckey HSD, $p < 0.05$). At Malgogne, 81.8% of samplings presented 4 species or more, with a maximum of 16 species in June and July 2006, whereas at Senaigerie 81.8% samplings presenting 4 species or less. Capitaine showed a significant lower species richness than both Malgogne and Senaigerie (Tuckey HSD, $p < 0.05$), with no gastropods during 3 months and only one species, *P. acuta*, the other months.

According to the gastropod taxa, the inter-station variations in abundance and species richness were more pronounced for prosobranchs than for pulmonates. The abundance of prosobranchs was significantly lower at Capitaine compared to Senaigerie and Malgogne (Anova, $F_{2,30} = 14.47$; Tuckey HSD, $p < 0.05$), whereas only Capitaine and Malgogne were significantly different for pulmonates (Anova, $F_{2,30} = 5.9$; Tuckey HSD, $p < 0.05$) (Fig. 5). Similar pattern was found concerning the species richness: significantly lower at Capitaine

compared to Senaigerie and Malgogne and at Senaigerie vs Malgogne for prosobranchs (Anova, $F_{2,30}$ = 21.62; Tuckey HSD, $p < 0.05$), whereas only Capitaine vs Malgogne and Senaigerie vs Malgogne were significantly different for pulmonates (Anova, $F_{2,30}$ = 18; Tuckey HSD, $p < 0.05$). Globally, pulmonates are more abundant and diverse than prosobranchs at each station during the whole study, i.e. 1262 (16 species) pulmonates vs 348 (6 species) prosobranchs in Malgogne, 807 (8 species) vs 108 (3 species) in Senaigerie, and 62 (1 species) vs 0 in Capitaine (Table 2, Fig. 5).

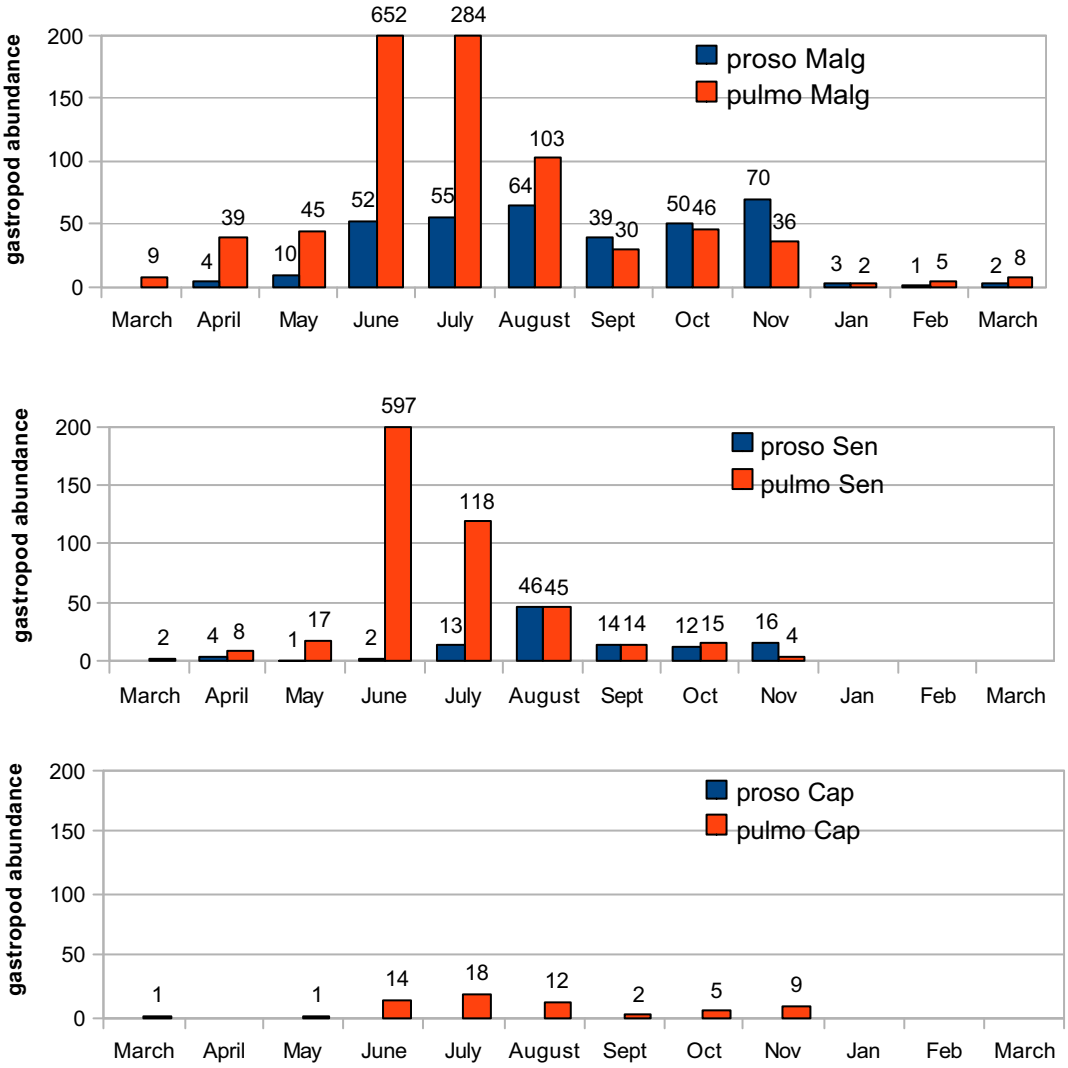


Figure 5: Taxon structure of gastropod community [number of prosobranchs (proso) and pulmonates (pulmo) individuals] between March 2006 and March 2007 in the 3 study-stations [Malgogne (Malg), Senaigerie (Sen), Capitaine (Cap)] of the Grand Lieu Lake.

Table 2. Structure of the gastropod community from March 2006 to March 2007 in the 3 stations (Malgogne, Senaigerie, Capitaine) of the Grand Lieu Lake (A = Abundance; %A = relative abundance i.e. percentage of snails of a species in all gastropod abundance, FO = frequency of occurrence: percentage of months a species is collected in 11 months).

Family	Species	**Malgogne**			**Senaigerie**			**Capitaine**		
		A	%A	FO (%)	A	%A	FO (%)	A	%A	FO (%)
Prosobranch	*Bithynia leachii* (Paasch)	33	1.3%	27.3%	0	0%	0%	0	0%	0%
Bithyniidae	*Bithynia tentaculata* (Linné)	5	0.2%	36.4%	0	0%	0%	0	0%	0%
Hydrobiidae	*Marstoniopsis scholtzi* (Schmidt)	20	0.8%	18.2%	0	0%	0%	0	0%	0%
Valvatidae	*Valvata cristata* (Müller)	192	7.4%	72.7%	76	2.9%	45.4%	0	0%	0%
	Valvata pulchella (Studer)	32	1.2%	54.5%	0	0%	0%	0	0%	0%
	Valvata piscinalis (Müller)	62	2.4%	63.6%	30	1.1%	36.4%	0	0%	0%
Viviparidae	*Viviparus lacustris* (Linné)	0	0%	0%	2	0.07%	9%	0	0%	0%
Pulmonate	*Ancylus fluviatilis* (Müller)	33	1.3%	27.3%	0	0%	0%	0	0%	0%
Ancylidae	*Ferissia wautieri* (Mirolli)	40	1.5%	9%	0	0%	0%	0	0%	0%
Lymnaeidae	*Lymnaea stagnalis* (Linné)	7	0.3%	27.3%	1	0.04%	9%	0	0%	0%
	Stagnicola palustris (Müller)	2	0.1%	9%	0	0%	0%	0	0%	0%
	Radix ovata (Draparnaud)	95	3.6%	36.4%	234	9%	27.3%	0	0%	0%
Planorbidae	*Armiger crista* (Linné)	11	0.4%	27.4%	0	0%	0%	0	0%	0%
	Anisus spirorbis (Linné)	16	0.61%	9%	0	0%	0%	0	0%	0%
	Anisus vortex (Linné)	58	2.2%	36.4%	26	1%	18.2%	0	0%	0%
	Bathyomphalus contortus (Linné)	122	4.7%	54.5%	4	0.1%	18.2%	0	0%	0%
	Gyraulus albus (Müller)	65	2.5%	63.6%	7	0.3%	27.3%	0	0%	0%
	Hippeutis complanatus (Linné)	4	0.1%	18.2%	0	0%	0%	0	0%	0%
	Planorbis carinatus (Müller)	1	0.04%	9%	0	0%	0%	0	0%	0%
	Planorbarius corneus (Linné)	39	1.5%	45.5%	17	0.6%	18.2%	0	0%	0%
	Planorbis planorbis (Linné)	269	10.3%	54.5%	247	9.5%	9%	0	0%	0%
	Segmentina nitida (Müller)	136	5.2%	72.7%	0	0%	0%	0	0%	0%
Physidae	*Physella acuta* (Draparnaud)	367	14.1%	100%	284	10.9%	81.8%	62	2.4%	72.7%

4.2.3. MC accumulation in gastropods

The MC values in gastropods ranged from 0 to 3.92 µg g^{-1} DW (maximum in *P. acuta* from Capitaine) and varied according to stations and species. The mean MC accumulation in all gastropods was higher at Capitaine (i.e. 0.82 ± 0.35 µg g^{-1} DW) and Senaigerie (i.e. 0. 55 ± 0.14 µg g^{-1} DW) compared to Malgogne (i.e. 0.13 ± 0.05 µg g^{-1} DW). Pulmonates of all stations tended to accumulate more MCs in their tissues, i.e. 0.51 ± 0.14 µg g^{-1} DW than prosobranchs, i.e. 0.35 ± 0.08 µg g^{-1} DW, but the difference was not significant.

In Malgogne, MC was detected only in 3 species (*V. cristata, V. pulchella and P. acuta*) among the 20 analysed from July to October 2006. In Senaigerie, 10 among 11 analysed species (excepted *Anisus spirorbis*) contained MCs from May to November 2006. In Capitaine, the only species sampled, *P. acuta*, contained MCs from March to November 2006. Only the pulmonate *P. acuta* occurred in all the stations, with the highest MC content compared to other gastropods. MC accumulation in *P. acuta* varied among stations and was significantly higher at Senaigerie (i.e. 1.19 ± 0.52 µg g^{-1} DW) and Capitaine (i.e. 0.82 ± 0.35 µg g^{-1} DW) (similar each other) than at Malgogne (i.e. 0.19 ± 0.03 µg g^{-1} DW) (Anova, respectively $F_{2,21}$ = 8,34; Tuckey HSD, $p < 0.05$).

4.2.4. MC accumulation in *L. stagnalis* and *P. antipodarum* encaged

Whatever species, all the snails encaged in May 2006 died in last week of August 2006 at all stations reducing the caging experiment to 4 months. During the study, the MC accumulation was much higher in *L. stagnalis* (0.19 ± 0.05 and up to 0.36 µg g^{-1} DW for the 3 stations) than in *P. antipodarum* (0.01 ± 0.00 and up to 0.03 µg g^{-1} DW).

For *L. stagnalis*, MC accumulation was different according to stations (0.07 ± 0.03 at Malgogne, 0.18 ± 0.04 at Capitaine and 0.27 ± 0.09 at Senaigerie). At Capitaine, MC content in snail tissues in June (i.e. 0.24 ± 0.05) was a probable consequence of the toxic cyanobacteria proliferation (mostly *P. agardhii* and in a lesser extent *M. aeruginosa)* and intracellular MC occurrence in May and June (Figs. 3, 4, 7). From July to August, MC content decreased, coinciding with decrease of both toxic cyanobacteria and intracellular MCs (Fig. 7). In August, *L. stagnalis* still contained MCs (i.e. 0.11 ± 0.02), whereas no MC was detected in cyanobacteria at that time (Fig. 3, 4, 7). At Senaigerie, *L. stagnalis* showed a

lower MC content in June compared to other months, probably in relation with the lowest MC content in cyanobacteria in June compared to July and August (Fig. 7). At Malgogne, MCs were detected in *L. stagnalis* only in July, when the MC content in cyanobacteria was the highest (i.e. 1.28 µg L^{-1}) (Fig. 3, 4, 7).

For *P. antipodarum*, no MC was detected in individuals from Malgogne and Capitaine. MC accumulation occurred only in July and August at Senaigerie (respectively 0.02 ± 0.00 and 0.01 ± 0.00), the station where MC content in cyanobacteria was the highest (among the 4-months of the caging experiment) (i.e. 1.54 µg L^{-1}).

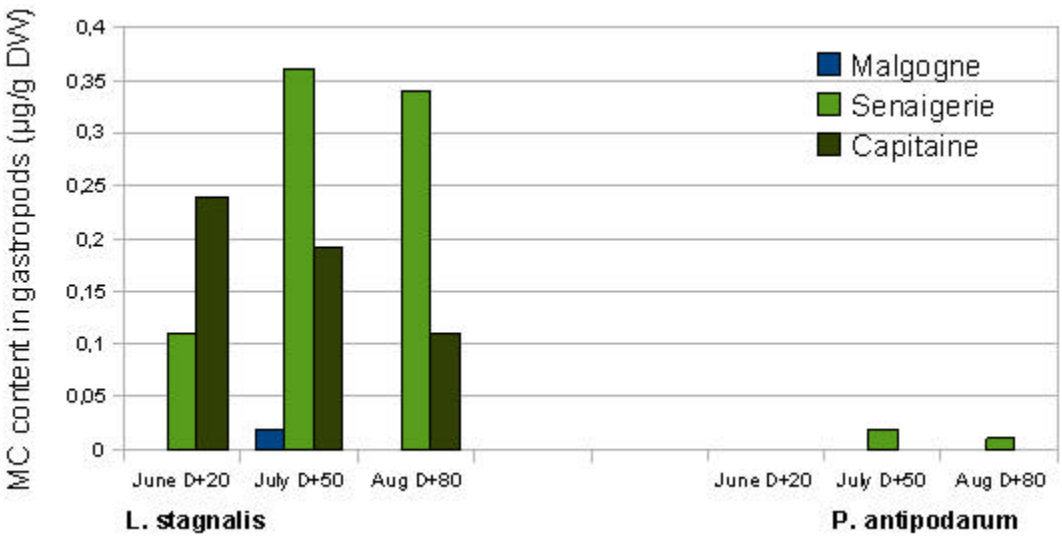


Figure 7 : MC accumulation (µg/g DW) in *L. stagnalis* and *P. antipodarum* encaged from May to September 2006 in the 3 study-stations (Malgogne, Senaigerie, Capitaine) of the Grand Lieu Lake

4.3. Discussion

Grand Lieu Lake is one of the richest and most particular ecosystem in France. However, the lake has become increasingly eutrophic since 1960s, mainly because of agricultural pollution and acting as an efficient trap for nutrients (Marion and Brient, 1998, 2000). Despite large beds of floating leaved plants that may limit phytoplankton production, the lake is turbid in the floating macrophyte area and exhibits yearly cyanobacterial blooms. Since 1980's, the submerged macrophytes almost completely disappeared while turbidity dramatically increased due to cyanobacteria (Vezie et al., 1998; Paillisson and Marion, 2002). During our investigation, cyanobacteria occurred at all 3 study-stations, with several proliferation episodes from April (more than 2 000 000 cells mL^{-1}) to October (322 000 cells mL^{-1}) 2006, and representing from 55 to 99% of phytoplankton communities. Among the 19 cyanobacterial species sampled, 7 were potentially producing MCs (*Anabaena flos aquae Microcystis aeruginosa, Microcystis flos aquae, Microcystis wesenbergii, Oscillatoria sp, Plankthotrix agardhi, Spirulina sp., Woronichinia sp*) (Chorus and Bartram, 1999). Even if it was not possible to establish with certainty which cyanobacterial species were responsible for MC production, comparison of species composition and MC concentration showed pronounced tendencies. In particular, intracellular MCs were mostly associated with proliferations of *P. agardhii*, the most abundant cyanobacteria species at all stations occurring in half of the samples, and to a lesser extent, of *Oscillatoria sp.* and *M. aeruginosa.* However, on several occasions, elevated MC-producing cyanobacteria densities did not correspond to high intracellular MC concentration (i.e. 280 000 cell mL^{-1} for *P. agardhii* and less than 2 µg MC-LReq L^{-1} in May at Capitaine). Intracellular MC concentration never over passed 7.2 µg L^{-1}. According to Briand et al. (2008), the concentration of MCs produced during *P. agardhii* blooms depends on variations in both the proportion of strains containing the genes involved in MC production [the *mcyA* genotype varied considerably (30 to 80%) during their two-year survey] and the MC cell quota in toxic strains. The changes in the proportions of the *mcyA* genotype appeared to be inversely correlated to changes in the *P. agardhii* density. Nevertheless, in our study, intracellular MCs were detected from April to October 2006 in two of the three stations, and dissolved MCs during the summer, up to 5.6 µg L^{-1}.

The 3 stations were chosen for their differences in cyanobacteria proliferations and intracellular MCs in phytoplankton recorded in the past few years (L. Brient, unpublished data). Capitaine was always the most contaminated site by MC-producing cyanobacteria, thereafter Senaigerie usually with relatively moderate cyanobacteria densities and MC occurrence, and Malgogne, almost never contaminated (Brient, unpublished data). Such differences between stations within one lake may be explained in part by nutrient input [the 3 stations are from 3 to 6 km away and receive flows from different types of landscapes (agricultural for Capitaine and Senaigerie, and unexploited grasslands for Malgogne) (L. Marion, personal communication)], and by the great spatiotemporal variability of cyanobacteria populations [the mosaic structure of blooms may induce great differences in stations only 10 meters far away (for review: Zurawell et al., 2005)]. During our investigation, Capitaine station was slightly more contaminated by MCs in the phytoplankton (intracellular) and in the water (dissolved) than Senaigerie, and Malgogne was the least contaminated station but showed more toxic cyanobacterial proliferation than before, suggesting a possible increase of the overall eutrophication of the lake. The MC accumulation in snails globally followed this pattern and was significantly higher in gastropods collected in high (i.e. mean of 0.82 ± 0.35 µg g DW in Capitaine and 0. 55 ± 0.14 µg g DW in Senaigerie) vs low (i.e. 0.13 ± 0.05 µg g DW in Malgogne) contaminated waters. These results are in accordance with the field study of Gérard et al. (2008) on several molluscan communities differently exposed to toxic cyanobacteria. Moreover, 100% of snails (one species, i.e. *P. acuta*, and 62 individuals) sampled in Capitaine were MC-intoxicated from March to November 2006, 90% in Senaigerie (May to November), against 15% in Malgogne (July to October).

Prosobranchs and pulmonates can be intoxicated via ingestion of toxic cyanobacteria (intracellular MCs) and extracellular MCs, dissolved (oral water uptake, trans-tegument penetration, gill or pulmonary breathing) or adsorbed on particles [e.g., sediment is ingested by depositivore prosobranchs to extract micro-organisms living inside (Dillon, 2000; Dorgelo & Leonards, 2001) and by pulmonates for trituration in the gizzard (Carriker, 1946)]. Numerous field studies found that MC accumulation in gastropods was related to intracellular MCs in the phytoplankton (and not to dissolved MCs), suggesting that intoxication was mainly due to ingestion of cyanobacteria (Zurawell et al. 1999, Yokoyama and Park, 2002; Chen et al., 2005; Xie et al., 2007). However, for the prosobranch *Bellamya aeruginosa*, MC intoxication was correlated to both intracellular and extracellular MCs (Zhang et al., 2007).

Prosobranchs are more closely related to sediment (whereas most of pulmonates are living in the entire water column), and are then expected to be also more intoxicated by adsorbed MCs. In the laboratory, the pulmonate *L. stagnalis* accumulated 1300 times more MCs by grazing toxic cyanobacteria (5 µg L^{-1}) than by exposure to dissolved MC-LR (33 µg L^{-1}), whereas the difference between the two intoxication routes was only of 1.5 times for the prosobranch *P. antipodarum* (Lance et al., 2006; Gérard et al., 2005; Gérard C., personal communication). All these results suggest that intoxication of prosobranchs occurs to the same extent with both exposure pathways, whereas pulmonates mainly accumulate MCs from ingestion of toxic cyanobacteria.

Based on field-studies, MC-accumulation seems to be inferior in prosobranchs (from 0.05 to 10 µg g^{-1} DW) than in pulmonates (from 3.46 to 140 µg g^{-1} DW) (Kotak et al., 1996; Ozawa, 2003; Chen et al., 2005; Gkelis et al., 2006; Zhang et al., 2007; Gérard et al., 2008; Gérard et al., 2009). Similarly, in the laboratory, *P. antipodarum* accumulated 1.3% of total ingested MCs with cyanobacteria against 61.0% for *L. stagnalis* (Lance et al., 2006, 2008). In our encaging experiment, *P. antipodarum* also accumulated less MCs (0.01 ± 0.00 µg g^{-1} DW) than *L. stagnalis* (0.19 ± 0.05 µg g^{-1} DW). However, differences in MC accumulation were not significant between pulmonates (i.e. 0.51 ± 0.14 µg g^{-1} DW) and prosobranchs (i.e. 0.35 ± 0.08 µg g^{-1} DW) sampled at the three stations in the natural populations, even if the same tendancy was observed. Accumulated MCs can be metabolized into less harmful compounds after conjugation with glutathione via glutathione-S-transferase, resulting in MC excretion or physiological degradation. Such detoxification processes have been shown to occur in various organisms (e.g., plants, invertebrates, and vertebrates) (Pflugmacher et al., 1998; Wiegand et al., 1999; Cazenave et al., 2006). MC metabolization and/or detoxification abilities are probably more or less efficient depending on MC-exposure routes and gastropod species, resulting in great variation in MC accumulation in their tissues.

Toxic effects of cyanobacteria on gastropods in the field may depend on their life history patterns (e.g., annual or perennial, iteroparous or semelpare), and their eco-physiology (e.g., aerial or aquatic respiration, close association to sediment). Indeed, those factors are susceptible to influence the cyanotoxin intoxication route (e.g., oral, gill, tegument) and the parameters of exposure to cyanobacteria (e.g., intensity, duration, frequency, timing). Our study confirms the dominance of pulmonates (vs prosobranchs), both in terms of species

richness and abundance, in waters highly exposed to cyanobacteria previously demonstrated by Gérard et al. (2009); prosobranchs never occurred in Capitaine, the highly contaminated station. This could be attributed to the fundamental broader physiological and ecological tolerances of pulmonates compared to prosobranchs, and to their greater genetic and phenotypic plasticity which allows them to vary trait expression in response to environmental changes (for reviews: Russel-Hunter, 1978; Aldridge 1983; MacMahon 1983; Dillon 2000). Previous experiments showed that the prosobranch *P. antipodarum* is susceptible to both extracellular dissolved MC exposure [decrease of survival, growth and fecundity (Gérard & Poullain, 2005)] and ingestion of toxic cyanobacteria [decrease of growth and fecundity (Lance *et al*., 2008)], whereas the pulmonate *L stagnalis* is more susceptible to ingestion of toxic cyanobacteria [decrease of growth and fecundity (Lance *et al*., 2006)] compared to dissolved MC exposure [only a decrease of fecundity (Gérard *et al*., 2005)]. Consequently, prosobranchs may be negatively affected by intracellular MCs during the cyanobacteria proliferation period and by extracellular MCs during bloom lyses, whereas pulmonates may be mainly impacted during the proliferation period of toxic cyanobacteria. Given these results, toxic blooms can lead to local extinctions of prosobranch populations that may explain their rarity in highly cyanobacterial contaminated waters.

As for MC accumulation patterns, deleterious effects on pulmonates and prosobranchs probably varied among species of each gastropod taxa. Toxic cyanobacterial proliferations may influence competitive interactions in favouring the most resistant or tolerant species to the detriment of the most sensitive ones. Among pulmonates, we showed that Planorbidae were more frequent than Physidae, possibly due to a lower susceptibility to nitrogen since a high nitrogen enrichment occurred in the lake (Marion and Brient, 1998, 2000) or to a higher tolerance to organic pollution (Mouthon, 1987), Nevertheless, among Physidae, *P. acuta* accumulated the highest amount of MCs (up to 3.92 µg g^{-1} DW) whatever stations and persisted alone in the highly contaminated one (Capitaine). According to Gérard et al. (2009), *P. acuta* was also the molluscan species accumulating the highest MC amount (up to 24.2 µg g^{-1} FW) among prosobranchs, pulmonates, and bivalves collected in the field. Moreover, only *P. acuta* occurred in the Combourg Lake (France) exposed for 8 years to recurrent proliferations of toxic cyanobacteria that have induced the disappearance of all other gastropod species (Gérard et al., 2008). Based on these results, *P. acuta* seems particularly resistant to MCs and would deserve further investigations since it can play the role of sentinel

species [i.e., bioindicator that accumulates toxins in their tissues without significant impact at environmental concentrations (Beeby, 2001)].

The structure of the gastropod communities showed great differences in terms of abundance and species richness according to the level of MC-contamination in the 3 study-stations. Capitaine, MC-contaminated for several years, presented the lowest species richness and abundance with 62 individuals belonging to one species. In Senaigerie, 82% of samplings had less than 4 species with 915 individuals, whereas in Malgogne more than 4 species and 1610 individuals were collected in 82% of samplings. The detrimental effect of MCs demonstrated on the gastropod life traits (i.e., survival, growth, and fecundity) (Gérard & Poullain, 2005; Gérard et al., 2005; Lance et al., 2007, 2008), but also on the fitness (hatching timing and success, survival of juveniles) (chapter 3), have probably cascade effects on the dynamics of gastropod populations. The breeding season of freshwater gastropods generally occurs in late spring or early summer (Russel-Hunter, 1978; Calow, 1978), coinciding with cyanobacteria population maxima. As we recently found that 1-week old juveniles ingested toxic cyanobacteria and accumulated MCs (chapter 3), most gastropods are potentially intoxicated by cyanotoxins from their birth, and thus, would have a decreased growth rate and survival, whereas the reproductive effort of adults would be altered (Gérard & Poullain, 2005; Gérard et al., 2005; Lance et al., 2007, 2008). To conclude, toxic cyanobacteria proliferations may constitute a determinant factor in the regulation of gastropod population dynamics, may change the structure of gastropod communities [as demonstrated in our study and by Gérard et al. (2009)], and even lead to their decline in case of severe and recurrent blooms (Gérard et al., 2008), with probable consequences on the functioning of the whole ecosystem.

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Chapitre 5 : Histopathologie et localisation des microcystines chez le pulmoné *L. stagnalis* exposé à une cyanobactérie toxique ou à de la microcystine-LR dissoute

Chapitre 5 : Histopathologie et localisation des microcystines chez le pulmoné *L. stagnalis* exposé à une cyanobactérie toxique ou à de la microcystine-LR dissoute

De précédents travaux (Gérard et al., 2005 ; Lance et al., 2006, 2007) ont montré que la quantité de microcystines (MCs) accumulée dans les tissus du gastéropode pulmoné *L. stagnalis* et l'impact sur ses traits de vie varient selon la voie d'intoxication (exposition aux MCs dissoutes ou intracellulaires), la présence ou non d'une autre source de nourriture non toxique, et l'âge du gastéropode (juvénile, adulte). D'autre part, l'impact des MCs sur les tissus de la glande digestive de la limnée semblait réversible (Lance et al., 2006). Nous avons souhaité approfondir l'étude de l'histopathologie de la glande digestive de *L. stagnalis* en la complétant par la localisation des MCs dans différents organes (glande digestive, glande génitale, pied, rein). L'objectif est de déterminer si la distribution des MCs dans l'organisme varie en fonction de la voie d'intoxication (ingestion de cyanobactéries toxiques vs exposition à de la MC-LR dissoute) et d'étudier les processus d'élimination des MCs et de régénération des tissus au cours de 3 semaines de dépuration suivant les 5 semaines d'intoxication. La localisation des MCs a été réalisée dans le laboratoire du Pr. D. Dietrich (Laboratoire de Toxicologie Environnementale, Université de Konstanz, Allemagne) à l'aide d'une technique d'immuno-histochimie utilisant des anticorps anti-MCs et mise au point à l'origine pour les tissus de poissons. Ce travail a été en partie subventionné par une Bourse du DAAD (Institut Académique d'Echanges Franco-Allemand).

La technique immuno-histologique utilisée met en évidence les MCs liées aux protéines phophatases (Ppases) ou à d'autres protéines/peptides contenant des acides aminés soufrés (e.g. cystéine, méthionine) comme les glutathions. La conjugaison des MCs avec les glutathions a été démontrée chez de nombreux organismes (plantes, invertébrés et vertébrés) et génère des composés plus hydrosolubles et moins toxiques, éliminés dans la bile et l'urine (Pflugmacher et al., 1998; pour revue : Cazenave et al., 2006). Par contre, les interactions entre les MCs et les Ppases (essentielles au maintien de l'intégrité cellulaire), qu'elles soient réversibles ou covalentes, inhibent ces dernières, engendrant une désorganisation du cytosquelette et des nécroses (for review: Dietrich and Hoeger, 2005; Hastie et al., 2005).

L'inhibition des Ppases, démontrée dans des hépatocytes de vertébrés, a probablement lieu dans les cellules digestives des gastéropodes, les Ppases étant communes à toutes les cellules eucaryotes.

Les résultats montrent que l'ingestion de cyanobactéries toxiques (5 µg MC-LReq L^{-1}) induit des altérations très sévères (nécroses, lésions) de la glande digestive : 95% des acini digestifs sont altérés en l'absence de salade et 67% en présence de salade. L'exposition à la MC-LR dissoute provoque une altération légère de 8% des acini à 33 µg L^{-1} et de 45% à 100 µg L^{-1}. Au cours de la dépuration, la glande digestive retrouve progressivement une structure normale chez tous les individus anciennement exposés à la toxine dissoute, et chez la moitié des individus anciennement exposés aux cyanobactéries toxiques, témoignant de la présence de cellules souches capables de régénérer l'épithélium des acini digestifs. L'accumulation de MCs liées, mise en évidence par immuno-histologie dans les zones altérées de la glande digestive, est proportionnelle au degré d'altération observé. De plus, les MCs sont principalement localisées dans le cytoplasme des cellules de la glande digestive après ingestion de cyanobactéries toxiques, suggérant qu'elles interagissent avec les Ppases et entraînent une nécrose de l'épithélium digestif. Après exposition à la MC-LR dissoute, les MCs liées sont localisées dans la lumière des acini digestifs, et provoquent moins de dommages dans la glande digestive, suggérant leur excrétion via des lysosomes. Dans la glande génitale, aucune pathologie n'est observée, mais des MCs liées sont détectées dans les ovocytes et les spermatozoïdes, quels que soient leur stade de développement et la voie d'intoxication. Ces résultats font l'objet d'une publication soumise prochaînement.

5. Histopathology and bound microcystin distribution in the tissues of *Lymnaea stagnalis* (Gastropoda) exposed to toxic cyanobacteria or dissolved microcystin-LR

Article prochainement soumis

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Abstract

Hepatotoxic microcystins (MCs) can accumulate in tissues in a free form (partly eliminated by conjugation with glutathiones) or in a bound form via a covalent linkage with phosphatase proteins (Ppases) (inducing their inhibition). Accumulation of free MCs by gastropods is higher by grazing toxic cyanobacteria than uptake of dissolved toxins. Up to now, accumulation of bound MCs has not been measured in these organisms. In this study, we used an immuno-histochemical method in order to examine and compare histopathological alterations and tissue distribution of covalently bound MCs in various organs (digestive gland, genital gland, kidney, foot) of the gastropod *Lymnaea stagnalis* exposed to MC-producing *Planktothrix agardhii* and dissolved MC-LR over a 5-week period. Intoxication was followed by a 3-week depuration period in order to investigate if MC elimination and tissue regeneration occur. The immuno-histochemical method predominantly detected covalently bound MCs to sulfhydryl-aminoacid (e.g. cysteine, methionine) containing peptides and proteins (e.g. Ppases or excretion/detoxification enzymes such as glutathiones). Severe and widespread necrotic changes and a strong presence of MCs within the cytoplasm of digestive cells co-occurred in the digestive gland of *L. stagnalis* after ingestion of toxic cyanobacteria (5 µg MC-LReq L^{-1}). Impact was far less severe and more reversible when snails were exposed to dissolved MC-LR at 100 µg L^{-1} and almost null at 33 µg L^{-1}, co-occurring with a moderate presence of MCs in the lumen of digestive acini. These results suggest that during exposure to dissolved MC-LR, toxins do not penetrate in the cytoplasm of digestive cells but are engulfed and further excreted via vacuoles, causing the cell death. During ingestion of toxic cyanobacteria, MCs penetrate in the cytoplasm of digestive cells where they can inhibit the Ppases leading to strong and irreversible digestive epithelium damages. In the genital gland, no histopathology occurred but MCs were detected in spermatozoids and oocytes of all exposed snails.

Introduction

Contamination of freshwaters by toxic cyanobacteria is a subject of serious international concern since several cyanobacterial metabolites, e.g., hepatotoxic microcystins (MCs), have been demonstrated to cause sublethal and lethal effects to diverse aquatic and terrestrial organisms (for review: Wiegand and Plufgmacher, 2005). MCs are cyclic heptapeptides of which about 80 structural variants have been identified (for review: Dietrich and Hoeger, 2005). They accumulate in hepatocytes where they can inhibit protein phosphatases (Ppases) (Hastie et al., 2005), which results in reorganization of cytoskeletal components and disruption of hepatic architecture, leading to severe and irreversible damage, and also comprehends tumor promoting potential. Similar but less severe damage can occur within the gastrointestinal tract and kidney (for reviews: Wiegand and Pflugmacher, 2005; Zurawell et al., 2005).

MCs can enter the aquatic food web through accumulation in various organisms including zooplankton, macroinvertebrates and vertebrates (for review: Zurawell et al., 2005). Freshwater gastropods inhabit the littoral area (Dillon, 2000) where cyanobacteria frequently form scums. Gastropods may therefore be intoxicated rapidly by ingestion of toxic cyanobacteria (intracellular toxins) or exposure to extracellular toxins released after cell lysis into the surrounding water (dissolved or adsorbed on particles). Indeed, field observations substantiate MC accumulation by gastropods (Kotak et al., 1996; Zurawell et al., 1999; Chen et al., 2005; Xie et al., 2007; Zhang et al., 2007; Gérard et al., 2008; Gérard et al., 2009). Furthermore, the ability of MCs to accumulate in tissues and to impair the digestive gland of the pulmonate *Lymnaea stagnalis* has been demonstrated after consumption of toxic cyanobacteria and exposure to dissolved MC-LR in experimental investigations (Gérard et al., 2005; Lance et al., 2006, 2007; Zurawell et al., 2006, 2007). After consumption of toxic cyanobacteria, 61.0% and 1.3% of total ingested MCs were accumulated by *L. stagnalis* and the prosobranch *Potamopyrgus antipodarum* respectively (Lance et al., 2006, 2007, 2008). These accumulations induced decreased life traits of both species, however suggested species specific differences in the capacity of MC assimilation and/or metabolization in the digestive system. Negative effects on life traits were also observed following immersion of *P. antipodarum* and *L. stagnalis* in 33 µg dissolved MC-LR L^{-1} but with a much lower MC

accumulation compared to ingestion of toxic cyanobacteria (Gérard et al., 2005; Gérard and Poullain, 2005). Previous investigations thus also suggest that accumulation in snails may depend on the exposure route.

MCs detected in organisms can be in a free form, partly eliminated by conjugation with glutathiones (Pflugmacher et al., 1998), and/or in a bound form via a covalent and irreversible linkage with Ppases (Hastie et al., 2005). Studies cited above disregard MCs covalently bound to Ppases, which might represent a considerably part of MCs accumulated in tissues (Dietrich and Hoeger, 2005; Ernst et al. 2005). Therefore, we adapted to the gastropod tissues an immuno-histochemical method which predominantly detects covalently bound MCs to sulfhydryl-aminoacid (e.g. cysteine, methionine) containing peptides and proteins (e.g. Ppases, glutathiones) (D. Dietrich, personal communication). The aim of this study was to examine histopathological alterations and tissue distribution of bound MCs in various organs (digestive gland, genital gland, kidney, foot) of the gastropod *L. stagnalis*, exposed to MC-producing *Planktothrix agardhii* and dissolved MC-LR in order to investigate:

- if cyanobacteria intracellular MCs and extracellular MC-LR induced histopathology, in particular in the digestive and genital glands of exposed gastropods;
- if MC elimination and tissue regeneration occur after a depuration period;
- and finally, if disruption of tissues was correlated to the occurrence of bound MCs in the digestive cells, according to the MC intoxication route (i.e. ingestion of MC-producing cyanobacteria or dissolved MC-LR exposure).

5.1. Material and methods

5.1.1. Biological material

Adults of *L. stagnalis* were obtained from a laboratory population in the Experimental Unit of the Institut National de Recherche en Agronomie (U3E INRA, Rennes). Prior to the experiment, *L. stagnalis* (25 ± 3 mm shell length) were isolated in glass containers of 35 mL (1 snail/container), acclimated to the experimental conditions (12/12 L/D, 20 ± 1°C) and fed on biological lettuce for 7 days. *P. agardhii* (strain PMC 75-02) was cultured as described in Lance et al. (2006). This filamentous cyanobacterium has been shown to produce three MC variants: dmMC-LR, dmMC-RR and MC-YR (Lance et al., in preparation, see in chapter 6). The *P. agardhii* suspension contained a total concentration of 5 µg MC-LR equivalents (MC-LReq) per litre. MC-LR was obtained from Alexis Corporation (USA) and solubilized with 0.1% MeOH in dechlorinated water for final MC-LR concentrations of 33 and 100 µg L^{-1}.

5.1.2. Experimental set up

After acclimation, snails were divided into 6 treatment groups according to diet and medium and were held in: 1) dechlorinated water with lettuce ad libitum (CONTR), 2) dechlorinated water without feeding (STARV), 3) dechlorinated water containing 33 µg MC-LR L^{-1} with lettuce ad libitum (033LT), 4) dechlorinated water containing 100 µg MC-LR L^{-1} with lettuce ad libitum (100LT) 5) *P. agardhii* suspension without additional feeding (CYANO), and 6) *P. agardhii* suspension with lettuce ad libitum (CYNLT). Each group consisted of 20 isolated individuals. Cyanobacterial suspensions as well as the medium of starved, control and MC-LR exposed snails were renewed twice a week. Each treatment was maintained for 5 weeks, after which all gastropods were maintained in dechlorinated water and fed solely on lettuce ad libitum for a 3-week depuration period.

5.1.3. Histopathology

After the treatment and depuration, four snails were removed from each treatment for histological investigations. Snail bodies were removed from their shells, and stomachs were

taken off due to the presence of sand that could impair the tissue during the sliding process. Possible shell residues on snail bodies were dissolved in EDTA (ethylene diamine tetraacetic acid) and prepared bodies were fixed in Bouin's fluid during 48h. Tissues were then processed as described in Lance et al. (2007), cut into serial 6-µm-thick longitudinal sections and stained with Hematoxylin & Eosin (H&E) (Martoja and Martoja-Pierson, 1967). Kidney, foot, digestive and genital glands were photographed in each section via an optic microscope using 40-200-fold magnification. Further quantitative and semi-quantitative parameters were measured in order to evaluate the pathology induced by MCs on the digestive gland, the predominantly impaired organ in this study. We created three parameters calculated from four snails per treatment, by evaluating 25 sections per individual:

1) Mean Area of the Digestive Lobule Epithelium (MADLE)

The digestive gland was assessed for variations in the thickness of the digestive lobule epithelium via comparison of the Mean Area of the Digestive Lobule Epithelium (MADLE). The area of the lobule epithelium was determined using microscopy images (magnification: 100) and the AnalySIS software (Soft Imaging System GmbH, Germany). The MADLE per treatment was determined via averaging the area of the lobule epithelium determined on 25 sections from each of the four snails per treatment.

2) Mean Degree of Pathological Changes (MDPC)

We classified the pathological changes observed in lobules of the whole digestive gland in 4 degrees of intensity in order to evaluate the impact of MCs. Pathological changes was classified as none (0), mild (1), moderate (2), strong (3) and severe (4). Validations of each group were combined to give the mean degree of pathological changes (MDPC) per treatment.

3) Percentage of Lobules with Intact Epithelium (PLIE)

The number of pathological inconspicuous lobules, i.e. lobules with intact epithelium, was determined in order to evaluate the number of lobules in which MC do no penetrate or has no impact. Accordingly, the percentage of lobules with intact epithelium (PLIE) corresponds to lobules classified to show no pathological alteration (degree 0). Counts of each treatment group were finally combined to give the PLIE per treatment.

5.1.4. Immuno-histochemistry

In order to localize MCs covalently bound in the snail tissue, four individuals from each treatment group were assessed immuno-histochemically after both treatment and depuration by analyzing five sections per individual. Snail sections were fixed as described above. Immuno-histochemical staining was carried out according to Ernst et al. (2007). Sections on polylysin-coated glass slides were deparaffinized in 100% xylol, rehydrated in descending ethanol concentrations (100%, 95% and 70%) and incubated with 1 mg ml^{-1} type XIV bacterial protease (Sigma-Aldrich, Germany) in PBS at 37°C for 10 min for antigen-demasking. Endogenous biotin was blocked using a commercial avidin/biotin blocking kit (BioGenex, USA). Slides were further blocked with a casein solution (Power Block™, BioGenex, USA) for 10 min at room temperature. Rabbit anti MC-LR antibodies (#2; kindly provided by Dr. John E. Eriksson, University of Turku, Finland) were diluted in Power Block™ (1:500) and applied to the tissue section in a humidified atmosphere for 16 h at 4°C. Antigen-antibody complexes were visualized using an IgAP-labelled, biotin-streptavidin amplified detection system (Super Sensitive™, BioGenex, USA), levamisole block (5 mM in MQ water) and Fast Red™ tablets (Roche, Germany). Sections were counter stained at room temperature for 6 min with Mayer's hematoxylin (Sigma-Aldrich, Germany), rinsed with tab water and mounted using Crystal/Mount™ (Biomeda, USA) and Shandon Histomount™ (Thermo Electron Corporation, Germany).

Immuno-histochemically stained slides were examined via an optic microscope using 40-200-fold magnification. Digestive lobules, gonadic acini, kidney and foot sections were classified to be MC-positive when MC-positive areas (recognized as a red color against an unstained background) were above background chromogen staining and positive staining areas were congruently observable in two independently stained serial sections from the same tissue sample. Further quantitative and semi-quantitative parameters were created in order to evaluate the intensity of the MC-intoxication of the digestive and genital glands, as these organs are known to be the major sites of MC accumulation (Chen et al., 2005; Xie et al., 2007; Zhang et al., 2007) and presented the most prominent MC-positive signal in this study. Parameters were calculated from 80 and 40 digestive lobules and gonadic acini pictures, respectively, taken from five sections of 4 snails per treatment, using identical conditions (i.e. constant magnification and illumination for all acquisitions) and 62.5-fold magnification.

1) Percentage of digestive lobules and gonadic acini with no MC (%NoMC)

Immunopositive staining was classified as none (0), sporadic (+), pronounced (++) and extensive (+++) in order to separate degrees of MC detection. Accordingly, the number of MC-immunonegative lobules and acini corresponds to lobules and acini classified to show no MC (degree 0). Counts of each treatment group were finally combined to give the percentage of digestive lobules or gonadic acini without MC-immunopositivity (%NoMC).

2) Mean intensity of the MC-positive staining (MIMCS)

MC-accumulation within the digestive and genital glands was quantified via colorimetric analysis of the MC-immunopositive staining using the image J software [Wayne Rasband, National Institute of Mental Health, Maryland, USA]. RGB images were converted to CIELab by means of the "color transformer" plugin [Maria E. Barilla, Electrical and Computer Engineering School, The University of Birmingham, UK]. Staining was analyzed in the "a" component (color transition from green to red). MC-positive staining intensity was measured as average pixel intensity over the whole picture as specimen area was constant over all the acquired samples. Measurements of each treatment group were finally combined to give the Mean Intensity of the MC Staining (MIMCS) in the digestive lobules and gonadic acini.

5.1.5. Statistical analysis

MDPC data are reported as median ± mean absolute deviation (MAD) of the individual ranks whereas other data are reported as mean ± standard error (SE). Data did not follow a normal distribution (according to the Kolmogornov-Smirnov test) and were thus analyzed for differences between all the different treatment groups using the Kruskall-Wallis (KW) test, and 2 by 2 treatment groups using 1) the Mann-Whitney U-test for MADLE, MDPC and MIMCS, 2) the Chi2 test for PLIE and %NoMC. Significant differences were determined at $p < 0.01$ and $p < 0.05$ level for all statistical analyses and were indicated as ** for $p < 0.01$ and * for $p < 0.05$.

5.2. Results

5.2.1. Histopathology

No significant pathological changes were observed in kidney, foot and genital gland of the snails. Oocytes and spermatozoids were always present in gonadic acini. The digestive gland of control snails consisted of a series of lobules from a single layer of cells (Fig. 1A and 1A'). In contrast to this, the epithelial cells in the digestive gland of starved snails appeared flattened and lumen of digestive lobules were enlarged after 5-week treatment (Fig. 1B). Snails exposed to 33 µg MC-LR L^{-1} showed slightly increased vacuolization of the digestive cells (Fig. 1C). Vacuolization was intensified when increasing the MC-LR exposure level to 100 µg L^{-1}, moreover causing few cell lysis and exudations into the lobule lumen (Fig. 1D). After 5 week-immersion in *P. agardhii* suspension containing 5 µg MC-LReq L^{-1}, severe necrosis occurred in all digestive lobules, including alterations of the cell shape, separation of the basal lamina from the cell, widespread cell lysis with release of cytoplasmic content to the lumen (Fig. 1E, 1F). The severity of necroses increased when snails were exposed to *P. agardhii* without feeding on lettuce, culminating in almost complete disruption of the digestive gland tissue integrity.

After the 3-week depuration, the digestive gland of the starvation group recovered (Fig. 1B') as well as most digestive lobules of snails previously exposed to MC-LR with few lobules still containing vacuolated or exfoliated cells (Figs. 1C', 1D'). However, necrotic changes in snails previously fed on toxic *P. agardhii* were still observable although less abundant than at the end of intoxication (especially for snails that ingested lettuce with cyanobacteria) (Figs. 1E' and 1F').

5.2.1.1. Mean Area of Digestive Lobule Epithelium (MADLE)

The MADLE differed significantly between the various treatment groups after both intoxication and depuration (Fig. 2). After the intoxication period, snails exposed to 33 µg MC-LR L^{-1} showed similar MADLE than control snails. However, *L. stagnalis* from the other treatment groups presented a significant reduced MADLE, and holding in *P. agardhii* without feeding of lettuce induced the most pronounced reduction of MADLE (Fig. 2). Ranking of

MADLE from the various treatments yielded the following order: CONTR = 033LT > 100LT, CYNLT > STARV > CYANO. After the depuration, the MADLE of treated snails was approximating to the one of the controls, although previously starved *L. stagnalis* showed significantly elevated MADLE and snails previously held in *P. agardhii* without lettuce still showed a decreased MADLE (Fig. 2).

5.2.1.2. Percentage of Lobules with Intact Epithelium (PLIE) & Mean Degree of Pathological Changes (MDCP)

After the intoxication period, PLIE and MDPC differed significantly between the different treatment groups. For snails exposed to 33 µg MC-LR L^{-1}, PLIE and MDPC were similar to that of control snails. Snails from the other treatment groups presented an increased number of pathological conspicuousness lobules, and thus a significant decrease of the PLIE and a significant increase of the MDPC (Table 1). Holding in *P. agardhii* without lettuce presented the lowest PLIE and the highest MDPC (Fig. 2).

After the depuration, only snails previously fed on toxic *P.agardhii* (i.e. treatment CYNLT & CYANO) presented significantly decreased PLIE and increased MDPC levels (Table 1).

5.2.2. MC-immunohistology

5.2.2.1. Localization of MCs in *L. stagnalis*

While snails of the unintoxicated groups (CONTR and STARV) showed a negligible amount of obviously false MC-immunopositivity (Figs. 3A and 3B), snails exposed to either *P. agardhii* suspension (CYANO & CYNLT) or dissolved MC-LR (033LT & 100LT) presented significantly MC-immunopositive staining. This staining was primarily observable in the digestive (i.e. lumen of digestive lobules, digestive cells; Fig. 3) and genital (i.e. oocytes, spermatozoids; Fig. 4) glands and in the digestive tract (i.e. lumen of the prointestine) after the intoxication period and was co-localized to histopathological changes in the serially sectioned tissues. MC immunopositivity was most prominent in snails exposed to toxic *P. agardhii* (Tab. 2), including strong staining in the cytoplasm of digestive cells and involving the whole digestive and genital gland sections (Figs. 3E; 3F; 4E and 4F). Snails

exposed to dissolved MC-LR presented lower MC-immunopositive staining (Tab. 2 & 3), localized in the lumen of digestive lobules and vacuoles of digestive cells in several isolated groups of lobules (Figs. 3B; 3C). MC-immunopositive staining was also observable after depuration, however decreased in intensity (Figs. 3 and 4). Minor MC-immunopositivity was detectable in the kidney and foot of exposed snails.

5.2.2.2. Percentage of digestive and gonadic Acini without MC-immunopositivity (%NoMC)

During the experiment, the %NoMC in the digestive and genital glands (Table 2) differed significantly between the various treatment groups. After treatment, snails exposed to either *P. agardhii* (CYANO & CYNLT) or high MC-LR concentrations (100LT) showed MC-intoxicated digestive and genital glands with significant smaller %NoMC than control snails (CONTR & STARV) (Table 2). Snails exposed to toxic *P. agardhii* had no MC-immunonegative lobules in the digestive gland, and only few MC-immunonegative acini in the genital gland, thus presented the lowest %NoMC.

After the depuration, snails previously exposed to dissolved MC-LR showed similar %NoMC than control snails in digestive and genital glands, whereas snails previously exposed to toxic *P. agardhii* still presented significantly lower %NoMC (Table 2).

5.2.2.3. Mean intensity of the MC-positive staining (MIMCS)

Treatment groups exposed to either *P. agardhii* or dissolved MC-LR showed significant higher MIMCS in the digestive and genital glands than control snails (i.e. groups CONTR & STARV), after both intoxication and depuration (Table 3). In both glands, snails exposed to toxic *P. agardhii* presented a significantly higher MIMCS than snails exposed to dissolved MC-LR (Table 3).

5.3. Discussion

5.3.1. Pathology in *L. stagnalis* according to MC-intoxication routes

No significant pathological changes were observed in kidney, foot and genital gland of the snails. However, after a 5-week exposure to MC-producing cyanobacteria (5 µg MC-LReq L^{-1}), the digestive gland of the gastropod *L. stagnalis* exhibited severe histopathological changes (i.e. 4.4% of intact digestive lobules vs 91.2% in control snails, the other lobules presenting widespread cell lysis and necrosis) associated to a strong presence of MCs. Such pathological changes have been described for *L. stagnalis* intoxicated with dissolved MC-LR (Zurawell et al., 2007) and aquatic vertebrates fed on MC-producing cyanobacteria (for review: Malbrouck and Kestemont, 2006; Ernst et al., 2006; 2007; Li et al., 2007). In the host cells, MCs can form non-covalent and covalent interactions with Ppases (PP1, 2A, 4, and 5) inhibiting their catalytic subunits (for review: Dietrich and Hoeger, 2005; Hastie et al., 2005). MC-Ppase interactions can induce hepatocyte degeneration and disorganization of the hepatic architecture, potentially leading to death of both mammals and fish (for reviews: Zurawell et al., 2005; Malbrouck and Kestemont, 2006). Moreover, Tencalla and Dietrich (1997) demonstrated that pathological changes in the liver of the rainbow trout *Oncorhynchus mykiss* gavaged with the toxic cyanobacterium *Microcystis aeruginosa* was a result of the MC absorption from the digestive tract into the blood, followed by a rapid and total inhibition of Ppase activities in the liver (recorded using a Ppase inhibition assay). In our study, the co-occurrence of bound MCs and severely destructed lobules thus suggests strong MC-Ppase interactions in the digestive gland of *L. stagnalis* after consumption of toxic cyanobacteria.

Over the 3-week depuration period, the necroses in the digestive gland of snails previously fed on toxic cyanobacteria were partially reversible (i.e. 50% of intact digestive lobules vs 4.4% after the intoxication period) and MCs were moderately eliminated (i.e. 76.5% of lobules containing MCs vs 95.6% after the intoxication period). The partial elimination of bound MCs may occur via detoxification pathways and/or via degradation and consequent elimination of damaged cells. Several observations support the latter hypothesis since: i) the covalent binding of MCs to Ppases is known to be irreversible (for review: Dietrich and Hoeger, 2005), and ii) the presence of regenerating lobules after depuration suggests the elimination of impaired cells [in the lumen of acini or by macrophages (Henry,

1987)] before the restoration of digestive epithelium. The digestive gland consists of a series of lobules from a single layer of cells containing digestive cells, excretory cells, immature cells and stem cells (Carriker, 1946; Charrier, 1995). The immature cells sequentially replace the digestive cells in normal conditions, whereas stem cells, highly protected, are capable of regenerating the epithelium in case of acute stress (Henry, 1987).

In contrast to ingestion of toxic cyanobacteria, exposure to dissolved MC-LR (100 µg L^{-1}) induced less severe impact (i.e. 55% of intact digestive lobules after the intoxication period, the other lobules presenting few cell lysis and increased vacuolization of the digestive cells), associated to a slight presence of bound MCs. Exposure to MC-LR at a lower dose (33 µg L^{-1}) induced only a slight increased vacuolization of digestive cells with almost no MCs in tissues. Over the 3-week depuration period, the changes were entirely reversible (i.e. respectively 87.4 and 93.5% of intact digestive lobules in snails previously exposed to 100 and 33 µg MC-LR L^{-1} vs 91.2% in control snails) and MCs were almost entirely eliminated from the digestive gland. Gehringer et al (2004) also provided evidence of a regeneration of the liver tissue of mice after a single sublethal dose of MC-LR, related to the removal of MC-LR from the liver by glutathione detoxification pathway. In the snail, the reversible cell vacuolization and the low amount of bound MCs suggest few MC-Ppase interactions in the digestive gland tissues of *L. stagnalis* exposed to dissolved MC-LR.

5.3.2. MC localization in the digestive gland of *L. stagnalis* according to exposure routes

5.3.2.1. Ingestion of toxic cyanobacteria

In order to appreciate the results, the particular functioning of the alimentary system of *L. stagnalis* must be considered. Particles ingested by gastropods are grinded in the gizzard and only particles = 4µm pass toward the digestive gland, the others are compacted in the gizzard string fraction of faeces and directed toward the prointestine (Carriker, 1946). The digestive gland is the primary site of secretion, intracellular (lysosomal) digestion, assimilation, accumulation, detoxification and metabolism (Charrier, 1995). Food material is engulfed in vacuoles by the digestive cells and further stored or excreted in the lumen of lobules to be then compacted in the digestive gland string fraction of faeces in the prointestine. To the light of these explanations and of what was observed in the present and

previous studies (Lance et al., 2006; Zurawell et al., 2006), intracellular MCs might follow these routes in *L. stagnalis*:

- (1) a disruption of cyanobacteria occurs in the gizzard (Carriker, 1946) and a fraction of the released MCs is eliminated in the gizzard string fraction of the faeces (Zurawell et al., 2006);

- (2) the other fraction of MCs enters the digestive gland and is engulfed in the digestive cells. Then, both MC excretion in the digestive gland string fraction of the faeces and MC accumulation after penetration in the cytoplasm of the digestive cells occur. Zurawell et al. (2006) observed that the digestive cells of *L. stagnalis* eliminated MCs between 3 and 30 days after their assimilation coinciding with vacuolated excretion of residues. Our study revealed MCs in the digestive tract during intoxication (when MCs can come both from gizzard and digestive gland string fraction of faeces) but also during depuration (when MCs only come from the digestive gland excretion). Otherwise, MC penetration in the cytoplasm of the digestive cells occurred since MC signal was strong not only in the lumen of the digestive lobules but also in the digestive cell cytoplasm.

5.3.2.2 Exposure to dissolved MC-LR

The digestive gland of snails exposed to dissolved MC-LR was partly unharmed and without MCs, whereas consumption of toxic cyanobacteria induced a massive MC intoxication involving all digestive lobules. The far higher gastropod intoxication by grazing toxic cyanobacteria than by uptake of dissolved toxins, which may occur via oral water uptake or transtegumental penetration, has been already shown (Kinnear et al., 2007; Kotak, 1996; Lance et al., 2006, 2008; Zurawell et al. 1999). However, according to the water ingestion rate of *L. stagnalis* (De With, 1996), the 5-week exposure to 100 MC-LR µg L^{-1} involves a ingestion of approximately 2 µg of toxin, identically that during the ingestion of toxic *P. agardhii* producing 5 µg MC-LReq L^{-1} (with dmMC-LR, dmMC-RR and MC-YR) (Lance et al., 2006). The difference in intoxication processes can thus be explained by an important excretion of dissolved MC-LR by the digestive cells after intoxication. Indeed, MC-LR was mainly detected in the lumen of both digestive lobules and tract, but rarely in the cytoplasm of digestive cells, suggesting that the toxin is engulfed and further eliminated in vacuoles. The cell lysis observed is more likely due to the vacuole excretion process that disrupts the epithelium (Carriker, 1946) than due to the direct toxic effect of MC-LR. The question why

MC-LR incorporated in digestive cells apparently remains sequestered into vacuoles, contrary to what happened with other MC variants produced by *P. agardhii* still remains. Moreover, the multiple compounds produced by cyanobacteria (identified toxins or not) might play a role in this difference of impact between crude extracts of cyanobacteria and purified toxins. According to Tencalla et al. (1994), severe liver damages occurred in the rainbow trout *O. mykiss* gavaged with *M. aeruginosa* producing MC-LR, whereas a gavage with pure MC-LR did not induce any impact. Differences in accumulation and impact in gastropod tissues between MC variants and between ingestion of cyanobacterial cells vs purified MCs need further studies.

5.3.3. Consequences on *L. stagnalis*

Our study is the first comparing impact of the two major intoxication routes for gastropods, already known to induce differences in MC accumulation, i.e. 1300 times more important in *L. stagnalis* after toxic cyanobacteria ingestion than after dissolved MC-LR exposure (Gérard et al., 2005; Lance et al., 2006). Both exposure routes are also known to induce negative impact on life traits (i.e. decreased fecundity and growth) of *L. stagnalis* (Gérard et al., 2005; Lance et al., 2007), probably due to the toxic effect of MCs inducing higher activities of the stem cells or to MC-detoxification processes, both leading to energy trade-offs. We found that snails exposed to 100 µg MC-LR L^{-1} or to toxic cyanobacteria with a concomitant non-toxic food consumption showed a diminution of the digestive epithelium area, as happened during starvation. Indeed, the digestive epithelium contains glycogen stores that can be mobilized by *L. stagnalis* during starvation (Livingstone and De Zwaan, 1983), inducing a reduction in its area, reversible when snails were fed on lettuce again. The stressful effect of MCs may thus alter the energy balance, as shown by Juhel et al. (2006) for zebra mussels exposed to toxic *M. aeruginosa*.

Moreover, whatever intoxication route, MCs occurred in all spermatozoids and oocytes (immature or mature), which are therefore likely to be damaged, possibly explaining the decrease in fecundity (Gérard et al., 2005; Lance et al., 2006). Processes by which MCs pass from the digestive to the genital gland, very close organs, are unknown but probably involve haemolymph transport. Even if few MCs were present in the kidney and in the foot of

intoxicated snails, MC distribution in different organs via haemolymph may impair the homeostasis of the snails.

In the field, freshwater gastropods may experience chronic ingestion of toxic cyanobacteria potentially producing different MC variants during the proliferation period, and acute exposure to extracellular MCs at the end of this period (Xie et al., 2007; Zhang et al., 2007; Zurawell et al., 1999). Consequently both intoxication routes are susceptible to have a negative impact on gastropod communities in a long term in case of recurrent toxic cyanobacteria proliferations as demonstrated by Gérard et al. (2008).

5.3.4. Conclusion

Due to the key role of gastropods in structuring freshwater communities as herbivorous grazers and preys of numerous predators (Dillon, 2000; Kerans et al., 2005), the negative impact of toxic cyanobacteria on gastropods may have potential cascading effects on the equilibrium and functioning of ecosystems. Moreover, according to our results, the risk of toxin transfer to higher trophic levels by gastropods, already suggested in numerous studies (Chen et al., 2005; Gérard et al., 2008; Kotak et al., 1996; Xie et al., 2007; Zhang et al., 2007; Zurawell et al., 1999), seems to be far higher due to the presence of covalently bound MCs (vs free MCs). Further investigations aim to quantify the proportion of free and covalent MCs in gastropod tissues, as well as to identify the mechanisms involved in toxicity in relation with MC variants.

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Table 1. Distribution (in percent) of lobules from the digestive gland of *L. stagnalis* according to the degree of pathological changes after 5-weeks treatment and 3-weeks depuration in various treatment groups

		Treatment group					
	medium food	**CONTR** dw let	**STARV** dw -	**033LT** 33 µg MC/l let	**100LT** 100 µg MC/l let	**CYNLT** P. agardhii let	**CYANO** P. agardhii -
Treatment	**0** (PLIE)	95.2 ± 2.05	75.0 ± 1.38*	91.6 ± 4.15	55.3 ± 3.35**	32.8 ± 1.25**	4.41 ± 0.82**
	1	2.90 ± 1.98	9.44 ± 0.72	5.09 ± 1.05	11.4 ± 2.71	14.1 ± 2.29	10.8 ± 2.51
	2	1.90 ± 0.00	9.40 ± 0.85	3.31 ± 0.55	11.4 ± 3.81	17.2 ± 1.81	23.5 ± 1.89
	3	0.00 ± 0.00	3.12 ± 0.67	0.00 ± 0.00	14.9 ± 1.67	20.3 ± 3.92	30.9 ± 3.49
	4	0.00 ± 0.00	3.04 ± 0.05	0.00 ± 0.00	7.02 ± 1.39	15.6 ± 2.62	30.4 ± 2.98
	MDPC	**0.12 ± 0.05**	**0.48 ± 0.15****	**0.27 ± 0.11**	**0.88 ± 0.13****	**1.74 ± 0.17****	**2.73 ± 0.16****
Depuration	**0** (PLIE)	91.2 ± 2.86	90.2 ± 2.92	93.5 ± 3.49	87.4 ± 3.21	62.6 ± 3.94**	50.0 ± 3.69**
	1	7.42 ± 0.48	7.34 ± 1.32	4.35 ± 1.83	4.23 ± 0.40	12.2 ± 1.83	12.2 ± 1.38
	2	1.38 ± 0.27	0.00 ± 0.00	2.15 ± 0.67	3.24 ± 1.05	11.4 ± 0.75	12.5 ± 2.09
	3	0.00 ± 0.00	2.46 ± 0.56	0.00 ± 0.00	5.13 ± 1.43	8.93 ± 1.76	10.8 ± 1.44
	4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.87 ± 0.65	14.5 ± 2.11
	MDPC	**0.13 ± 0.02**	**0.15 ± 0.08**	**0.11 ± 0.03**	**0.17 ± 0.05**	**0.54 ± 0.10****	**1.38 ± 0.28****

Pathological changes in lobules were classified as none (0), mild (1), moderate (2), strong (3) and severe (4). Values are presented as mean ± MAD. Four snails per treatment were assessed, by evaluating 25 sections per individual. Validations of each treatment group were combined to give the percentage of lobules with intact epithelium (PLIE) and the mean degree of pathological changes (MDPC) per treatment group. The PLIE and the MDPC of control and treated snails were analysed for statistical differences using the Chi2 test and the Mann-Whitney U-test respectively and indicated as ** for $p < 0.01$ and * $p < 0.05$.

Table 2. Distribution (in percent) of lobules from the digestive and the genital glands of *L. stagnalis* according to the degree of MC-immunopositive staining after 5-weeks treatment and 3-weeks depuration in various treatment groups

			Treatment group					
		medium food	**CONTR** dw let	**STARV** dw -	**033LT** 33 µg MC/l let	**100LT** 100 µg MC/l let	**CYNLT** P. agardhii let	**CYANO** P. agardhii -
Treatment	digestive gland	**0** (%NoMC) + ++ +++	95.1 ± 3.71 4.90 ± 1.58 0.00 ± 0.00 0.00 ± 0.00	94.7 ± 2.91 5.30 ± 0.95 0.00 ± 0.00 0.00 ± 0.00	68.6 ± 4.08* 25.5 ± 1.42 5.90 ± 1.47 0.00 ± 1.05	25.0 ± 3.39** 37.5 ± 4.15 27.5 ± 2.94 10.0 ± 1.07	0.00 ± 0.00** 18.6 ± 2.87 40.6 ± 3.64 40.8 ± 3.52	0.00 ± 0.00** 4.7 ± 0.86 4.8 ± 1.09 90.5 ± 3.99
	genital gland	**0** (%NoMC) + ++ +++	97.9 ± 3.21 2.10 ± 0.76 0.00 ± 0.00 0.00 ± 0.00	96.7 ± 2.91 3.29 ± 0.45 0.00 ± 0.00 0.00 ± 0.00	95.0 ± 4.83 5.00 ± 1.12 0.00 ± 0.00 0.00 ± 0.00	50.0 ± 3.07* 21.4 ± 2.00 21.2 ± 1.77 7.40 ± 0.85	23.8 ± 2.46** 26.7 ± 3.75 21.5 ± 1.99 28.0 ± 3.09	0.00 ± 0.00** 0.00 ± 0.00 4.87 ± 0.76 95.1 ± 4.39
Dépuration	digestive gland	**0** (%NoMC) + ++ +++	97.2 ± 4.25 2.80 ± 0.18 0.00 ± 0.00 0.00 ± 0.00	94.9 ± 3.07 5.10 ± 0.73 0.00 ± 0.00 0.00 ± 0.00	89.6 ± 3.05 5.23 ± 1.27 5.18 ± 1.94 0.00 ± 0.00	66.4 ± 3.22 8.82 ± 1.54 20.2 ± 2.95 4.60 ± 1.35	40.0 ± 2.55** 40.6 ± 3.87 13.3 ± 1.76 6.10 ± 0.00	23.5 ± 2.05** 46.6 ± 3.19 21.0 ± 2.95 8.88 ± 1.39
	genital gland	**0** (%NoMC) + ++ +++	97.2 ± 4.25 2.80 ± 0.18 0.00 ± 0.00 0.00 ± 0.00	95.8 ± 4.15 4.19 ± 0.51 0.00 ± 0.00 0.00 ± 0.00	95.3 ± 3.33 4.76 ± 1.87 0.00 ± 0.00 0.00 ± 0.00	85.7 ± 2.92 14.3 ± 0.99 0.00 ± 0.00 0.00 ± 0.00	54.5 ± 2.05* 45.5 ± 3.72 0.00 ± 0.00 0.00 ± 0.00	35.7 ± 3.91** 64.3 ± 3.54 0.00 ± 0.00 0.00 ± 0.00

dw = deionised water; lt = lettuce

MC-immunopositive staining was classified as none (0), sporadic (+), pronounced (++) and extensive (+++). Values are presented as mean ± SE. Four snails per treatment were assessed, by evaluating 5 sections per individual. Validations of each treatment group were combined to give the percentage of digestive Acini with no MC (%NoMC). The %NoMC of control and treated snails was analysed for statistical differences using the Chi2 test and indicated as ** for $p < 0.01$ and * $p < 0.05$.

Table 3. Distribution of lobules from the digestive and genital glands of *L. stagnalis* according to the intensity of MC-immunopositive staining after 5-weeks treatment and 3-weeks depuration in various treatment groups

		Treatment group					
	medium food	**CONTR** dw let	**STARV** dw -	**033LT** 33 µg MC/l let	**100LT** 100 µg MC/l let	**CYNLT** P. agardhii let	**CYANO** P. agardhii -
Treatment	digestive gland	6.28 ± 0.85	8.02 ± 0.71	19.5 ± 1.65*	38.0 ± 2.56*	70.2 ± 2.88**	91.6 ± 4.70**
	genital gland	5.64 ± 1.01	5.37 ± 2.22	13.9 ± 2.14*	26.9 ± 2.55**	49.2 ± 3.69**	68.6 ± 4.93**
Depuration	digestive gland	4.50 ± 1.04	6.44 ± 1.67	10.1 ± 0.94*	17.3 ± 1.08*	36.2 ± 3.19**	63.5 ± 3.65**
	genital gland	3.69 ± 0.33	5.19 ± 1.49	7.44 ± 0.49*	8.64 ± 0.48*	15.9 ± 1.67**	27.4 ± 4.20**

dw = deionised water; lt = lettuce

Values are presented as mean ± SE. Four snails per treatment were assessed, by evaluating 5 sections per individual. Validations of each treatment group were combined to give the mean intensity of MC-positive staining (MIMCS). The MIMCS of control and treated snails was analysed for statistical differences using the the Mann-Whitney U-test and indicated as ** for $p < 0.01$ and * $p < 0.05$.

Figure captions:

Figure 1. Histopathological changes in the digestive gland tissue of *Lymnaea stagnalis* held in various treatment groups (A = CONTR; B = STARV; C = 033LT, D = 100LT; E = CYNLT and F = CYANO) after 5 weeks of treatment (A, B, C, D, E, & F) and 3 weeks of depuration (A', B', C', D', E' & F'). Sections were stained with H&E and observed by light microscopy at 62.5-fold magnification.

Normal structure is given in capital letters: BL = basal lamina, CT = connective tissues, DC = digestive cells, DL = digestive lobule, L = lumen, SE = structured epithelium, V = vacuoles; damaged structures are labelled with small letters: cd = cytoplasmic debris, cl = cell lysis, fdc = flattened digestive cells, ibl = impaired basal lamina, idc = impaired digestive cells, idl = impaired digestive lobules, re = regenerating epithelium, vdc = vacuolated digestive cells.

Figure 2. Mean area of the digestive lobule epithelium (MADLE) in the digestive gland of *Lymnaea stagnalis h*eld in various treatment groups (A = CONTR; B = STARV; C = 033LT, D = 100LT; E = CYNLT and F = CYANO) after 5 weeks of treatment (Treatment) and 3 weeks of depuration (Depur).Values are given as mean ± S.E. The MADLE of control and treated snails were analysed for statistical differences using the Mann-Whitney U-test and indicated as ** for $p < 0.01$ and * $p < 0.05$.

Figure 3. Immunohistochemical determination of microcystin (MC) in the digestive gland tissue of *Lymnaea stagnalis* held in various treatment groups (A = CONTR; B = STARV; C = 033LT, D = 100LT; E = CYNLT and F = CYANO) after 5 weeks of treatment (A, B, C, D, E, & F) and 3 weeks of depuration (A', B', C', D', E' & F'). Sections were stained using MC-antibodies and observed by light microscopy at 62.5-fold magnification.

MC accumulations dyed red (light colouration are additional highlighted by circles). Normal structure is given in capital letters: DC = digestive cells, DL = digestive lobule, L = lumen; damaged structure is labelled with small letters: idc = impaired digestive cells, idl = impaired digestive lobules.

Figure 4. Immunohistochemical determination of microcystin (MC) in the genital gland tissue of *Lymnaea stagnalis* held in various treatment groups (A = CONTR; B = STARV; C = 033LT, D = 100LT; E = CYNLT and F = CYANO) after 5 weeks of treatment (A, B, C, D, E, & F) and 3 weeks of depuration (A', B', C', D', E' & F'). Sections were stained using MC-antibodies and observed by light microscopy at 62.5-fold magnification.

MC accumulations dyed red (light colouration are additional highlighted by circles). Normal structure is given in capital letters: GA = gonadic acini, IO = immature oocytes, MO = mature oocytes, IS = immature spermatozoids, MS = mature spermatozoids; no histopathological damage was detected in the genital gland.

Figure 1

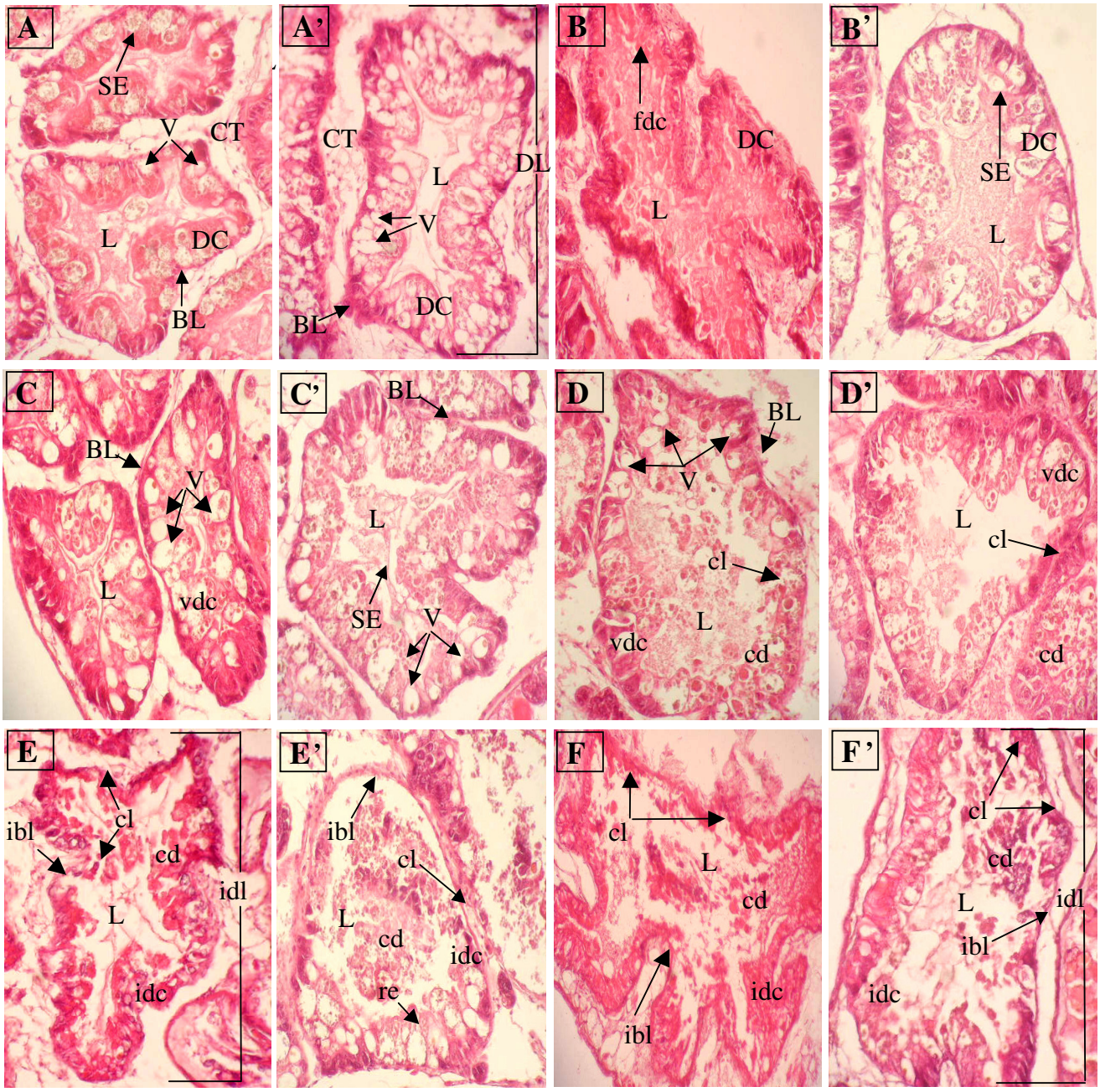
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Figure 2

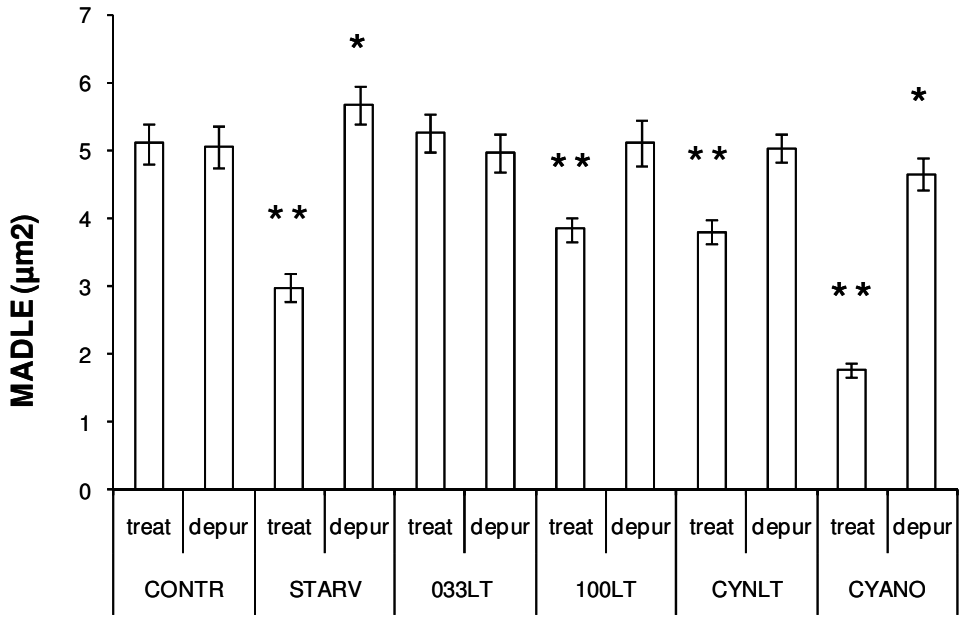
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Figure 3

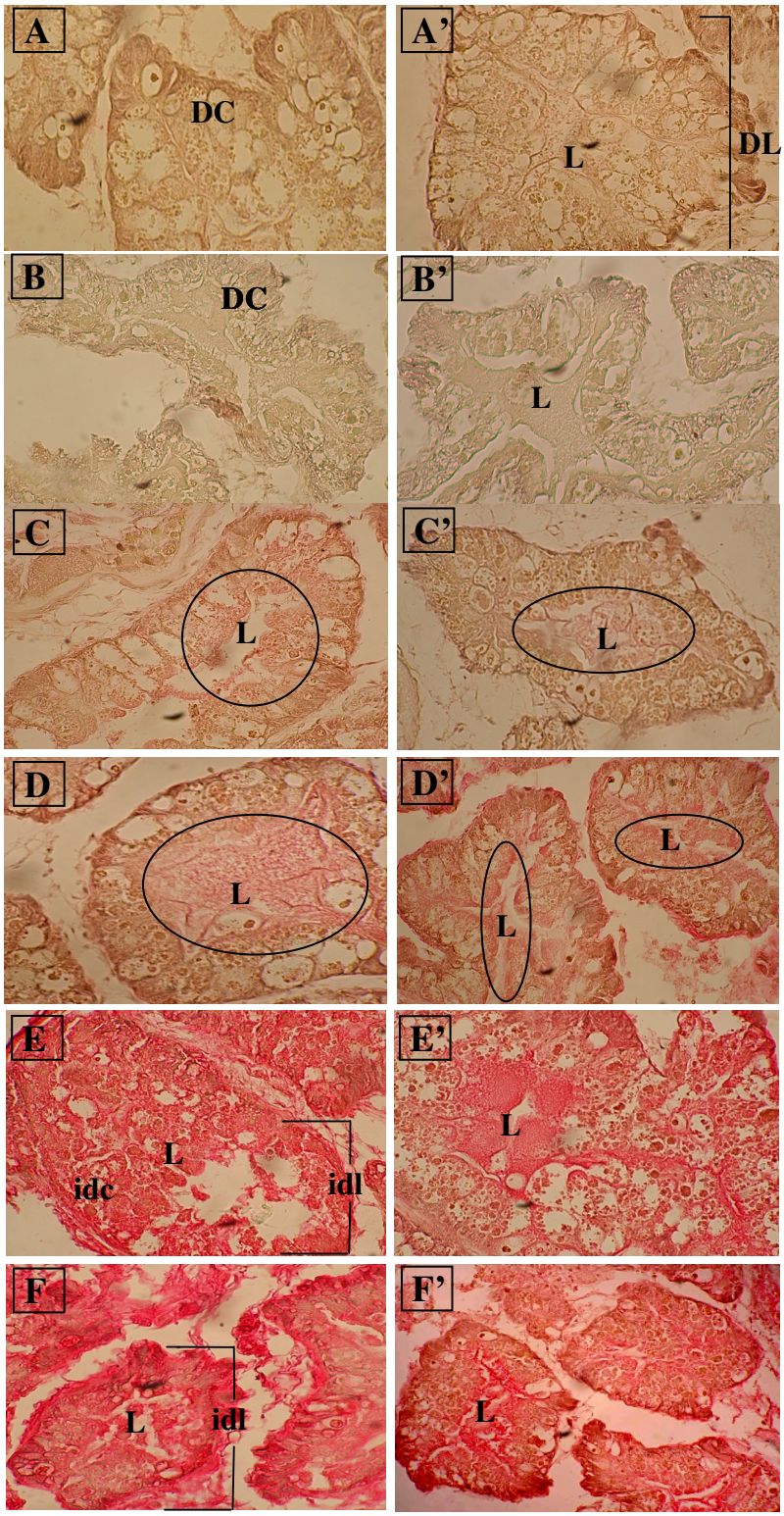
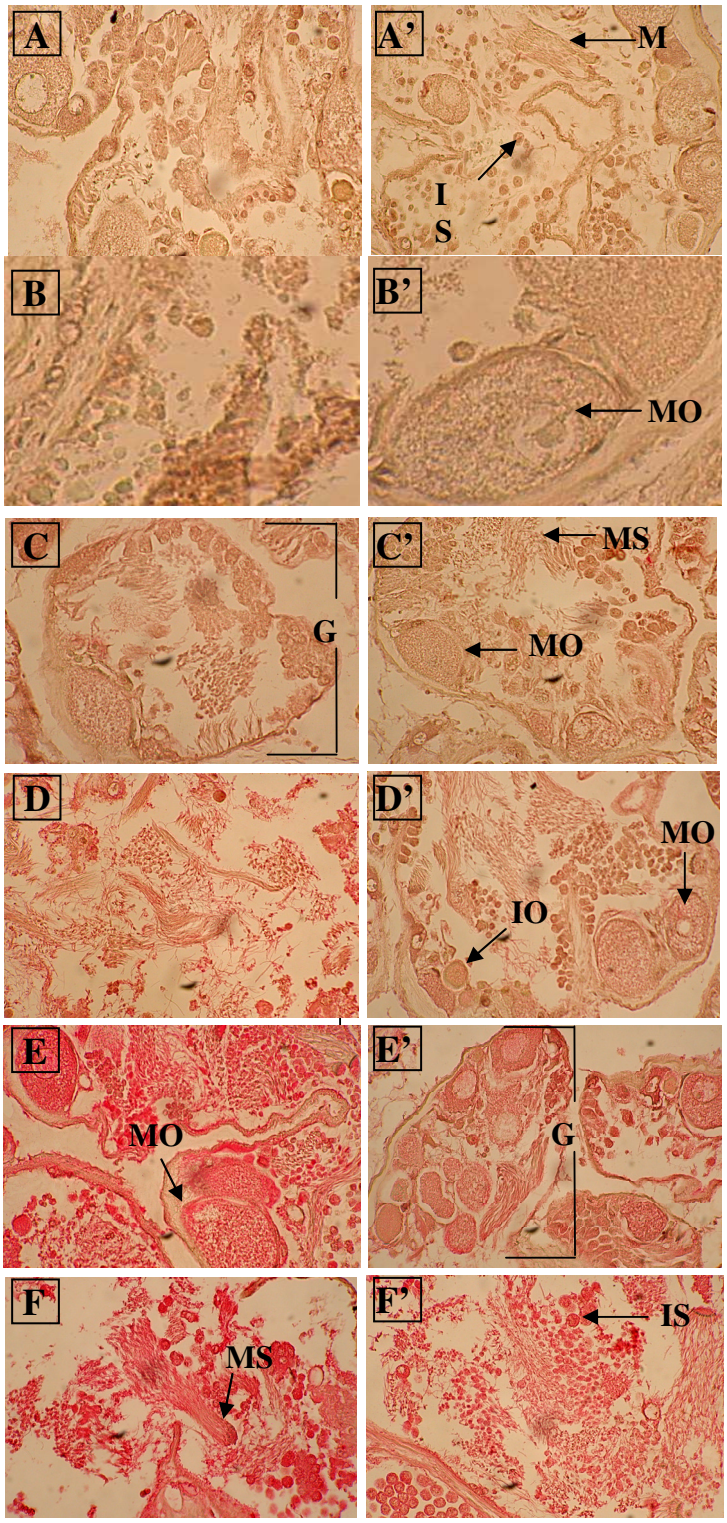


Figure 4



Chapitre 6 : Accumulation de microcystines libres et liées chez le pulmoné *L. stagnalis* exposé à une cyanobactérie toxique ou à de la MC-LR dissoute

Chapitre 6 : Accumulation de microcystines libres et liées chez le pulmoné *L. stagnalis* exposé à une cyanobactérie toxique ou à de la MC-LR dissoute

De précédents travaux (Gérard et al., 2005 ; Lance et al., 2006, 2007) ont montré que l'accumulation de microcystines (MCs) dans les tissus du gastéropode pulmoné *L. stagnalis* varie selon la voie d'intoxication (i.e. 1300 fois plus importante après exposition aux cyanobactéries toxiques vs à la MC-LR dissoute), la présence ou non d'une autre source de nourriture non toxique, et l'âge du gastéropode (juvénile, adulte). Cependant, ces travaux ne prennent en compte que l'accumulation des toxines libres, éliminées par les gastéropodes lors des processus de détoxication (Lance et al., 2006, 2008), alors qu'une partie des MCs se lie de manière covalente et irréversible aux protéines phosphatases (Ppases), devenant ainsi impossible à extraire par les solvants organiques comme le méthanol et indétectable par le test ELISA classiquement utilisé. Dans le chapitre 5, la technique d'immuno-histochimie mise au point avec l'équipe du Pr. D. Dietrich nous a permis de relier les dommages histopathologiques sévères observés après ingestion de cyanobactéries toxiques par la limnée à la présence de MCs liées dans le cytoplasme des cellules endommagées.

Le but de cette étude est de mesurer l'accumulation de MCs libres et liées de manière covalente chez *L. stagnalis* en fonction des modalités d'intoxication. Les limnées ont été exposées pendant 5 semaines à des cyanobactéries (*P. agardhii*) productrices de MCs (dmMC-LR, dmMC-RR and MC-YR) à 2 concentrations (5 et 30 µg L^{-1}) et à de la MC-LR dissoute (30 et 100 µg L^{-1}). L'expérience impliquant l'ingestion de cyanobactéries toxiques a été renouvelée en utilisant la même souche de *P. agardhii* mais produisant les 3 variants de MCs dans des proportions différentes, afin de déterminer si celles-ci influencent la quantité de MCs totales (liées et libres) et la proportion des variants de MCs libres accumulées dans la limnée. Chaque intoxication a été suivie de 3 semaines de dépuration afin de déterminer dans quelles proportions les MCs libres et liées sont éliminées. Le dosage des toxines libres a été réalisé grâce à deux méthodes, le test ELISA et la Chromatographie Liquide à Haute Performance couplée à la Spectrographie de Masse (HPLC MS), afin de comparer les résultats obtenus avec ces deux techniques et d'évaluer l'efficacité du test Elisa pour le

dosage des MCs libres chez les gastéropodes. De plus, l'HPLC MS permet d'identifier et de doser les différents variants de MCs produits par les cyanobactéries et accumulés par les gastéropodes. Le dosage des MCs liées est basé sur une coupure chimique de la molécule de MC par oxydation au niveau d'un groupement non impliqué dans la liaison avec les Ppases. Ainsi, toutes les MCs, libres ou liées, sont concernées par cette réaction et donnent une nouvelle molécule, le MMPB (acide 2-methyl-3-methoxy-4-phenylbutirique), dosée par HPLC MS. Nous obtenons alors la quantité de MCs totales qui nous permet de déduire la quantité de MC liées à partir de la quantité de MCs libres. Ces dosages ont été réalisés dans le laboratoire de Biochimie et Pharmacologie du Dr J. Meriluoto (Turku, Finlande), lors d'un séjour de 3 mois financé par le Ministère des Affaires Etrangères Franco-Finlandais.

Les résultats montrent qu'en fin d'intoxication, les mollusques exposés à de la MC-LR dissoute à 30 et 100 µg L^{-1} ont accumulé uniquement des MCs libres avec un maximum de respectivement 0.07 et 0.26 µg MCs libres g DW^{-1}, alors que ceux exposés à *P. agardhii à* 5 et 33 µg MC-LReq L^{-1} accumulent respectivement jusqu'à 33.82 et 69.90 µg MCs totales g DW^{-1} dont 17.7 à 66.7% de MCs liées. Après 3 semaines de dépuration, les MCs sont entièrement éliminées des gastéropodes préalablement exposés à la MC-LR dissoute. Par contre, chez les gastéropodes préalablement exposés aux cyanobactéries toxiques, une moyenne de 90.2 ± 3.3% des MCs libres et entre 0 et 59.2% des MCs liées sont éliminées. A la fin de la dépuration, en moyenne 3.5 ± 0.9 µg g DW^{-1} de MCs liées (maximum de 15.3 µg g DW^{-1}) sont encore détectées, représentant jusqu'à 91% de la quantité de MCs totales. Par ailleurs, l'accumulation de MCs chez les gastéropodes varie en fonction des proportions de variants de MCs produits par *P. agardhii.* En effet, les mollusques exposés à la suspension de cyanobactéries produisant 65% de MC-YR (7.7% de dmMC-LR, 27.4% de dmMC-RR) accumulent 74.5% de MC-YR parmi les MCs libres, et accumulent 7.8 fois plus de MCs totales que les mollusques exposés à la suspension produisant 90.6% de dmMC-RR (2.5% de MC-YR, 6.9% de dmMC-LR) et présentant 83% de dmMC-RR dans leurs tissus.

Ces résultats suggèrent que la proportion relative des différents variants de MC produits par les cyanobactéries peut influencer l'accumulation de MCs libres et covalentes chez les gastéropodes. D'autre part, l'importante accumulation de MCs liées est à prendre en considération pour mesurer le risque de transfert de MCs dans le réseau trophique à partir des gastéropodes.

Accumulation of free and covalently bound microcystins in tissues of *Lymnaea stagnalis* (Gastropoda) exposed to toxic cyanobacteria or dissolved microcystin-LR

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Keywords: *Lymnaea stagnalis*, cyanobacteria, free and covalently bound microcystins (MCs), accumulation, detoxification

Abstract

Accumulation of the hepatotoxic microcystins (MCs) has been demonstrated in the freshwater gastropod *Lymnaea stagnalis* mainly by ingestion of toxic cyanobacteria, and in a lesser extent, by exposure to dissolved MC-LR. Previous studies reported accumulation of free MCs in gastropods but did not consider MCs covalently bound to protein phosphatases. Here, we measure the accumulation of both free and covalently bound MCs over a 5-week period in *L. stagnalis* exposed to: i) dissolved MC-LR (33 or 100 µg L^{-1}), and ii) two *Planktothrix agardhii* suspensions producing 5 or 33 µg MC-LReq L^{-1} (with a repetition of these treatments few months later). Intoxication was followed by a 3-week depuration period. Free MCs in snails were quantified both with the ELISA test and the Liquid Chromatography ElectropSpray Ionization tandem Mass Spectrometry (LC-ESI-MS/MS). Covalently bound MCs were analysed using the MMPB (2-methyl-3-methoxy-4-phenylbutiric acid) method with LC-ESI-MS/MS.

Snails exposed to dissolved MC-LR at 33 and 100 µg L^{-1} accumulated respectively up to 0.07 and 0.26 µg total MCs g DW^{-1} with covalently bound MCs remaining below the detection limit, whereas those exposed to MC-producing *P. agardhii* at 5 and 33 µg MC-LReq L^{-1} accumulated respectively up to 33.8 and 69.9 µg total MCs g DW^{-1} among them between 17.7 and 66.7% of covalently bound MCs. After 3 weeks of depuration, MCs were entirely eliminated from snails previously exposed to dissolved MC-LR. For snails previously exposed to toxic cyanobacteria, a mean of 90.2 ± 3.3% of free MCs and between 0 and 59.2% of covalently bound MCs were eliminated. After depuration, 3.5 ± 0.9 µg g DW^{-1} of covalently bound MCs (up to 15.3 µg g DW^{-1}) were still detected, representing up to 91% of total MCs.

In addition, MC-accumulation in these gastropods was different according to the proportion of MC-variants in *P. agardhii*. Snails exposed to the suspension producing 65% of MC-YR (with 7.7% of dmMC-LR and 27.4% of dmMC-RR) presented 74.5% of MC-YR among free MCs in their tissues, and accumulated 7.8 times more total MCs than snails exposed to the suspension with 90% of dmMC-RR (with 2.5% of MC-YR and 6.9% of dmMC-LR) presenting 83% of dmMC-RR in their tissues. It suggests that the proportion of MC variants produced by toxic cyanobacteria in the field could influence free and covalently bound MC accumulation in gastropods. The results are discussed in terms of potential MC transfer from gastropods to the food web.

Introduction

Massive cyanobacterial blooms in freshwaters worldwide have become a serious threat to human health and to aquatic biota due to the production of potent toxic metabolites (e.g., hepatotoxic microcystins) (for review: Wiegand and Plufgmacher, 2005). The microcystins (MCs), intracellular cyclic heptapeptides of which 80 structural variants have been identified (for review: Dietrich and Hoeger, 2005), are preferentially taken up by hepatocytes where they can specifically interact with the protein phosphatases (Ppases) (Hastie et al., 2005). Covalent or non-covalent MC-Ppases interactions result in enzyme inhibition, reorganization of cytoskeletal components and disruption of hepatic architecture, leading to severe and irreversible damages, and potentially death (for reviews: Zurawell et al., 2005, Wiegand and Pflugmacher, 2005).

MCs can enter the aquatic food web through accumulation in various organisms including zooplankton, macroinvertebrates and vertebrates (for review: Zurawell et al., 2005). Freshwater gastropods are essential in food web as direct consumers of phytoplankton and as preys of numerous invertebrates and vertebrates (Dillon, 2000). They inhabit the littoral area (Dillon, 2000) where cyanobacteria frequently form scums, and can therefore be intoxicated by ingestion of toxic cyanobacteria (intracellular toxins) or exposure to toxins released after cell lysis into the surrounding water. MC accumulation by gastropods has been demonstrated in the field (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005; Xie et al., 2007; Zhang et al., 2007; Gérard et al., 2008, 2009; Zurawell et al., 1999). In the laboratory, the consumption of MC-producing cyanobacteria (5 $\mu g\ L^{-1}$) induced severe impact on the life traits and MC accumulation in the pulmonate *Lymnaea stagnalis* and the prosobranch *Potamopyrgus antipodarum* (Lance et al., 2006, 2007, 2008). Negative effects on life traits were also observed following exposure to dissolved MC-LR at 33 $\mu g\ L^{-1}$ in both species but with minor MC accumulation (Gérard et al., 2005; Gérard and Poullain, 2005).

Nevertheless, these studies only reported free MC accumulation and did not consider accumulation of MCs irreversibly covalently bound to the tissue e.g. to the target proteins Ppases (Goldberg et al., 1995; Maynes et al., 2006). The ability of these toxins to bind covalently to the tissue has been demonstrated in some organisms [e.g., bivalves (Williams et al., 1997b,c; Dionisio Pires et al., 2004)] and the covalently bound toxins may play an important role as a source of MC that are transferred through the food web. To evaluate

covalently bound MC in gastropods is essential since gastropods are consumed daily by invertebrates (e.g. crayfish, leeches, aquatic insects as adult coleopterans or larval tabanids) and vertebrates (e.g. fish, waterfowl) (for review: Michelson, 1957), which in turn are consumed by aquatic or terrestrial predators (i.e. fish, amphibians, musk rats and birds).

Here, a chemical method has been adapted to detect total (bound plus free) MC content in snail tissues, through the formation of 2-methyl-3-methoxy-4-phenylbutiric acid (MMPB) as an oxidation product of the MCs (Sano et al., 1992; Harada, 1996; Williams et al., 1997b,c; Ott and Carmichael, 2006), and the detection of MMPB molecule by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The free, extractable, MC content in snail tissues using two detection methods, enzyme-linked immunosorbent assay (ELISA) and LC-ESI-MS/MS was also assessed.

This study examines the accumulation of covalently bound and free MCs in adult *L. stagnalis* exposed to cyanobacteria producing MCs (concentrations corresponding to 5 and 33 µg of MC-LR, referred to as MC-LR equivalents, (MC-LReq) L^{-1} with MC-YR, dmMC-LR and dmMC-RR) (*Planktothrix agardhii*, PMC 75-02) or to dissolved MC-LR (33 and 100 µg L^{-1}) for a period of 5 weeks. MC concentrations used were observed in eutrophic waters during cyanobacteria proliferations and after cyanobacterial lysis (for review: Chorus and Bartram, 1999). Experiment was repeated few months later with the same strain of *P. agardhii* (5 and 33 µg MC-LReq L^{-1}). We assessed the proportion of free and covalently bound MCs as well as the percentage of various MC variants in *P. agardhii* and snail tissues during 5 weeks of exposure. For all experiments, the intoxication period was followed by a 3-week depuration period in order to determine the potential decrease of both bound and free MCs in gastropod tissues.

The discussion focuses on:

- the comparison in the accumulation of covalently bound and free MCs in snails according to both intoxication pathways (toxic cyanobacteria ingestion vs dissolved MC exposure) and exposure doses,
- the change in the accumulation of covalently bound and free MCs in snails depending on the proportion of MC-variants in toxic cyanobacteria,
- and finally, the consequences in terms of potential MC transfer in the food web.

6.1. Material and methods

6.1.1. Biological material

Adult *L. stagnalis* were obtained from a laboratory population in the Experimental Unit of the Institut National de Recherche en Agronomie (U3E INRA, Rennes). Prior to the experiment, *L. stagnalis* (25 ± 3 mm shell length) were isolated in glass containers of 35 mL (1 snail/container), acclimated to the experimental conditions (12/12 L/D, 20 ± 1°C) and fed on biological lettuce for 7 days. *P. agardhii* (strain 75-02), originating from the recreational watersport site of Viry (Essone, France), was cultured as described in Lance et al. (2006). This filamentous cyanobacterium has been shown to produce three microcystin (MC) variants: dmMC-LR, dmMC-RR and MC-YR in various proportions (Yéprémian et al., 2007). The *P. agardhii* suspension provided twice a week to the gastropods contained a total concentration of 5 or 33 µg MC-LReq L^{-1} measured by high pressure liquid chromatography with UV diode array detection (HPLC-DAD) using the method described in Lance et al. (2006). The MC-LR (Alexis Corporation, USA) was solubilised with MeOH (1 mL L^{-1}) in dechlorinated water for final MC-LR concentrations of 33 and 100 µg L^{-1}.

6.1.2. Experimental set up

After acclimation, snails were divided into various treatment groups according to diet and medium and were held in:

- dechlorinated water with lettuce ad libitum (CONTR);
- dechlorinated water containing 33 µg of dissolved MC-LR L^{-1} (D33+LT) or 100 µg MC-LR L^{-1} (D100+LT) with lettuce ad libitum;
- two *P. agardhii* suspensions, P1 and P2 (same strain cultured in same conditions but at different times), both diluted in order to obtain two MC concentrations:
  - 5 µg MC-LReq L^{-1} without additional feeding (CYAN5) and with lettuce ad libitum (CYAN5+LT);
  - 33 µg MC-LR eq L^{-1} without additional feeding (CYAN33) and with lettuce ad libitum (CYAN33+LT, only with the P2 suspension).

Each group consisted of 20 isolated individuals. Cyanobacterial suspensions as well as the medium of starved, control and MC-LR exposed snails were renewed twice a week. Each treatment was maintained for 5 weeks, after which all gastropods were maintained in dechlorinated water and fed solely on lettuce ad libitum for a 3-week depuration period.

6.1.3. Quantitative analysis of free MC-LReq in exposed snails using the enzyme linked immunosorbent assay (ELISA)

Four snails were randomly chosen every week in the groups CYAN33 and CYAN33+LT exposed to the second *P. agardhii* suspensions (P2), and at the end of the intoxication and depuration periods in all other groups. Snails were starved for ca. 24h to empty their gut contents (Carriker, 1946) to ensure that the MC measurement reflected only assimilated toxins and did not include MCs in the undigested filaments of *P. agardhii*. MC extraction from tissues and analysis by immuno-assay were performed as described in Lance et al. (2006) with an ELISA Microcystin Plate Kit (Envirologix, Portland, ME, USA) with detection threshold of 0.05 µg L^{-1} and to the nearest 0.01 µg L^{-1} (Gilroy et al., 2000). The amount of detected MCs was expressed in µg MC g^{-1} dry weight (DW). The values were calculated by taking into account extraction recovery and possible matrix-induced signal enhancement or suppression with the ELISA test because of unspecific binding to the antibodies. The recovery for the extraction and the matrix effect (i.e. effect of snail tissue) were assessed as described in Lance et al. (2006). The average recovery was 68 ± 3.9% and matrix effect was negligible (from 0.28 to 7.5% of differences between matrix and methanol results with an average of 2.89 ± 0.37%). Similar results about the recovery for the extraction and the matrix effect with the ELISA test were already observed for *L. stagnalis* (Lance et al., 2006).

6.1.4. Quantitative analysis of free MCs in exposed snail tissues by LC-ESI-MS/MS

6.1.4.1. Sample preparation: extraction of MCs from snail tissues

Water was purified to 18.2 Ω cm with Milli Q Synthesis purification system (Molsheim, France). Methanol (HPLC grade) was purchased from Rathburn (Walkerburn, Scotland, UK) and butanol from Merck, (Darmstadt, Germany).

For extraction of free microcystins 10 mg of freeze-dried tissue material sample was extracted with butanol: methanol: water; 5:20:75, and sonicated. The extract was cleaned-up and concentrated by solid-phase extraction Oasis HLB 30mg cartridges (Waters, Milford, MA, USA).

6.1.4.2. LC-ESI-MS/MS Analysis

Acetonitrile (HPLC S grade) was purchased from Rathburn (Walkerburn, Scotland, UK). Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). The chromatographic separation was achieved with Agilent 1100 LC, Purospher Star C-18e 4 mm x30 mm column with 3µm particles. The gradient mobile phase consisted of A) 0.1% formic acid in water and B) acetonitrile. The gradient was from 25% B to 90% B over 8 min with flow rate 0.5 ml/min, and the column re-equilibrated to 25% B for 4 min with flow rate 1 ml/min. The compounds were detected with a Micromass Quattro Micro triple-quadrupole instrument with the single ion recording (SIR) and multiple reactant monitoring (MRM) on positive ion mode. The relevant ion recordings were dmMC-RR *m/z* 512.8 [M+2H]++, dmMC-LR *m/z* 981.5 [M+H]+, MC-YR *m/z* 1045.5 [M+H]+ and on the MRM mode the transitions from these ions to fragment *m/z* 135.1 [M+H]+.

6.1.4.3. Quantification of MCs and signal response calculations

The quantification was based on spiked control tissues extracted and treated in the same way as the sample. The samples were spiked with an extracted natural bloom sample. The concentrations of the toxins present in the spiking mixture had been determined by HPLC-DAD analysis (Meriluoto et al. 2000, Meriluoto and Spoof 2005). The matrix from the samples caused severe signal suppression and the signal response fluctuated during the sample series. The effects were corrected by using external standards imbedded into sample series to normalise the signal response. The amount of free MCs was expressed in µg MC g^{-1} DW.

6.1.5. Quantitative analysis of total MCs in exposed snails using the MMPB (3-methoxy-2-methyl-4-phenylbutyric acid) method and quality control of MC measurement

6.1.5.1. Sample preparation: Lemieux oxidation of snail tissues

For the MMPB reaction the reagents potassium permanganate, sodium metaperiodate, sodium carbonate anhydrous, sodium dihydrogen phosphate and the di-sodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Sodium bisulphite solution was purchased from Merck, trypsin (10 × solution, 25 g /l) from Sigma-Aldrich Chemie (Steinheim, Germany) and sulphuric acid from J.T. Baker (Deventer, the Netherlands). The MMPB standard used was a kind gift from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and Prof. K.-I. Harada.

For MMPB analysis the tissue sample was trypsinated for two hours in pH 8.5 at 37°C and oxidised for three hours with solution containing 0.1 M $KMnO_4$ and 0.1 M $NaIO_3$ in pH 9.0 at room temperature. The reaction was ended with sodium bisulphite solution and acidified with sulphuric acid. The solution cleaned-up and concentrated by solid-phase extraction using Oasis HLB 30mg cartridges.

6.1.5.2. LC-ESI-MS/MS Analysis and quantification MMPB

Instrument and mobile and stationary phase specifications as described above (in 2.4.2.). The gradient was from 40% B to 70% B over 3 min and then rapidly taken to 90% B for 1 min, with flow rate 0.5 ml/min, and the column re-equilibrated back to 40% B for 4 min with flow rate 1 ml/min. The MMPB molecule was detected with transitions from *m/z* 209.2 [M+H]+, to *m/z* 91, 131 and 191.

6.1.5.3. Covalently bound MC calculation

Quantification of MCs with the MMPB method gives the total MC content present in the snail sample. We calculated the covalently bound MC content in each homogenised snail tissue sample by the expression: bound MCs = total MCs –free MCs.

6.1.6. Data and statistical analysis

6.1.6.1. Index calculation

A mean free MC content in snails from the cyanobacterial treatments at the end of both intoxication and depuration periods was calculated by combination of free MC measurements from ELISA and LC-ESI-MS/MS methods after validation of each group by statistical comparisons (see section 3.2.2.a). Based on this mean free MC content (freeMC), we calculated for each group exposed to cyanobacteria:

1) the percentage of free MC elimination from snails between the cyanobacterial treatments and the depuration periods (%elimfreeMC) by the expression:

   $\%elimfreeMC = 100 \times [freeMC_{treatment} - freeMC_{depuration}] / freeMC_{treatment}$;

2) the covalently bound MC content in each snail sample by the expression:

   boundMCs = total MCs –freeMCs,

3) the percentage of covalently bound MC elimination from snail tissues between the treatment and the depuration periods (%elimboundMC) by the expression: $\%elimboundMC = 100 \times [boundMC_{treatment} - boundMC_{depuration}] / boundMC_{treatment}$.

The 8-week follow-up of the percentage of bound MCs in snails exposed to the second cyanobacterial suspension (P2 at 33 µg MC-LReq L^{-1}) with and without lettuce was calculated from free MCs measured with LC-ESI-MS/MS due to difference between LC-ESI-MS/MS and ELISA measurements for 2 couples among the 16 couples of values (section 3.2.2.a).

6.1.6.2. Statistical analysis

The data that did not follow a normal distribution (according to the Kolmogorov-Smirnov test) were compared using the Kruskall-Wallis (KW) test and 2 by 2 using: 1) the Mann-Whitney U-test for free and bound MC content in snail tissues (µg MC g DW^{-1}); 2) the Chi2 test for the percentage of each MC variant accumulated in snails, the %boundMC, the %elimfreeMC and the %elimboundMC. Data are reported as mean ± standard error (± S.E.). Differences were considered to be statistically significant at $\underline{P} < 0.05$.

6.2. Results

6.2.1. MC production by *P. agardhii*

The *P. agardhii* strain 75-02 always produced 3 MC variants (MC-YR, dmMC-LR and dmMC-RR) identified by LC-ESI-MS/MS. The proportions for the variants varied when the strain was cultured in the same condition but at different times. Both *P. agardhii* cultures P1 and P2 presented a stable proportion of MC variants during the 5-week treatment period, as quantified by HPLC-DAD and LC-ESI-MS/MS analysis throughout the treatment period (i.e. P2 2.48 ± 0.44% of MC-YR, 6.98 ± 0.83% of dmMC-LR and 90.5 ± 0.91% of dmMC-RR; and P1 64.8 ± 3.06% of MC-YR, 7.76 ± 2.27% of dmMC-LR and 27.38 ± 2.58% of dmMC-RR).

6.2.2. Accumulation of free MCs in exposed snails

6.2.2.1. Proportion of the different MC variants accumulated in snails:

After both intoxication and depuration periods and regardless of the total MC concentration exposure (5 and 33 µg MC-LReq L^{-1}), snails exposed to the *P. agardhii* suspension P2 showed significantly different proportion of MC variants (i.e. mean 82.9 ± 1.1% of dmMC-RR in all treatment groups after intoxication period) than snails exposed to the *P. agardhii* suspension P1 (i.e. mean 74.5 ± 7.3% of MC-YR in all treatment groups after intoxication period) (Table. 1). Moreover, for both *P. agardhii* suspensions and MC concentrations (5 and 33 µg L^{-1}), the proportion of MC variants was significantly modified from the end of intoxication to the end of depuration, i.e. from 74.5 ± 7.3% to 95.0 ± 2.4% of MC-YR in all snails exposed to the *P. agardhii* suspension P1, and from 2.68 ± 0.8 to 14.4 ± 5.4% of MC-YR in snails exposed to the *P. agardhii* suspension P2 (Table 1).

Table 1. Proportion (mean ± ES %) of free MC variants (dmMC-RR, dmMC-LR, MC-YR) in *Lymnaea stagnalis* tissues after 5 weeks of exposure to two separate cultures of the same *Planktothrix agardhii strain* (PMC 75-02), and after 3 weeks of depuration in various treatment groups. ne = not evaluated.

% of each MC variant		***P. agardhii* suspension P1**			***P. agardhii* suspension P2**		
	MC s in snail tissues	dmMC-RR	dmMC-LR	**MC-YR**	**dmMC-RR**	dmMC-LR	MC-YR
END OF TREATMENT PERIOD	CYAN5	11.9 ± 6.43	11.8 ± 7.91	**76.3 ± 6.71**	**81.8 ± 2.44**	14.2 ± 3.22	4.05 ± 0.78
	CYAN5+LT	12.0 ± 2.84	17.8 ± 8.39	**70.2 ± 9.65**	**84.4 ± 0.60**	13.7 ± 1.15	1.86 ± 0.99
	CYAN33	11.5 ± 1.57	11.5 ± 4.26	**77.0 ± 5.81**	**84.6 ± 0.68**	13.2 ± 1.20	2.22 ± 0.74
	CYAN33+LT	ne	ne	ne	**80.8 ± 0.52**	16.6 ± 0.86	2.59 ± 0.66
END OF DEPURATION PERIOD	CYAN5	ne	ne	ne	**63.7 ± 5.41**	21.9 ± 11.6	14.3 ± 3.64
	CYAN5+LT	3.74 ± 2.74	0.00 ± 0.00	**96.2 ± 2.65**	**67.8 ± 12.1**	28.6 ± 15.7	3.60 ± 3.60
	CYAN33	3.08 ± 2.02	3.00 ± 0.17	**93.9 ± 2.19**	**52.2 ± 3.68**	21.1 ± 5.48	26.6 ± 4.81
	CYAN33+LT	ne	ne	ne	**46.9 ± 6.27**	39.7 ± 15.6	13.3 ± 9.39

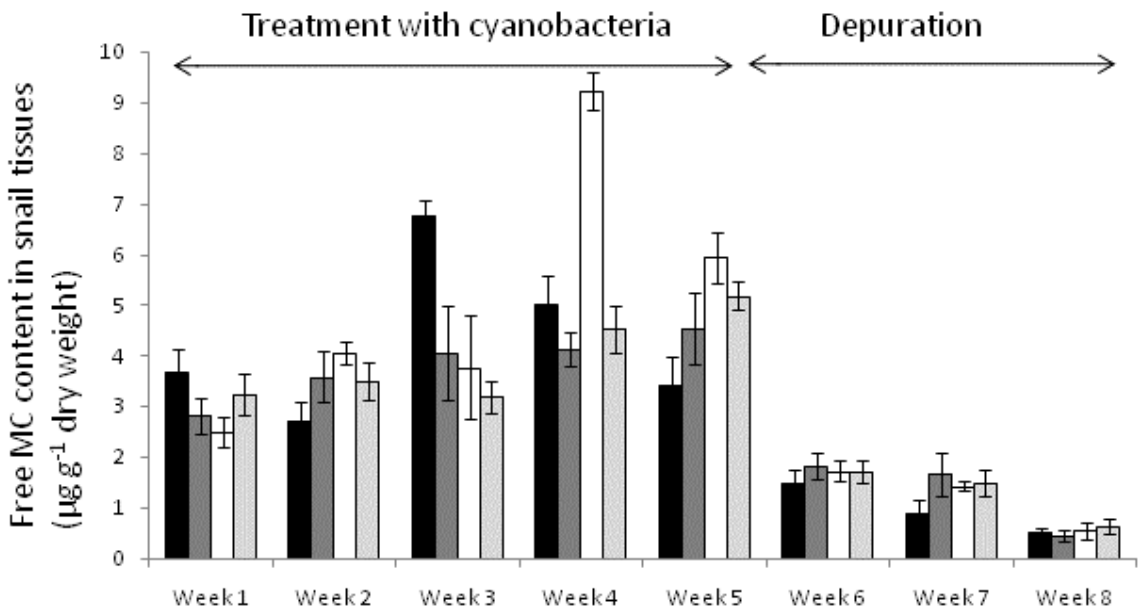
6.2.2.2. MC quantification

a. Comparison between the ELISA test and LC-ESI-MS/MS

No free MCs were detected with both ELISA test and LC-ESI-MS/MS method in starved and control snails after treatment or depuration period. After the treatment period, the free MC content in snails exposed to 33 or 100 µg MC-LR L^{-1} (D33+LT and D100+LT) was different according to the method: respectively 0.07 ± 0.01 and 0.26 ± 0.03 µg g^{-1} DW with ELISA test, and no free MC detected with LC-ESI-MS/MS. After the depuration, no free MCs were detected with both methods in those groups. The free MC content measured in *L. stagnalis* held in *P. agardhii* was similar between the two measurement methods at the end of the treatment and depuration periods, regardless of the proportion of MC variants (dmMC-LR, dmMC-RR and MC-YR) and the final MC concentration (5 or 33 µg L^{-1}) in the cyanobacterial suspension. The 8-week follow-up of MC content in snails exposed to the cyanobacterial suspension P2 (33 µg L^{-1}) with and without lettuce revealed similar values between LC-ESI-MS/MS and ELISA methods for each week, except for the groups CYAN33

at the week 3 and CYAN33+LT at the week 4 for which LC-ESI-MS/MS measurement were higher than ELISA measurement (Fig. 1).

Figure 1. Follow-up of free MC-LReq accumulated in *L. stagnalis* tissues (µg g DW-1) (± S.E.) fed on MC-producing (33 µg L-1) *P. agardhii* P2 without and with lettuce with MC measurement by LC-ESI-MS (respectively CYAN33 in black and CYAN33+LT in white or by ELISA test (respectively CYAN33 in deep grey and CYAN33+LT in light grey) during 5-week intoxication and 3-week depuration.



b. Comparison between treatment groups

The free MC content in snails differed significantly between groups after both treatment and depuration. During the entire experiment, snails exposed to 33 and 100 µg of dissolved MC-LR L^{-1} presented a significantly lower MC content than snail exposed to *P. agardhii*. Ranking of MC content from the various treatments yielded the following order: CONTR = STARV < D33+LT < D100+LT < CYAN5+LT < CYAN5 < CYAN33+LT ≤ CYAN33 (Table 2). For both MC concentrations (5 and 33 µg L^{-1}) and after treatment and depuration periods, snails exposed to the *P. agardhii* suspension P1 showed a significantly higher mean free MC accumulation than snails exposed to the *P. agardhii* suspension P2 (i.e. 7.5 times superior at 5 µg L^{-1} and 8.2 times at 33 µg L^{-1} without feeding of lettuce at the end of the intoxication period) (Table 2).

Table 2. Accumulation of free and covalently bound MCs in *L. stagnalis* tissues (µg g DW-1) (± S.E.) and percentage of covalently bound MCs among total MCs (%boundMCs) after 5-week exposure to two MC-producing *P. agardhii* suspensions P1 and P2 and after 3-week depuration. ne = not evaluated.

		with *P. agardhii* P1			with *P. agardhii* P2		
		MC s (µg g-1 DW)		% boundMCs	MC s (µg g-1 DW)		% boundMCs
		free MCs	bound MCs		free MCs	bound MCs	
END OF TREATMENT PERIOD	CYAN5	24.6 ± 2.74	9.22 ± 2.69	27.2 ± 2.61	3.27 ± 0.27	6.56 ± 1.00	66.7 ± 3.69
	CYAN5+LT	11.9 ± 0.84	2.57± 0.93	17.7 ± 2.08	3.39 ± 0.48 (only elisa)	ne	ne
	CYAN33	32.4 ± 2.62	37.5 ± 3.93	53.6 ± 3.57	3.97 ± 0.57	5.13 ± 1.24	56.3 ± 3.98
	CYAN33+LT	ne	ne	ne	5.56 ± 0.49	3.90 ± 0.51	41.2 ± 2.79
END OF DEPURATION PERIOD	CYAN5	1.93 ± 0.15 (only elisa)	ne	ne	0.29 ± 0.03	< dl	ne
	CYAN5+LT	0.93 ± 0.61	1.72 ± 0.11	64.7 ± 4.63	0.19 ± 0.02	< dl	ne
	CYAN33	5.28 ± 2.01	15.3 ± 2.59	74.3 ± 6.94	0.48 ± 0.09	5.11 ± 0.36	91.3 ± 3.79
	CYAN33+LT	ne	ne	ne	0.55 ± 0.17	4.65 ± 0.62	89.3 ± 4.17

6.2.3. Accumulation of covalently bound MCs in exposed snails

The total MC content in snails exposed to dissolved MC-LR evaluated with the MMPB method always remained below the detection limit. At the end of treatment and depuration periods, snails from the CYAN33 group exposed to P1 or P2 *P. agardhii* suspensions presented higher amount of bound MCs than of free MCs (Table 2). The highest amount of bound MCs (37.5 ± 3.9 µg g^{-1} DW) was found in snails from the CYAN33 group exposed to the cyanobacterial suspension P1. At the end of the intoxication period, the percentage of bound MCs among total MCs (%boundMC) varied from 17.7 ± 2.1% to 66.7 ± 3.6% (Table 2).

The %boundMC increased between the depuration and the treatment period in snails exposed to the P1 (CYAN5+LT and CYAN33) and the P2 (CYAN33 and CYAN33+LT) *P. agardhii* suspension. At the end of depuration, the %boundMC varied from 64.7 ± 4.6% to 91.3 ± 3.7% (Table 2). The global trends of the %boundMC can only be viewed as tentative

as statistical comparisons could not have been performed in all groups due to the lack of data (Table 2).

The 8-week follow-up of the percentage of bound MCs in *L. stagnalis* exposed to the cyanobacterial suspension P2 (at 33 µg L^{-1}) with and without lettuce (Fig. 2) shows that the proportion of bound MCs rapidly increased during the first week of exposure and reached the maximum of the treatment period (i.e. 69.5 ± 6.3%) at the end of the second week. During the depuration, the percentage of bound MCs linearly increased in both groups until a maximum of 90.9 ± 3.8% in snails exposed to *P. agardhii* without lettuce at the end of depuration.

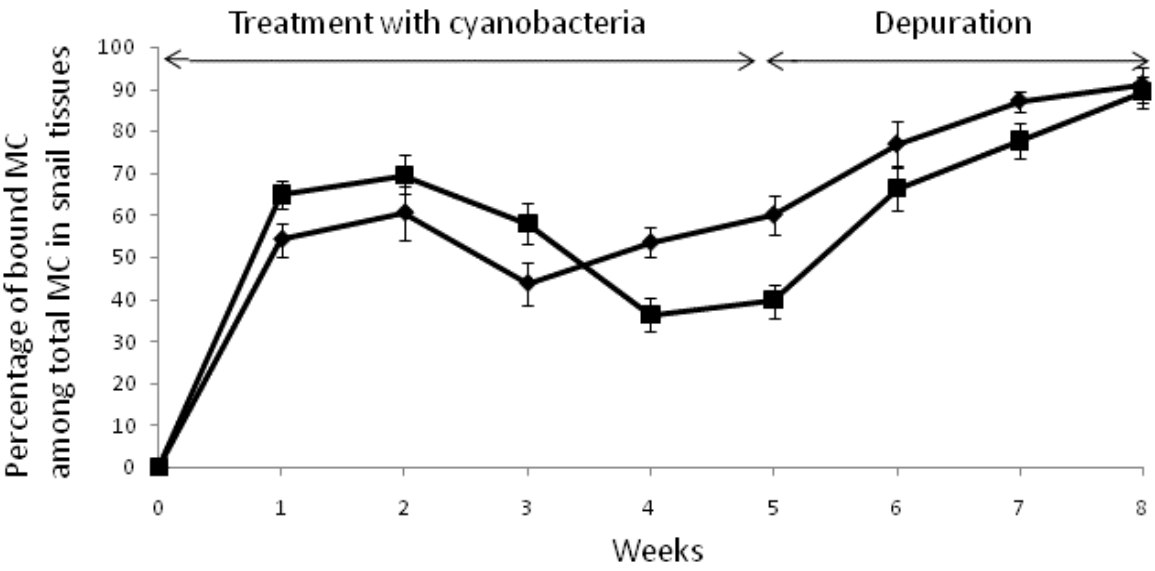


Figure 2. Follow-up of the percentage (%) (± S.E.) of covalently bound MCs among total (free + bound) MCs accumulated in *L. stagnalis* exposed to MC-producing (33 µg MC-LReq L^{-1}) *P. agardhii* suspension P2 without (triangle) and with lettuce (square) during 5-week intoxication and 3-week depuration.

6.2.4. Percentage of elimination of free and covalently bound MCs during the depuration

Snails exposed to 33 and 100 µg dissolved MC-LR L^{-1} eliminated the free MC content to below limit of detection from their tissues during the 3-week depuration period. The elimination of free MCs from *L. stagnalis* previously exposed to toxic cyanobacteria was far higher than the elimination of bound MCs regardless of the treatment group and of the *P. agardhii* suspension (Table 3). The %elimfreeMC was similar in all groups from the two

different cyanobacterial suspensions with an average of 90.2 ± 3.3%. The %elimboundMC was different between the groups and was higher for snails exposed to the *P. agardhii* suspension P1 than for snails exposed to the suspension P2, i.e. respectively 59.2 ± 2.9% vs 0.39 ± 0.1% for the CYAN33 group (only one statistical comparison has been performed due to the lack of data) (Table 3).

Table 3. Percentage (%) of free and covalently bound MC elimination from *L. stagnalis* tissues after 3 weeks of depuration; snails were previously exposed during 5 weeks to two MC-producing *P. agardhii* suspensions P1 and P2, at 5 or 33 µg L^{-1} MC-LReq with (CYAN+LT) or without lettuce (CYAN). ne = not evaluated.

	***P. agardhii* suspension P1**		***P. agardhii* suspension P2**	
	free MCs	bound MCs	free MCs	bound MCs
CYAN5	92.1 ± 4.36	ne	91.1 ± 2.08	ne
CYAN5+LT	92.2 ± 2.91	33.1± 1.84	94.4 ± 3.41	ne
CYAN33	83.7 ± 4.19	59.2 ± 2.97	87.9 ± 2.77	0.39 ± 0.06
CYAN33+LT	ne	ne	90.1 ± 3.54	0.00 ± 0.00

6.3. Discussion

6.3.1. Free MC accumulation with different intoxication routes and MC variants

After 5 weeks of intoxication, the gastropod *L. stagnalis* exposed to MC-producing cyanobacteria (5 and 33 µg MC-LReq L^{-1}) exhibited stronger free MC accumulation (i.e. respectively maximum of 24.6 µg g^{-1} DW and 32.4 µg g^{-1} DW) than snails exposed to dissolved 100 µg MC-LR L^{-1} (i.e. 0.26 µg g^{-1} DW). These results are in accordance with field studies (Kotak, 1996; Zurawell et al. 1999; Yokoyama and Park, 2002; Chen et al., 2005; Xie et al., 2007) concluding that gastropods accumulated free MCs mainly by grazing toxic phytoplankton, and to a lesser extent via uptake of dissolved toxins. In the laboratory, the two intoxication routes were already known to induce differences in free MC accumulation by *L. stagnalis*, i.e. 1300 times more important in *L. stagnalis* after toxic cyanobacteria (5 µg MC-LReq L^{-1}) ingestion than after dissolved MC-LR exposure (33 µg L^{-1}) (Gérard et al., 2005; Lance et al., 2006). Moreover, we recently showed using an immunohistological method (Lance et al., in preparation), that the amount of MCs penetrating the cytoplasm of digestive gland cells was important after ingestion of toxic cyanobacteria (producing MC-YR, dmMC-LR and dmMC-RR) by *L. stagnalis*, whereas negligible after exposure to dissolved MC-LR (excreted into lysosomal vacuoles). Two hypotheses are therefore possible: i) gastropod intoxication (i.e. MC penetration in the snail) is far higher by grazing toxic cyanobacteria than by uptake of dissolved toxins, and ii) uptake of MCs across membranes differs between structural MC congeners (variants). Even if it was not the purpose of the present study, it is worthy to note that a part of the results seems to support the last hypothesis. Indeed, *L. stagnalis* exposed to *P. agardhii* producing 33 µg MC-LReq L^{-1} with 90.5% of dmMC-RR accumulated low amount of free MCs (i.e. maximum of 5.56 µg g^{-1} DW) with a high proportion of dmMC-RR (82.9%) in their tissues. On the other hand, when exposed to the same cyanobacterial strain producing 33 µg MC-LReq L^{-1} but with 64.7% of MC-YR, snails accumulated 8.2 times more free MCs with a high proportion of MC-YR (74.5%) (and 7.3 times more bound MCs). It thus appears that *L. stagnalis* accumulated MC-YR in a higher extent than other variants involved in this study (dmMC-RR, dmMC-LR and MC-LR). Moreover, the proportion of MC-YR in snails exposed to the two *P. agardhii* suspensions increased after the depuration period, regardless of the proportion after the intoxication period, suggesting that MC-YR is less eliminated by snails than dmMC-RR. However, these

results about the difference of accumulation and depuration between MCs variants in snails can only be viewed as tentative and would need to be confirmed by more detailed investigations.

6.3.2. Comparison of detection methods of free MCs in gastropods

Our results report that *L. stagnalis* exposed for 5 weeks to 33 µg dissolved MC-LR L^{-1} accumulated a maximum of 0.07 µg g DW^{-1} in accordance with a similar study of Gérard et al. (2005) (maximum of 0.06 µg g DW^{-1} after a 6-week exposure), whereas no free MC accumulation was found when measured by LC-ESI-MS/MS. ELISA measurements may give an overestimation of toxin concentrations due to cross reactivity by immunoaffinity with metabolised MCs (i.e. conjugated with glutathione and cysteine) (Metcalf, 2000) that have no or a much reduced toxicity (Ibelings and Chorus, 2005). Studies on the accumulation of the hepatotoxic nodularin in organisms of the Baltic Sea express the amount of toxin by the term TEH (i.e. Total Extractable Hepatotoxins) which includes the non-toxic or less toxic biotransformation products. TEH measured by ELISA commonly exceed the concentration of untransformed hepatotoxins (analysed on LC-MS) (Lehtonen et al, 2003; Kankaanpaa et al., 2005). However, cystein conjugates were not detected by LC-ESI-MS/MS in this study, as also found by Dionisio Pires et al. (2004) in mussels. Moreover, no free MCs and thus no false positives were detected with the ELISA test in starved and control snails. The absence of MCs when measured by LC-ESI-MS/MS was probably due to the higher detection threshold of this method (Msagati et al., 2006). When MC contents increased in snails exposed to *P. agardhii*, values were similar between the two methods at the end of intoxication and depuration periods. In spite of the fact that the ELISA test cannot detect accurately the total MC content due to the variation in the specificities of the antibodies, (Msagati et al., 2006), this method gives a good report of the MC content in *L. stagnalis* tissues.

6.3.3. Accumulation of covalently bound MCs

After penetration in the cytoplasm of the host cells, MCs can interact with the catalytic subunit of Ppases (PP1, 2A, 4 and 5) in a two-step mechanism involving a rapid and reversible binding (within minutes to a few hours) and inactivation of PPases, followed by a covalent bound after several hours (Hastie et al., 2005; Maynes et al., 2006). Since MCs

covalently bind to the Ppases and because the MCs cannot be extracted from the covalent complex by organic solvent, detection of MCs in animal tissues has been limited in free and probably also conjugated MCs with glutathione and cysteine (Craig et al., 1996). MC accumulation by gastropods reported in previous field (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003; Chen et al., 2005; Xie et al., 2007; Zhang et al., 2007; Gérard et al., 2008, 2009) and laboratory (Zurawell et al., 2006, 2007; Lance et al., 2006, 2007) studies were thus most likely underestimated.

Using an oxidation procedure adapted from previously developed methods (Sano et al., 1992; Harada, 1996; Williams et al., 1997a, b; Ott and Carmichael, 2006) and followed by a detection of oxidation products by LC-ESI-MS/MS, we provided direct evidence for the existence of covalently bound MCs in gastropod tissues. On average, 44% of total MCs were MCs covalently bound to Ppases in *L. stagnalis* exposed to *P. agardhii* during 5 weeks, regardless of the final MC concentration (5 or 33 µg MC-LReq L^{-1}) in the cyanobacterial suspension and of the presence or absence of a concomitant non-toxic food (lettuce). Moreover, the proportion of bound MCs rapidly increased during the first week of exposure to *P. agardhii* (33 µg MC-LReq L^{-1}) and reached the maximum of the treatment period (i.e. 69 % of total MCs corresponding to 222% of free MCs) at the end of the second week. The existence of covalently bound MCs, using a similar method, has been demonstrated in tissues of fish [salmon livers (Williams et al., 1997a)] and of molluscs [but only in some bivalves (Williams et al., 1997b and c; Dionisio Pires et al., 2004)]. In zebra mussels, covalently bound MC-LR was generally lower than free unbound MC-LR, but could reach 38% of the total amount of toxin (Dionisio Pires et al., 2004).

A previous study followed the consumption of *P. agardhii* containing MCs (5 µg MC-LReq L^{-1}) during 5 weeks and reported that an average of 47% of ingested MCs was measured in adult *L. stagnalis* (and 66% in juveniles) by methanol extraction (i.e. free MCs) (Lance et al., 2006). It suggests that the other 53% of ingested MCs could have been eliminated in the gizzard or the digestive gland fraction of the faeces, or accumulated in the digestive gland in a covalent form. According to our present results, 37.2% of accumulated MCs were covalently bound MCs in the tissues of *L. stagnalis* similarly exposed [i.e. 5-weeks exposure to *P. agardhii* (5 µg MC-LReq L^{-1})]. From these studies, it can be stated that at least 84% of ingested intracellular MCs are accumulated in a free or covalent form by adult *L. stagnalis* (and probably almost 100% in juveniles) after consumption of toxic cyanobacteria.

6.3.4. Consequences of the total MC accumulation by gastropods for the food web

6.3.4.1. Toxin transfer through the food web

The total MC content (free and covalently bound MCs) needs to be considered in order to assess the contamination risk of the food web from gastropods. The present study reveals for the first time an important accumulation of total MCs (a maximum of 69.9 µg g^{-1} DW after 5 weeks) by gastropods after a 5-week exposure to toxic cyanobacteria, with a high proportion (maximum of 66.7%) of covalently bound MCs. Moreover, the percentage of covalently bound MCs increased during the depuration period and reached 91.3% of the total MC content. This increase is both due to a high free MC elimination (mean of 90% of elimination in all groups) and to a lesser bound MC elimination, varying from 0 to 59% according to treatments. Indeed, free MCs accumulated can be metabolized into less harmful compounds after conjugation with gluthatione via glutathione-S-transferase, resulting in MC excretion or physiological degradation in several organisms (Pflugmacher, 1998; Wiegand et al., 1999, Pietsch et al., 2001). However, the elimination process of covalently bound MCs has never been studied so far. Williams et al. (1997b, c) found that mussels rapidly cleared the covalently bound MCs when transferred in untreated salt water: the total MC content dropped from 336.9 µg MC g^{-1} FW to 11.3 µg MC g^{-1} FW during 4 days, and was undetectable after these 4 days. As the covalent binding of MCs to Ppases is known to be irreversible and induce cell necrosis (for review: Dietrich and Hoeger, 2005), their elimination might occur via degradation and consequent elimination of damaged cells and/or during the Ppase renewal.

Nevertheless, at the end of the 3-week depuration period, *L. stagnalis* tissues still contained up to 20.6 µg g^{-1} DW of total MCs (with 15.3 µg g^{-1} DW of covalently bound MCs), suggesting that gastropods can represent a vector for MC transfer in the food web. However, as questioned by Williams et al. (1997) and Ibelings et Chorus (2007), the covalent complex MC-Ppases is probably not toxic as an intact entity or not bioavailable for the next trophic level (there is possibly no enzymes capable of reversing the covalent linkage to liberate free toxic MCs). Hence, investigations are required to demonstrate the toxicity of covalently bound MCs and their transfer patterns through the food web.

6.3.4.2. Impact on the natural communities of gastropods

In mammal and fish exposed to dissolved MC-LR or fed on MC-producing cyanobacteria, MCs are known to induce severe disorganization of the hepatic architecture during acute poisoning, and hepatocyte degeneration during chronic exposure (for reviews: Zurawell et al., 2005; Malbrouck and Kestemont, 2006; Ernst et al., 2007; Li et al., 2007). A recent study (Lance et al., in preparation) suggests that the impact of MC on the digestive gland structure (i.e. severe and widespread necrotic changes) of the gastropod *L. stagnalis* is likely also to be attributed to the MC-Ppases interaction in the cytoplasm of digestive gland cells. Fisher and Dietrich (2000) studied the relationship between hepatotoxic injury and MC localisation in rainbow trout (*Oncorhynchus mykiss*) gavaged with toxic cyanobacteria and showed that Ppase inhibition and hepatocyte necrosis appeared to be associated with the reversible interaction MCs-Ppases, blocking access to the active centre of enzymes, whereas apoptotic cell death results from the covalent interaction. The present study reveals that both MC-Ppases interactions occurs in *L. stagnalis* during a 5-week exposure to toxic cyanobacteria, leading to the severe impact on the tissues as well as the negative impact on the life traits (i.e. survival, growth and fecundity) previously observed (Lance et al., 2006; 2007; in preparation).

According to the life cycle of freshwater gastropods and the structure of their populations during the proliferation periods of cyanobacteria [i.e. the main breeding season takes place in late spring or early summer (Calow, 1978), and coincides with the beginning of the cyanobacteria population maxima (Chorus and Bartram, 1999)], it is highly probable that toxic cyanobacteria have a strong negative impact on natural gastropods communities in eutrophic waters. A decrease in gastropod abundance will have indirect negative consequences on the populations of the predator organisms and indirect positive consequences on the proliferation of toxic cyanobacteria as gastropods may largely control algal and cyanobacterial biomass and productivity by grazing (Mc Collum et al., 1998; Liess & Hillebrand, 2004 for review). The sublethal effects of MCs on gastropod populations may thus have potential cascading effects on the equilibrium and functioning of the whole aquatic ecosystem.

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Chapitre 7 : Transfert trophique des microcystines du gastéropode *Lymnaea stagnalis* au poisson *Gasterosteus aculeatus* (épinoche) et impact chez le poisson

Chapitre 7 : Transfert trophique des microcystines du gastéropode *Lymnaea stagnalis* au poisson *Gasterosteus aculeatus* (épinoche) et impact chez le poisson[1].

L'accumulation de microcystines (MCs) a été démontrée chez le gastéropode pulmoné *Lymnaea stagnalis* principalement suite à l'ingestion de cyanobactéries productrices de MCs (maximum de 80 µg g^{-1} DW) (Lance et al., 2006), et dans une moindre mesure après exposition à de la MC-LR dissoute (maximum 0.26 µg g^{-1} DW exposé à 100 µg L^{-1}) (Lance et al., en préparation, voir chapitre 6). Les concentrations de MCs enregistrées dans les tissus des limnées sont probablement sous-estimées par rapport à la réalité dans la mesure où seules les MCs libres ont été prises en compte dans ces dosages. En effet, une partie des MCs peut se lier de manière irréversible aux protéines phosphatases (Ppases) et devenir ainsi indétectable par les techniques de dosage classiques (Elisa) (Maynes et al., 2006). Les MCs liées peuvent représenter jusqu'à 38% des MCs totales chez certains mollusques bivalves (Williams et al., 1997; Dionisio Pires et al., 2004), et entre 27 et 67% (jusqu'à 37.5 µg g^{-1} DW) chez *L. stagnalis* après ingestion de cyanobactéries toxiques (aucune MC liée n'est détectée après exposition à de la MC-LR dissoute) (Lance et al., chapitre 6). Environ 90% des MCs accumulées sous forme libre par *L. stagnalis* sont éliminées après 3 semaines de dépuration (Lance et al., 2006), probablement grâce à des processus de détoxication mis en évidence chez d'autres organismes (e.g. végétaux, zooplancton, poissons, mammifères) et impliquant des glutathions (Wiegand et al., 1999; Cazenave et al., 2006). En ce qui concerne les MCs liées, la détoxication, qui a lieu probablement lors du renouvellement des Ppases, est nettement moins efficace : seules 0 à 59% des MCs liées sont éliminées en 3 semaines de dépuration, période pendant laquelle leur proportion augmente jusqu'à 90% des MCs totales (Lance et al., en préparation). La présence de MCs libres et liées dans les tissus de *L. stagnalis*, y compris après l'arrêt de la contamination, sous-entend que cette espèce peut jouer le rôle de vecteur de MCs dans la chaîne trophique et est susceptible de contaminer de nombreux prédateurs (e.g. sangsues, écrevisses, insectes aquatiques, poissons, oiseaux) (Michelson, 1957). Jusqu'à

[1] Cette partie a été réalisée avec Anais Petit, stagiaire de Master 1 à l'université de Rennes 1 d'avril à juin 2008, et en collaboration avec Wielfried Sanchez, ingénieur de recherche à l'Institut National de l'Environnement Industriel et des Risques de Verneuil sur Halatte (France).

présent, les expériences réalisées au laboratoire ont impliqué du zooplancton en tant que vecteur de MC (Lauren-Määttä et al. , 1995; Smith et Haney, 2006). En milieu naturel, le transfert trophique des MCs a été démontré entre des bivalves et des poissons malacophages mais sans qu'il y ait de biomagnification [i.e. transfert trophique d'une substance résultant en une plus forte concentration dans les tissus du consommateur que dans sa nourriture (Ibelings et Chorus, 2007] (Ibelings et al., 2005). Le transfert de la nodularine (NODLN) dissoute, une autre hépatotoxine, a également été mis en évidence avec de faibles taux d'accumulation chez des larves de brochet (*Esox lucius*) et des Mysidacés (*Neomysis integer*) après consommation de zooplancton préalablement contaminé (Karjalainen et al., 2005).

Nous avons voulu mesurer au laboratoire le risque de transfert de MCs le long de la chaîne trophique impliquant des cyanobactéries toxiques (*Planktothrix agardhii*), des gastéropodes (*L. stagnalis*) et des poissons (l'épinoche *Gasterosteus aculeatus*).
L'épinoche est un petit poisson carnassier euryhalin des zones tempérées de l'hémisphère Nord, utilisé comme espèce bioindicatrice de par sa large répartition et sa sédentarité (Sanchez et al., 2007). Son régime alimentaire est considéré comme omnivore (e.g., macroinvertébrés benthiques, mésoplancton, œufs de poissons) et peut inclure des mollusques selon leur disponibilité dans le milieu (Bruslé et Guignard, 2001). Proie de nombreux poissons piscivores (Sipia et al., 2007), l'épinoche peut à son tour être vectrice de MCs dans le réseau trophique. Plus précisément, les objectifs majeurs de notre étude sont 1) de démontrer la réalité du transfert de MCs des limnées aux épinoches en suivant les cinétiques d'accumulation et d'élimination des MCs dans différents organes du poisson (foie, muscle, rein, branchie) et 2) de déterminer si les MCs libres et liées sont transférées de façon similaire des limnées aux épinoches. Pour cela, les épinoches sont nourries lors d'une phase d'intoxication avec des glandes digestives de *L. stagnalis* présentant 2 concentrations différentes de MCs avec différentes proportions de MCs libres et liées, puis lors d'une phase de dépuration avec des glandes digestives de limnées saines. D'autre part, nous avons également étudié l'impact de la contamination sur les épinoches en mesurant : 1) le stress oxydant induit par dosage de l'activité d'une enzyme détoxifiante, la glutathion-S-transferase (GST), et de 2 enzymes antioxidantes : la glutathion peroxydase (GPx) et la superoxyde dismutase (SOD), 2) les effets histopathologiques sur le foie, organe cible des MCs, 3) les modifications de la fréquence ventilatoire et des comportements alimentaire et locomoteur connus pour être perturbés lors de l'exposition des épinoches à divers polluants (Wibe et al, 2004; Craig et Laming, 2004).

Les résultats montrent que les épinoches ingèrent les glandes digestives de limnées de la même manière qu'elles soient contaminées ou non par les MCs. L'intoxication des épinoches se traduit par une accumulation de MCs dans le foie, les muscles, les reins et les branchies des épinoches (maximum de 3.96 ± 0.14 µg g^{-1} DW dans le foie), suivie lors de la phase de dépuration par une élimination totale des toxines dans les branchies et les reins et partielle dans le foie et les muscles. Malgré cette élimination, aucune induction de l'enzyme de détoxification GST n'est mise en évidence. Par contre, l'enzyme antioxydante GPx est activée lors de l'intoxication du poisson, suggérant que la consommation de glandes digestives de *L. stagnalis* contenant des MCs (libres et liées) entraîne un stress oxydant et la production de ROS chez l'épinoche. Cette consommation a un impact histopathologique modéré sur les épinoches ayant ingéré les glandes digestives de limnées contenant le plus de MCs totales (libres et liées) et ne modifie pas leur fréquence ventilatoire. Par contre, les épinoches intoxiquées restent significativement plus longtemps immobiles que les témoins pendant l'intoxication, puis se déplacent significativement plus pendant la dépuration, probablement en raison respectivement d'une moins bonne condition générale du poisson, puis d'une recherche de nourriture plus active pour compenser les pertes énergétiques engendrées par la métabolisation des toxines. Enfin, la concentration de MCs (seules les MCs libres ont été dosées pour le moment) dans le foie des épinoches est 1.7 fois inférieure à celle présente dans les glandes digestives de limnée les plus riches en MCs (mais avec 64% de MCs liées), et est équivalente lorsque les glandes digestives de limnées contiennent moins de MCs mais avec 94% de MCs liées. Ces résultats démontrent la réalité du transfert des MCs des gastéropodes aux poissons, mais sans qu'il y ait de biomagnification. D'autre part, ils suggèrent des différences de transfert entre MCs libres et liées à prendre en considération dans les mesures de prévention en milieu naturel, compte tenu du risque probablement supérieur induit par les MCs liées (vs libres) en raison d'une élimination moins efficace.

Chapitre 7 : Trophic transfert of MCs from the gastropod *Lymnaea stagnalis* to the fish *Gasterosteus aculeatus* (three-spined stickleback) and impact on the fish

Introduction

Our previous studies showed that the microcystin (MC) exposure of the gastropod pulmonate *Lymnaea stagnalis* lead to an accumulation of free MC, in an higher extent after cyanobacterial cell consumption (*P. agardhii*, 5 µg L^{-1}) (up to 60% of ingested MCs are accumulated) (Lance et al., 2006), than after a dissolved MC-LR exposure (33 µg L^{-1}) (i.e. 1300 less) (Gérard et al., 2005). We hypothesized that these accumulations were underestimated due to the potent covalent linkage between MCs and phosphatase proteins (Ppases), preventing from a MC extraction by organic solvents classically used (Hastie et al., 2005; Maynes et al., 2006). We recently demonstrated that *L. stagnalis* exposed to dissolved MC-LR (33 and 100 µg L^{-1}) accumulated only free MCs (up to 0.26 µg MCs g DW^{-1}), whereas those exposed to MC-producing *P. agardhii* (5 and 33 µg L^{-1}) accumulated free and bounds MCs (up to 69.9 µg total MCs g DW^{-1}) (Lance et al., unpublished data). These results suggest that adult *L. stagnalis* may accumulate at least 85% of ingested MCs during toxic cyanobacteria consumption, with up to 67% of covalently bound MCs. Although 90% of free MCs can be eliminated after a 3-weeks depuration period [probably by detoxification processes involving glutathiones enzymes (Wiegand et al., 1999; Cazenave et al., 2006)], the elimination of covalently bound MCs [probably occurring during the Ppase renewal] is lower (from 0 to 59%) and their proportion among total MCs increases (up to 90%) during the depuration period (Lance et al., in preparation). Consequently, *L. stagnalis*, which accumulate high amount of bound MCs (i.e. up to 37.5 µg g^{-1} DW), is a potential vector of MCs to higher consumers (e.g. crayfish, leeches, aquatic insects as adult coleopterans or larval tabanids, fish, waterfowl) (for review: Michelson, 1957), which in turn are consumed by aquatic or terrestrial predators (i.e. fish, amphibians, musk rats and birds).

The three-spined stickleback, *Gasterosteus aculeatus* (Gasterosteidae), is an euryhalin fish from the temperate areas of the Northern hemisphere, used as an indicative species due to its widespread distribution and its sedentary (Sanchez et al., 2007). The three-spined stickleback is omnivorous (e.g., macroinvertebrates, mesoplankton, fish eggs) and ingest snails depending on their availability (Bruslé and Guignard, 2001). As they live both in the

open sea and in the littoral zone, where cyanobacteria may accumulate after wind events and where gastropods live, a contamination of fish via ingestion of MC-contaminated gastropods appears to be environmentally realistic. Numerous studies (for review: Malbrouck and Kestemont, 2006; Ernst et al., 2006, 2007) reported a MC accumulation in fish, associated with pathological changes in the liver and behavioural changes (i.e. increased ventilation rates) following toxic cyanobacteria consumption, but more rarely following MC-contaminated preys (Smith and Haney, 2006). A few studies have focused on the transfer of cyanotoxins (MCs or nodularin) from invertebrates to higher trophic levels, but involved zooplankton as vector of toxin (Engström-Öst et al., 2002; Karjalainen et al., 2005; Smith and Haney, 2006). Moreover, consumed by piscivorous fish (Sipia et al., 2007), *G. aculeatus* could thereafter transfer MCs in the food web. The trophic transfer of MCs in freshwater ecosystems between omnivorous or planktivorous fish to carnivorous ones has been suggested in the field (Ibelings et al., 2005, for review: Ibeling and Chorus, 2007).

The aim of this study is to evaluate if free and covalently bound MCs accumulated in *L. stagnalis* tissues after toxic cyanobacteria consumption are transferred to the three-spined stickleback. Fish were firstly fed on digestive glands of *L. stagnalis* containing two different total MC concentrations, with different proportions of free and bound MCs, (i.e. intoxication period), then with digestive glands of non exposed snails (i.e. depuration period). The impact of the ingestion of MC-contaminated snails on the three-spined stickleback was followed by measuring: 1) the accumulation and elimination of MCs in several organs (liver, muscle, kidneys, gills), 2) the potential oxidative stress response via the activity of 3 detoxifying enzymes: glutathion peroxydase, glutathion-S-transferase and superoxyde dismutase, 3) the histopathological impact on the liver, target organ of MCs, 4) the behavioural changes, via the gill ventilation rate, the feeding and locomotary activities known to be affected in the three-spined sticklebacks following exposure to various pollutants (Wibe et al, 2004; Craig et Laming, 2004).

7.1. Material and methods

7.1.1. Biological material

The filamentous cyanobacterium *Planktothrix agardhii* (Oscillatoriale) (strain PMC 75-02), originating from the recreational watersport site of Viry (Essone, France) and isolated by the National Museum of Natural History (Paris, France), was maintained in a modified medium (20 mL of liquid BG11 per litre of dechlorinated water). The *P. agardhii* suspension provided twice a week to the gastropods contained a total concentration of 33 µg MC-LReq L^{-1} measured by HPLC using the method described in Lance et al. (2006).

The gastropod *Lymnaea stagnalis* (Pulmonata, Lymnaeidae) was obtained from a laboratory population in the Experimental Unit of the Institut National de Recherche en Agronomie (U3E INRA, Rennes). Prior to the experiment, adult snails (25 ± 3 mm shell length) were isolated in glass containers of 35 mL (1 snail/container), acclimated to the experimental conditions (12/12 L/D, 20 ± 1°C) and fed on biological lettuce for 7 days.

The three-spined stickleback *Gasterosteus aculeatus* (Teleostei, Gasterosteidae) was obtained from a laboratory population in the Experimental Unit of the Institut National de Recherche en Agronomie (U3E INRA, Rennes). Prior to the experiment, three-spined sticklebacks were isolated in aquarium of 24 cm by length, 15 cm by height and 13 cm by depth, with filtered and oxygenated water, and acclimated to the experimental conditions (12/12 L/D). The temperature, pH, and oxygen concentration were recorded daily and remained stable during the experiment, with mean values (± SE) of respectively 18.4 ± 0.2°C, 8.03 ± 0.6 and 8.67 ± 0.2 mg L^{-1}.

7.1.2. Experimental set up

7.1.2.a. Intoxication of the gastropod *L. stagnalis*

During a 4-week intoxication period, 200 *L. stagnalis* adults were exposed to *P. agardhii*. At the end of the intoxication period, 100 snails were sacrificed and their digestive gland was removed and frozen in order to feed the first group of fish. The other 100 snails was placed in dechlorinated water and fed on dried lettuce *ad libitum*, during a 4-week depuration period in order to feed the second group of fish.

7.1.2.b. Intoxication of the fish *G. aculeatus*

Portions of *L. stagnalis* digestive glands (30 mg fresh weight, FW) were used in order to feed the fish. During the 5-day intoxication period, *G. aculeatus* were divided in 3 groups (21 individuals per group) according to diet: *L. stagnalis* digestive glands 1) without MCs ("contr snail"), 2) sampled at the end of the intoxication period of the snail ("intox snail"), 3) sampled at the end of the depuration period of the snail ("depur snail"). The fish intoxication was followed by a 5-day depuration period, during which all fish were fed on non-toxic digestive glands of snails.

7.1.3. MC accumulation in snails and three-spined sticklebacks

At the end of their respective periods of intoxication and depuration, 10 three-spined sticklebacks and 5 gastropods of each group were sampled, weighted and sacrificed. The MC accumulation was measured in the snail digestive gland, and in the liver, kidneys, muscles and gills of fish. Fish organs and snail digestive glands were placed in liquid nitrogen prior to be frozen at -80°C, then freeze-dried and crushed in powder. Free and bound MCs accumulated in the digestive gland of *L. stagnalis* were measured on 10 mg of freeze dried tissues. The method used detects total (bound plus free) MC content in snail tissues, through the formation of 2-methyl-3-methoxy-4-phenylbutiric acid (MMPB) as an oxidation product of the MCs, and the detection of MMPB by Liquid Chromatography Electro Spray Ionization tandem Mass Spectrometry (LC-ESI-MS/MS) (more details in the chapter 6, Lance et al., in preparation). The free MC content in snail tissues with LC-ESI-MS/MS was also assessed, and the bound MC content was thus estimated by subtracting the free MCs from the total MC content. Free MC extraction from tissues of fish and snails was performed on 5 mg of freeze dried tissue with 1 mL of 100% methanol, and analysis by immuno-assay was realized as described in Lance et al. (2006) with an ELISA Microcystin Plate Kit (Envirologix INC) with detection threshold of 0.05 µg L^{-1} and to the nearest 0.01 µg L^{-1} (Gilroy et al., 2000). Free and bound MC content in snails and fish were expressed in µg g^{-1} dry wet (DW). After the 5-day depuration period, the percentage of elimination of MCs (%detox) from the different organs of the fish (liver, kidneys, muscles and gills) was calculated for each group as followed:

% détox = 100 x [MC content after the intoxication – MC content after the depuration] / MC content after the intoxication

7.1.4. Behavioural observations and ventilation rate of the fish

7.1.4.a. Feeding delay and ventilation rate

Ten fish of each group were observed daily. The ventilation rate was determined by counting the opercula movement for 15 sec, multiplied by four in order to obtain the ventilation rate per min. The feeding delay (i.e. time required for the fish to ingest its food) was evaluated for 6 min, right after the introduction of the snail digestive gland in the aquariums.

7.1.4.b. Fish locomotion measurements

The locomotor activity was estimated through 5 degrees: 1) total immobility, 2) active immobility (movements without change of location in the aquarium), 3) low mobility, 4) medium mobility and 5) fast mobility. The total duration of each of these degrees in the activity was calculated for 10 individuals of each group, observed and filmed 5 min daily during the 10 days of the experiment (5-day intoxication and 5-day depuration).

7.1.5. Measurement of enzymatic activities involved in protection against oxydative stress and biotransformation

The gills, kidney and muscles of 8 individuals, and livers of 2 individuals per group were sampled, stored in liquid nitrogen and biochemical measurements were performed in the National Institute of Industrial Environnement and Risks (Verneuil sur Halatte, France). Prior to enzyme activity measurements, organs were homogenized in ice-cold phosphate buffer (100 mM, pH 7.8) supplemented with 20% v/v glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The homogenate was centrifuged at 10,000 g, 4°C, for 15 min and the supernatant was used for biochemical assays. Total proteins were previously determined using the method of Bradford (1976) with bovine serum albumin (Sigma) as a standard. SOD and total GPx activities were assessed according to the methods of Paoletti et al. (1986) and Paglia and Valentine (1967) respectively, using purified bovine enzymes (Sigma) as standards. For GST activity determination, chlorodinitrobenzene was used as substrate (Habig et al., 1974) and purified GST from equine liver (Sigma) as a standard. All assays were adapted for the three-spined stickleback as described by Sanchez et al. (2005) and measures were carried out at room temperature in microtiter plates, using a microplate reader (Power Wavex – Bio-Tek instruments).

7.1.6. Histology of fish livers

Two livers of each group were sampled at the end of the intoxication and depuration periods, and fixed in Bouin's fluid during 48h. Tissues were then processed as described in Lance et al. (2007), cut into serial 5-µm-thick longitudinal sections and stained with Hematoxylin & Eosin (H&E) (Martoja and Martoja-Pierson, 1967). Histological sections were photographed via an optic microscope using 40-200-fold magnification.

7.1.7. Statistical analysis

Some data did not follow a normal distribution (according to the Shapiro-Wilk test) and were thus analysed for differences between treatment groups using the Kruskall-Wallis (KW) and the Friedman tests and 2 by 2 treatment groups using the Mann-Whitney U-test for the feeding delays, the ventilation rates, the mobility degrees, the MC content in snail and some fish organs (gills, muscles, kidneys), and the concentration of enzymes in all organs. The Wilcoxon test was used for comparisons in mobility degrees between the intoxication and depuration periods and in MC content between fish organs. The MC content in fish liver followed a normal distribution and was then analysed for differences between groups using the Student t test. Significant differences were determined at $p < 0.05$ for all statistical analyses. Data are reported as mean ± standard error (± SE).

7.2. Results

7.2.1. MC accumulation in gastropods and fish

7.2.1.a. MC accumulation in *L. stagnalis*

The digestive gland of *L. stagnalis* contained 6.82 ± 0.24 µg g^{-1} DW of free MCs and 11.61 ± 1.63 µg g^{-1} DW of covalently bound MCs at the end of the intoxication period, and 0.44 ± 0.05 µg g^{-1} DW of free MCs and 6.25 ± 0.85 µg g^{-1} DW of covalently bound MCs at the end of the depuration period. The covalently bound MCs represented 63.3% of total MCs at the end of the intoxication period, and 93.9% at the end of the depuration period.

7.2.1.b. MC accumulation in *G. aculeatus*

During the 5-day intoxication, each stickleback ingested 30 mg FW [= 3.9 mg DW, (Lance et al., 2006)] of *L. stagnalis* digestive gland per day, corresponding for the whole intoxication to respectively for the groups "depur snail" and 'intox snail" to 0.008 ± 0.00 µg and 0.130 ± 0.00 µg of total free MCs ingested, and to 0.02 ± 0.00 µg and 0.04 ± 0.00 µg of total bound MCs ingested.

The control sticklebacks did not accumulate MCs regardless of the organ and period (Table 1). The sticklebacks fed on the most contaminated digestive glands ('intox snail") accumulated free MCs in all the organs, in a significant higher amount than sticklebacks fed on the less contaminated digestive glands ("depur snail"), at the end of intoxication ($t = 26.76$; $ddl = 8.22$; $p < 0.05$) and depuration periods ($t = -3.84$; $ddl = 6$; $p < 0.05$) (Table 1).

The sticklebacks fed on the most contaminated digestive glands containing 63.3% of bound MCs ('intox snail"), showed a significant lower free MC content in the liver than the free MC content in the digestive gland of the snail (i.e. 3.9 ± 0.1 vs 6.8 ± 0.2 µg g^{-1} DW). However, the sticklebacks fed on the less contaminated digestive glands but containing 93.9% of bound MCs ('depur snail"), showed a similar free MC content in the liver than the MC content in the snail digestive gland (i.e. 0.3 ± 0.1 and 0.4 ± 0.0 µg.g^{-1}).

Table 1 : Free MC accumulation (µg.g^{-1} DW) (mean ± SE) in different organs of three-spined sticklebacks fed on high ("intox snail") or low ('depur snail") MC-contaminated snail digestive glands at the end of intoxication and depuration periods.

		Intoxication period		Depuration period	
Fish treatments		depur snail	intox snail	depur snail	intox snail
Fish organs	Liver	0.33 ± 0.12	3.96 ± 0.14	0.43 ± 0.09	0.32 ± 0.09
	Gills	0.16 ± 0.05	2.54 ± 0.11	nd	nd
	Kidneys	0.36 ± 0.04	1.69 ± 0.15	nd	0.34 ± 0.01
	Muscles	0.17 ± 0.08	1.05 ± 0.09	0.16 ± 0.09	0.44 ± 0.04

Regardless of the organ, the MC content in fish tissues was lower than 1 µg g^{-1} DW at the end of the depuration period. Differences occurred in MC accumulation and elimination according to the organ (liver, gills, kidneys, and muscles) and intoxicated diet (concentrations of free and covalent MCs). The liver of fish from the «intox snail» group contained significantly less MCs at the end of the depuration period than at the end of the intoxication period ($t = 26.99$, ddl = 8.06, $p < 0.05$) (Table 1), with a percentage of MC elimination ("%detox") of 92%. Interestingly, the MC content was similar between the two periods in the liver of fish from the «depur snail» group ($t = - 5.07$, ddl = 6, $p = 0.09$), and no MC was eliminated, even after 3 weeks of depuration. In gills, the MCs were entirely eliminated at the end of the depuration period, for both groups. In kidneys, the percentage of MC elimination was respectively 80% and 100% for "intox snail" and "depur snail" groups. In muscles, 58% and 6% of MCs accumulated were eliminated respectively for "intox snail" and "depur snail" groups after the 3-week depuration.

7.2.2. Histopatholy of fish liver

In the liver of control sticklebacks, the parenchyma architecture was regular, consisting of hepatocytes constituting a cellular string (Fig 1B). Between hepatocytes are localized sinusoïd capillary (SC) (Fig 1C) coming from the central vein (V) in which are visible erythrocytes (E) (Fig 1A). The liver of intoxicated three-spined sticklebacks was globally similar to that of controls, but presented few disintegration of the parenchyma architecture localized near the blood vessel, for both "intox snail" et "depur snail" groups (Fig 1).

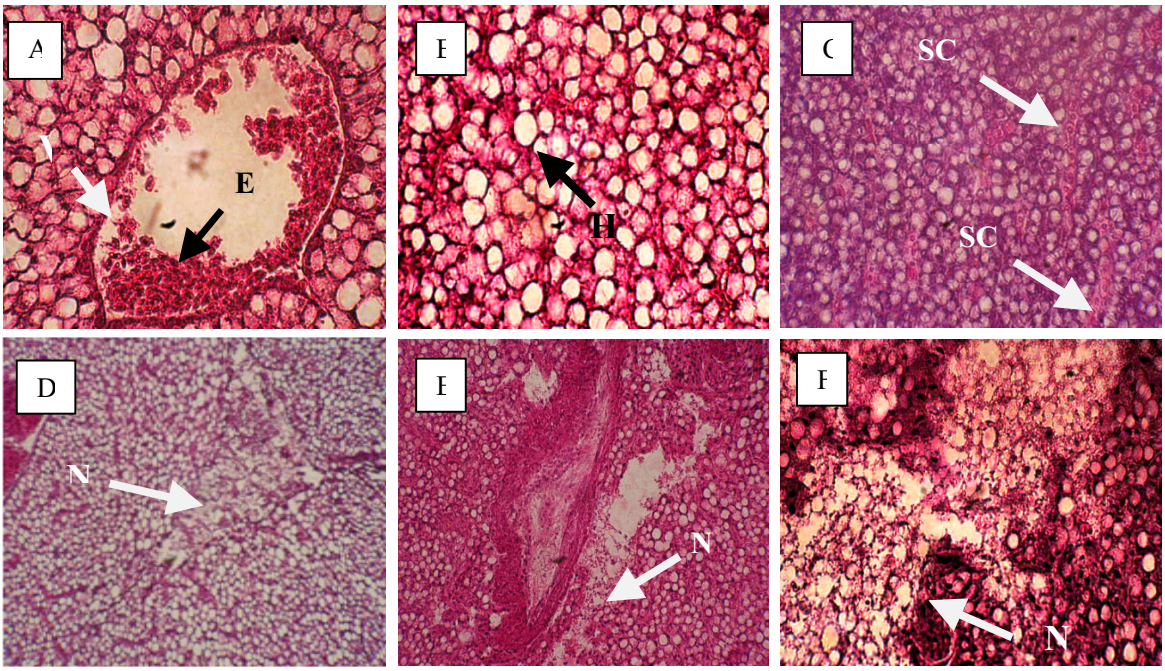

Figure 1: Hematoxylin and Eosin stained sections of *G. aculeatus* liver after various treatments: A (x40), B (x40) , C (x20) = control; D (x10) = fed on low MC-contaminated *L. stagnalis* ('depur snail") and E (x10), F (x40) = fed on high MC-contaminated *L. stagnalis* ("intox snail"). H = Hepatocyte; V = Vein; E = Erythrocytes; SC = Sinusoïd Capillary; N = Necrosis

7.3.3. Biotransformation and oxidative stress enzymes

The induction of the activity of the gluthation peroxydase (GPx) was observed in the liver and gills of fish from the "intox snail" group ($p<0.05$), but not in both kidneys and muscles. No change was recorded in GPx activity for fish of the “depur snail” group (Table 2). The activities of gluthation-S-transférase (GST) and superoxyde dismutase (SOD) were similar between all groups (Table 2).

Table 2 : Mean (±SE) enzymatic activity of GPx (Gluthation peroxydase) (U g^{-1}), GST (Gluthation-S-transferase) (U mg^{-1}), SOD (Superoxyde dimutase) (U g^{-1}) according to organs, treatments and period. Significant differences between controls and treated fish are indicated by (*p<0,05).

	intoxication				depuration			
organ	Liver	Gills	Kidney	Muscles	Liver	Gills	Kidney	Muscles
GPx								
Control snail	85,3 ± 15,2	73,4 ± 10,3	26,4 ± 5,2	59,4 ± 10	78,6 ± 12,8	78,1 ± 9,1	28,4 ± 4,6	64,9 ± 9,2
Depur snail	96,1 ± 25,3	65,2 ± 8,5	27,4 ± 5,9	65,8 ± 13,4	91,6 ± 20,1	81,6 ± 6,4	31,2 ± 3,3	54 ± 7,6
Intox snail	143,7 ± 24,6*	94,3 ± 3,4*	35,1 ± 6,1	51,3 ± 8,5	93,5 ± 17,7	83,5 ± 5,5	27,3 ± 7,5	61,7 ±10,2
GST								
Control snail	1,94 ± 0,5	2,43 ± 1,02	1,36 ± 0,23	1,49 ± 0,81	2,05 ± 0,63	2,25 ± 1,11	1,12 ± 0,65	1,68 ± 0,54
Depur snail	1,46 ± 0,71	2,55 ± 0,98	1,54 ± 0,62	1,99 ± 0,94	1,98 ± 0,72	1,96 ± 1,34	1,28 ± 0,76	1,75 ± 0,67
Intox snail	2,63 ± 0,68	2,61 ± 1,56	1,78 ± 0,95	1,73 ± 1,03	2,16 ± 0,58	2,51 ± 1,58	1,43 ± 0,91	1,42 ± 0,41
SOD								
Control snail	11,5 ± 2,5	31,6 ± 11,5	13,8 ± 4,6	14,6 ± 5,4	10,9 ± 2,1	29,4 ± 8,7	10,9 ± 3,2	15,1 ± 5,6
Depur snail	13,8 ±1,9	29,5 ± 12,2	16,7 ± 5,1	16,5 ± 7,5	14,8 ± 3,5	23,1 ± 13,7	8,4 ± 3,6	21,4 ± 8,9
Intox snail	9,4 ± 3,8	24,7 ± 8,6	11,8 ± 3,9	18,3 ± 7,3	15,7 ± 4,4	35,1 ±12,9	11 ± 2,8	17,2 ± 4,1

7.2.4. Behavioural observations and gill ventilation

7.2.4.a. Feeding delay and ventilation rate

The feeding delay and the number of opercula movements were similar in all groups at the end of both intoxication (respectively KW: H = 0.95; ddl = 2; p = 0.62 and H = 1.57, ddl = 2; p = 0.46) and depuration (respectively KW: H = 1.12; p = 0.57 and H = 1.128; ddl = 2; p = 0.57) periods (Table 3).

Table 3: Mean (± SE) feeding delay (sec) and ventilation rate (number of opercula movements per min) in fish fed on non toxic *L. stagnalis* ("control snail"), high MC-intoxicated *L. stagnalis* ("intox snail"), and low MC-intoxicated *L. stagnalis* ("depur snail"), at the end of intoxication and depuration periods.

	Feeding delay		Ventilation rate	
	intoxication	depuration	intoxication	depuration
"Control snail"	223.2 ± 20.78	195.6 ± 20.25	106.96 ± 4.53	117.84 ± 5,88
"Intox snail"	200.2 ± 21.95	213.2 ± 23.14	96.64 ± 7.07	111.12 ± 5.27
"Depur snail"	235.9 ± 19.92	183.5 ± 21.68	102.88 ± 4.78	108.94 ± 5.36

7.2.4.b. Fish mobility

During the intoxication period, the total duration of immobility was significantly different between groups (KW: H = 12.11; ddl = 2; $p < 0.05$). All intoxicated sticklebacks ("intox snail" and "depur snail") remained immobile during a significant longer time than controls (respectively U = 409.5 and 788; $p < 0.05$). Fish remained immobile during 78 ± 11 sec in a 5-min period in the control group, against respectively 107 ± 9 and 132 ± 12 sec in the "depur snail" and "intox snail" groups, that presented similar duration of immobility (U = 714; p = 0,32) (Fig. 2A). The intoxication also induced an impact on the duration of rapid mobility between groups (KW: H = 6.29; ddl = 2; $p < 0.05$). The most intoxicated sticklebacks ("intox snail") moved rapidly significantly less often than controls (U = 1651; $p < 0.05$) and than less intoxicated fish ("depur snail") (U = 395; $p < 0.05$) (Fig. 2A).

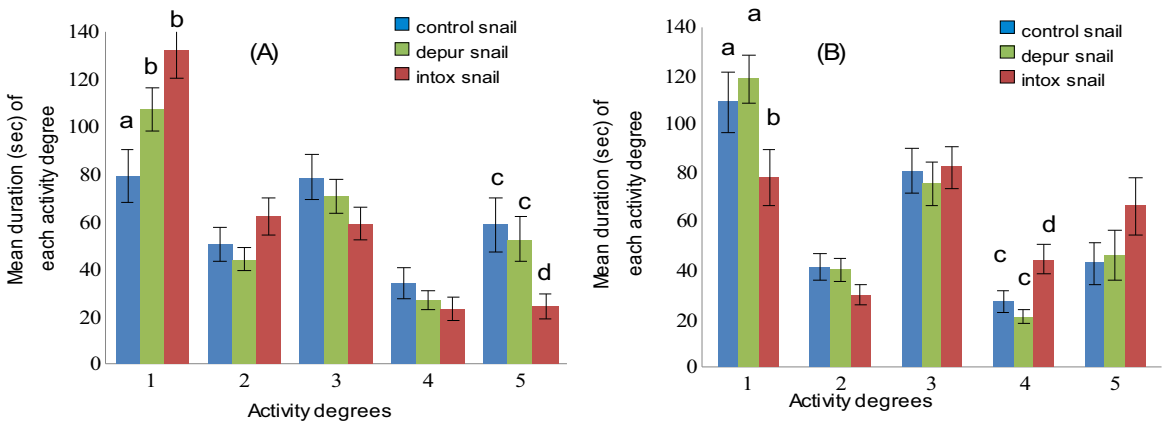

Figure 2 : Mean (± SE) duration (sec) of each activity degree: 1) total immobility, 2) active immobility (movements without change of location in the aquarium), 3) low mobility, 4) medium mobility and 5) fast mobility, for G *aculeatus* fed on non toxic *L. stagnalis* ("control snail"), high MC-intoxicated *L. stagnalis* ("intox snail"), and low MC-intoxicated *L. stagnalis* ("depur snail"), at the end of the intoxication (A) and depuration (B) periods. Different letters (a, b, c, d) indicate significant differences.

After the depuration (Fig. 2B), the total duration of immobility was significantly different between groups (KW: H = 8.46; ddl = 2; $p < 0.05$). The fish from «intox snail» group remained significantly less immobile than fish from control or "depur snail" groups (respectively U = 1558.5 and 389.5; $p < 0.05$). The total duration of the medium mobility was also significantly different between groups (KW: H = 8.08; ddl = 2; $p < 0.05$), and was significantly higher in "intox snail" group than in control (U = 902.5; $p < 0.05$) and "depur snail" groups (U = 389.5; $p < 0.05$) (Fig. 2B).

The duration of each mobility degree was significantly different between the intoxication and depuration periods in the "intox snail" group. The sticklebacks significantly reduced their immobility time and increased their low, medium and rapid mobility times during the depuration (respectively T = 1016 and 310.5, 418.5; 327; $p < 0.05$).

7.3. Discussion

MC trophic transfer from gastropods to fish

Our results provide direct evidence of the MC transfer from the gastropod *L. stagnalis* to the three-spined stickleback *G. aculeatus*, and the subsequent MC accumulation in all the fish organs (liver, kidneys, muscles and gills). After a 5-day consumption of MC-rich *L. stagnalis* digestive glands (total of 0.17 µg MC ingested, in which 63.3% of covalently bound MCs), an average of 3.96 ± 0.14 µg g^{-1} DW of free MCs accumulated in the fish liver. Considering a mean dry weight of 10 mg per fish liver, approximately 23% of total MCs (free and bound) ingested was accumulated under a free form in the fish liver. In the laboratory, Engström-Öst *et al.* (2002) demonstrated the accumulation of the cyanobacterial hepatotoxin nodularin by mysid shrimps (*Mysis relicta*) and three-spined sticklebacks (*G. aculeatus*) (up to 1.4 µg g^{-1} DW after 5 days), fed on MC-intoxicated copepods. Few studies have suggested a trophic MC transfer to omnivorous and carnivorous fish in the field (Williams *et al*., 1997; Ibelings *et al*., 2005; Gkelis *et al*., 2006; Xie *et al*, 2005; 2007). To our knowledge, only the study of Smith and Haney (2006) have reported the free MC transfer from zooplankton to the sunfish *Lepomis gibbosus* under controlled laboratory conditions [resulting in a lesser MC accumulation (up to 11.2 ng g DW in the liver) than in our study], and no studies have investigated the potent transfer of MCs covalently bound to phosphatase proteins (Ppases).

After a 5-day ingestion of *L. stagnalis* digestive glands containing less MCs (total of 0.03 µg MC ingested) but with 93.9% of covalently bound MCs, the three-spined sticklebacks accumulated 0.33 ± 0.12 µg g^{-1} DW of free MCs in the liver, corresponding to approximately 11% of total MCs (free and bound) ingested. In this case, accumulated MCs in the fish may provide from: i) free MCs (6%) among total MCs in the digestive gland of the snail (i.e., 0.008 µg during 5 days), suggesting that 41% of the free MCs in the digestive gland of the snail are transferred to the liver of the consumer; ii) bound MCs (94%) among total MCs in the digestive gland of the snail (i.e., 0.02 µg during 5 days), suggesting a MC release from Ppases during digestion by fish. This metabolization of the MC-Ppase complex may have released at least a part of the MCs containing the Adda group, as the free MCs in the fish were measured with the Elisa test (Msagati et al., 2006; Tillmanns et al., 2007). The percentage presented above only includes MCs in the liver of the three-spined sticklebacks. However, MCs also accumulated in other organs, and the total (free and bound) MC concentration in the fish was not assessed (only free MCs), but may be superior to the total amount of free MCs ingested. Thus, our results do not allow us to state whether the covalently bound MCs in the

snails were involved in the fish contamination. In their review, Ibelings and Chorus (2007) suggest that MCs covalently bound to Ppases may not be readily available as the digestive tract of the predator probably do not release them. Investigations need to be conducted to determine if covalently bound MCs accumulated in preys are released or not in the digestive tract of consumers and if changes can occur in their toxicity.

Histological and behavioural MC-impact on the fish

In vertebrates, MCs accumulate in hepatocytes where they can interact with and inhibit the Ppases (Hastie et al., 2005), resulting in reorganization of cytoskeletal components and disruption of hepatic architecture. Histopathology in the liver due to direct contamination by intracellular or dissolved MCs has been demonstrated for several fish species: in the carps *Cyprinus carpio and Hypophtalmichthys molitrix* (Råbergh et al, 1991; Fischer et Dietrich, 2000; Li et al, 2007), in the rainbow trout *Oncorhynchus mykiss* (Tencalla and Dietrich, 1994) and the whitefish *Coregonus lavaretus* (Ernst et al, 2007). In those fish, MCs mainly induced hepatocyte dissociation, disintegration of the parenchyma liver architecture and necrosis, predominantly peripheral to central veins, as observed in a lesser extent in our most intoxicated three-spined sticklebacks. In contrary to the study of Ernst et al (2007), no dilated sinusoid capillary was found. However, these authors reported that the severity of pathological changes was depending on exposure time, and increased between 7 and 28 days of *Planktothrix rubescens*-exposure. Our results did not reveal severe liver pathology but suggest that a consumption of MC-contaminated snails longer than 4 days may induce a stronger impact. Similarly, Ibelings et al. (2005) demonstrated the occurrence of liver damages in 37% of perch and ruffe exposed to MC-contaminated zooplankton and bivalves in the field. Moreover, Ernst et al (2007) showed that the ingestion of MC-producing *P. rubescens* induced a physiological stress (increased ventilation) in the whitefish after 10 days. In our study, the ventilation rate was unchanged, probably due to a limited physiological impact and/or to the shorter duration of experiment.

Changes in the mobility of fish can also be indicative to assess and measure the behavioural impact of MC intoxication. All MC-intoxicated fish remained immobiles during a significantly longer time than controls. Moreover, the ingestion of the highest MC-rich snail digestive glands significantly decreased the rapid moving of the three-spined sticklebacks [as observed by Craig et Laming (2004) during ammonium exposure], whereas the ingestion of the lower MC-contaminated ones did not modify the speed of fish movements. An inverse dose-dependant effect of MC-LR (i.e. increased activity at the lowest toxin concentration)

was observed in the zebrafish (Baganz et al., 1997). The decreased locomotion of MC-intoxicated fish might be induced by an energy trade-off allowing MC-metabolization and/or excretion, as suggested for the gastropods (Lance et al, 2007; 2008). During the depuration period, we observed that the MC-exposed fish moved during a longer time than controls, possibly interpreted as an active research for food in order to compensate energy losses. Nevertheless, the feeding delays were similar during MC-intoxication and depuration, and not increased as for three-spined sticklebacks exposed to Dichlorodiphenyl-Dichloro-Ethylen (Wibe et al., 2004). The similarity in the time required by fish for the ingestion of both MC-rich and non toxic digestive glands of *L. stagnalis* supposes an unchanged appetency, and then, that food toxicity was not detected. In the same way, Smith and Haney (2006) also reported that sunfish continued to consume MC-toxic zooplankton at the same rate throughout the experiment. Even if the consumption of MC-producing cyanobacteria has been demonstrated for several fish species (for review: Malbrouck et Kestemont, 2006) and gastropods (Zurawell et al., 2006; Lance et al., 2006; 2008), some phytoplanktivorous fish species (e.g. carp, tilapia) are able to discriminate between toxic and non-toxic cyanobacterial strains (Beveridge et al., 1993 ; Keshavanath et al., 1994). According to Engström-Öst et al. (2006), three-spined sticklebacks are able to move in cyanobacterial proliferations potentially toxic in order to be hidden from predators (increased turbidity), and thus do not seem to detect their toxicity.

Oxidative stress induced by MCs in the fish

MC accumulation in the liver, kidneys, gills and muscles of fish is the consequence of toxin absorption through the digestive tract during the digestion of the MC-contaminated snail digestive glands and of MC distribution in the entire organism via blood circulation. MC transport by the blood was already hypothesized by Cazenave *et al* (2005) in *Corydoras paleatus* presenting a MC-LR accumulation in gills. Moreover, Tencalla and Dietrich (1997) showed both MC absorption through the digestive tract, mediated by the bile acid carrier, and rapid MC transport by the blood flow in the rainbow trout fed on toxic *Microcystis aeruginosa*. The MC distribution in the whole organism may possibly impair the homeostasis as shown for fish species (e.g., perturbation of the metabolism, induction of physiological stresses, histopathological and vascular damages) (for review: Malbrouck and Kestemont, 2006). In vertebrates (rat and fish), MCs enter cells and induce the production of reactive oxygen species (ROS), which may also contribute to their toxicity (Cazenave et al., 2006).

ROS are extremely toxic for cells because they react with cellular macromolecules leading to enzymatic inhibition, lipid peroxydation and DNA damages. The overproduction of ROS in cells generally activates the detoxification and the antioxydant processes acting to reduce their amount. The oxidative stress can be evaluated in 3 ways: the measurement of the ROS production, of lipid peroxydation products and changes in antioxydant mechanisms (Cazenave et al., 2006). In this study, we investigated such stress induced in the three-spined stickleback by the consumption of MC-contaminated snail digestive glands by measuring the activity of the biotransformation/detoxification enzyme glutathion-S-transferase (GST), and of two antioxydant enzymes, glutathion peroxydase (GPx) and superoxyde dismutase (SOD). Indeed, free accumulated MCs can be metabolized into less harmful compounds after conjugation with glutathione, via GST, resulting in MC excretion or physiological degradation. Such detoxification processes have been shown to occur in various organisms such as bivalves (Dreissena sp.), copepods (Daphnia sp.), fish (*Danio rerio*), and mammals (mice, rat) (Pflugmacher, 1998; Wiegand et al., 1999, Pietsch et al., 2001). The activity of the GST in fish can be increased (Jos et al, 2005; Wiegand et al., 1999), inhibited (Cazenave et al., 2006) or unchanged (Li et al., 2003) by MC exposure, depending on the timing and the intensity of contamination. However, no induction of the GST activity has been demonstrated in our study. Moreover, both ROS and lipid peroxydes are known to be reduced by the activity of GPx and SOD, acting as a defence mechanism against oxidative stress (Wiegand et al., 1999; Gehringer et al, 2004; Cazenave et al., 2006). Our results show an induction of GPx activity in the liver of MC-intoxicated three-spined sticklebacks suggesting a metabolization of MCs in this organ, as observed for numerous other fish species [embryos of zebrafish (Wiegand et al., 1999) and hepatocytes of *Cyprinus caprio* (Li et al., 2003) both exposed to MC-LR, *C. paleatus* exposed to MC-RR (Cazenave et al., 2006), and tilapia fed on MC-producing cyanobacteria (Jos et al., 2005)]. However, we observed that GPx activity was also induced in the gills of *G. aculeatus*, contrary to *C. paleatus* (Cazenave et al., 2006) and tilapia (Jos et al., 2005).

Implication for the food web and human health risks

Biomagnification refers to a toxin transfer from food to an organism, resulting in a higher concentration in the organism than in its diet, whereas for biodilution, decreased toxin levels are observed with each increase in trophic level in the food web (toxins subject to metabolization and excretion at every level) (for review: Ibelings and Chorus, 2007). Our

study assesses the MC transfer from the gastropod *L. stagnalis* to the fish *G. aculeatus*, but without biomagnification (rather a slight biodilution). Indeed, the most contaminated fish presented in the liver a concentration of free MCs 1.7 times less important than those in the digestive gland of the gastropod (i.e. 4.0 ± 0.1 vs 6.8 ± 0.2 µg free MCs g^{-1} DW), and the less contaminated fish presented in the liver a similar free MC concentration than in the snail digestive gland (0.3 ± 0.1 vs 0.4 ± 0.0 µg g^{-1} DW). Consequently, the biomagnification factor ranged from 0.58 to 0.82 (only considering MC accumulation in equivalent organs, i.e. digestive gland of the snail and liver of the fish). Similarly, in their 3-year investigation in the lake Ijsselmeer (The Netherland), Ibelings *et al*. (2005) demonstrated a MC transfer by assessing the MC contents in several trophic levels (cyanobacteria, zooplankton, bivalves, planktivorous and carnivorous fish), but no biomagnification occurred. These authors explained the absence of biomagnification by the capacity of aquatic organisms for MC-detoxification.

Concerning human health risks, our study demonstrated accumulation of MCs followed by their partial elimination in the liver and kidneys of the fish, and total in the gills after depuration. However, MC also accumulated in muscles of the three-spined sticklebacks (1.05 µg g^{-1} DW) after a 5-day ingestion of MC-contaminated digestive gland of snails, and remained (0.44 µg g^{-1} DW) after 5 days of depuration. In their experiment on the MC transfer from zooplankton to the sunfish, Smith and Haney (2006) also reported that fish accumulated MCs in the liver (up to 11.2 ng g WW) and the muscles (up to 0.2 ng g WW), still detectable after a 15 days of depuration (up to 0.1 ng g WW). Numerous studies on other fish species (for reviews: Malbrouck et Kestemont, 2006; Ernst *et al*, 2007; Xie *et al*., 2007) reported the liver as the organ that accumulated the highest amount of MCs after MC-producing cyanobacteria. Our study shows that the muscles and liver of *G. aculeatus* contained more MCs than gills and kidneys at the end of the depuration, in contrary to the results reported by Xie *et al* (2005; 2007) for *Carassius auratus*, *Silurus glanis* and *C. carpio* in which muscles presented the lowest MC content. Even if no biomagnification occurs from snails to three-spined sticklebacks, our results suggest a probable MC-tranfer to the predators of intoxicated three-spined sticklebacks, among them some species (e.g. Salmonidae) usually consumed by human beings.

Conclusion

The presence of free MCs in several organs of the three-spined sticklebacks after consumption of MC-intoxicated snails suggest the possibility of a transfer in the food web, both aquatic and terrestrial. The negative impact of MCs we demonstrate on three-spined sticklebacks following intoxication remained limited probably due to the low intensity and duration of MC-exposure. In the field, risks are expected to be higher for 3 reasons:

- i) Fish may be chronically contaminated during an extended period because toxic cyanobacteria can proliferate from April to October in the temperate regions of the Northern hemisphere, with a highest MC production than in our study. Moreover, gastropods are able to accumulate up to 80% of ingested MCs under a free or covalently bound form, and thus may represent a significant MC vector in natural conditions (Lance et al., 2006, in preparation).
- ii) The multitude of other stresses that co-occur in the field with cyanobacteria proliferations (e.g., anthropogenic pollution, predation, hypoxia) may reduce the capacity of fish to detoxify MCs.
- iii) The MC content in fish tissues is probably underestimated due to the potent covalent binding of MCs with Ppases, preventing from a MC extraction by methanol (the research of covalently bound MCs in *G. aculeatus* samples of this study will be soon realized).

This study needs to be strengthen by deeper investigations on the potential covalently bound MCs transfer, and their toxicity, as there are less eliminated from organisms than free MCs (Lance et al., in preparation) and may persist in organisms of the food web after the bloom collapse. Consequently, if covalently bound MCs are still toxic for consumers, the MC contamination of fish may represent a higher health hazard.

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Chapitre 8 :
Discussion générale et perspectives

Chapitre 8 : Discussion générale et perspectives

L'analyse bibliographique présentée en partie 1 de ce manuscrit a permis de déterminer le cadre conceptuel de nos travaux, et de mettre en évidence le manque d'études concernant les interactions entre cyanobactéries et gastéropodes, malgré la réalité de leur confrontation en milieu naturel, particulièrement dans les eaux eutrophes. L'objectif de cette thèse était donc d'explorer différents aspects concernant l'impact des cyanobactéries toxiques sur les gastéropodes par une approche essentiellement expérimentale, mais aussi en milieu naturel, afin de : i) comprendre comment les gastéropodes répondent à l'exposition aux cyanotoxines et dans quelle mesure ils en sont affectés à l'échelle de l'organisme (traits de vie, fitness, activité locomotrice), de la population et de la communauté, ii) de suivre le devenir des cyanotoxines au sein des organismes exposés en termes d'accumulation et de détoxication à travers l'exemple des microcystines (MCs), iii) de mesurer au laboratoire le risque de transfert des MCs dans le réseau trophique aquatique à partir des gastéropodes. Les paragraphes qui suivent présentent un récapitulatif des résultats de nos travaux et des réponses apportées à certaines de nos questions, ainsi que des perspectives de recherches qu'ils ouvrent sur cette problématique.

8.1. Contamination par ingestion de cyanobactéries toxiques et accumulation de MCs par les gastéropodes pulmonés et prosobranches

La plupart des gastéropodes d'eau douce sont à la fois brouteurs et détritivores, et ont un régime alimentaire généraliste, qui peut inclure des cyanobactéries dans des proportions variables, en fonction des saisons et de la disponibilité des ressources alimentaires (Pinel-Alloul & Magnin, 1979, Reavell, 1980, Brendelberger, 1997). Les expériences mettant en jeu l'exposition des gastéropodes à la souche de *P. agardhii* (PCC 75-02) productrice de MCs (5 µg MC-LReq L^{-1}) ont montré l'ingestion de cyanobactéries toxiques par le prosobranche *P. antipodarum* et le pulmoné *L. stagnalis*, quels que soient leur âge et la présence ou non d'une autre source alimentaire non toxique, le facteur déterminant étant la disponibilité de cyanobactéries. Le taux d'ingestion est du même ordre pour 5 individus de *P. antipodarum* (taille comprise entre 2 et 4 mm) que pour un individu de *L. stagnalis* (taille comprise entre 15 et 25 mm), avec respectivement 70% et 63% des cyanobactéries disponibles consommées les premières 24h (Lance et al., 2006, 2008). Quelle que soit l'espèce, les gastéropodes ne

semblent pas percevoir la toxicité des cyanobactéries, qu'ils sont donc susceptibles d'ingérer en milieu naturel.

Après ingestion, les cyanobactéries toxiques sont probablement lysées chez les prosobranches grâce au stylet cristallin assurant une digestion à la fois mécanique par sa rotation rapide, et chimique par les enzymes qu'il contient. Chez les pulmonés, la digestion mécanique se fait par trituration grâce au sable présent dans le gésier, et digestion chimique par les enzymes déversées par la glande digestive (Carriker, 1946; Dillon, 2000). Les MCs libérées, qui résistent à la digestion dans le tractus gastro-intestinal comme chez tout eucaryote (Msagati et al., 2006), sont ensuite dirigées vers la glande digestive organisée en acini, puis probablement endocytées depuis la lumière des acini par les cellules de l'épithélium digestif où s'opère une digestion intracellulaire (Carriker, 1946; Zurawell, 2001). Zurawell (2001) a élucidé certains aspects du devenir de *Microcystis aeruginosa* (cyanobactérie formant des colonies) et de ses MCs chez le pulmoné *L. stagnalis*, et montre que 57% des MCs libres détectées dans les limnées proviennent des cyanobactéries intactes dans le tractus alimentaire, éliminées dans les fèces dans les 8 heures après ingestion, limitant ainsi l'accumulation dans la glande digestive à 17%. Nos résultats montrent que *L. stagnalis* accumule, sous forme libre, 61% des MCs ingérées lors de la consommation de la cyanobactérie filamenteuse *P. agardhii*. Nous avons soumis les gastéropodes à 24h de jeûne alimentaire après exposition aux cyanobactéries afin de ne pas inclure dans les mesures d'accumulation les MCs restées dans les cellules non digérées. Les différences s'expliquent en grande partie par la présence d'un mucus entourant les colonies de *M. aeruginosa* et limitant leur lyse dans l'estomac des gastéropodes. Ainsi, en milieu naturel, la contamination des gastéropodes par ingestion de cyanobactéries peut varier en fonction des caractéristiques morphologiques des espèces qui prolifèrent. De plus, les gastéropodes consomment préférentiellement les cellules de tailles moyennes et faibles (Cattaneo et Kalff, 1986), et en laboratoire, ils broutent plus intensément les cyanobactéries filamenteuses que les cyanobactéries coloniales (Mac Collum et al., 1998). Ainsi, on peut supposer une forte consommation et donc potentiellement une forte accumulation de MCs dans les lacs peu profonds des régions tempérées où *P. agardhii* est l'espèce qui prolifère le plus souvent (Scheffer et al., 1997).

L'accumulation de MCs peut aussi varier en fonction des espèces de gastéropodes : *P. antipodarum* exposé à *P. agardhii* n'accumule sous forme libre que 2.6 % des MCs ingérées avec les cyanobactéries toxiques, contre 61% pour *L. stagnalis*, supposant que la digestion des cyanobactéries dans l'estomac du prosobranche pourrait être moins efficace que dans celui des pulmonés. En milieu naturel, l'accumulation de MCs tend à être plus faible chez les prosobranches que chez les pulmonés : de 0.05 à 10.0 µg g DW^{-1} chez les prosobranches et de 3.46 à 140 µg g DW^{-1} chez les pulmonés (Kotak et al., 1996; Ozawa, 2003; Chen et al., 2005; Gkelis et al., 2006; Zhang et al., 2007; Gérard et al., 2008; Gérard et al., 2009). De la même façon, au laboratoire, *P. antipodarum* accumule un maximum de 0.18 µg g^{-1} poids sec, alors que *L. stagnalis* est capable d'accumuler jusqu'à 80 µg g^{-1} poids sec (Gérard et Poullain, 2005; Lance et al., 2006). Nous observons également cette tendance lorsque ces 2 espèces sont placées dans des enclos grillagés en milieu naturel, le pulmoné accumule environ 20 fois plus de MCs que le prosobranche pour un même site. Cependant, dans notre étude sur les gastéropodes du lac de Grand Lieu, la différence d'accumulation de MCs entre les 2 taxons n'est pas significative, probablement en raison de différences dans la composition spécifique de la communauté de gastéropodes.

Par ailleurs, l'efficacité d'élimination des toxines varie entre espèces au laboratoire. Lorsque les gastéropodes ne sont plus exposés aux cyanobactéries toxiques, les MCs sont totalement éliminées chez *P. antipodarum* alors qu'elles restent détectables après 3 semaines chez *L. stagnalis*. De façon générale, les MCs libres peuvent être métabolisées via les enzymes de détoxication impliquant la conjugaison avec les glutathions et générant des composés plus hydrosolubles et moins toxiques, plus facilement éliminés dans la bile et l'urine (Pflugmacher et al., 1998; pour revue : Cazenave et al., 2006). Cependant, les MCs sont rarement totalement éliminées et nous avons mis en évidence des capacités de détoxication qui varient selon l'âge et la disponibilité des ressources énergétiques. Ainsi, le pourcentage de MCs accumulées est toujours plus important, d'une part, chez les juvéniles (vs adultes), probablement en raison de leur système de détoxication immature et donc moins compétent, et d'autre part, chez les gastéropodes qui ne disposent pas d'autres ressources alimentaires que les cyanobactéries toxiques (vs avec salade), un apport énergétique supplémentaire permettant d'effectuer une détoxication plus efficace (see Lance et al., 2006, 2008).

8.2. Réponse des gastéropodes pulmonés et prosobranches (traits de vie, fitness) aux MCs en fonction de la contamination

Contamination par les MCs intracellulaires ou dissoutes et impact sur les traits de vie de Potamopyrgus antipodarum et Lymnaea stagnalis

De nombreux auteurs ont reporté que la quantité de MCs accumulée dans les tissus des gastéropodes en milieu naturel est proportionnelle à celle de MCs intracellulaires dans les cyanobactéries, et non corrélée à la quantité de MCs dissoutes dans le milieu, suggérant que la principale source de contamination serait l'ingestion de cyanobactéries toxiques (Zurawell et al. 1999, Yokoyama and Park, 2002; Chen et al., 2005; Xie et al., 2007). Cependant, Zhang et al. (2007) ont montré que la quantité de MCs accumulées chez le prosobranche *Bellamya aeruginosa* peut être corrélée à la fois aux MCs dissoutes et aux MCs intracellulaires. Au laboratoire, la comparaison de nos résultats avec ceux de Gérard et Poullain (2005) indique que *P. antipodarum* accumule seulement 1.3 fois plus de MCs en ingérant des cyanobactéries toxiques (5 µg MC-LReq L^{-1} pendant 5 semaines) vs par exposition aux MCs dissoutes (33 µg MC-LR L^{-1} pendant 6 semaines). Chez *L. stagnalis,* ce rapport est de 1300 (Lance et al., 2006, unpublished data in chapitre 6). Il semblerait donc que les 2 voies de contamination n'aient pas la même importance ni le même impact chez le prosobranche et le pulmoné en termes d'accumulation, ce que corroborent les résultats obtenus sur les traits de vie. Chez le pulmoné, l'ingestion de souches toxiques de *P. agardhii* entraîne un ralentissement de la croissance quel que soit l'âge (plus marquée chez les juvéniles), et une réduction de la fécondité dans le cas des adultes. Ces réductions de croissance et fécondité sont plus marquées lorsque le gastéropode ingère des cyanobactéries seule que lorsqu'il est maintenu à jeun, démontrant la forte toxicité de *P. agardhii* chez *L. stagnalis*. De plus, les effets négatifs sont encore observables après la reprise d'une alimentation normale. Chez le prosobranche, l'impact est similaire (réduction de croissance et de fécondité) mais moins intense (le jeune alimentaire entraine une baisse des traits de vie plus importante) et réversible (reprise de croissance similaire aux témoins et hausse de fécondité après la reprise d'une alimentation normale). Par contre, *P. antipodarum* semble plus sensible que *L. stagnalis* aux MCs dissoutes dans la mesure ou il présente une baisse de survie, croissance et fécondité alors que *L. stagnalis* ne présente qu'une baisse de fécondité (Gérard et Poullain, 2005; Gérard et al. , 2005). Ainsi, même si le prosobranche accumule toujours de faibles quantités de MCs, sa sensibilité aux 2 types de contamination est démontrée, alors que le pulmoné s'avère nettement plus sensible à

la contamination par les cyanobactéries toxiques que par les MCs dissoutes, différence qui se répercute sur l'accumulation de MCs dans ses tissus.

Impact des MCs intracellulaires ou dissoutes sur la fitness de L. stagnalis

L'expérience sur la fitness révèle que l'exposition à la MC-LR dissoute (33 µg L^{-1}) a un impact négatif presque aussi important que l'ingestion de cyanobactéries toxiques (10 µg L^{-1}) sur la descendance de *L. stagnalis* en termes de taux d'éclosion des œufs, de développement embryonnaire et de survie des néonates. En effet, les deux modes de contamination des adultes entraînent non seulement une diminution significative du nombre d'œufs pondus (déjà démontrée par Gérard et al., 2005 et Lance et al., 2006), mais aussi une précocité d'éclosion pour les pontes placées en eau saine après oviposition. D'autre part, la survie des néonates est réduite lorsque les parents ont été exposés aux cyanobactéries toxiques mais pas lorsqu'ils ont été exposés à de la MC-LR dissoute. De plus, quand les pontes et les néonates sont laissés en milieu parental toxique (vs eau saine), les effets délétères s'intensifient et les descendants viables de *L. stagnalis* sont encore moins nombreux. Plus précisément, les pourcentages d'éclosion et de survie des néonates sont réduits en présence de MC-LR dissoute, suggérant une perméabilité des pontes à la toxine dés l'oviposition et donc, une contamination au cours de l'embryogenèse. La réduction de survie des néonates est encore plus marquée en présence de cyanobactéries toxiques et les néonates accumulent des MCs dès la première semaine après leur naissance, suggérant une ingestion très précoce de *P. agardhii* par *L. stagnalis*. Ces résultats ouvrent des perspectives de recherche sur la sensibilité (en termes d'accumulation de MCs et de répercussions sur les traits de vie) à une nouvelle contamination à l'état juvénile ou adulte, pour des néonates qui ont survécu après une double exposition (parentale et des pontes).

8.3. Conséquences de la présence de MCs intracellulaires ou dissoutes sur les populations de gastéropodes prosobranches et de pulmonés en milieu naturel

Ces résultats laissent présager qu'en milieu naturel, les pulmonés seront principalement affectés pendant la saison de prolifération des cyanobactéries (période estivale sous nos latitudes), alors que les prosobranches seront affectés à la fois pendant la prolifération et lors de la sénescence du bloom (automne sous nos latitudes) qui coïncide avec

une libération massive de cyanotoxines dans le milieu. Notre suivi mensuel réalisé pendant un an sur 3 sites du lac de Grand Lieu exposés différemment aux cyanobactéries toxiques, montre une accumulation de MCs et une réduction de la richesse spécifique et de la diversité des gastéropodes dans les sites où prolifèrent des cyanobactéries productrices de MCs. De plus, les pulmonés sont plus abondants que les prosobranches dans les 3 sites, et aucun prosobranche n'a été prélevé dans le site le plus contaminé à la fois par les cyanobactéries toxiques et les MCs, et qui présente des proliférations de cyanobactéries récurrentes depuis plusieurs années (Brient et al., unpublished data). Des résultats similaires ont été reportés par Gérard et al. (2009) dans 6 sites présentant un gradient de contamination par les cyanobactéries : la densité de cyanobactéries influence significativement l'abondance relative des taxons de mollusques (prosobranches, pulmonés et bivalves) et les pulmonés constituent le taxon dominant dans les sites les plus contaminés. D'autre part, Gérard et al. (2008) ont mis en évidence lors du suivi à long terme d'une communauté de gastéropodes lacustre que le déclin de celle-ci était lié aux proliférations récurrentes de cyanobactéries toxiques. Alors que les prosobranches ont une origine aquatique et sont très dépendants des conditions abiotiques, les pulmonés, d'origine terrestre, ont colonisé secondairement le milieu aquatique et présentent un degré de plasticité élevé qui leur permet de survivre dans les milieux plus instables. La forte plasticité des pulmonés s'explique par leur grande diversité à la fois intra-spécifique, éco-phénotypique et génétique (Russel-Hunter, 1978; Aldridge 1983; MacMahon 1983; Dillon 2000). Par ailleurs, la plupart des pulmonés vivent dans toute la colonne d'eau (et remontent généralement à la surface pour respirer), et sont moins inféodés au milieu aquatique que les prosobranches qui vivent sur le substrat, voire dans les couches supérieures de celui-ci, et respirent l'oxygène dissous. Au niveau des surfaces respiratoires, il se peut que la pénétration de la toxine dissoute soit plus massive par la branchie des prosobranches constamment immergée que par le poumon des pulmonés. D'autre part, en tant que dépositivores, les prosobranches ingèrent régulièrement du sédiment pour en extraire les nutriments et sur lequel des MCs peuvent être adsorbées, alors que les pulmonés n'ingèrent des particules minérales que pour permettre la trituration des aliments dans leur estomac dépourvu de stylet cristallin. Enfin, les prosobranches qui tendent à être itéropares et pérennes, sont potentiellement exposés à un plus grand nombre de blooms toxiques au cours de leur vie que les pulmonés qui sont généralement semelpares et annuels.

De nouvelles études, en particulier à l'échelle cellulaire, sont nécessaires pour élucider les mécanismes de détoxication (métabolisation, excrétion) et de résistance au stress qui

conduisent à des différences d'accumulation et de sensibilité aux cyanotoxines entre prosobranches et pulmonés. De même, il serait intéressant d'approfondir les réponses des gastéropodes aux cyanobactéries toxiques i) à travers des modèles d'étude pouvant jouer le rôle d'espèce sentinelle comme le pulmoné *Physella acuta*, qui se maintient dans des milieux où prolifèrent régulièrement des cyanobactéries, tout en accumulant de très fortes quantités de MCs dans leurs tissus (Lance et al., unpublished data, Gérard et al., 2008; Gérard et al., 2009), et ii) via une approche à la fois expérimentale et en milieu naturel à long terme, afin de mieux comprendre les mécanismes d'adaptation qui peuvent exister chez certains pulmonés.

8.4. Histopathologie et localisation des MCs libres et liées chez *L. stagnalis*

Contamination de la glande génitale de L. stagnalis par les MCs

L'étude immuno-histologique de la localisation des MCs chez *L. stagnalis* permet de montrer que les spermatozoïdes et les ovocytes sont contaminés intensément après ingestion de cyanobactéries toxiques (5 µg MC-LReq L^{-1}), plus modérément après exposition à la toxine dissoute à 100 µg L^{-1}, et faiblement à 33 µg L^{-1}. Ces résultats montrent une contamination de la glande génitale, et probablement des organes associés impliqués dans la formation des pontes (e.g. glande albumineuse synthétisant le liquide périvitellin des oeufs, oviducte). La technique utilisée met en évidence les MCs liées aux protéines phophatases (Ppases) ou à d'autres protéines/peptides contenant des acides aminés soufrés (e.g. cystéine, méthionine) comme les glutathions. Les Ppases sont essentielles au maintien de l'intégrité cellulaire et sont présentes dans toute cellule eucaryote. Les interactions entre les MCs et les Ppases, qu'elles soient réversibles ou covalentes, inhibent ces dernières, engendrant une désorganisation du cytosquelette et une nécrose cellulaire (for review: Dietrich and Hoeger, 2005; Hastie et al., 2005). Cette inhibition, démontrée dans des hépatocytes de vertébrés, peut également avoir lieu également dans les cellules germinales des gastéropodes. Si les MCs détectées dans les gamètes sont liées aux Ppases, ceux-ci ont toutes les chances d'être non viables. Comme nous n'avons pas relevé d'histopathologie de la glande génitale, nous ne pouvons cependant pas affirmer que les MCs détectées sont liées aux Ppases, et non à d'autres protéines.

MCs liées dans les tissus de la glande digestive de L. stagnalis et histopathologie

Les MCs liées sont principalement localisées dans la glande digestive de *L. stagnalis,* engendrant une histopathologie moins sévère lors de l'exposition à la MC-LR dissoute vs aux cyanobactéries toxiques. En effet, l'ingestion de cyanobactéries toxiques induit des altérations délétères (nécroses, lésions) de la glande digestive (jusqu'à 95% des acini digestifs sont altérés), associées à une présence importante de MCs liées dans le cytoplasme des cellules digestives. L'exposition à la MC-LR dissoute provoque une altération nettement moins marquée des acinis digestifs (vacuolisation accrue, quelques nécroses pour 8% des acini à 33 µg L^{-1} et 45% à 100 µg L^{-1}), associée à une présence de MCs liées principalement dans la lumière des acini. Nous supposons que lors de l'exposition à la MC-LR dissoute, la toxine est majoritairement excrétée après conjugaison avec des enzymes de détoxication, provoquant cependant quelques nécroses [chez *L. stagnalis*, l'exocytose des vacuoles excrétrices entraîne la mort cellulaire (Carriker, 1946)]. Par contre, la destruction sévère des tissus après ingestion de cyanobactéries semble être due à une pénétration des MCs dans les cellules digestives, suivie par une liaison des MCs aux Ppases.

Selon une première hypothèse, cette différence de métabolisation entre les deux modes d'exposition à la toxine est liée à une variation dans l'intensité de contamination. Sur la base du taux d'ingestion d'eau, i.e., 8-12 µl / g / h pour *L. stagnalis* (De Witt et al., 1996), et pour une concentration de 100 µg MC-LR L^{-1}, on suppose qu'environ 2 µg de MC-LR pourrait pénétrer chez *L. stagnalis* par l'eau de boisson, ce qui est similaire à la quantité de MCs ingérées lors de l'exposition à la souche toxique de *P. agardhii* produisant 5 µg MC-LReq L^{-1} (Lance et al., 2006). Cependant, l'observation des coupes immuno-histologiques montre que les limnées ayant ingéré des cyanobactéries toxiques ont une glande digestive entièrement contaminée par les MCs, alors que la glande digestive des limnées exposées à la toxine dissoute présente entre 25% (à 100 µg L^{-1}) et 69% (à 33 µg L^{-1}) d'acini sans aucune trace de MC. Cette observation permet de confirmer que la MC-LR dissoute ne pénètre pas massivement chez *L. stagnalis* et qu'elle est probablement excrétée au fur et à mesure, ce qui expliquerait pourquoi elle n'a pas le temps d'interagir avec les Ppases.

La deuxième hypothèse met en cause des différences dans les interactions des variants de MCs avec la membrane des cellules digestives et/ou avec les Ppases. Il se peut en effet que le variant MC-LR reste piégé dans les vacuoles d'endocytose et/ou se lie préférentiellement aux enzymes de détoxication, alors que certains des variants produits par *P. agardhii* (MC-

YR, dmMC-LR et dmMC-RR) pénètrent plus facilement dans les cellules digestives et se lient donc plus facilement aux Ppases. Les dosages effectués par HPLC-ESI-MS/MS ont montré que les limnées exposées à la MC-LR dissoute à 30 et 100 µg L^{-1} accumulent de très faibles quantités de MCs libres (respectivement 0.07 et 0.26 µg MCs libres g DW^{-1}) sans présence de MCs liées, alors que celles exposées à *P. agardhii* à 5 et 33 µg L^{-1} accumulent respectivement un maximum de 33.8 et 69.9 µg MCs totales g DW^{-1}, comprenant de 17.7 à 66.7% de MCs liées. Les quelques MCs liées révélées par immunohistochimie chez les limnées exposées à la toxine dissoute sont absentes lors du dosage par HPLC/MS/MS, probablement en raison du seuil élevé de détection (également observé avec le test Elisa pour des accumulations < 0.5 µg g DW^{-1}), confirmant leur faible présence chez le mollusque. De plus, l'accumulation de MCs libres et liées varie en fonction de la proportion de variants de MCs produits par *P. agardhii*. En effet, l'accumulation total de MCs est 7.8 fois plus importante lorsque *P. agardhii* produit une majorité de MC-YR vs dmMC-LR, dmMC-RR (avec 74.5% de MC-YR parmi les 3 variants dans les tissus de *L. stagnalis*) que lorsque *P. agardhii* produit une majorité de dmMC-RR (avec 83% de dmMC-RR dans les tissus de *L. stagnalis*). Il semblerait ainsi que *L. stagnalis* accumule la MC-YR en plus grande quantité que les autres variants (dmMC-RR, dmMC-LR et MC-LR). Dans leur revue, Ditriech et Hoeger (2005), suggèrent que des modifications structurales mineures entre les variants de MCs peuvent avoir des effets majeurs sur l'absorption (e.g. différences d'affinité avec les transporteurs utilisés par les MCs pour pénétrer dans les cellules), la métabolisation et l'excrétion des MCs. Ces résultats suggèrent que la proportion relative des différents variants de MC produits par les cyanobactéries en milieu naturel peut influencer l'accumulation de MCs libres et covalentes chez les gastéropodes.

Au vu de nos résultats sur la fitness (i.e., altération du développement embryonnaire même lors de l'exposition à 33 µg L^{-1} de MC-LR dissoute) et de l'histopathologie de la glande digestive de *L. stagnalis* après injection per os de MC-LR (Zurawell et al., 2006), le moindre impact observé chez *L. stagnalis* après exposition à la MC-LR dissoute (vs ingestion de cyanobactéries toxiques) semble plutôt relié à une faible pénétration de la toxine permettant une excrétion efficace (première hypothèse). Néanmoins, nous ne pouvons rejeter la deuxième hypothèse impliquant les types de variants et qui ouvre de nouvelles perspectives de recherche sur les différences d'accumulation et de liaison aux Ppases/enzymes de détoxication entre les variants de MCs. Un marquage de la MC-LR [e.g. culture de cyanobactéries avec du ^{14}C, comme réalisé par Wiegand et al. (1999)] ou un test d'inhibition

de l'activité des Ppases en présence de MC-LR [e.g. en suivant leur activité de déphosphorylation d'une phophorylase marquée au ^{33}P, comme réalisé par Tencalla et Dietrich (1994)], ainsi qu'une contamination par la MC-YR dissoute pour comparaison, pourraient être très utiles et permettraient en plus de déterminer si les MCs contaminent la glande génitale via l'hémolymphe.

Persistance des MCs liées et reconstruction des tissus après exposition

Nous avons démontré que 90% des MCs libres accumulées par *L. stagnalis* après ingestion de cyanobactéries toxiques sont éliminées après 3 semaines de dépuration (résultats confirmés dans l'étude plus récente effectuée en Finlande) (Lance et al., 2006). D'après les connaissances actuelles, aucune élimination par les enzymes de détoxication ne peut avoir lieu pour les MCs liées de manière covalente (irréversible). Cependant, dans nos deux études, entre 0 et 59.2% des MCs liées sont éliminées (dosage par HPLC MS MS après oxydation des tissus) et l'intensité du signal des MCs dans les cellules des glandes digestive et génitale diminue de moitié (immunohistochimie) après 3 semaines de dépuration chez les gastéropodes préalablement exposés aux cyanobactéries toxiques. Nous supposons que cette élimination a lieu lors du renouvellement des Ppases et/ou des cellules endommagées suite à leur inhibition. Cette hypothèse semble confirmée par l'observation des coupes histologiques montrant que la glande digestive retrouve une structure normale chez la moitié des individus anciennement exposés aux cyanobactéries toxiques, i.e., 50% de lobules digestifs intacts après 3 semaines de dépuration vs 4.4% après 5 semaines d'intoxication. La présence de lobules en phase de régénération témoigne de l'élimination des cellules nécrosées [dans la lumière des acinies et par les macrophages (Henry, 1987)] et de l'activité des cellules souches, très protégées au sein de l'épithélium digestif et capables de régénérer les acini (Carriker, 1946; Charrier, 1985).

8.5. Risques de contamination du réseau trophique par les MCs : de *Lymnaea stagnalis* à *Gasterosteus aculeatus*

Malgré l'élimination partielle des MCs, *L. stagnalis* anciennement exposés aux cyanobactéries toxiques contient encore jusqu'à 15.3 µg g DW^{-1} de MCs liées, représentant jusqu'à 91% de la quantité de MCs totales après 3 semaines de dépuration. Cette expérience,

associée aux premiers résultats sur l'ingestion de cyanobactéries par *L. stagnalis* (Lance et al., 2006), permet de déduire qu'au moins 83% des MCs intracellulaires ingérées sont accumulées sous forme libres ou liées chez les limnées adultes, et presque 100% chez les juvéniles. Ces résultats suggèrent une forte implication des gastéropodes pulmonés dans le transfert de MCs à leurs prédateurs. L'hypothèse d'une éventuelle contamination des poissons par transfert trophique de MCs a déjà été formulée pour plusieurs espèces de poissons omnivores et carnivores dans des eaux où prolifèrent des cyanobactéries toxiques (Williams *et al*., 1997; Ibelings *et al*., 2005; Gkelis *et al*., 2006; Xie *et al*, 2005; 2007). A notre connaissance, seule l'étude de Smith et Haney (2006) a mis en évidence le transfert de MCs libres du zooplancton au poisson *Lepomis gibbosus* en laboratoire, et aucune étude n'a pris en compte l'éventuel transfert de MCs liées, nécessitant une métabolisation du complexe MC-Ppases dans l'estomac du prédateur et une libération d'une partie des MCs. D'autre part, nous avons montré que les fèces de *L. stagnalis* contiennent des MCs libres (1.39 µg g^{-1} DW). Or, les faeces des gastéropodes dulcicoles constituent une ressource de bonne qualité nutritive et de quantité non négligeable pour la faune coprophage (Schreiber et al., 2002), qui est donc susceptible d'être contaminée suite à la consommation de cyanobactéries par les gastéropodes.

Notre étude démontre le transfert de MCs libres du gastéropode *L. stagnalis* à l'épinoche *Gasterosteus aculeatus,* et suggère un transfert de MCs liées. Ce transfert se traduit par l'accumulation de MCs libres dans tous les organes étudiés (foie, reins, muscles et branchies), après seulement 4 jours d'ingestion journalière par le poisson de 40 mg (poids frais) de glande digestive de limnée contaminée. Nous n'avons par mis en évidence de biomagnification dans la mesure où la quantité de MCs libres dans le foie des épinoches est équivalente ou 1.7 fois inférieure (ingestion de glandes digestives contenant respectivement 94% et 64% de MCs liées) à celle présente dans la limnée. La contamination du poisson suggère un passage de la toxine dans la circulation sanguine via le tractus gastrointestinal lors de la digestion, pouvant induire des perturbations de l'homéostasie comme décrites par Malbrouck et Kestemont (2006). Nos résultats montrent l'induction légère d'un stress oxydant (augmentation de l'activité de l'enzyme antioxydante Glutathion peroxidase) dans le foie de l'épinoche. Par ailleurs, l'activité locomotrice des poissons est diminuée pendant l'intoxication, probablement en raison d'une réallocation des ressources énergétiques en faveur des processus de métabolisation et d'excrétion des MCs. L'élimination des MCs après 4 jours de dépuration est totale dans les branchies des épinoches, très importante dans le foie et les reins (92%), mais seulement partielle dans les muscles (de 6 à 58%) qui contiennent

encore 1.05 µg MCs libres g^{-1} DW). De plus, l'accumulation totale de MCs chez le poisson est probablement supérieure dans la mesure où il faut ajouter la quantité de MCs liées (analyses en cours). L'accumulation de MCs dans les muscles de l'épinoche à partir de limnées contaminées laisse supposer un risque non négligeable de transfert dans le réseau trophique pouvant aboutir à l'homme.

Il paraît nécessaire d'approfondir les aspects de transfert des MCs, libres ou liées, entre les gastéropodes et leurs prédateurs, à la fois pour prédire les risques de contamination des réseaux trophiques à partir des gastéropodes vecteurs, mais aussi dans un contexte de prévention et de gestion des risques sanitaires. Des études en laboratoire, complétées par des investigations à long terme en milieu naturel, sont nécessaires pour suivre le devenir des MCs le long des chaînes trophiques en mesurant la quantité de MCs dans les cyanobactéries, les gastéropodes et les prédateurs malacophages comme l'écrevisse, l'anguille ou le brochet, potentiellement consommés par l'homme. Une étude à laquelle nous avons participé a par exemple mis en évidence la contamination des anguilles (consommatrices de mollusques) en milieu naturel (Acou et al., 2008). De plus, dans la mesure ou nous avons mis en évidence que plus de la moitié des MCs accumulées chez *L. stagnalis,* après ingestion de cyanobactéries, l'étaient sous forme liée, il paraît nécessaire d'évaluer l'éventuelle toxicité de ces MCs liées. Jusqu'à présent, aucune étude n'a encore mis en évidence la métabolisation du complexe MC-Ppases dans l'estomac des prédateurs, ni la potentielle toxicité des résidus issus de cette métabolisation.

8.6. Conclusion

L'exposition des gastéropodes aux proliférations de cyanobactéries toxiques peuvent être fréquentes en régions tempérées, particulièrement dans les zones littorales des lacs eutrophes (Zurawell et al., 1999; Xie et al., 2007; Gérard et al., 2008). En milieu naturel, les gastéropodes peuvent se contaminer par ingestion de cyanobactéries toxiques pendant la période chaude qui favorise les proliférations (généralement de Mai à Octobre), et par absorption des cyanotoxines (dissoutes ou adsorbées) libérées massivement lors de la lyse des blooms (à la fin de l'été et au début de l'automne). Les concentrations de toxines produites par les cyanobactéries varient énormément en fonction des conditions environnementales, e.g., de 1 à 8600 µg L^{-1} pour les MCs (Christoffersen, 1996 for review), suggérant que les gastéropodes peuvent être exposés à des concentrations bien plus fortes que celles utilisées dans notre étude (5, 10 ou 33 µg L^{-1}). L'ensemble des résultats apportés par cette thèse suggèrent d'une part, un impact négatif des cyanobactéries toxiques sur les communautés de gastéropodes dulcicoles (en termes de dynamique et de composition), et d'autre part, un risque de contamination des organismes malacophages.

Les effets négatifs des MCs et les différences de sensibilité entre prosobranches et pulmonés démontrés par nos résultats peuvent induire des changements dans l'abondance et la composition des communautés de gastéropodes. Les deux voies principales de contamination des gastéropodes, i.e., par les MCs extracellulaires (eau de boisson, pénétration transcutanée), et plus particulièrement par les MCs intracellulaires (ingestion de cyanobactéries), agissent probablement en synergie en milieu naturel. Les gastéropodes sont directement affectés à l'échelle individuelle (mortalité, réduction de croissance, histopathologie), mais aussi à l'échelle spécifique via leur potentiel reproducteur et leur fitness (diminution de fécondité, contamination des gamètes, perturbation du développement embryonnaire, réduction de la survie des néonates). Sachant que les MCs dissoutes pénètrent dans les pontes, et que les juvéniles à peine éclos ingèrent des cyanobactéries et accumulent des MCs, l'impact des proliférations récurrentes de cyanobactéries toxiques va se répercuter sur l'ensemble de la population quelle que soit sa structure d'âge, et fortement affecter la dynamique des populations. A chaque saison de prolifération, les cyanobactéries productrices de MCs vont donc réduire de manière drastique le nombre d'individus capables de se reproduire, et à terme conduire potentiellement au déclin des communautés de gastéropodes comme démontré par Gérard et al. (2008). L'impact sévère des proliférations de cyanobactéries toxiques sur les

communautés de gastéropodes (en termes d'abondance et de composition avec une disparition des espèces sensibles) a également été démontré lors de notre étude sur le lac de Grand Lieu (44) et dans plusieurs autres sites en Bretagne (Gérard et al., 2008 ; Gérard et al., 2009).

Par ailleurs, les MCs que nous avons étudiées ne sont pas les seules toxines produites par les cyanobactéries, et la plupart produisent d'autres types de toxines (e.g. neurotoxines, lipopolysaccharides, cyanopeptolides, microviridines…), et les extraits bruts de cyanobactéries sont souvent plus toxiques que les toxines purifiées (Gkelis et al., 2006 ; pour revues : Wiegand et Pflugmacher, 2005 ; Ibelings & Chorus, 2007). Les multiples composés produits par les cyanobactéries peuvent donc avoir des effets additifs ou synergiques sur les gastéropodes dans les eaux contaminées par plusieurs espèces et souches de cyanobactéries. De même, la contamination par les MCs peut rendre les gastéropodes moins résistants aux autres perturbations environnementales (e.g., sécheresse, parasitisme, xénobiotiques), et ainsi engendrer des impacts encore plus sévères sur leur traits d'histoire de vie ou leur capacités de détoxication.

L'impact négatif des cyanobactéries toxiques sur les communautés de gastéropodes peut avoir des répercussions considérables sur tout le réseau trophique, car ils jouent un rôle clé dans la structuration des écosystèmes dulcicoles et représentent un lien important entre les producteurs primaires et les consommateurs secondaires (Kerans et al., 2005). En tant que consommateurs primaires et brouteurs efficaces, les gastéropodes dulcicoles peuvent contrôler la biomasse algale (périphyton et cyanobactéries) (Biggs and Lowe, 1994; Mc Collum et al., 1998 ; Liess & Hillebrand, 2004; Suren, 2005). De plus, ils favorisent indirectement les macrophytes en broutant le périphyton présent à leur surface et en réduisant ainsi la compétition (Brönmark, 1990; Steinman et al., 1987 ; Weber et Lodge, 1990). Les gastéropodes peuvent aussi avoir un impact positif à la fois sur les macrophytes et le périphyton par les nutriments qu'ils excrètent (sous forme dissoute ou dans les faeces) (Liess & Haglund, 2007). L'étude du cycle de la matière et du transfert d'énergie dans les eaux douces montre que les gastéropodes accélèrent efficacement la circulation de la matière et jouent un rôle dominant dans les réseaux alimentaires benthiques des eaux courantes et stagnantes (Reavell, 1980; Dillon, 2000). Des modifications de l'abondance et de la structure des communautés de gastéropodes peuvent donc avoir des répercussions sur le fonctionnement de l'ensemble de l'écosystème dulcicole et indirectement aggraver la fréquence et la sévérité des proliférations de cyanobactéries.

Dans les eaux régulièrement contaminées par les cyanobactéries toxiques, les espèces de gastéropodes sensible tendent à disparaître, tandis que celles qui persistent (e.g., *P. acuta*) sont celles qui accumulent le plus de MCs, jouant ainsi le rôle de vecteurs de MCs pour de nombreuses espèces (e.g., oiseaux d'eau, poissons, amphibiens, sangsues, écrevisses, insectes) (Michelson, 1957, Turner & Chislock, 2007), risquant de contaminer les réseaux trophiques à la fois aquatiques et terrestres. Dans un contexte de gestion des écosystèmes aquatiques, ces espèces de gastéropodes (e.g., *P. acuta*) peuvent constituer des espèces sentinelles (i.e., moniteurs biologiques qui accumulent les cyanotoxines dans leurs tissus sans effet négatif significatif aux concentrations ambiantes) dans la mesure où elles sont relativement ubiquistes, abondantes, et de taille suffisante pour procurer un matériel d'analyse suffisant (Beeby, 2001).

Selon Lydeard et al. (2004), les gastéropodes et les bivalves sont probablement les macroinvertébrés aquatiques les plus mis en en danger par les activités humaines. De plus, le lien récemment suggéré entre le réchauffement climatique et l'intensification de la fréquence et de la sévérité des blooms de cyanobactéries (Paerl & Huisman, 2008), associé au fait que la résilience des communautés de gastéropodes relative au réchauffement climatique est faible (Mouthon & Daufresne, 2006), suggèrent qu'au moins certaines espèces de mollusques sont menacées d'extinction. Il devient donc urgent de mettre en place des actions pour limiter les proliférations de cyanobactéries (e.g., par réduction des causes anthropiques de l'eutrophisation) pour préserver la biodiversité et rétablir un meilleur fonctionnement des écosystèmes aquatiques.

Annexes

Annexe 1 : Liste non exhaustive des espèces de cyanobactéries potentiellement toxiques et de leurs toxines (N.I.= toxine présente mais non identifiée). Les espèces ayant déjà été observées en France apparaissent en caractères gras. Tableau tiré du document « Evaluation des risques liés à la présence de cyanobactéries et de leur toxines dans les eaux destinées à l'alimentation, à la baignade et autres activité récréatives », Agence Française de Sécurité Sanitaire des Aliments, Juillet 2006.

Cyanobactéries toxiques	Toxines
Anabaena affinis	N.I.
Anabaena circinalis	Anatoxine-a, Saxitoxines, Microcystines
Anabaena flos-aquae	Anatoxines (-a, -a(s), -b, -b(s), -c, -d), Microcystines
Anabaena hassallii	N.I.
Anabaena lemmerman	Microcystines, Anatoxine-a(s)
Anabaena planctonica	Anatoxine-a
Anabaena spiroides	Anatoxine-a, Microcystines
Anabaena torulosa	N.I.
Anabaena variabilis	N.I.
Anabaena sp.	Anatoxine-a
Anabaenopsis milleri	Microcystines
Aphanizomenon flos-aquae	Anatoxine-a, Saxitoxines
Aphanizomenon ovalisporum	Cylindrospermopsine
Aphanizomenon sp.	Anatoxine-a
Coelosphaerium naegelianum	Hépatotoxine
Cylindrospermopsis raciborskii	Cylindrospermopsine, Saxitoxines
Cylindrospermum sp.	Anatoxine-a
Fischerella epiphytica	N.I.
Gloeotrichia echinulata	N.I.
Gloeotrichia pisum	N.I.
Hapalosiphon hibernicus	Microcystines
Lyngbya birgei	N.I.
Lyngbya gracilis	Debromoaplysiatoxine
Lyngbya major	N.I.
Lyngbya majuscula	Lyngbyatoxine-a
Lyngbya wollei	Saxitoxines
Microcoleus lyngbyaceus	N.I.
Microcystis aeruginosa	Microcystines
Microcystis botrys	Microcystines
Microcystis farlowian	Ichtyotoxine
Microcystis flos-aquae	Microcystines
Microcystis panniformis	Microcystines
Microcystis toxica	N.I.
Microcystis viridis	Microcystines, Microviridine
Microcystis wesenbergii	Microcystines
Microcystis sp.	Anatoxine-a
Nodularia spumigena	Nodularines
Nostoc paludosum	N.I.
Nostoc rivulare	N.I.
Nostoc sp.	Microcystines

Annexe 1 : suite

Cyanobactéries toxiques	Toxines
Oscillatoria formosa	Homoanatoxine-a
Oscillatoria lacustris	N.I.
Oscillatoria limosa	Microcystines
Oscillatoria tenuis	Microcystines
Oscillatoria nigroviridis	Oscillatoxine-a
Oscillatoria sp.	Anatoxine-a
Phormidium favosum	Anatoxine-a
Planktothrix agardhii	Microcystines
Planktothrix mougeotii	Microcystines
Planktothrix rubescens	Microcystines
Planktothrix sp.	Anatoxine-a
Pseudanabaena sp.	Neurotoxine
Raphidiopsis sp.	Cylindrospermopsine
Schizothrix calcicola	Aplysiatoxines
Scytonema hofmanni	Scytophycines a et b
Scytonema pseudohofmanni	Scytophycines a et b
Spirulina subsalsa	N.I.
Symploca hydnoides	N.I.
Symploca muscorum	Aplysiatoxine
Synechococcus sp.	N.I.
Trichodesmium erythraeum	Neurotoxine
Umezakia natans	Cylindrospermopsine
Woronichinia naegeliana anciennement *Gomphosphaeria naegelianum*	Anatoxine-a

Annexe 2. Valeurs toxicologiques pour les microcystines pour différentes voies d'administration (orale, IP : intrapéritonéale, IN : intranasale, IT : intratrachéale). MC : Microcystine. Tableau tiré du document « Evaluation des risques liés à la présence de cyanobactéries et de leur toxines dans les eaux destinées à l'alimentation, à la baignade et autres activité récréatives », Agence Française de Sécurité Sanitaire des Aliments, Juillet 2006.

Toxicité	Voie administration	Valeurs repères	Toxine	Source
Aiguë	Orale	DL50 = 5 mg.kg^{-1} (souris, gavage)	MC-LR	(Fawell *et al.* 1994, Fawell *et al.* 1999a)
		DL50 = 10,9 mg.kg^{-1} (souris, gavage)	MC-LR	(Yoshida *et al.* 1997)
	IP	25 µg.kg^{-1} < DL50 < 150 µg.kg^{-1}	MC-LR	(Kuiper-Goodman et *al.* 1999)
	IN	36 µg.kg^{-1} < DL50 < 122 µg.kg^{-1}	MC-LR	(Fitzgeorge *et al.* 1994)
	IT	50 µg kg^{-1} < DL50 < 100 µg.kg^{-1}	MC-LR	(Ito *et al.* 2001)
Subchronique	Orale	DSENO = 40 µg.kg^{-1}.j^{-1} (souris, gavage, 13 semaines)	MC-LR	(Fawell *et al.* 1994, Fawell *et al.* 1999a)
		DMENO = 50 µg.kg^{-1}.j^{-1} (rat, eau de boisson, 28 jours)	MC-LR	(Heinze 1999)
		DMENO = 100 µg/kg p.c. j^{-1} (porc, eau de boisson, 44 jours)	Extrait de *M. aeruginosa*	(Falconer 1994)
		DSENO = 333 µg.kg^{-1}.j^{-1} (souris, alimentation, 43 jours)	Extrait d'*A. flos-aquae*	(Schaeffer *et al.* 1999)
Embryotoxicité	Orale	DSENO = 600 µg.kg^{-1}.j^{-1} (souris, gavage, 10 jours)	MC-LR	(Fawell *et al.* 1994, Fawell *et al.* 1999a)
		DSENO = 333 µg.kg^{-1}.j^{-1} (souris, alimentation, pendant la gestation et la lactation)	Extrait d'*A. flos-aquae*	(Schaeffer *et al.* 1999)
Tératogénèse	Orale	DSENO = 333 µg.kg^{-1}.j^{-1} (souris, alimentation, pendant la gestation et la lactation)	Extrait d'*A. flos-aquae*	(Schaeffer *et al.* 1999)

Annexe 3. Accumulation de microcystines (MCs) observées en milieu naturel dans les tissus, le sang ou les faeces de poissons, en fonction des concentrations de MCs enregistrées dans les cyanobactéries ou dans l'eau. Tableau tiré de la revue de Malbrouck, C., Kestemont, P. (2006).

Table 2. Microcystin (MC) accumulation observed in field conditions in fish tissues, blood, or feces, depending on the toxin concentration in water or in cyanobacterial cells

Species	Field study	Toxin concn. in cyanobacterial cells (dry wt) or water (per liter)	Method of detection	Toxin accumulation in tissues, blood, or feces	References
Whitefish (*Coregonus lavaretus* L.)	Field: fish caught in a Lake Ammersee hatchery (Germany) during blooms of *Planktothrix* sp. in winter 1998 and 2000	In *Planktothrix* extracts: MCs varied between 0.5 and 6.5 μg/mg dry weight In water: MCs varied between 0 and 78 ng/L	Immunoprobing (MC-antibodies)	MCs in gut contents MC-protein adducts in liver homogenates	[25]
Tilapia (*Tilapia rendalli*)	Field: fish caught in the shallow coastal Jacarepagua Lagoon (Rio de Janeiro, Brazil) every two weeks from August 1996 to November 1999	In water, MC varied from 0 to 980 μg/L	HPLC	MCs detected in fish livers (0–31.1 μg/g), viscera (0–71.6 μg/g), and muscle tissue (2.9–337.3 ng/g)	[44]
Flounder (*Platichthys flesus*)	Field: fish caught in the western Gulf of Finland during July and August 1999		ELISA	Time-dependent accumulation maximum concentration in liver: 399 ± 5 ng nodularin or MC/g dry weight; no hepatotoxins in muscle	[46]
Hemiculter eigenmanni and *Culter brevicauda*	Field: fish caught in eutrophic Hoedong Reservoir (South Korea), feeding on *Microcystis* spp.	No MC detected in water		Elevated levels of MC in body tissues and feces, generally higher in feces than in body tissues	[47]
Not cited	Field: fish caught in Sepetiba Bay (Rio de Janeiro, Brazil)	Highest MC value in water: 0.12 μg/L	ELISA	MCs detected in muscle fish tissues 19% of animal samples above 0.04 μg/kg/d Maximum value in fish muscle: 39.6 μg/kg	[45]

[a] HPLC = high-performance liquid chromatography; ELISA = enzyme-linked immunosorbent assay.

Annexe 4. Accumulation de microcystines (MCs) observées en laboratoire dans les tissus, le sang ou les faeces de poissons, en fonction des concentrations de MCs administrées. Tableau tiré de la revue de Malbrouck, C., Kestemont, P. (2006).

Table 3. Microcystin (MC) accumulation observed in experimental conditions in fish tissues, blood, or feces, depending on the toxin co administered[a]

Species	Experimental study	Toxin concn. in cyanobacterial cells (dry wt) or water (per liter)	Method of detection	Toxin accumulation in tissues, blood, or feces	R
Common carp (*Cyprinus carpio*)	Experimental: fish (322 ± 36 g) fed with bloom scum at a dose of 50 μg MC/kg body weight during 28 d	Bloom dominated by *Microsystis aeruginosa* (91.3%), containing MC-RR (73.7%), -LR, and -YR, total toxin concn.: 357.3 ± 26.8 μg/g dry weight	PPIA	In hepatopancreas: 261.0 ± 108.3 ng MC-LR equivalent/g fresh weight In muscle: 38.3 ± 12.3 ng MC-LR equivalent/g fresh weight	
Goldfish (*Carassius auratus*)	Experimental: fed juveniles injected IP with 125 μg MC-LR/kg body weight		PPIA	Max. concn. in liver: 0.3 μg/g tissue after 8 h	
	Experimental: fed and fasted juveniles injected IP with 125 μg MC-LR/kg body weight		PPIA	Max. concn. in liver after 48 h: 0.3 μg/g tissue in fasted individuals and 0.15 μg/g tissue in fed individuals	
	Experimental: isolated hepatocytes incubated with 10 μg MC-LR/L during 4 h		PPIA	Rapid accumulation (within 1 h) Accumulation rate higher in the fasted treatments Max. concn. in fasting treatments: 0.009 μg/10^6 cells Max. concn. In feeding treatments: 0.015 μg/10^6 cells	
Silver carp (*Hypophthalmichthys molitrix*)	Experimental; two treatments: feeding 80 d with *Microcystis viridis* cells/ feeding 40 d with *M. viridis* cells and then 40 d with artifcial carp feed	MC-LR varied between 268 and 580 μg/g dry weight MC-RR varied between 110 and 292 μg/g dry weight	HPLC Measure of MC-LR and MC-RR every 20 d	Maximum MC-RR in blood, liver, and muscle: 49.7, 17.8, and 1.77 μg/g dry weight No MC-LR detectable in muscle and blood samples	
	Experimental: fish directly exposed to monoclonal *M. aeruginosa* strains (NIES 44, 88, and 99)			MC detected in body tissues (0.6–2.5 μg/g dry wt) and feces	
Tilapia rendalli juveniles	Experimental; feeding with:		ELISA		
	Fish food plus intact toxic cells of *M. aeruginosa* strain NPLJ-4 (20.4 μg MC/fish/d) during 15 d and fish food without toxic cells during the next 15 d			Max. concn. in liver (0.6 μg/g) during the accumulation period Max. concn. in muscle (0.05 μg/g) during the depuration period MCs in feces during the depuration period (max. 0.07 μg MC/g feces/p fish)	
	Only intact toxic cells during 28 d (14.6 μg MC/ fish/d)			Max concn. in liver: 2.8 μg/g; average concn. in liver: 0.93 ± 1.2 μg/g; in muscle: 0.06 ± 0.018 μg/g	
	Fish Food plus disrupted toxic cells of (29.2 μg MC/fish/d) during 42 d			Average concn. in liver: 0.92 ± 0.5 μg/g; in muscle: 0.05 ± 0.028 μg/g	

[a] MCLR = microcystin-LR; MC-RR = microcystin-RR; MC-YR = microcystin-YR; PPIA = protein phosphatase inhibition assay.

Annexe 5 : Présence de MCs dans la faune aquatique consommée par l'homme. Tableau tiré de la revue « Accumulation of cyanobacterial toxins in freshwater seafood and its consequences for public health : A review», Ibelings et Chorus, 2007.

Table 1
Cyanobacterial toxins in human diet, examples taken from literature

Organism	Organ(s)/tissue	Toxin conc. ($\mu g\ g^{-1}$)	Daily intake ($\mu g\ d^{-1}$)	×Lifetime TDI	References
Fish					
Odontesthes bonariensis	Muscle	0.05/0.34	5.0/34.0	1.8/12.1	Cazenave et al., 2005
Hypophthalmichthys molitrix	Muscle	0.00025–0.097	0.0025–0.97	0.009–3.5	Chen et al., 2005
Cyprinus carpio	Muscle	0.038	3.8	1.6	Li et al., 2004
Tilapia rendalli	Muscle	0.002–0.337	0.72–101.1	0.3–42.1	Magalhaes et al., 2001
Unidentified fish spp.	Muscle	0.04	11.9	5.0	Magalhaes et al., 2003
Oreochromis niloticus	Muscle	0.102	10.2	3.6	Mohamed et al., 2003
Hypophthalmichthys molitrix	Muscle	0.0016	0.79	0.3	Shen et al., 2005
Platichthys flesus	Muscle	0.0005–0.1	0.075–1.5	0.03–0.6	Sipia et al., 2006
Rutilus rutilus	Muscle	0.0004–0.2	0.06–3	0.05–1.3	Sipia et al., 2006
Unidentified fish spp.	Muscle	0.0007–0.025	0.08–1.9	0.03–1	Van Buynder et al., 2001
Oncorhynchus mykiss	Muscle	0.035	10.5	3.7	Wood et al., 2006
Mussels					
Anodonta woodiania	Foot/muscle	0.009/0.026	2.7/7.8	1.1/3.3	Chen and Xie, 2005a
Hyriopsis cumingii	Foot/muscle	0.022/0.039	6.6/11.7	2.8/4.9	Chen and Xie, 2005a
Cristaria plicata	Foot/muscle	0.01/0.023	3.0/6.9	1.3/2.9	Chen and Xie, 2005a
Lamprotula leai	Foot/muscle	0.021/0.058	6.3/17.4	2.6/7.3	Chen and Xie, 2005a
Anodonta woodiania	Whole	0.064	19.2	8	Chen and Xie, 2005a
Hyriopsis cumingii	Whole	0.188	56.4	23.5	Chen and Xie, 2005a
Cristaria plicata	Whole	0.096	28.8	12	Chen and Xie, 2005a
Lamprotula leai	Whole	0.131	39.6	16.5	Chen and Xie, 2005a
Unidentified mussels	Whole	0.247	4.8	1.7	Saker et al., 2004
Unidentified mussels	Whole	2.5	0.8–48.2	0.3–7.2	Van Buynder et al., 2001
Crayfish					
Unidentified crab spp.	Muscle	0.103	31.0	12.9	Magalhaes et al., 2003
Procambarus clarkia	Muscle	0.005/0.010	1.5/10.8		Chen and Xie, 2005b
Shrimps					
Palaemon modestus	Muscle	0.006/0.026	1.8/7.8	0.8/3.3	Chen and Xie, 2005b
Macrobrachium nipponensis	Muscle	0.004/0.012	1.2/3.6	0.5/1.5	Chen and Xie, 2005b
Palaemon modestus	Whole	0.114	34.2	14.3	Chen and Xie, 2005b
Macrobrachium nipponensis	Whole	0.051	15.3	6.4	Chen and Xie, 2005b
Unidentified prawns	Flesh	0.205	2.1	1.8	Saker et al., 2004
Unidentified prawns	Flesh	0.005–0.022	0.13–0.55	0.05–0.2	Van Buynder et al., 2001

See references listed in the table for assumptions or conversion factors used in calculation of daily intake. All toxin concentrations are expressed as MCYST-LR equivalents, with the exception of (Cazenave et al., 2005): MCYST-RR and (Saker et al., 2004): CYN. The table shows toxin concentrations for those parts of animals that are eaten. If, for instance, viscera are removed prior to consumption of fish the table shows values in muscle tissue. If however, organisms are eaten whole, like mussels, the data shown are based upon concentrations in whole organisms. Toxin concentrations presented here are the mean, minimum or maximum concentrations encountered in surveys, expressed per gram of wet (fresh) weight. From papers by Chen and Xie (2005a,b) both seasonal mean and maximum concentrations found in muscles are presented (maximum only when eaten whole), likewise for Cazenave et al. (2005) but here the maximum is the value in muscle tissue, whilst for fish in the study of Magalhaes et al. (2001) and Sipia et al. (2006) ranges (seasonal low–high) are given. The numbers for Mohamed et al. (2003), Saker et al. (2004) and Wood et al. (2006) are the maximum concentrations encountered. Data from Van Buynder et al. (2001) represent minimum and maximum values. Daily intake is typically based upon consumption of 100–300 g of seafood (all as indicated by the respective authors). Van Buynder et al. (2001) and Saker et al. (2004) use cumulative food intake over a period of 14 days. For comparison we have recalculated the food intake in their study to a value corresponding to a daily intake. The column ×TDI indicates by how many times the provisional WHO TDI for MCYST-LR of 0.04 $\mu g\ kg^{-1}\ bw\ d^{-1}$ (0.03 for CYN, as proposed by Humpage and Falconer, 2003) is exceeded in a 60–70 kg adult. While this assessment relates to a TDI for lifetime daily exposure, see Table 2 for TDIs proposed for single or seasonal exposure patterns.

Annexe 6 : Présentation des organes du système digestif de *L. stagnalis,* ainsi que leur fonction. D'après les données de Carriker (1946).

Organes ou parties d'un organe	Fonctions
Œsophage :	Premier site de digestion enzymatique par les sécrétions des glandes salivaires
(estomac = jabot, gésier et pylore)	
Jabot :	Stockage de nourriture et premier site de digestion mécanique
Gésier :	Site principal de digestion mécanique, aidée par la présence de sable et de fines particules. Digestion chimique par les enzymes des glandes salivaires et digestives
Pylore :	Lieu de triage des particules alimentaires en fonction de leur taille et leur digestibilité. Les particules de taille > 0.4 µm sont dirigées dans le prointestin (excrétion) par un conduit ventral. Les particules de taille < 0.4 µm de diamètre sont dirigées vers le vestibule hépatique par un conduit dorsal.
Vestibule hépatique :	Dirige sélectivement les particules solubles en suspension de taille < 0.4 µm vers les conduits de la glande digestive, et les particules non digestibles vers le caecum.
Glande digestive :	Site d'endocytose, de digestion intracellulaire (lysosomale), d'excrétion vacuolaire des résidus non digestibles. Sécrétion d'enzymes digestives déversées dans le gésier.
Caecum :	Reçoit les particules non digestibles du vestibule hépatique. Consolide la fraction caecale des fèces, dirigée ensuite vers le prointestin.
Prointestin :	Absorption d'eau. Consolidation finale des fèces (avec les parties provenant du gésier, de caecum et de la glande digestive) par sécrétion d'un cément.
Intestin moyen et rectum:	Production de mucus et élimination des fèces.

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