UNIVERSITÉ DU QUÉBEC À RIMOUSKI

NÉOPLASIE HÉMIQUE CHEZ LA MYE COMMUNE (*MYA ARENARIA*) : IMPLICATION D'UNE CONTAMINATION ANTHROPIQUE

Thèse présentée

dans le cadre du programme de doctorat en océanographie

en vue de l'obtention du grade de *philosophiae doctor*

PAR

© **Julie Pariseau**

Mars 2012

Composition du jury :

Céline Audet, présidente du jury, Université du Québec à Rimouski

Réjean Tremblay, directeur de recherche, Université du Québec à Rimouski

Jean-Marie Sévigny, codirecteur de recherche, Université du Québec à Rimouski

Franck C.J. Berthe, codirecteur de recherche, University of Prince Edward Island

Jean-Michel Danger, examinateur externe, Université du Havre

Dépôt initial le 3 août 2011 Dépôt final le 5 mars 2012

À mes parents, merci pour TOUT ce que vous avez fait pour moi. Si j'ai ce diplôme aujourd'hui c'est grâce à vous !!! Je vous aime xxxx

REMERCIEMENTS

Je désire remercier, tout d'abord, mon directeur le Dr Réjean Tremblay (UQAR-ISMER) pour m'avoir permis de réaliser ce projet. J'ai bénéficié de ta grande expérience dans le domaine de la recherche et pour cela je t'en serai toujours reconnaissante. Merci pour tes conseils judicieux, ta rigueur scientifique, tes corrections et ton appui exceptionnel tout au long de cette thèse.

Je voudrais également remercier mon codirecteur le Dr Franck Berthe (UPEI-AVC) pour m'avoir permis d'utiliser ses laboratoires et ses équipements. Je te remercie également pour ta rigueur scientifique, tes bons conseils, tes corrections et ton appui indéfectible. De plus, grâce à mon séjour à l'Île-du-Prince-Édouard, je peux maintenant me débrouiller en anglais, et pour ceci, tu auras toujours ma reconnaissance.

Je tiens à remercier mon codirecteur le Dr Jean-Marie Sévigny (MPO-IML) dont la contribution à la réalisation de cette thèse a été essentielle. Merci pour vos conseils, vos corrections rigoureuses et votre soutien.

Je voudrais remercier le Dr Émilien Pelletier qui m'a été d'une aide précieuse pour la réalisation de cette thèse. Merci d'avoir mis à ma disposition tous les appareils nécessaires aux analyses chimiques et merci également pour la rigueur de tes corrections ainsi que tes bons conseils.

Je voudrais remercier le Dr T. Jeffery Davidson pour m'avoir initiée à la néoplasie hémique ainsi que pour son aide très appréciée. Merci Jeff!

J'exprime ma reconnaissance au Dr Richard St-Louis pour son aide précieuse en chimie. Merci Richard pour tes bons conseils qui mon permis d'acquérir une expertise pour les techniques d'analyse spectrométrique (GC-MS et ICP-MS). Merci au Dre Maryse Delaporte pour son expertise en cytométrie en flux. Je tiens à te remercier pour ton soutien, tes conseils et ton amitié. Je remercie également le Dr Ahmed Siah qui m'a fait découvrir le merveilleux monde de la biologie moléculaire. Ahmed, merci pour ton aide, tes conseils, ton soutien et ton amitié. Tu m'as beaucoup appris et je t'en serai toujours reconnaissante.

Je remercie le Dr Jean-Michel Danger, professeur-chercheur à l'Université du Havre (France) d'avoir accepté de faire partie de mon jury de thèse comme membre externe malgré un horaire très chargé. Merci Jean-Michel!

Je tiens également à remercier Patricia McKenna et Garth Arsenault pour leur aide précieuse au laboratoire ainsi qu'à la salle des bassins de l'Atlantic Veterinary College de l'Université de l'Île-du-Prince-Édouard. Merci Patty pour ton aide dans l'analyse des échantillons en cytométrie en flux et merci Garth pour l'installation des bassins et de tout mon matériel ce fut très apprécié!

Je remercie Allan Anderson, Lise Chevarie, Iften Redjah et Jean-François Mallette pour leur aide dans la collecte des myes ainsi que Laurie Gauthier et Nathalie Côté pour leur aide dans les analyses chimiques.

Ce travail a été subventionné par le Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG), les Chaires de recherche du Canada (CRC), l'Agence de promotion économique du Canada Atlantique (APECA), la Fondation canadienne pour l'innovation (FCI) et le Réseau Aquaculture Québec (RAQ).

Je remercie ma famille du plus profond de mon cœur pour son soutien moral et financier. C'est grâce à votre aide précieuse que cette thèse a pu être complétée. Merci à mes parents Gilles et Suzanne, mes frères Olivier et Jean-Philippe et mon copain Phasouk que j'aime tant!!

Enfin, je remercie mes amis Andréane, Annick, Annie, Benoit, Geneviève, Isabel, Joël, Marie-Christine, Simon et Yanick. Merci d'avoir toujours été là pour moi. Vous avez su me soutenir dans les moments les plus difficiles et je vous en remercie.

AVANT-PROPOS

Le corps de la thèse comprend 3 chapitres rédigés sous forme d'articles qui ont déjà été publiés dans des revues scientifiques ou qui sont destinés à l'être sous peu. Les références pour ces articles sont les suivantes:

Chapitre 1:

Pariseau, J., M. Delaporte, R. Tremblay, J.-M. Sévigny, P. McKenna, M. AboElkhair, T. J. Davidson, F.C.J. Berthe. A laboratory experiment on heamic neoplasia transmission in soft-shell clams (*Mya arenaria*). À soumettre à Marine Biology.

Chapitre 2:

Pariseau J., Saint-Louis R., Delaporte M., AboElkhair M., McKenna P., Tremblay R., Davidson T.J., Pelletier E., Berthe F.C.J. 2009. Potential link between exposure to fungicides chlorothalonil and mancozeb and haemic neoplasia development in soft-shell clam *Mya arenaria*: A laboratory experiment. Marine Pollution Bulletin, 58 (4): 503-514.

Chapitre 3:

Pariseau, J., P. McKenna, M. AboElkhair, R. Saint-Louis, É. Pelletier, T.J. Davidson, R. Tremblay, F.C.J. Berthe & A. Siah. 2011. Effects of pesticide compounds (chlorothalonil and mancozeb) and benzo [*a*] pyrene mixture on aryl hydrocarbon

receptor, p53 and ubiquitin gene expression levels in haemocytes of soft-shell clams (*Mya arenaria)*. Ecotoxicology, 20 (8): 1765-1772.

RÉSUMÉ

L'origine et le mécanisme de développement de la néoplasie hémique sont encore inconnus, mais plusieurs facteurs ont été suggérés tels que la pollution par des substances anthropiques, la présence d'un agent pathogène et l'influence de la génétique. L'objectif général de cette étude est de contribuer à comprendre le développement de la néoplasie hémique chez la mye commune *Mya arenaria*. Tout d'abord, il semble y avoir une transmission entre les individus atteints et ceux qui sont en bonne santé. Le but du chapitre 1 est de déterminer si, en mésocosme, la néoplasie hémique est une maladie transmissible entre des myes malades provenant de North River (Île-du-Prince-Édouard, Canada) et des myes saines provenant de différentes populations de l'Est du Canada: Anse Saint-Étienne (QC), Baie de Métis (QC), Havre-aux-Maisons (QC), Parc National de Kouchibouguac (NB) et Barasway Bay (NF). L'hypothèse de base pour tenter d'expliquer une possible transmission entre les individus est l'implication d'un agent infectieux. Les résultats obtenus au cours de cette expérience montrent qu'après une cohabitation de 62 jours, aucune des myes saines n'avait développé la maladie. La présence potentielle d'un agent infectieux seul ne semble donc pas expliquer le développement de la néoplasie hémique. Cependant son action pourrait être amplifiée par d'autres facteurs tels que la pollution par des substances anthropiques toxiques. Le but du chapitre 2 est de déterminer si une exposition chronique des myes en laboratoire aux deux fongicides (chlorothalonil et mancozèbe) les plus utilisés par les producteurs de pommes de terre de l'Île-du-Prince-Édouard peut induire la néoplasie hémique chez des myes négatives provenant d'un environnement positif. Pour cette étude, la formulation commerciale des fongicides a été utilisée. L'exposition des myes négatives de North River au fongicide Bravo 500® n'a pas induit un pourcentage élevé de cellules tétraploïdes et les analyses de la glande digestive et du manteau n'ont pas révélé de niveau détectable de chlorothalonil. Pour le Manzate 200 DF®, quelques myes ont révélé un niveau élevé de cellules tétraploïdes mais aucune différence n'a été observée entre les myes traitées et les myes contrôles. L'analyse du manganèse dans la glande digestive et le manteau n'a pas révélé de différence significative. Il n'y a donc pas de néoplasie hémique qui a pu être induite par la présence de fongicides même si une forte association entre la présence de néoplasie hémique et la contamination par des substances anthropiques est faite dans la littérature. À cette étape du travail, nous avons décidé d'aborder l'étude de la néoplasie hémique en utilisant non plus la tétraploïdie comme indicateur de la néoplasie, mais plutôt l'expression de certains gènes susceptibles de réagir à une contamination. Nous avons donc tenté, au chapitre 3, de déterminer l'effet de l'exposition de la mye commune à un mélange de benzo[*a*]pyrène, chlorothalonil et mancozèbe sur le niveau d'expression des gènes codant pour la p53, l'ubiquitine et le récepteur AhR. L'hypothèse de notre étude était qu'un mélange de composés cancérigènes

de l'environnement (pesticides et HAP) induirait l'expression des gènes impliqués dans le cycle cellulaire des hémocytes de *M. arenaria*. Les résultats de notre étude ont mis en évidence une augmentation significative de l'expression du gène AhR après 72 heures d'exposition et une diminution significative de celle du gène codant pour la p53 après trois jours d'exposition. De plus, le profil d'expression du gène codant pour l'ubiquitine était similaire à celui du gène codant pour la p53 sans toutefois présenter de différences significatives entre les temps d'expositions. Les résultats obtenus au cours de cette thèse viennent conforter l'hypothèse d'une étiologie multifactorielle. La présence possible d'un agent infectieux seul ainsi que la présence seule de contamination ne semblent pas expliquer le développement de la néoplasie hémique chez la mye. Si la tétraploidïe reste une manifestation de la maladie sur laquelle il est possible de s'appuyer pour définir le statut d'une population vis-à-vis de la néoplasie hémique, notre travail a montré que cet indicateur devient moins efficace dans le modèle expérimental que nous avons utilisé. La tétraploidïe signale une transformation cellulaire qui précède une cascade d'évènements moléculaires; en tant que tel, son intérêt dans le modèle expérimental est limité. Dans cette perspective, il nous apparait qu'élucider les mécanismes moléculaires impliqués dans le développement de la néoplasie hémique pourrait contribuer significativement à la compréhension de la maladie.

Mots clés : *Mya arenaria*, néoplasie hémique, cytométrie en flux, chlorothalonil, mancozèbe, benzo [*a*] pyrène, p53, récepteur aryl hydrocarbone, ubiquitine, gènes de références

ABSTRACT

The aetiology and the mechanism of haemic neoplasia are mainly unknown but many causative factors are suggested such as pollution by anthropogenic substances, pathogens and genetics. The aim of this thesis is to contribute to a better understanding of haemic neoplasia development in the soft-shell clam (*Mya arenaria*). First of all, it seems that a transmission between diseased and healthy clams is possible. The aim of the chapter 1 was to determine whether haemic neoplasia can be transmitted between haemic neoplasia-positive clams from North River (Prince Edward Island, Canada) and haemic neoplasia-negative clams from different populations from Eastern Canada: Anse Saint-Etienne (QC), Metis Bay (QC), Havre-aux-Maisons lagoon (QC), Kouchibouguac National Park (NB) and Barasway Bay (NF). The hypothesis for this experiment was the implication of a virus in haemic neoplasia transmission. The results obtained in this experiment showed that after a cohabitation of 62 days, none of the HN-negative clams developed the disease. The fact that no transmission was obtained suggests that a possible virus alone cannot explain the development of HN. However, the action of the virus could be amplified by other factors like pollution by anthropogenics substances. The aim of the chapter 2 was to determine if, under chronic exposure, two major pesticides (chlorothalonil and mancozeb) which are used in potato production could induce haemic neoplasia. For this experiment, the commercial formulation was used. Long term exposure to fungicide Bravo 500® did not induce high tetraploidy levels on negative clam from North River and the analysis of the digestive gland and the mantle did not reveal any detectable level of chlorothalonil. In the Manzate 200 DF®, some clams revealed high level of tetraploid cells but no difference were observed between the treatments and the control. The analysis of the digestive gland and the mantle for manganese did not highlight any significant difference in tissue concentration. Fungicides tested in this experiment did not induce haemic neoplasia although a strong association was made in literature. At this point, we decide to use gene expression involved in contamination instead of tetraploidy status as an early indicator of cell cycle disruption. The aim of chapter 3 was to investigate the effect of a mixture of benzo [*a*] pyrene, chlorothalonil and mancozeb on p53, ubiquitin and aryl hydrocarbon receptor gene expression levels. The hypothesis was that carcinogenic compounds (pesticides and polycyclic aromatic hydrocarbon) induced gene expression in haemocytes of *M. arenaria*. The results showed a significantly high expression of AhR after 72 h of exposure. P53 gene expression seems to be up-regulated by the mixture after 48 h, however not significantly; but the level of p53 mRNA is down-regulated by the xenobiotics between 48 and 72 h after exposure. However, there was no significant difference for ubiquitin gene expression level during exposure, while it followed the p53 gene expression pattern. The results obtained from this thesis support the multifactorial aetiology hypothesis. A potential

virus alone and pollution alone cannot explain the development of haemic neoplasia in soft shell clam. If tetraploidy remains effective to define haemic neoplasia population status, our work showed that this indicator becomes less effective in the experimental model that we used. Tetraploidy shows cellular transformation, which precedes molecular events; as a consequence, its interest in the experimental model is limited. In this perspective, it seems that elucidation of the molecular mechanisms involved in haemic neoplasia development could contribute significantly to the disease understanding.

Keywords : *Mya arenaria*, haemic neoplasia, flow cytometry, chlorothalonil, mancozeb, benzo[*a*]pyrene, p53, aryl hydrocarbon receptor, ubiquitin, housekeeping genes

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ABRÉVIATIONS, DES SIGLES ET DES ACRONYMES

2N diploïde.

2,4,5 T acide 2,4,5-trichlorophénoxyacétique.

4N tétraploïdie.

ADN acide désoxyribonucléique.

AhR récepteur aryl hydrocarbone.

ARN acide ribonucléique.

AhR Receptor aryl hydrocarbon.

BaP benzo[*a*]pyrene.

bHLH basic helix-loop-helix.

BPC composés biphényles polychlorés.

CL_{50} concentration léthale 50.

DMSO diméthylsulfoxyde.

DN disseminated neoplasia.

EPA US Environmental Protection Agency.

EST expressed sequence tags.

FCM flow cytometry method.

FWALG Freshwater Aquatic Life Guidelines.

GC-MS gaz chromatography-mass spectrometry.

HAP hydrocarbure aromatique polycyclique.

HN haemic neoplasia.

Hsp70 heat shock proteins.

ICP-MS inductively coupled plasma mass spectrometry.

IPE Île-du-Prince-Édouard.

MPO-IML Ministère des Pêches et Océans-Institut Maurice Lamontagne.

PBS phosphate buffer saline.

PERT product enhances reverse transcriptase.

PI propidium iodide.

psu practical salinity unit.

Q RT-PCR quantitative real time polymerase chain reaction.

TCDD 2,3,7,8 tetrachlorodibenzo-p-dioxine.

UQAR-ISMER Université du Québec à Rimouski-Institut des Sciences de la mer.

Xb xénobiotique.

INTRODUCTION GÉNÉRALE

CONTEXTE DE L'ÉTUDE

MYA ARENARIA

La mye commune (*Mya arenaria*) est un bivalve fouisseur suspensivore et microphage appartenant à l'embranchement des Mollusques, l'ordre des Eulamellibranches et la famille des Myidés (Ruppert & Barnes, 1994). Cette espèce endobenthique fréquente les substrats meubles des battures sablo-vaseuses de la zone intertidale des estuaires des côtes de l'hémisphère nord de Cape Hatteras, en Caroline du Nord, jusqu'au Labrador (Hanks, 1963; Roseberry, 1988; MPO 1999). *Mya arenaria* est une des espèces de la communauté boréo-atlantique à *Macoma balthica* décrite pour différentes régions de l'estuaire et du golfe du Saint-Laurent (Desrosiers & Brêthes, 1984). La mye est également présente sur les côtes du nord de la Norvège à la Baie de Biscay en France (Hanks, 1963). Elle a été introduite dans le Pacifique et sa distribution s'étend de la Californie à l'Alaska avec quelques populations dans le sud des îles japonaises (Hanks, 1963; Newell, 1991). Les populations ont une distribution spatiale régie par le comportement grégaire des larves, la pression de prédation et les facteurs hydrodynamiques du milieu (Matthiessen, 1960; Emerson, 1990; Newell, 1991).

La mye commune est une espèce eurytherme et euryhaline. En effet, les conditions idéales sont : 25 à 35 psu pour la salinité et une température entre 6 et 20°C (MPO, 1999). Elle se nourrit de phytoplancton et zooplancton en suspension et peut filtrer jusqu'à 2 litres d'eau à l'heure à des températures d'environ 10°C (Riisgård & Seerup, 2003). La croissance de la mye est influencée par la température de l'eau, la disponibilité de nourriture et la consommation d'oxygène (Lewis & Cerrato, 1997). Sur la côte est canadienne, la croissance des juvéniles est évaluée entre 8 et 15 mm par année (MPO, 1999).

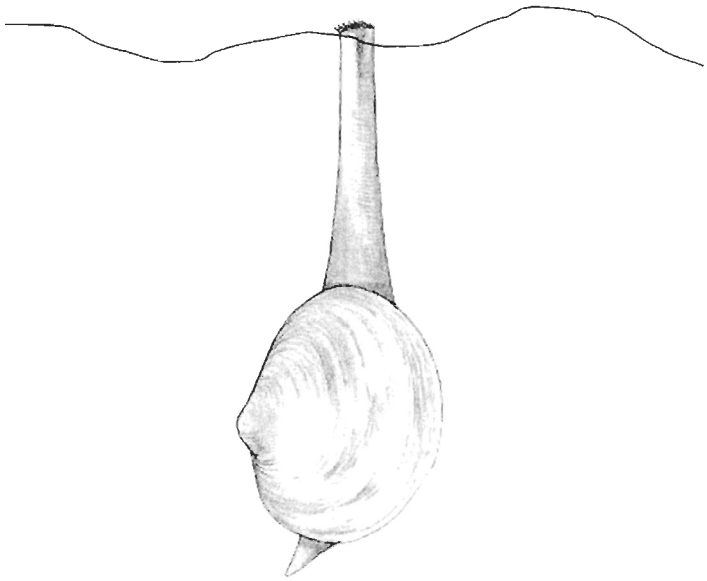


Figure 1. *Mya arenaria* (Mollusque : Bivalve)

La période de reproduction a lieu généralement entre mai et juin avec la possibilité d'une deuxième période de reproduction à l'automne (Roseberry *et al.*, 1991; Etchian *et al.*, 2004). La reproduction chez les bivalves est influencée par des facteurs environnementaux comme la température et la disponibilité de nourriture (Stickney, 1964; MPO, 1999). La mye peut atteindre la maturité sexuelle entre l'âge de 4 mois et 2 ans (Coe & Turner, 1938). La maturation des gamètes est complète en juin et le déclenchement de la ponte se fait généralement par un stimulus tel que la hausse de la température (Stickney, 1963). La fécondation est externe (Stickney, 1963) et les larves se déplacent dans la colonne d'eau de 2 à 4 semaines avant de se métamorphoser en myes juvéniles pour se fixer sur le fond et ultimement s'enfouir dans le sédiment (Newell & Hidu, 1986).

EXPLOITATION DE LA MYE

EXPLOITATION PAR LA PÊCHE

Sur le continent américain, l'exploitation des espèces bivalves fouisseurs est dominée par la palourde américaine (*Mercenaria mercenaria)* sur la côte est américaine (Malouf &

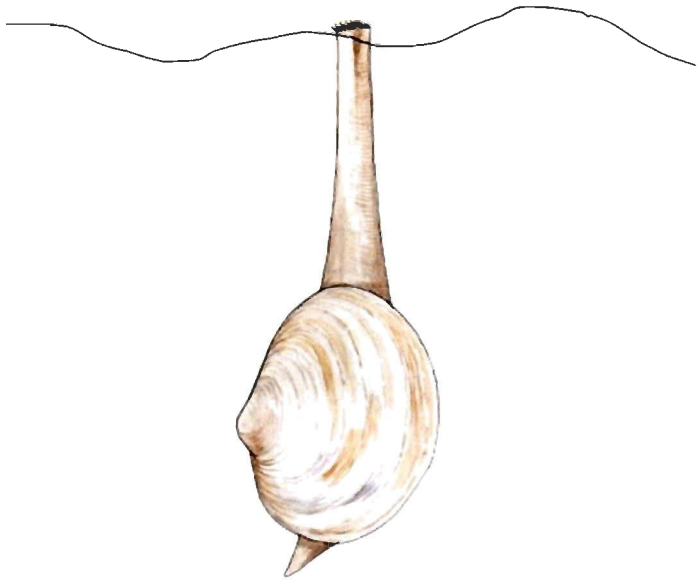


Figure 1. *Mya arenaria* (Mollusque : Bivalve)

La période de reproduction a lieu généralement entre mai et juin avec la possibilité d'une deuxième période de reproduction à l'automne (Roseberry *et al.*, 1991; Etchian *et al.*, 2004). La reproduction chez les bivalves est influencée par des facteurs environnementaux comme la température et la disponibilité de nourriture (Stickney, 1964; MPO, 1999). La mye peut atteindre la maturité sexuelle entre l'âge de 4 mois et 2 ans (Coe & Turner, 1938). La maturation des gamètes est complète en juin et le déclenchement de la ponte se fait généralement par un stimulus tel que la hausse de la température (Stickney, 1963). La fécondation est externe (Stickney, 1963) et les larves se déplacent dans la colonne d'eau de 2 à 4 semaines avant de se métamorphoser en myes juvéniles pour se fixer sur le fond et ultimement s'enfouir dans le sédiment (Newell & Hidu, 1986).

EXPLOITATION DE LA MYE

EXPLOITATION PAR LA PÊCHE

Sur le continent américain, l'exploitation des espèces bivalves fouisseurs est dominée par la palourde américaine (*Mercenaria mercenaria)* sur la côte est américaine (Malouf &

Siddall, 1985; Petrovits, 1985; Kraus, 1988; Buckner, 1988) et par la palourde japonaise (*Tapes philippinarum*) sur la côte ouest (Chevarie & Myrand, 2002). Pour la mye commune, des ensemencements ont été effectués, chaque année, depuis 1987 sur des gisements publics surtout au Maine et également au Massachusetts dans le but de favoriser son exploitation (Beal *et al.,* 1995; Parker *et al.*, 1998; Beal *et al.*, 1999; Beal & Kraus, 2002). Au Canada, la mye commune a une valeur économique importante avec des débarquements à l'Île-du-Prince-Édouard évalués à 1,4 millions de dollars en 2008 et 3,3 millions de dollars en 2009 (MPO, 2010). Le prix moyen était de 1,50 $ le kg en 2006 et 1,45 $ le kg en 2007 (MPO, 2008).

Exploitation par l'aquaculture

La myiculture peut devenir une activité économique très importante pour plusieurs petites localités de l'est des États-Unis et du Canada (Evans *et al.*, 2002). Le développement de cet élevage est favorisé par le déclin des populations sauvages causé par la surexploitation et la dégradation de l'habitat (Evans *et al.,* 2002). Cette espèce a également une importance écologique, car elle niche dans la zone intertidale, un endroit d'alimentation pour plusieurs espèces d'oiseaux, de poissons et de crabes (Newell & Hidu, 1986). C'est une espèce sentinelle utilisée régulièrement dans les programmes de biosurveillance (Blaise *et al.*, 2002). Au Canada, cette culture fait l'objet de travaux dans les provinces du Québec, du Nouveau-Brunswick et de l'Île-du-Prince-Édouard (Provencher, 2003).

Actuellement, la culture de la mye soulève de l'intérêt au Canada en tant qu'espèce à l'essai en vue d'une production commerciale à grande échelle (MPO, 2005). Depuis 1998, le centre maricole des Îles-de-la-Madeleine (CeMIM) et l'entreprise «Élevage de myes PGS Noël inc.» collaborent au développement de la myiculture aux Îles-de-la-Madeleine (Bourque *et al.*, 2001; Chevarie *et al.*, 2009, Chevarie *et al.*, en préparation). Dans le cadre du programme de recherche-développement en myiculture aux Îles-de-la-Madeleine, des travaux ont été réalisés sur la caractérisation des sites et des myes, l'approvisionnement en myes juvéniles, l'entreposage et le prégrossissement des myes avant l'ensemencement, les

ensemencements, la production et la rentabilité économique (Chevarie *et al.*, 2009; Chevarie *et al.*, en préparation).

En juillet 1999, des épisodes de mortalité massive de myes ont été notés sur une ferme expérimentale de Darnley Basin à l'Île-du-Prince-Édouard (McGladdery *et al.*, 2001a). L'analyse des tissus a révélé que plus de 95% des individus étaient atteints de néoplasie hémique (McGladdery *et al.*, 2001a). L'étude de McGladdery *et al.* (2001a) est le premier cas documenté de mortalité massive de mye commune associée à la néoplasie hémique au Canada Atlantique.

NÉOPLASIE HÉMIQUE

DESCRIPTION DE LA MALADIE

Distribution de la néoplasie

Les néoplasies constituent une catégorie de maladies invasives et progressives qui affectent plus de 15 espèces de bivalves (Barber, 2004). Les néoplasies ont été observées chez des espèces de bivalves épibenthiques, telles que la moule (*Mytilus edulis*) (Farley, 1989; Kent *et al.*, 1989; Krishnakumar *et al.*, 1999) et endobenthiques, comme la mye (Brousseau, 1987; Brousseau & Baglivo, 1991 a et b; McGladdery, 2001a), dans plusieurs types d'habitat (intertidaux et subtidaux) autant sur les côtes des océans Pacifique qu'Atlantique. Ces maladies ont été généralement documentées dans les régions tempérées, plus particulièrement en Amérique du Nord sur les côtes Atlantique et Pacifique, en Europe sur les côtes Atlantique, Baltique et Adriatique (Méditerranée). Quelques cas ont été rapportés dans le golfe du Mexique, en Australie et en Amérique du Sud (Lansdberg, 1996). La capacité de recherche plus réduite des pays du sud pourrait expliquer qu'il y ait moins de publication sur cette maladie dans ces régions.

Différents types de néoplasies

Les premiers cas documentés de néoplasie l'ont été sur l'huître américaine (*Crassostrea virginica*), l'huître japonaise (*C. gigas*) et la moule bleue (*M. edulis*) dans les années 1960 (Farley, 1969 a et b). Il y a deux types prédominants de néoplasies qui peuvent affecter les bivalves : la néoplasie gonadale et la néoplasie disséminée ou dite hémique. La néoplasie gonadale est une maladie qui apparaît dans l'épithélium germinal et qui a une origine gonadique (Brousseau, 1987). Cette maladie a un impact négatif sur la reproduction en réduisant la fécondité (Alonso *et al.*, 2001). La néoplasie disséminée est caractérisée par la présence de cellules tumorales en circulation dans l'hémolymphe. Cette néoplasie est similaire à certaines néoplasies des vertébrés (Leavitt *et al.*, 1990; Strandberg *et al.*, 1999; Walker *et al.*, 2006). Les cellules tumorales se divisent rapidement pour, ultimement, infiltrer les tissus et, à des stades avancés, tuer l'hôte (Landsberg, 1996). Il y aurait possiblement engorgement des tissus ce qui diminuerait leur fonctionnalité et permettrait l'apparition d'infections secondaires (Barber, 2004). La mort des organismes peut également résulter de la perte des hémocytes qui jouent un rôle primordial dans la défense immunitaire chez les bivalves via la phagocytose ou l'encapsulation (Sinderman, 1990).

La néoplasie disséminée implique la prolifération de cellules d'origine inconnue. Chez la mye commune, cette condition est appelée néoplasie hémique car il semble que les cellules néoplasiques proviennent des hémocytes (Couch & Harshbarger, 1985; Brousseau, 1987, Smolowitz *et al.*, 1989; McGladdery *et al.*, 2001a; Bower, 2004; Smolowitz, 2005). En fait, le tissu d'origine des cellules néoplasiques est pour le moment inconnu. Selon Farley (1969a), Rasmussen (1986) et Ford & Tripp (1996), les cellules néoplasiques seraient d'origine gonadique chez l'huître américaine (*C. virginica*). Cette affirmation est basée sur l'observation de la présence de cellules néoplasiques affichant un haut niveau mitotique près des gonades. Par contre, plusieurs études privilégient l'origine hémique. Smolowitz *et al.* (1989) ont trouvé qu'un anticorps produit contre les cellules néoplasiques de *M. arenaria* réagissait positivement non seulement avec les cellules néoplasiques, mais aussi avec les hémocytes normaux et les tissus conjonctifs. Selon Smolowitz *et al.* (1989), ces résultats suggèrent que les hémocytes normaux et les cellules néoplasiques ont des

antigènes communs et proviennent des tissus conjonctifs. Noël *et al.* (1991) ont obtenu des résultats similaires avec la moule bleue (*M. edulis*). En effet, un anticorps produit contre les cellules néoplasiques a réagi positivement avec les cellules néoplasiques et les hémocytes normaux. Ceci pourrait donc indiquer que les hémocytes normaux et les cellules néoplasiques sont ontogénétiquement reliés dans le cas de la néoplasie disséminée.

Prévalence et variabilité

Selon certains auteurs (Farley *et al.*, 1986; Brousseau, 1987), la néoplasie hémique présente sur la côte est des États-Unis aurait un cycle saisonnier avec un maximum de prévalence à la fin de l'automne et au début de l'hiver et un minimum à la fin du printemps et au début de l'été. Selon d'autres auteurs, la prévalence de cette maladie serait plutôt diphasique avec des maximums en décembre et avril et des minimums en février et durant l'été (Cooper *et al.*, 1982; Leavitt *et al.*, 1990). L'utilisation de différents critères pour établir les stades de la maladie et les différents tests pour le diagnostic serait possiblement en cause dans l'interprétation du cycle saisonnier (Brousseau & Baglivo, 1991b). Selon Barber (2004), l'augmentation de l'intensité de la néoplasie hémique entraînerait automatiquement une forte mortalité ce qui diminuerait, par conséquent, la prévalence de la maladie. On a émis l'hypothèse que les variations saisonnières seraient corrélées avec les périodes de reproduction (Elston *et al.*, 1992). Pour le moment, il est impossible de préciser si le cycle saisonnier de cette maladie le long de la côte est des États-Unis est monophasique ou diphasique. Cependant, les pourcentages élevés de prévalence de la maladie se retrouvent durant les mois d'hiver tandis que les bas pourcentages se retrouvent durant les mois d'été (Brousseau, 1987; Barber, 1990; Brousseau & Baglivo, 1991b). Pour ce qui est du Canada Atlantique, des myes atteintes de néoplasie hémique ont été trouvées en juillet à l'Île-du-Prince-Édouard (McGladdery *et al.*, 2001b) et au mois d'août en Nouvelle-Écosse et au Nouveau-Brunswick (Morrison *et al.*, 1993). Il semblerait que l'eau froide de cette région peut amener un délai dans la progression de la maladie (Morrison *et al.*, 1993). Il a été suggéré que les basses températures pourraient ralentir le développement et la progression de la maladie (Appeldoorn *et al.*, 1980; Leavitt *et al.*, 1990).

La néoplasie hémique atteint des niveaux épizootiques dans les populations de mye commune (*M. arenaria*) aux États-Unis et à l'Île-du-Prince-Édouard, coque commune (*Cerastoderma edule*) en Irlande, Espagne et en France et de moule bleue (*M. trossulus*) aux États-Unis tandis que les populations d'huîtres *C. virginica* aux États-Unis, *Ostrea edulis* en Espagne et de moule *M. edulis* aux États-Unis sont plus faiblement atteintes (Barber, 2004).

Taux de mortalité

La diminution du nombre d'individus portant la maladie durant certaines périodes de l'année pourrait être causée par la mort des organismes sévèrement atteints et/ou par la rémission des individus faiblement atteints (Barber, 1990). Le pourcentage de myes atteintes de néoplasie hémique diminue au cours de l'année, mais la maladie ne disparaît jamais. La néoplasie hémique, qui franchit un stade sévère, devient une maladie qui conduit à la mort de l'individu dans la majorité des cas. Selon Barber (1990), lorsque le pourcentage de cellules néoplasiques atteint 23%, la mort de l'individu est quasi certaine. La néoplasie hémique peut également être une maladie chronique. La maladie peut toutefois être moins intense chez certains individus et, dans ce cas, des rémissions peuvent survenir (Cooper *et al.*, 1982; Leavitt *et al.*, 1990; Barber, 2004). Lorsqu'il y a rémission, trois processus pour l'élimination du néoplasme ont été avancés : (1) la production d'une matrice extracellulaire qui serait apparemment sécrétée par les hémocytes pour envelopper les cellules néoplasiques, (2) la phagocytose et (3) la production d'une cytotoxine hémolytique (Brousseau & Baglivo, 1991a). Des études en laboratoire ont montré que le taux de mortalité des bivalves atteint de néoplasie hémique sévère pouvait atteindre 100% en 5-6 mois (Brousseau, 1987; Brousseau & Baglivo, 1991b; Reno *et al.*, 1994).

HÉMOCYTES ET CELLULES NÉOPLASIQUES

Les bivalves ont un système circulatoire ouvert avec un cœur tubulaire qui pompe l'hémolymphe vers les vaisseaux et les organes (Canesi *et al.*, 2002). Les myes saines

possèdent deux types majeurs d'hémocytes : granulaires et agranulaires (hyalinocytes) responsables de l'absorption de nutriments, du traitement des déchets, du transport d'oxygène, de l'osmose et de la défense contre les pathogènes (Huffman & Tripp, 1982; Canesi *et al.*, 2002). Ces cellules sont allongées, possèdent un, parfois deux noyaux et leur rapport noyau/cytoplasme est faible (Hine, 1999) (Figure 2A). La classification des hémocytes chez les bivalves est difficile à effectuer, car le site d'hématopoïèse et les différentes étapes de maturation cellulaire sont inconnus. Par exemple, Mix (1976) propose qu'il n'y ait qu'un seul type cellulaire et que les hyalinocytes deviendront des cellules granulocytes à maturité. Cependant, Cheng (1981) suggère que les granulocytes et agranulocytes sont deux types cellulaires distincts avec les rhodocytes comme troisième type cellulaire. Récemment, Synard (2007) a distingué cinq types d'hémocytes : deux types de cellules granulocytes et trois types de cellules agranulocytes. Ces types cellulaires ont été classés selon le diamètre du noyau, le diamètre de la cellule, le ratio noyau/cytoplasme, la présence ou l'absence de granules et l'affinité avec la coloration cellulaire. Ces types cellulaires pourraient représenter différents stades de développement (Synard, 2007).

Les myes atteintes de néoplasie hémique sont caractérisées par une augmentation de cellules dites «néoplasiques» qui sont des cellules morphologiquement altérées qui circulent dans l'hémolymphe. Ces cellules d'une possible origine hémocytique sont hypertrophiées (avec un diamètre 2 à 4 fois plus grand que le diamètre des hémocytes), ont un ou plusieurs noyaux, ont peu ou pas de pseudopodes, un ratio noyau/cytoplasme plus élevé que la normale et un taux de mitose élevé (Mix, 1976; Peters, 1988; Barber, 1990; Moore *et al.*, 1992; Morrison *et al.*, 1993; Barber, 2004) (Figure 2B). Le matériel génétique des cellules néoplasiques est altéré avec un niveau d'ADN distinct tétraploïde (4N) (Reno *et al.*, 1994; Moore, 2003; Synard, 2007; Delaporte *et al.*, 2008) et un plus grand nombre de chromosomes (44-80 comparés à 26-39 pour les cellules normales) (Reno *et al.*, 1994; Barber, 2004). Une variation du statut de ploïdie en présence de néoplasie hémique a également été observée chez d'autres espèces de bivalves. Chez la moule bleue (*M. edulis*) atteinte de néoplasie hémique, des cellules néoplasiques tétraploïdes et pentaploïdes avec de 2,03 à 2,64 fois plus d'ADN que les hémocytes normaux ont été observées (Elston *et al.*,

1990). Chez la coque commune (*Cerastoderma edule*), de l'hypodiploïdie (absence d'un chromosome du nombre normal diploïde) et de l'hyperdiploïdie (présence d'un ou plusieurs chromosomes en sus du nombre normal diploïde) des cellules néoplasiques ont été démontrées (da Silva *et al.*, 2005). Ces cellules néoplasiques présentent approximativement 10% de chromosomes acrocentriques et télocentriques (centromère près d'une extrémité) comparativement à une majorité de chromosomes métacentriques pour les individus en bonne santé (Reno *et al.*, 1994). La présence de chromosomes acrocentriques et télocentriques pourrait indiquer une cassure au niveau chromosomal près du centromère (Reno *et al.*, 1994). Ces cellules hémiques néoplasiques ont perdu leurs fonctions de phagocytose, d'adhésion et de neutralisation des particules présentes dans l'hémolymphe (Kent *et al.*, 1989; Leavitt *et al.*, 1990; Beckmann *et al.*, 1992; Dugan *et al.*, 2002). Ceci pourrait être le résultat d'une perturbation au niveau du cytosquelette due à une diminution de l'actine dans les cellules néoplasiques (Moore *et al.*, 1992).

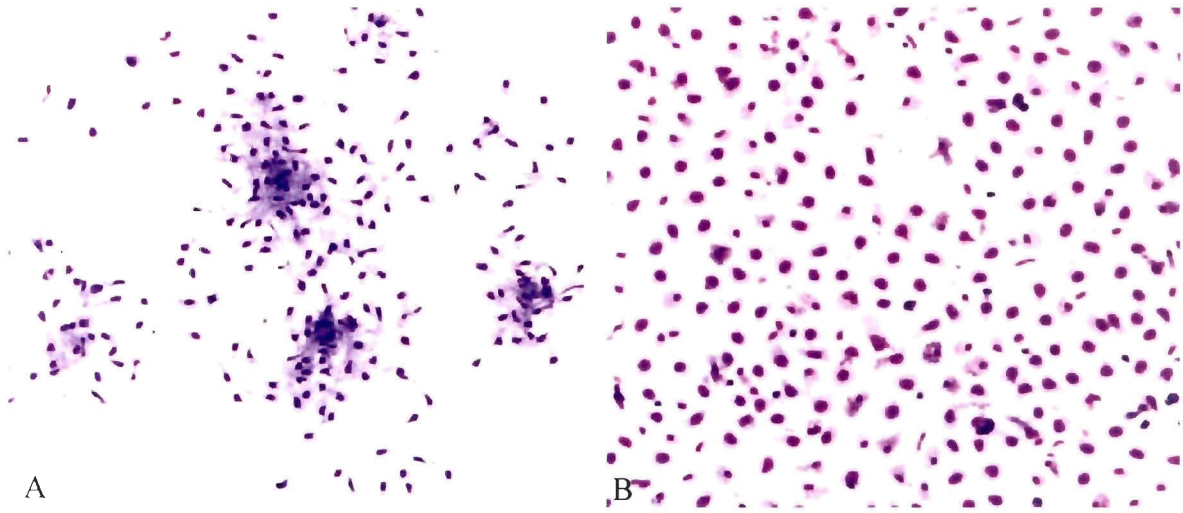


Figure 2. A) Hémocytes normaux. B) Cellules néoplasiques (Synard, 2007)

Il y a deux types de cellules hémiques néoplasiques chez *M. arenaria* (Brown *et al.*, 1971; Yevich & Barszcz, 1976; Farley *et al.*, 1986; Leavitt *et al.*, 1990; Elston *et al.*, 1992; Strandberg *et al.*, 1999; Synard, 2007) : les cellules de types A sont grandes, anaplasiques, ont peu de cytoplasme, plusieurs noyaux et un taux de mitose élevé (Figure 3A) alors que

1990). Chez la coque commune (*Cerastoderma edule*), de l'hypodiploïdie (absence d'un chromosome du nombre normal diploïde) et de l'hyperdiploïdie (présence d'un ou plusieurs chromosomes en sus du nombre normal diploïde) des cellules néoplasiques ont été démontrées (da Silva *et al.*, 2005). Ces cellules néoplasiques présentent approximativement 10% de chromosomes acrocentriques et télocentriques (centromère près d'une extrémité) comparativement à une majorité de chromosomes métacentriques pour les individus en bonne santé (Reno *et al.*, 1994). La présence de chromosomes acrocentriques et télocentriques pourrait indiquer une cassure au niveau chromosomal près du centromère (Reno *et al.*, 1994). Ces cellules hémiques néoplasiques ont perdu leurs fonctions de phagocytose, d'adhésion et de neutralisation des particules présentes dans l'hémolymphe (Kent *et al.*, 1989; Leavitt *et al.*, 1990; Beckmann *et al.*, 1992; Dugan *et al.*, 2002). Ceci pourrait être le résultat d'une perturbation au niveau du cytosquelette due à une diminution de l'actine dans les cellules néoplasiques (Moore *et al.*, 1992).

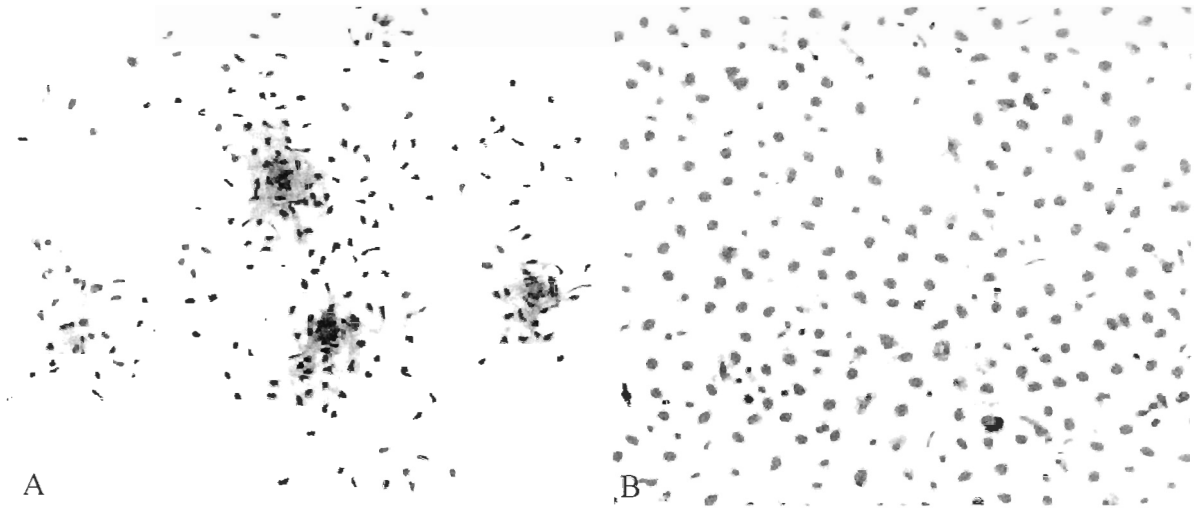


Figure 2. A) Hémocytes normaux. B) Cellules néoplasiques (Synard, 2007)

Il y a deux types de cellules hémiques néoplasiques chez *M. arenaria* (Brown *et al.*, 1971; Yevich & Barszcz, 1976; Farley *et al.*, 1986; Leavitt *et al.*, 1990; Elston *et al.*, 1992; Strandberg *et al.*, 1999; Synard, 2007) : les cellules de types A sont grandes, anaplasiques, ont peu de cytoplasme, plusieurs noyaux et un taux de mitose élevé (Figure 3A) alors que

celles de type B sont rondes, un peu plus petites avec un seul noyau (Brown *et al.*, 1971) (Figure 3B). Des observations similaires ont été décrites chez l'huître américaine *C. virginica*, l'huître Olympia *Ostrea lurida* (Farley & Sparks, 1970) et la moule bleue *M. edulis* (Lowe & Moore, 1978; Green & Alderman, 1983).

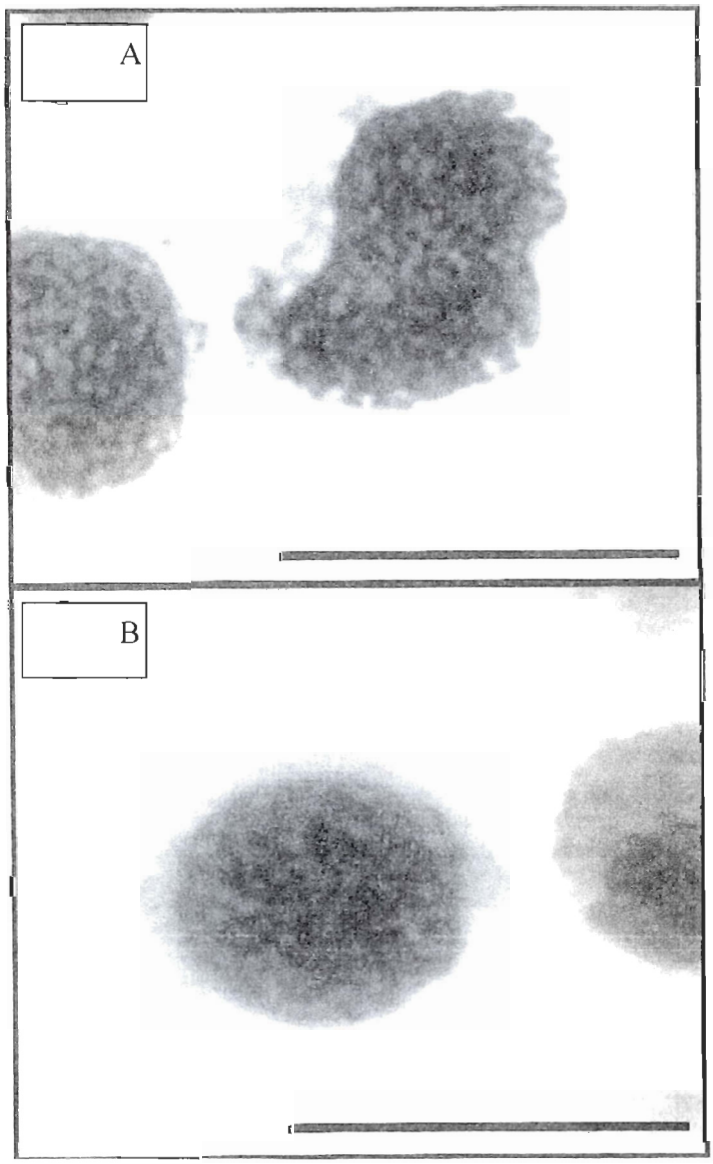


Figure 3. Cellules néoplasiques de *Mya arenaria*.Coloration modifié de Wright Gemsa. A) Cellules néoplasiques de type A. B) Cellules

celles de type B sont rondes, un peu plus petites avec un seul noyau (Brown *et al.*, 1971) (Figure 3B). Des observations similaires ont été décrites chez l'huître américaine *C. virginica*, l'huître Olympia *Ostrea lurida* (Farley & Sparks, 1970) et la moule bleue *M. edulis* (Lowe & Moore, 1978; Green & Alderman, 1983).

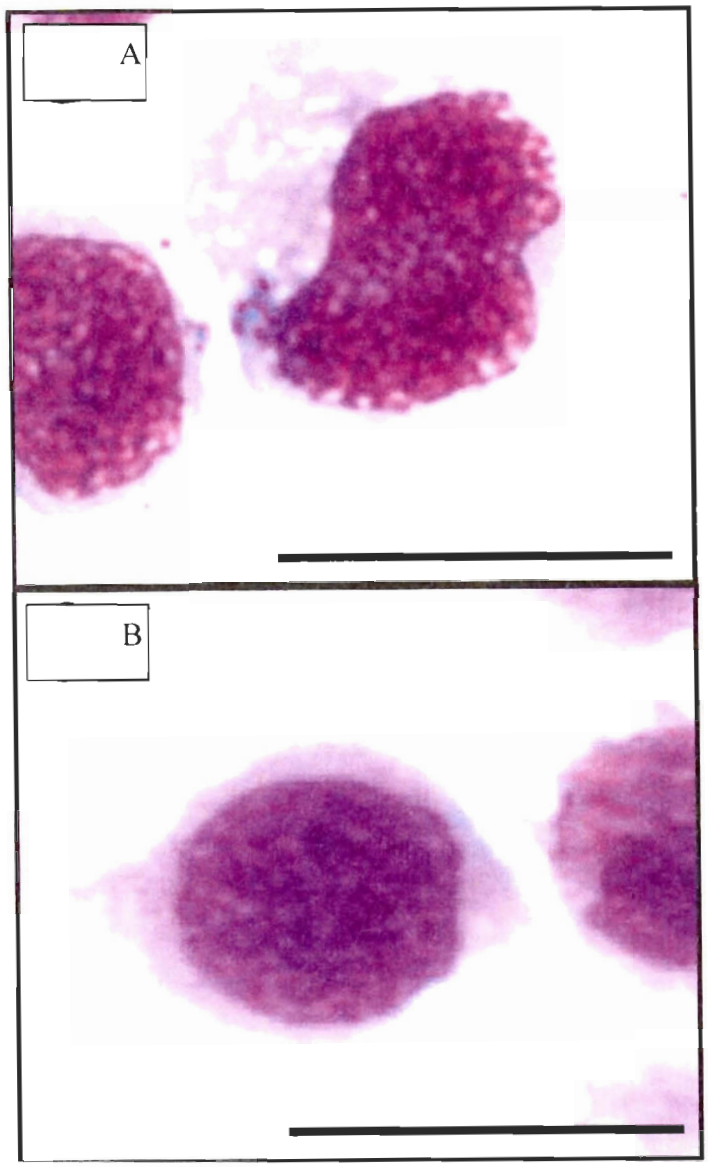


Figure 3. Cellules néoplasiques de *Mya arenaria*.Coloration modifié de Wright Gemsa. A) Cellules néoplasiques de type A. B) Cellules

néoplasiques de type B. Échelle : 20 µm (Synard, 2007)

Durant la progression de la maladie, le nombre de cellules néoplasiques augmente et le nombre d'hémocytes normaux, et plus spécifiquement celui des hyalinocytes, diminue (Peters, 1988; Elston *et al.*, 1992). Cooper *et al.*, (1982) ont établi cinq niveaux de sévérité de la néoplasie hémique chez *M. arenaria* basés sur le nombre de cellules néoplasiques par ml d'hémolymphe : le niveau 1 contient $< 10^4$ cellules néoplasiques par ml et le niveau 5 $> 10^7$ cellules néoplasiques par ml. De plus, le nombre de cellules néoplasiques par millilitre était positivement corrélé avec la sévérité des lésions histopathologiques. Kent *et al.*, (1989) ont noté que les myes saines ou au début de la maladie étaient capables de nettoyer jusqu'à 90% d'une suspension bactérienne préalablement injectée dans le muscle adducteur postérieur, tandis que chez les myes affichant des stades avancés de la néoplasie hémique seulement 44 et 83% des bactéries ont pu être nettoyées. Ceci suggère une diminution de l'efficacité des mécanismes de défense cellulaire de l'organisme atteint de néoplasie hémique.

DIAGNOSTIC DE LA NÉOPLASIE HÉMIQUE

Les cellules néoplasiques peuvent être détectées de plusieurs façons. Le diagnostic de la néoplasie hémique est souvent basé sur la technique de l'hématocytologie couplée ou non avec l'analyse des tissus par histopathologie (Brousseau, 1987; Elston *et al.*, 1988; McGladdery *et al.*, 2001a; Villalba *et al.*, 2001).

HÉMATOCYTOLOGIE

L'hématocytologie consiste en l'examen de l'hémolymphe au microscope en contraste de phase ou après une coloration Geimsa ou autres colorations pour décrire les caractéristiques morphologiques des cellules (Brown *et al.*, 1977; Peters, 1988; Elston *et al.*, 1992). Une extraction de 0,1 à 0,5 ml d'hémolymphe est nécessaire dans le muscle

adducteur ou la cavité péricardiale. C'est une méthode simple, rapide qui n'entraîne pas la mort de l'animal (Farley *et al.*, 1986; Farley *et al.*, 1991). Un grand nombre d'individus peut être analysé dans une période de temps relativement courte. Un même animal peut également être analysé plusieurs fois pour étudier la progression de la maladie. L'hématocytologie est une méthode utilisée avec succès, car le ratio cellules néoplasiques et cellules normales augmente avec la progression de la maladie (Cooper *et al.*, 1982). Plusieurs critères de classification ont été suggérés pour décrire la progression de la maladie, par exemple Brousseau et Baglivo (1991b) ont classé les myes en trois groupes selon l'intensité de la maladie : les myes négatives, les myes faiblement atteintes (1-50% de cellules néoplasiques) et les myes sévèrement atteintes (>51% de cellules néoplasiques).

Histopathologie

L'histopathologie est une procédure qui consiste en un examen microscopique d'une coupe histologique après coloration. Cette technique donne une information sur la progression et la localisation de la maladie dans les différents tissus (Yevich & Barszcz, 1976; Mix, 1983). Par contre, l'animal doit être sacrifié pour le diagnostic. Récemment, Synard (2007) a défini trois groupes pour classifier les myes : 1) les myes avec moins de 20% de cellules néoplasiques, 2) les myes présentant entre 20 et 50% de cellules néoplasiques et 3) les myes avec plus de 50% de cellules néoplasiques. Dans les groupes 1 et 2, les cellules néoplasiques ont été observées majoritairement dans les branchies et la glande digestive tandis que dans le groupe 3, les cellules néoplasiques ont été identifiées dans les branchies, le manteau, la gonade, la glande digestive, les reins et le pied (Synard, 2007).

L'hématocytologie et l'histopathologie sont des techniques qui n'analysent qu'un nombre limité de cellules par lame et elles sont très coûteuses (Delaporte *et al.*, 2008). Au début des années 1990, la méthode de cytométrie en flux a été établie comme outil de diagnostic pour la néoplasie hémique (Reno *et al.*, 1994; da Silva *et al.*, 2005; Delaporte *et al.*, 2008).

CYTOMÉTRIE EN FLUX

La cytométrie en flux est une méthode plus puissante et précise due à la possibilité d'analyser une grande quantité de cellules et d'obtenir des données multiparamétriques sur chacune d'elles (taille, granulométrie et intensité de la fluorescence) (Delaporte *et al.*, 2008). Cette technique permet de compter et de séparer les cellules de l'hémolymphe selon leur niveau de ploïdie soit 2N pour les hémocytes normaux et 4N pour les cellules néoplasiques (Reno *et al.*, 1994; da Silva *et al.*, 2005; Delaporte *et al.*, 2008). La cytométrie en flux permet également d'analyser les différentes phases du cycle cellulaire : G0/G1, S et G2/M, par coloration de l'ADN (Ashton-Alcox & Ford, 1998; Allam *et al.*, 2002). Reno *et al.*, (1994) ont démontré que la néoplasie hémique est caractérisée par la tétraploïdie. Des études chez *M. edulis* ont également montré que la néoplasie peut être caractérisée par la tétraploïdie (Elston *et al.*, 1990; Moore *et al.*, 1991). Différents seuils expérimentaux de cellules tétraploïdes ont été établis pour diagnostiquer la néoplasie hémique. Selon Reno *et al.*, (1994), les myes négatives peuvent avoir entre 4 et 6% de cellules tétraploïdes et les myes positives ont plus de 10% de cellules tétraploïdes. Cette technique a été utilisée pour l'évaluation du contenu en ADN pour plusieurs études des cellules néoplasiques et des hémocytes normaux chez plusieurs espèces de mollusques (Elston *et al.*, 1990; Moore *et al.*, 1991; Reno *et al.*, 1994; da Silva *et al.*, 2005; Delaporte *et al.*, 2008).

Le principal problème pour le diagnostic de la néoplasie hémique à de faibles pourcentages est la difficulté de discriminer entre la néoplasie hémique et d'autres conditions physiologiques qui auraient les mêmes caractéristiques (Elston *et al.*, 1992). En effet, le manque d'information sur le site d'hématopoïèse chez les bivalves et les stades de développement des hémocytes y sont pour beaucoup. Par exemple, des cellules qui affichent les mêmes caractéristiques morphologiques que les cellules néoplasiques, mais qui ne sont pas atteintes d'une pathologie ont pu être observées (Elston *et al.*, 1992).

Dans le cadre d'une étude utilisant différentes populations de *M. arenaria* (Delaporte *et al.*, 2008), nous suggérons un mode de diagnostic par cytométrie en flux pour définir la néoplasie hémique chez l'individu ainsi qu'au niveau de la population. Suite à cette étude, des seuils expérimentaux ont été établis. Les myes affichant des pourcentages d'hémocytes

tétraploïdes (phase S-4N) de plus de 20% ont toutes été confirmées comme positives à la néoplasie par hématocytologie. Des myes avec moins de 5% de cellules tétraploïdes sont, quant à elles, considérées négatives. Ce résultat est en accord avec celui de Reno *et al.*, (1994) qui proposent que les myes négatives aient en moyenne 6% et moins de cellules en phase S-4N. En terme de population, le seuil pour que celles-ci soit considérées positives est de 15% (15% des individus doivent avoir plus de 20% de cellules tétraploïdes).

FACTEURS INFLUENÇANT LE DÉVELOPPEMENT DE LA NÉOPLASIE HÉMIQUE

Plusieurs facteurs ont été mis en cause pour tenter d'expliquer l'étiologie de cette maladie (Barber, 2004). Quelques études suggèrent l'implication d'un rétrovirus comme facteur dominant (Oprandy *et al.*, 1981; Romalde *et al.*, 2007) tandis que d'autres suggèrent que l'étiologie de cette maladie serait plutôt multifactorielle (l'implication d'un rétrovirus combinée à la présence de pesticides) (Elston *et al.*, 1992; McGladdery *et al.*, 2001a). Par contre, aucune de ces études n'a pu démontrer de résultats reproductibles pour expliquer le développement de la néoplasie hémique (Elston *et al.*, 1992).

Un des facteurs abondamment discutés dans la littérature est l'exposition à des substances anthropiques tel que les composés biphényles polychlorés (BPC), les hydrocarbures aromatiques polycycliques (HAP) et les herbicides (Brown *et al.*, 1979; Brown, 1980; Walker *et al.*, 1981; Couch & Harshbarger, 1985). Les effets de la dégradation de l'habitat et la présence de contaminants chimiques sont complexes, mais il est clair qu'ils nuisent à la santé des bivalves. Le développement des néoplasies, chez les bivalves, présente de fortes corrélations avec les environnements contaminés (Elston *et al.*, 1992; Harper *et al.*, 1994). Par exemple, les myes retrouvées dans le site de New Bedford Harbor un site pollué situé dans la région du Massachusetts surveillé par la U.S. Environmental Protection Agency (EPA), sont en grande partie atteintes de néoplasie hémique, tandis que les myes retrouvées dans le site non pollué de Buzzards Bay en sont exemptes (Leavitt *et al.*, 1990). Le site de New Bedford Harbor est contaminé par de hauts niveaux de BPC et la prévalence élevée de néoplasie hémique (22 à 90 %) a relancé la

question d'un possible lien entre la qualité de l'environnement et cette maladie (Reinisch *et al.*, 1984; Brousseau, 1987; Miosky *et al.*, 1989; Brousseau & Baglivo, 1991b, Strandberg *et al.*, 1999; McGladdery *et al.*, 2001b). Yevich et Barszcz (1977) ont examiné plusieurs sites aux États-Unis et ont trouvé des myes positives à la néoplasie hémique seulement dans des sites pollués aux hydrocarbures légers. Farley *et al.*, (1991) ont trouvé une corrélation positive entre la prévalence de néoplasie hémique et les niveaux de chlordane, un insecticide organochloré, dans les tissus de la mye commune.

Par contre, la néoplasie hémique a également été retrouvée chez des bivalves vivant dans des sites non pollués (Oprandy *et al.*, 1981; Couch & Harshbarger, 1985). Quelques études ont tenté sans succès d'établir un lien entre la prévalence de la néoplasie hémique et la contamination. En effet, il n'y a pas de lien clair qui a pu être obtenu entre la prévalence de la maladie chez la mye commune et la pollution d'un site par les hydrocarbures (Brown *et al.*, 1977; Appeldoorn *et al.*, 1984). De plus, une étude réalisée par Krishnakumar *et al.*, (1999) n'a pu trouver de lien entre la quantité de contaminants tel que les HAP, BPC et les métaux lourds ainsi que la prévalence de la néoplasie hémique chez la moule *M. trossulus*. Il semble donc que la pollution environnementale ne soit pas la cause unique et directe de la maladie, mais pourrait être un des facteurs principaux (Oprandy *et al.*, 1981). Le développement de la néoplasie hémique pourrait également être dû à un effet synergique de plusieurs contaminants (Elston *et al.*, 1992; Barber, 2004).

Des études sur la contamination et la néoplasie gonadale ont également été réalisées chez la mye commune. Ces expériences ont été menées avec l'utilisation de la dioxine TCDD (2,3,7,8 tetrachlorodibenzo-p-dioxine) un composé persistant dans l'environnement et faiblement métabolisé par les organismes (Nebert *et al.*, 2000). Le TCDD est un sous produit de la synthèse de l'acide trichlorophénoxyacétique (2,4,5 T) fréquemment utilisé comme herbicide dans les endroits où la néoplasie gonadale a été identifiée (Butler *et al.*, 2001; Butler *et al.*, 2004). Les résultats n'ont montré aucune induction de la néoplasie gonadale chez la mye commune, mais une apparition de cellules non différenciées dans la gonade (Butler *et al.*, 2004).

Un autre facteur à considérer pour le développement de la néoplasie hémique est l'influence de la génétique (Barber, 2004). Des études chez les vertébrés ont montré que l'utilisation de la dioxine TCDD induit l'expression de gènes tel que le récepteur aryl hydrocarbone (AhR) (Butler *et al.*, 2004) impliqué dans la métabolisation des xénobiotiques via le cytochrome P450 et l'ubiquitine (van Beneden, 1997; Rhodes *et al.*, 1997) utilisé dans la dégradation des protéines. De plus, l'ubiquitine se lierait avec la protéine p53 en présence de contaminants pour sa dégradation dans le protéasome (Maki *et al.*, 1996; Rhodes *et al.*, 1997). L'altération du gène p53 ou sa séquestration en région cytoplasmique pourrait contribuer au développement de la néoplasie hémique de la mye en contact avec de l'eau ou des sédiments pollués (Barker *et al.*, 1997).

Au début des années 1990, le gène codant pour la protéine p53 a été décrit comme un gène de suppression des tumeurs qui est communément muté ou altéré chez de nombreux cancers humains (Kelley *et al.*, 2001; Hofseth *et al.*, 2004; Slee *et al.*, 2004; Walker *et al.*, 2006). Ce gène serait impliqué dans la régulation du cycle cellulaire et dans l'apoptose et il faciliterait la réparation de l'ADN. Son rôle dans la promotion de la croissance et la division cellulaires est bien connu (Hofseth *et al.*, 2004). Selon Donehower *et al.*, (1992), une mutation dans le gène p53 de la souris serait susceptible de favoriser les tumeurs. Les anomalies du gène p53 seraient associées avec le développement de cellules tétraploïdes. La transition entre la diploïdie et la tétraploïdie serait associée à la surexpression du gène codant pour la protéine p53 (Watanabe *et al.*, 2004). Lorsque l'ADN subit des dommages, il doit y avoir réparation avant de poursuivre le cycle cellulaire. La cellule possède donc trois points de contrôle du cycle cellulaire utilisé par la p53 : un point à la fin de la phase G_1 (cellules 2N) qui empêche l'entrée en phase S (réplication de l'ADN), un à la fin de la phase G_2 (cellules 4N) qui empêche l'entrée en mitose et un autre au début de la mitose (Alberts *et al.*, 2004).

Deux principales hypothèses impliquant le gène p53 ont été suggérées dans la littérature pour expliquer la présence de néoplasie hémique chez la mye commune (Barker *et al.*, 1997; Walker *et al.*, 2006). Selon la première hypothèse (Barker *et al.*, 1997), une mutation du gène p53 serait liée à la présence de néoplasie hémique. Les travaux de Barker

et al., (1997) ont identifié une mutation de type transversion à la fin de l'exon 6 du gène p53 chez des myes atteintes de néoplasie hémique. Il s'agirait de la substitution d'une cytosine pour une guanine située à la première position du codon (Barker *et al.*, 1997). Par contre, Barker *et al.*, (1997) n'ont observé cette mutation que sur deux échantillons sur onze porteurs de néoplasie. Barker *et al.*, (1997) ont également trouvé une mutation au niveau de la protéine p53 par une technique d'immunofluorescence à l'aide de l'anticorps monoclonal PAb 240. Cet anticorps reconnait la p53 mutée en condition non dénaturante. Cette mutation sur la protéine se retrouverait entre les acides aminés 213 et 217 chez cinq individus sur onze identifiés comme porteurs de la maladie. Une des principales critiques à propos de cette étude est le manque de reproductibilité des résultats et le fait que la mutation du gène p53 ne correspondait pas à la mutation de la protéine p53.

La deuxième hypothèse émise par Walker *et al.*, (2006) suggère que la protéine p53 est séquestrée dans le cytoplasme par la protéine mortaline. En effet, leurs résultats démontrent que les protéines p53 et mortaline formeraient un complexe dans le cytoplasme des cellules néoplasiques. D'autres études montrent également que pour les cellules néoplasiques, les protéines p53 et p73 (protéine de la même famille que p53) seraient séquestrées dans le cytoplasme plutôt que dirigées vers le noyau comme c'est le cas pour les cellules saines (Kelley *et al.*, 2001; Walker *et al.*, 2006). La mortaline, une protéine membre de la famille des hsp70 (*heat shock proteins*), séquestrerait la protéine p53 dans le cytoplasme près du centriole ce qui empêcherait son déplacement vers le noyau et donc son action (Walker *et al.*, 2006; Böttger *et al.*, 2008). Par conséquent, les cellules néoplasiques ne seraient pas reconnues par la protéine p53 mutée ou séquestrée dans le cytoplasme et continueraient de se diviser sans qu'il y ait arrêt du cycle cellulaire ou apoptose (Reno *et al.*, 1994; Alberts *et al.*, 2004). Les altérations de la protéine p53 pourraient favoriser une croissance cellulaire incontrôlée (Barker *et al.*, 1997). Chez la mye commune en présence de néoplasie hémique, les cellules cancéreuses tétraploïdes se divisent continuellement dans l'hémolymphe (Barker *et al.*, 1997). Chez l'humain, l'inactivation de la protéine p53 arrêterait la mitose et entraînerait la formation de cellules tétraploïdes (Watanabe *et al.*, 2004). *In vitro*, l'inactivation de la protéine p53 favoriserait la formation de cellules

tétraploïdes chez des cellules humaines et des cellules de souris (Fujiwara *et al.*, 2005). De plus, une étude réalisée par Stephens *et al.*, (2001) a montré que les myes atteintes de néoplasie hémique exprimaient la protéine p73 et les myes négatives exprimaient la protéine p97 (même famille que la p53).

Plusieurs facteurs affectant le niveau d'évolution de la néoplasie hémique ont été identifiés: température de l'eau, ponte, âge et sexe des organismes. La prévalence la plus importante de cette maladie chez la mye commune semble subvenir à deux moments différents, soit lorsque la température de l'eau se situe entre 5°C et 10°C et lorsqu'il y a ponte (Cooper *et al.*, 1982). Farley *et al.*, (1991) suggèrent que la maladie touche en premier les myes adultes de grande taille (>56mm) pour s'étendre aux juvéniles par la suite. Il y aurait moins de juvéniles atteints que d'adultes (Appeldoorn *et al.*,1980; Farley *et al.*, 1991). Leavitt *et al.*, (1990) suggèrent que la fréquence de la maladie est significativement moins importante chez les myes de moins de deux ans (<30mm) et de plus de quatre ans (>70 mm) que chez les myes de trois-quatre ans (40-70 mm). Certains travaux suggèrent également que la prévalence de la néoplasie hémique serait significativement plus faible chez les femelles que chez les mâles (Brown *et al.*, 1979).

Transmission

L'origine et le mécanisme de développement de la néoplasie hémique sont encore inconnus, mais, selon certaines études, il semble y avoir une transmission entre les individus atteints et ceux qui sont en bonne santé (Oprandy *et al.*, 1981; McGladdery *et al.*, 2001b; Dungan *et al.*, 2002). Plusieurs expériences suggèrent que la néoplasie hémique peut être transmise d'un individu malade vers un individu sain via l'eau environnante. En effet, Appeldoorn *et al.*, (1984) ont indiqué qu'après six mois de cohabitation 72% des myes saines ont développé la maladie. De plus, House (1997) a mis huit groupes de myes saines en contact avec un groupe de myes positives à la néoplasie et après 14 mois d'expérience sept groupes ont affiché une transmission de la néoplasie hémique. Les travaux de McGladdery *et al.*, (2001a) ont montré que 20% des myes étaient atteintes de néoplasie hémique après une expérience de 36 jours de cohabitation. Après 93 jours de

cohabitation, les résultats obtenus par MacCallum *et al.*, (2003) ont montré que 17% des myes présentaient des cellules néoplasiques.

Des résultats similaires ont également été observés chez la moule. Elston *et al.*, (1988) ont montré une transmission via l'eau chez la moule bleue *M. edulis*. Vingt moules saines ont été placées avec 50 moules provenant d'un site positif à la néoplasie hémique et 40% des moules saines avaient développé la maladie en 231 jours. Certaines critiques peuvent être faites sur les résultats des expériences mentionnées ci-haut. Tout d'abord, les diagnostics de la néoplasie hémique ont été établis par hématocytologie ou histopathologie. Ces techniques ne permettent que l'analyse d'un nombre limité de cellules par lame et sont moins puissantes que la cytométrie en flux qui permet non seulement l'analyse d'un nombre beaucoup plus élevé de cellules, mais aussi d'obtenir des données multiparamétriques pour chacune d'elles. Les diagnostics établis par hématocytologie et histopathologie sont donc basés sur la morphologie cellulaire avec des pourcentages de classification différents, selon les auteurs, pour déterminer si une mye est négative ou positive à la néoplasie hémique. Par exemple, certains auteurs considèrent une mye positive avec plus de 0,1% de cellules néoplasiques tandis que d'autres auteurs considèrent une mye comme étant positive lorsque celle-ci a plus de 1% de cellules de type néoplasiques.

L'étude de House (1997) suggère qu'il peut y avoir transmission de la néoplasie hémique chez *M. arenaria* par ingestion de cellules néoplasiques. Au cours de cette expérience, les myes ont été mises en contact avec une suspension d'algues ($7x10^5$ d'un mélange 50/50 de *Isochrysis galabanea* et *Cheatoserous calcitrans* par ml) et deux concentrations de cellules néoplasiques (10^5 et 10^6 de cellules néoplasiques par litre), ce qui s'est traduit par l'apparition de myes néoplasiques après 5 mois. Une hypothèse suggérée pour expliquer ces résultats est que les myes pourraient relâcher des cellules néoplasiques dans l'environnement (McLaughlin 1994), et que celles-ci auraient la capacité de survivre 48 heures dans l'eau (Sunila & Farley 1989). De plus, avec un diamètre de 12 µm (Appeldoorn *et al.*, 1984), les cellules néoplasiques se situent dans la classe de taille de particules qui peuvent être filtrées et ingérées par la mye (Langdon & Siegfried, 1984). Par

contre, à notre connaissance, il n'y a pas d'autres études qui ont été réalisées pour corroborer ces résultats ce qui fait que ceux-ci doivent être considérés avec précautions.

D'autres modes de transmission ont également été étudiés pour la néoplasie hémique. Une transmission a été obtenue par injection d'hémolymphe contenant des cellules néoplasiques chez *M. arenaria* (Farley, 1987; McLaughlin, 1994; Weinberg *et al.*, 1997; House *et al.*, 1998; Walker *et al.*, 2009), chez *Mytilus* spp. (Elston *et al.*, 1988; Kent *et al.*, 1991; Moore, 2003) et *Cerastoderma edule* (Twomey & Mulcahy, 1988; Collins & Mulcah,y 2003). De plus, chez la mye commune, une transmission de la néoplasie hémique par injection d'un filtrat d'hémolymphe préparé à partir de cellules néoplasiques a pu être obtenue (Oprandy *et al.*, 1981). Collins & Mulcahy (2003) ont obtenu les mêmes résultats chez *C. edule*. Par contre pour ces études, il est impossible de déterminer s'il y a eu une réelle transmission de la maladie ou seulement une transplantation de cellule néoplasique (MacCallum *et al.*, 2003).

Plusieurs études ayant pour but de démontrer la transmission de la néoplasie hémique ont obtenu des résultats négatifs (Appeldoorn *et al.*, 1984; Kent *et al.*, 1991; MacCallum *et al.*, 2003). Le fait que certaines populations de bivalves soient plus susceptibles que d'autres au développement de la néoplasie pourrait être relié à des facteurs génétiques (Barber, 2004). Toutefois, il n'existe que peu d'information sur une possible étiologie génétique de cette maladie. Pour la néoplasie hémique, à notre connaissance il n'y a pas d'étude plus récente que celle de Frierman & Andrews (1976) sur l'huître américaine *C. virginica* qui suggère une prédisposition génétique à développer la maladie avec des prévalences de 8,4% pour les animaux de laboratoire (31/369) par rapport à 0,08 % pour les huîtres sauvages (39/51000). Les huîtres de laboratoire étaient caractérisées par un déficit en hétérozygotie mesuré sur la variabilité allélique de différentes allozymes. Les auteurs suggèrent que cette baisse en hétérozygotie augmenterait la susceptibilité des huîtres à développer la néoplasie (Frierman & Andrews, 1976). Le niveau d'hétérozygotie mesuré sur les allozymes a souvent été mis en relation avec la performance et la résistance des bivalves à différents types de stress (Zouros, 1987; Mitton, 1995; Myrand *et al.*, 2002). Pour la néoplasie gonadale, quelques études sur la palourde (*Mercenaria* spp.) suggèrent

une prédisposition génétique dans le développement de la maladie (Bert *et al.*, 1993; Eversole & Heffernan, 1995; Arnold *et al.*, 1996). Les populations de palourde de l'Indian River (Floride, États-Unis) sont situées dans une zone d'hybridation de deux espèces constituées selon ces études de 68% *M. mercenaria*, 4% *M. capechiensis* et 28% d'hybrides. La prévalence de la néoplasie gonadale diffère selon ces 3 génotypes avec une augmentation significative chez les hybrides (21,6%) par rapport à *M. mercenaria* (6,5%) et *M. capechiensis* (11,8%) (Bert *et al.*, 1993; Eversole & Heffernan, 1995; Arnold *et al.*, 1996). L'hypothèse suggérée est que le taux de mutation élevé chez les organismes hybrides pourrait être en cause dans le développement de la maladie (Barber, 2004).

Un autre facteur qui pourrait expliquer la transmission de la néoplasie hémique est la présence d'un agent infectieux non identifié dans l'eau qui serait filtrée par l'organisme (Brousseau, 1987; Farley, 1989; Barber, 2004). L'épisode de mortalité massive de mye commune observée à l'Île-du-Prince-Édouard (une population qui n'était pas affectée par la néoplasie hémique au préalable) en 1999 suggère une introduction soudaine d'un agent infectieux pathogène dans une population intacte (Farley *et al.*, 1986; McGladdery *et al.*, 2001a). Aucune étude n'a montré une transmission de la néoplasie hémique d'une espèce à une autre. Ceci indique que l'agent serait spécifique à chaque espèce (Kent *et al.*, 1991; MacCallum *et al.*, 2003).

Des données expérimentales suggèrent l'implication d'un agent viral dans le développement de la néoplasie hémique chez *M. arenaria*. Brown (1980) a rapporté la présence d'oncornavirus (déterminé par gradient de sucrose) ainsi que l'activité de l'enzyme transcriptase inverse (indicateur de retrovirus) uniquement chez les myes néoplasiques. L'enzyme transcriptase inverse joue un rôle fondamental dans la réplication des rétrovirus, par la transcription virale de l'ARN en ADN qui sera intégré dans le génome de la cellule pour la création de nouvelles particules virales (Flint *et al.*, 2004). De plus, un virus dont les caractéristiques physiques et morphologiques se rapprochant de celles d'un rétrovirus de type B a été observé par gradient de sucrose chez les myes malades ce qui suggère que la néoplasie pourrait être transmise par un rétrovirus (Appeldoorn *et al.*, 1980; Oprandy *et al.*, 1981; Cooper *et al.*, 1982; Oprandy & Chang, 1983; Morrison *et al.*, 1993;

House *et al.*, 1998; Dugan *et al.*, 2002; Romalde *et al.*, 2007). Les rétrovirus sont des virus de 80-100 nm de diamètre enveloppés dans une capside icosahédrique. Le génome est diploïde et composé de deux molécules d'ARN à simple brin (Gelderblom, 2008). Le rétrovirus a pu être isolé, injecté chez des myes saines et ré-isolé (Oprandry *et al.*, 1981) tout en observant l'activité de la transcriptase inverse. Une étude menée par Oprandy et Chang (1983) a démontré que la néoplasie hémique et la réplication virale ont été induites chez la mye commune par le 5-bromodeoxyuridine, un nucléoside synthétique analogue à la thymidine. Ce composé initie la différenciation des cellules néoplasiques (Kotzin & Baker, 1972) et est connu pour induire la réplication virale. La néoplasie hémique a été induite après quatre jours de traitement et des particules virales ont pu être observées. Ces résultats ont, par contre, été critiqués, car d'autres études ont échoué dans la détection de particules virales (Elston *et al.*, 1992; House *et al.*, 1998; Barber 2004). Deux études ont été effectuées dans le but de détecter la présence d'un rétrovirus chez les myes atteintes de néoplasie hémique. La première étude avait pour objectif de rechercher la présence d'un rétrovirus dans plusieurs tissus de myes par quantification de l'activité transcriptase inverse à l'aide de la technique PERT (*Product Enhanced Reverse Transcriptase*). Les résultats n'ont pas mis en évidence de particules rétrovirales (AboElkhair *et al.*, 2009a). La deuxième étude visait à déterminer s'il y a un lien entre les niveaux de transcriptase inverse et la tétraploïdie des myes atteintes de néoplasie hémique. Une corrélation positive entre l'activité de la transcriptase inverse et la tétraploïdie a été obtenue (AboElkhair *et al.*, 2009b). La présence de l'activité de l'enzyme transcriptase inverse sans particule rétrovirale identifiée pourrait indiquer une source endogène de cet enzyme au lieu d'une source exogène provenant d'un rétrovirus.

Romalde *et al.*, (2007) ont observé des particules qui ressemblent à un virus dans le cytoplasme de cellules néoplasiques chez la coque commune. Ce sont des particules de 120 nm de taille observées par microscopie électronique à transmission. L'activité de l'enzyme transcriptase inverse a été détectée grâce à la technique PERT. Ces résultats supportent l'hypothèse de l'étiologie virale de la néoplasie hémique. Par contre, l'image présentée dans cette étude ne montre pas plusieurs particules virales et ces particules ne démontrent

pas les caractéristiques morphologiques d'un rétrovirus (Figure 4). De plus, les rétrovirus ne sont pas les seules sources d'activité de la transcriptase inverse (House *et al.*, 1998).

Il y a donc quelques données en faveur d'une étiologie virale de la néoplasie hémique. Même si la présence d'un virus demeure un très bon candidat, la nature de l'agent pathogène n'est pas claire (Elston *et al.*, 1992; Barber, 2004). Il est donc possible qu'à part la présence d'un agent infectieux, d'autres facteurs tels que la pollution par des activités anthropiques puissent intervenir dans le développement de la néoplasie.

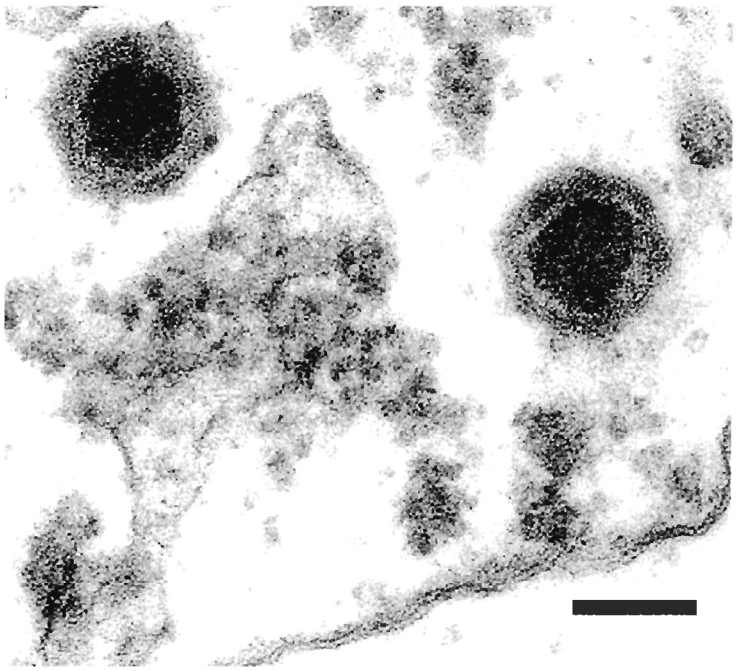


Figure 4. Particules ressemblant à des particules virales observées chez les cellules néoplasiques de *Cerastoderma edule*. Échelle : 100 nm (Romalde *et al.*, 2007)

CONTAMINATION

Certains auteurs ont suggéré que la mortalité massive de la mye commune reliée à la néoplasie hémique à la fin des années 1990 à l'Île-du-Prince-Édouard aurait été causée par l'utilisation de pesticides par les producteurs de pomme de terre. L'Île-du-Prince-Édouard est une province où les pesticides sont les plus utilisés au pays par habitant (Dunn, 2004)

avec un achat en 2008 de plus d'un demi-million de kg de pesticides (PEI, 2009). L'utilisation de pesticides est importante, car cette province abrite le plus grand groupe de producteurs de pommes de terre du Canada Atlantique (White *et al.*, 2006). La production de pommes de terre est une culture qui dépend énormément des pesticides avec environ 15 applications par année (Mutch *et al.*, 2002). En réponse à ce constat, plusieurs suivis de pesticides ont été lancés (Mutch *et al.*, 2002; White, 2004). Le suivi de Mutch *et al.*, (2002) pour la période entre 1994 et 1999 a permis de détecter 169 pesticides présents dans l'eau environnante de l'Île-du-Prince-Édouard. De tous ces pesticides, seul le chlorothalonil a été trouvé en excès (1,34 µg/L pour une valeur recommandée de 0,18 µg/L) (CCME, 1999). Entre 1994 et 2006, l'Île-du-Prince-Édouard a connu 16 évènements de mortalité de poissons qui ont été reliées aux pesticides et 26 autres causés possiblement par des pesticides (Gormley *et al.*, 2005; White, 2004). Différentes classes de pesticides ont été impliquées dans ces évènements de mortalité de poissons : l'azinphos-methyl, un insecticide organophosphoré, le chlorothalonil, un fongicide organochloré dérivé du benzène et le mancozèbe, un fongicide dithiocarbamate de manganèse et de zinc sont les principaux présumés coupables.

Dans la province de l'Île-du-Prince-Édouard, le chlorothalonil et le mancozèbe sont deux fongicides utilisés par les agriculteurs (PEI, 2009). Ces fongicides sont appliqués chaque semaine durant la saison de production de pommes de terre pour contrôler les maladies fongiques. Le chlorothalonil (tetrachloroisophtalonitrile) est un fongicide non systémique toxique pour les poissons, les invertébrés aquatiques et les organismes marins. Les valeurs de CL_{50} sont de 0,1 mg/L pour la truite arc-en-ciel (*Oncorhynchus mykiss*), 5,9 mg/L pour la moule bleue (*M. edulis*) et 35 mg/L pour la mye commune (*M. arenaria*) (Ernst *et al.*, 1991). *In vitro*, le chlorothalonil affecte la fonctionnalité des hémocytes du tunicier, *Botryllus schlosseri*, en prévenant la phagocytose (Cima *et al.*, 2008). Il supprime également la production d'oxygène des hémocytes d'huître (Baier-Anderson & Anderson, 2000). Le mancozèbe est un fongicide de la famille des éthylenedithiocarbamates avec le zinc et le manganèse comme co-métaux. Ce produit est modérément toxique pour les poissons et les organismes aquatiques avec une CL_{50} de 2,2 mg/l pour la truite arc-en-ciel

(*O. mykiss*) (Tomlin 2000). Cependant, il semble que le mancozèbe soit un agent cancérigène chez les mammifères (Belpogi *et al.*, 2002) et qu'il induit des dommages à l'ADN chez des cellules exposées *in vitro* (Calviello *et al.*, 2006; Domico *et al.*, 2007).

L'eau des rivières potentiellement contaminées par des pesticides se déverse vers la mer et les organismes marins retrouvés dans les sédiments sont exposés à ces pesticides. Cependant, aucune documentation sur d'éventuels liens directs entre les pesticides et le développement de la néoplasie n'a pu être trouvée. En 2004, des échantillons de myes communes retrouvées dans des endroits connus pour l'utilisation de pesticides n'ont pas montré de contamination reliée à l'agriculture (Pariseau *et al.*, 2009, données non publiées). Une des explications possibles serait la dégradation de ces pesticides dans l'eau par hydrolyse et/ou photolyse (Putman *et al.*, 2003; Kwon & Armrust, 2006; Chaves *et al.*, 2008) ou une élimination rapide des pesticides par les bivalves, comme c'est le cas chez la moule bleue (Ernst *et al.*, 1991). L'exposition aux pesticides ou aux produits de dégradation toxiques de ces pesticides peut induire des effets néfastes chez les organismes.

Les mécanismes moléculaires

Comme les fongicides (chlorothalonil et mancozèbe), les hydrocarbures aromatiques polycycliques (HAP) sont toxiques pour les organismes (Ernst *et al.*, 1991; Tomlin, 2000; Xu *et al.*, 2009). En effet, les HAP, et plus spécifiquement le benzo [*a*] pyrène (BaP), sont considérés comme des composés cancérigènes (Xu *et al.*, 2009). Des études ont montré que le BaP agit comme un xénobiotique immunosuppresseur avec les lymphocytes et monocytes des humains (Grevenynghe *et al.*, 2004). En effet, le BaP altère la différenciation des monocytes (Grevenynghe *et al.*, 2003) et les fonctions des lymphocytes T et B (Davila *et al.*, 1996).

Les mécanismes moléculaires qui agissent dans l'immunotoxicité des pesticides et des HAP impliquent le récepteur aryl hydrocarbone (AhR) (Hankinson, 1995). Le AhR est un récepteur membre de la famille des protéines transcriptionnelles (*helix-loop-helix* (bHLH)) impliqué dans le métabolisme des contaminants (Butler *et al.*, 2001). Chez les vertébrés, le AhR lie les xénobiotiques (Xb) pour former le complexe AhR-Xb. Ce

complexe se déplace vers l'ADN et s'y fixe pour entraîner l'induction de gènes impliqués dans le métabolisme des xénobiotiques comme le P4501A1 (Ma & Whitlock, 1996). Le métabolisme des xénobiotiques implique certaines formes du cytochrome p450 tel que le CYP1A1 (Ioannides & Parke, 1993). Le cytochrome p450 métabolise les composés en produits immunotoxiques déclenchant l'apoptose des cellules impliquées dans les processus immunitaires (Mann *et al.*, 1999). En effet, les HAP induisent l'apoptose des précurseurs des lymphocytes B (Page *et al.*, 2003) et conséquemment altèrent leur prolifération en affectant les processus mitotiques (Davila *et al.*, 1996).

Décrite comme le gardien du génome, la protéine p53 agit en régulant l'intégrité génomique durant le cycle cellulaire (Hofseth *et al.*, 2004). En condition anormale de prolifération cellulaire, due par exemple à un stress génotoxique, l'expression du gène p53 permettra l'arrêt du cycle cellulaire ou l'apoptose (Toledo & Wahl, 2006). Chez l'humain, le BaP induit l'expression du gène p53 dû à la prolifération de cellules anormales (Binkova *et al.*, 2000; Jiao *et al.*, 2008). En plus, le BaP influence l'expression du gène p53 dans les tissus du corégone (*Coregonus lavaretus*) (Brzuzan *et al.*, 2006). Ce poisson est utilisé comme modèle pour étudier le mécanisme moléculaire impliqué dans l'initiation de tumeurs par des composés cancérigènes (Brzuzan *et al.*, 2006). Chez la mye commune, le gène p53 a déjà été cloné et séquencé (Kelley *et al.*, 2001). La structure moléculaire du gène p53 chez la mye est similaire à celle du gène p53 chez l'humain suggérant un haut niveau de conservation des fonctions durant l'évolution (Kelley *et al.*, 2001). Récemment, chez la mye commune, une relation entre l'expression du gène p53 et la progression de la néoplasie hémique a été démontrée (Siah *et al.*, 2008a). De plus, chez des myes exposées à la dioxine (TCDD), un composé cancérigène persistant dans l'environnement, on a observé une induction de l'expression de certains gènes tel que l'ubiquitine (van Beneden, 1997; Rhodes *et al.*, 1997). L'ubiquitine est un marqueur de protéines cibles pour une dégradation par le protéasome 26S (Olberding *et al.*, 2004). Il semble que l'ubiquitine soit impliquée dans la dégradation de la p53 en relation avec des contaminants (Rhodes *et al.*, 1997).

L'ensemble de ces résultats suggère que le mélange de composés environnementaux cancérigènes (pesticides, HAP) induirait l'expression de certains gènes des hémocytes de

M. arenaria. Une des hypothèses est que les pesticides et HAP initient la progression du cycle cellulaire en cellules tumorales. Il est bien connu que ces contaminants induisent le récepteur aryl hydrocarbone. Ce récepteur métaboliserait les composés en produits cancérigènes qui induiraient ensuite l'expression des gènes p53 et l'ubiquitine.

OBJECTIFS ET HYPOTHÈSES DE RECHERCHE

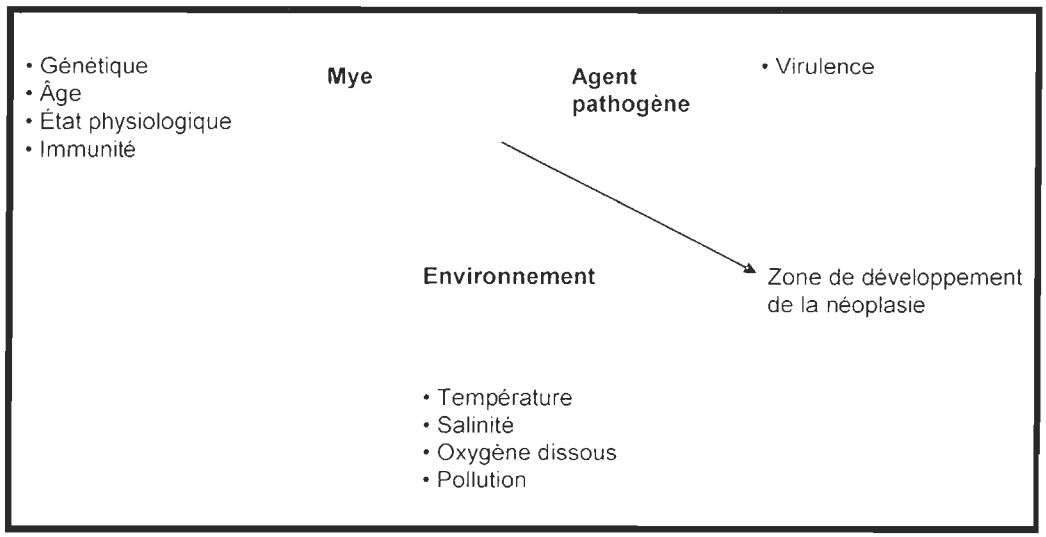


Figure 5. Représentation schématique des facteurs agissant sur la développement de la néoplasie hémique

L'hypothèse générale qui sous-tend la présente étude est qu'il y a convergence entre trois groupes de facteurs (hôte, agent pathogène et environnement) pour assurer le succès du développement de la néoplasie hémique (Figure 5). La figure 5 montre que, selon l'hypothèse, la zone de développement de la néoplasie hémique se situerait à la jonction des trois facteurs dans ce cas-ci la mye, l'agent pathogène et les conditions de l'environnement.

L'objectif général de cette étude est de contribuer à comprendre le développement de la néoplasie hémique chez la mye commune *M. arenaria*. Plus spécifiquement, cette étude permettra, en premier lieu, de déterminer si, en mésocosme, la néoplasie hémique est une

maladie transmissible d'un organisme malade vers un organisme sain en utilisant pour la première fois un outil de diagnostic précoce et puissant, le cytomètre en flux. Par la suite, nous déterminerons si, dans des conditions de laboratoire, une exposition chronique des myes aux fongicides chlorothalonil et mancozèbe les plus utilisés par les producteurs de pommes de terre de l'Île-du-Prince-Édouard, induit la néoplasie hémique chez des myes saines provenant d'un site où la maladie est présente. Finalement, nous déterminerons chez les hémocytes de la mye les niveaux d'expression des gènes codant pour la p53, l'ubiquitine et le récepteur aryl hydrocarbone après l'injection d'un mélange de chlorothalonil et mancozèbe et d'un hydrocarbure aromatique polycyclique (benzo [*a*] pyrene).

Les hypothèses suivantes seront vérifiées :

1. chez la mye commune, la néoplasie hémique est une maladie transmissible d'un individu malade vers un individu sain;
2. le chlorothalonil est un fongicide qui induit la néoplasie hémique chez les myes négatives qui ont déjà été en contact avec cette maladie;
3. le mancozèbe est un fongicide qui induit la néoplasie hémique chez les myes négatives qui ont déjà été en contact avec cette maladie;
4. le niveau d'expression du gène p53 des hémocytes de myes augmentent après injection d'un mélange de deux fongicides, le chlorothalonil et le mancozèbe, et d'un hydrocarbure aromatique polycyclique, le benzo [*a*] pyrene;
5. le niveau d'expression du récepteur aryl hydrocarbone des hémocytes de myes augmente après l'injection d'un mélange de deux fongicides (chlorothalonil et mancozèbe) et d'un hydrocarbure aromatique polycyclique (benzo [*a*] pyrene);
6. le niveau d'expression du gène de l'ubiquitine des hémocytes de myes augmente après l'injection d'un mélange de deux fongicides (chlorothalonil et mancozèbe) et d'un hydrocarbure aromatique polycyclique (benzo [*a*] pyrene).

L'hypothèse 1 a été testée dans le cadre des expériences décrites au chapitre 2 qui sera soumis dans la revue scientifique Marine Biology. Les hypothèses 2 et 3 ont été testées lors d'expériences décrites au chapitre 3 et publiées dans Marine Pollution Bulletin, tandis que le chapitre 4 est centré sur les hypothèses 4, 5 et 6 qui ont fait l'objet d'une étude publiée dans Ecotoxicology.

CHAPITRE 1
UNE ÉTUDE EN LABORATOIRE SUR LA TRANSMISSION DE LA NÉOPLASIE HÉMIQUE CHEZ LA MYE COMMUNE (*MYA ARENARIA*)

1.1 RÉSUMÉ

L'origine et le mécanisme de développement de la néoplasie hémique sont encore inconnus. Cependant, il semble y avoir une transmission entre les individus atteints et ceux qui sont en bonne santé. Le but de cette étude est de déterminer si, en mésocosme, la néoplasie hémique est une maladie transmissible entre des myes malades provenant de North River (Île-du-Prince-Édouard, Canada) et des myes saines provenant de différentes populations de l'Est du Canada: Anse Saint-Étienne (QC), Baie de Métis (QC), Havre-aux-Maisons (QC), Parc National de Kouchibouguac (NB) et Barasway Bay (NF). Les résultats obtenus au cours de cette expérience montrent qu'après une cohabitation de 62 jours, aucune des myes saines n'a développé la maladie. De plus, peu de myes ont affiché un pourcentage de tétraploïdie appartenant au groupe intermédiaire (15-50%) et de ce nombre, aucune mye n'a développé la maladie. Dans la présente étude, la technique de cytométrie en flux, un outil puissant et précis, a été utilisée pour le diagnostic. Cette technique permet de compter et de séparer les cellules de l'hémolymphe selon leur niveau de ploïdie soit 2N pour les hémocytes normaux (G0/G1) et 4N pour les cellules néoplasiques (G2/M) à l'aide de l'iodure de propidium. Cette technique permet également d'analyser une grande quantité de cellules et d'obtenir des données multiparamétriques sur chacun d'elles. Cette expérience a été réalisée en laboratoire et toutes les conditions ont été contrôlées réduisant au minimum l'impact des effets environnementaux. Le fait qu'aucune transmission n'a été obtenue suggère qu'un virus seul ne peut expliquer le développement de la néoplasie hémique. Cette maladie est probablement d'origine multifactorielle impliquant un agent viral, des conditions environnementales précises et un hôte spécifique.

Ce premier article, intitulé « *A laboratory experiment on haemic neoplasia transmission in soft-shell clams (*Mya arenaria*)* », fut corédigé par moi-même ainsi que par le Dre Maryse Delaporte, le Dr Réjean Tremblay, le Dr Jean-Marie Sévigny, Mme Patricia McKenna, mon collègue Mohammed AboElkair, le Dr T. Jeffrey Davidson et le Dr Franck C.J. Berthe. Cet article est en cours de préparation pour soumission à la revue *Marine Biology*. En tant que premier auteur, ma contribution à ce travail fut l'essentiel de la recherche sur la transmission de la néoplasi, l'exécution de l'expérience et la rédaction de l'article. Le Dre Maryse Delaporte, deuxième auteur, a développé la méthodologie. Les Drs Réjean Tremblay, Jean-Marie Sévigny, T. Jeffrey Davidson et Franck C.J. Berthe ont fourni l'idée originale et ont contribué à la rédaction et à la révision de l'article. Finalement, Mme Patricia McKenna et mon collègue Mohammed AboElkhair ont contribué à l'exécution de l'expérience. Une version abrégée de cet article a été présentée à la réunion annuelle du *Réseau Aquaculture Québec* à l'automne 2007.

1.2 A LABORATORY EXPERIMENT ON HAEMIC NEOPLASIA TRANSMISSION IN SOFT-SHELL CLAMS (*MYA ARENARIA*)

1.2.1 ABSTRACT

The aetiology and the mechanism of haemic neoplasia (HN) are mainly unknown. However, it seems that a transmission between diseased and healthy clams is possible. The aim of this study was to determine whether HN can be transmitted between HN-positive clams from North River (Prince Edward Island, Canada) and HN-negative clams from different populations from Eastern Canada: Anse Saint-Etienne (QC), Metis Bay (QC), Havre-aux-Maisons lagoon (QC), Kouchibouguac National Park (NB) and Barasway Bay (NF). The results obtained by this experiment showed that after a cohabitation of 62 days, none of the HN-negative clams developed the disease. Few clams showed a tetraploidy in the intermediate group but none developed HN. In the present study, the flow cytometry technique (FCM), a powerful and accurate tool, was used for the diagnosis. This technique can discriminate between single normal diploid cells (G0/G1) and neoplastic tetraploid cells (G2/M) using propidium iodide fluorescence signal and analyzed a high number of cells in a few seconds with acquisition of multi-parametric data per cells. The experiment was conducted in laboratory and all the conditions were controlled reducing the impact of environmental effects. The fact that no transmission was obtained suggests that a virus alone cannot explain the development of HN. This disease has probably a multifactorial aetiology involving a viral agent, environmental conditions and a specific host.

Keywords: *Mya arenaria*, haemic neoplasia, flow cytometry, tetraploidy, transmission, cohabitation experiment.

1.2.2 Introduction

The soft-shell clam, *Mya arenaria*, is an important species for the North American seafood market. Since 1995 in Prince Edward Island (PEI, Canada), the growers have reported any abnormal clams' mortalities to provincial and federal fisheries departments. Based on a monitoring program, a massive mortality of soft-shell clams was observed in 1999 at the site of Darnley Basin, PEI. After histological analyses, more than 95% of clams showed an advanced haemic neoplasia status (McGladdery *et al.*, 2001b).

Haemic neoplasia (HN) in soft-shell clams is an invasive and progressive disease characterized by proliferative neoplastic cells that may be of haemocyte origin (McGladdery *et al.*, 2001a; Bower, 2004; Smolowitz, 2005). The neoplastic haemocytes have a rounded shape with no pseudopod (Moore *et al.*, 1992), a large pleomorphic nucleus containing one or more nuclei (Reno *et al.*, 1994), a high nucleus/cytoplasm ratio (Reno *et al.*, 1994) and a high mitotic pattern (Kent *et al.*, 1991). The neoplastic haemocytes have lost their adherence, phagocytosis, and defence abilities (Leavitt *et al.*, 1990; Beckmann *et al.*, 1992; Dugan *et al.*, 2002). The neoplastic cells can also be characterized by an altered ploidy status (Reno *et al.*, 1994). Indeed, the neoplastic haemocytes showed tetraploid DNA content (4N) (Reno *et al.*, 1994; Moore, 2003; Delaporte *et al.*, 2008) with a higher number of chromosomes ranging from 44 to 80 instead of 26-39 (Reno *et al.*, 1994; Barber, 2004). Given the tetraploid status, some protocols based on flow cytometry technique have been suggested to diagnose HN (Reno *et al.*, 1994; da Silva *et al.*, 2005; Delaporte *et al.*, 2008). Those protocols analyzed the different phases of the cell cycle (G0/G1, S and G2/M phases) using a fluorescent DNA stain, the propidium iodide. This fluorescent stain allows the determination of the percentage of 2N cells (normal haemocytes) and 4N cells (neoplastic haemocytes) (Reno *et al.*, 1994; da Silva *et al.*, 2005; Delaporte *et al.*, 2008). Reno *et al.*, (1994) demonstrated that HN can be characterized by tetraploid status.

Several factors should be taken into consideration in order to explain the disease. The most important appears to be: environmental contamination (Brown *et al.*, 1979; Brown, 1980; Walker *et al.*, 1981; Couch & Harshbarger, 1985), viral infection (Farley *et al.*, 1991; McGladdery *et al.*, 2001a; Bower, 2004), genetic susceptibility of the organisms (Frierman

& Andrews, 1976; McGladdery *et al.*, 2001a, Bower, 2004) and other stressors like the presence of several species of toxic dinoflagellates (Landsberg, 1996), eutrophisation (Towney & Mulcahy, 1988), a high density of organisms (Ford *et al.*, 1997) and the maximal spawning activity with peak infestation of the parasitic copepod, *Mytilicola refringens* (Balouet *et al.*, 1986). However, none of these factors has been clearly identified to date (Barber, 2004).

The aetiology and the mechanism of HN are unknown but it seems that a transmission between diseased and healthy individuals is possible (Oprandy *et al.*, 1981; McGladdery *et al.*, 2001a; Dungan *et al.*, 2002). The results obtained by McGladdery *et al.*, (2001 a) showed that the disease can be transmitted between soft-shell clams from Atlantic Canada by injection of whole haemocytes and a cohabitation exposure. Several studies on *M. arenaria* have demonstrated a transmission of HN by cohabitation. Indeed, Appeldoorn *et al.*, (1984) showed that after 6 months of cohabitation, 72% of clams developed the disease. House (1997) obtained positive clams after 14 months of cohabitation. In the study carried out by McGladdery *et al.*, (2001a), 20% of the clams were positive after 1 month of cohabitation. MacCallum *et al.*, (2003) found 17% of positive clams after a 3-month experiment. A positive cohabitation transmission was also obtained for blue mussels, *Mytilus edulis* (Elston *et al.*, 1988) and *M. trossulus* (Brooks & Elston, 1991).

In addition, the transmission of HN in soft-shell clams was demonstrated by inoculation of neoplastic haemocytes (Appeldoorn *et al.*, 1984; Farley, 1987; Farley *et al.*, 1986; Elston *et al.*, 1988; Sunila & Farley, 1989; McLauglin, 1994; McLauglin *et al.*, 1992; Sunila, 1992; House *et al.*, 1998; Collins & Mulcahy, 2003; Walker & Böttger, 2008; Walker *et al.*, 2009). A positive transmission of HN has also been shown in several bivalve species by transplantation of neoplastic cells in *Cerastoderma edule* (Twomey & Mulcahy, 1988), *M. edulis* (Elston *et al.*, 1988) and *M. trossulus* (Kent *et al.*, 1991). The transmission was observed within the same bivalve species but not between different bivalve species (Kent *et al.*, 1991; MacCallum *et al.*, 2003).

Some studies did not reveal a transmission of the disease (Appeldoorn *et al.*, 1984; Kent *et al.*, 1991; MacCallum *et al.*, 2003). It seems that certain bivalve species are more

prone to the development of HN. This fact could be based on genetic aspects, but only little information is available on the genetic aetiology for the disease. For instance, Frierman & Andrews (1976) found the highest prevalence of haemic neoplasia among laboratory-produced strains of oysters, *Crassostrea virginica*, compared to wild oysters suggesting that the laboratory strains were genetically predisposed to disease development.

In order to test the hypotesis that some soft-shell clam stocks may be genetically more susceptible to develop HN, transmission by cohabitation tests were carried out between HN-positive clams from PEI and HN-negative clams from different area to determine wheter HN can be transmitted. Results obtained by St-Onge *et al.*, (in prep) showed very strong regional differentiation between Northern Gulf of St. Lawrence, Southern Gulf of St. Lawrence, Magdalen Archipelago and Southern Atlantic Canada using microsatellite markers. We made this transmission test to verify if this strong regional differentiation will have an impact in HN development.

1.2.3 Material and methods

1.2.3.1 Animals

Adult soft-shell clams *M. arenaria* (5-6 cm in length) were sampled from 5 sites of Eastern Canada (200 clams per population) during June 2005 (Figure 6): Anse Saint-Etienne Bay (Québec), Metis Bay (Québec), Kouchibouguac National Park (New-Brunswick), Havre-aux-Maisons lagoon (Magdalen Islands, Québec) and Barasway Bay (Newfoundland). Soft-shell clams were transported alive in coolers to the laboratory in Atlantic Veterinary College-University of Prince Edward Island, where each population was kept separately in tanks (122 x 184 x 30 cm) equipped with two Fluval® pumps and biofilters and supplied with static seawater at 18°C, salinity 28 and saturated oxygen by air supply manifolds. Soft-shell clams were held in tanks for 2 weeks before the beginning of the experiment. During this time, the clams were fed 3 times a week with a microalgae paste (Innovative Aquaculture Products®) at 3% of clam dry mass. All clams were placed inside of foam to mimic the pressure of the sediment. Inside the foam, a stainless steel nut was placed to maintain the clam on the bottom of the tank.

1.2.3.2 Transmission experiment (62 days)

This cohabitation experiment was set up to determine if a transmission of HN between a diseased clam and a healthy clam is possible. The experiment was conducted between June 30 and August 30, 2005. For the study, 4 tanks were used: 1 for the control clams and 3 for the treated clams. In each treatment tank, 20 clams per population were mixed with an average of 10 HN-positive clams from North River, (46°15'01''N, 63°10'42''O) near Charlottetown (Prince Edward Island). These HN-positive clams had more than 50% of 4N cells. In the control tank, HN-positive clams were substituted by HN-negative clams from Havre-aux-Maisons. During the experiment, the water temperature and salinity, oxygenation, feeding and foam installation conditions were the same as above.

Figure 6. Map of the Gulf of St. Lawrence showing the sampling sites of the soft-shell clam (*Mya arenaria*) used in the HN transmission study

1.2.3.2 Transmission experiment (62 days)

This cohabitation experiment was set up to determine if a transmission of HN between a diseased clam and a healthy clam is possible. The experiment was conducted between June 30 and August 30, 2005. For the study, 4 tanks were used: 1 for the control clams and 3 for the treated clams. In each treatment tank, 20 clams per population were mixed with an average of 10 HN-positive clams from North River, (46°15'01''N, 63°10'42''O) near Charlottetown (Prince Edward Island). These HN-positive clams had more than 50% of 4N cells. In the control tank, HN-positive clams were substituted by HN-negative clams from Havre-aux-Maisons. During the experiment, the water temperature and salinity, oxygenation, feeding and foam installation conditions were the same as above.

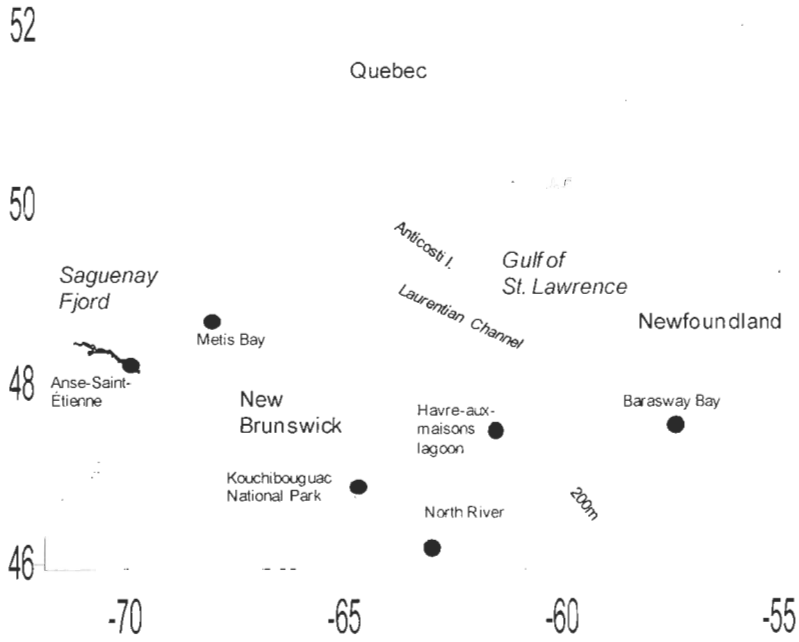


Figure 6. Map of the Gulf of St. Lawrence showing the sampling sites of the soft-shell clam (*Mya arenaria*) used in the HN transmission study

1.2.3.3 Collection of haemolymph and flow cytometry detection of haemic neaoplasia

After a 2-month experiment, haemolymph was drawn from the adductor muscle sinus, using 3ml plastic syringe fitted with a 25-gauge needle. Haemolymph was transferred into microtubes and kept on ice. An aliquot of 500 µl of haemolymph was fixed in absolute ethanol (1:5) and stored at -20°C until the analysis (da Silva *et al.*, 2005).

The haemolymph samples fixed in ethanol was prepared for flow cytometry analysis following the protocol of da Silva *et al.*, (2005) and Delaporte *et al.*, (2008). Fixed haemolymph samples were centrifuged to pellet the cells at 400 g for 10 min and at room temperature. Cells were re-suspended, re-hydrated for 30 min and washed twice with 0.01 M phosphate buffer saline (PBS) before being filtered through an 80 µm nylon mesh while being transferred to a flow cytometer tube. Samples were then treated with DNase-Free RNase A (0.005%) and stained with propidium iodide (PI, 0.005%) for 1 hour. PI fluorescence, which is related to the DNA content of each cell, was detected on the FL2 detector (orange light, at 550-600 nm) of a FACSCalibur flow cytometer (Becton Dickinson). For each sample, more than 3000 cells were counted at a low flow rate (15 µl min^{-1}).

For each clam, the results were represented on cytograms with the width and area of PI fluorescence signal that distinguished between single normal diploid cells (G0/G1), neoplastic tetraploid cells (G2/M) and doublets (Figure 7a). After gating on the single cell region (R1), PI fluorescence of single cells was plotted on a FL2 area histogram to calculate the number of diploid and tetraploid cells in this region R1 (Figure 7b).

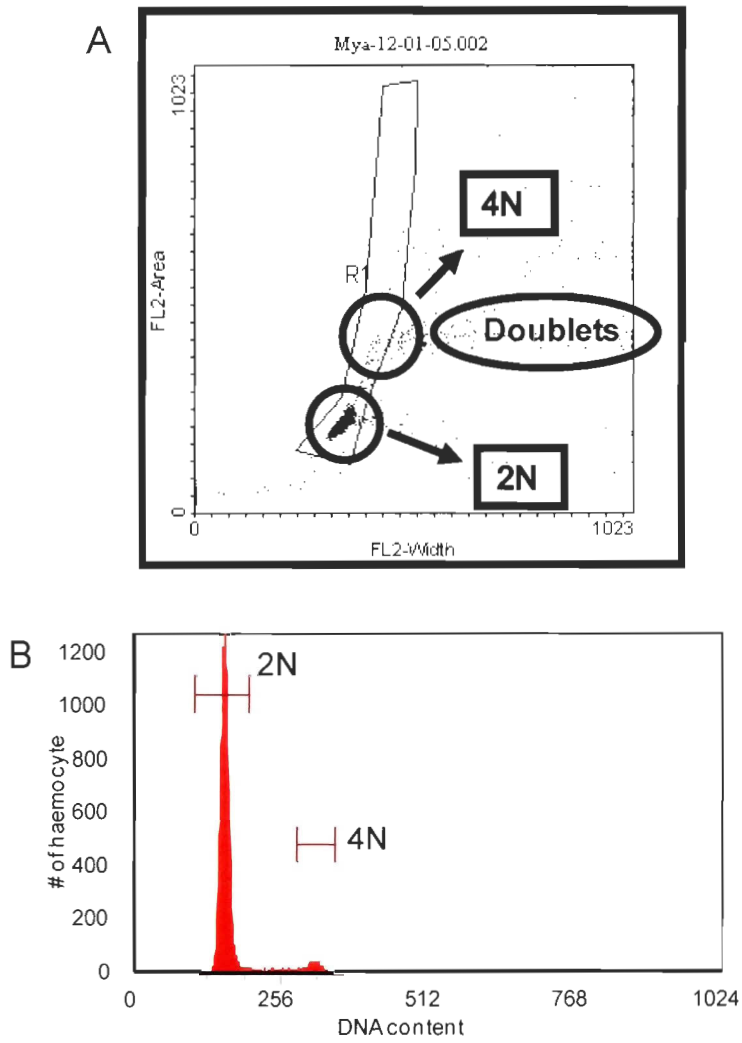


Figure 7. Flow cytometry analysis of diploid (G0/G1) and neoplastic tetraploid (G2/M) cells from a single clam sampled at North River, PEI, Canada. A) Cytogram representing diploid (2N) and tetraploid (4N) single cells on a width vs area FL2. B) Histogram of propidium iodide fluorescence. Markers 2N represent diploid peak and 4N tetraploid peak

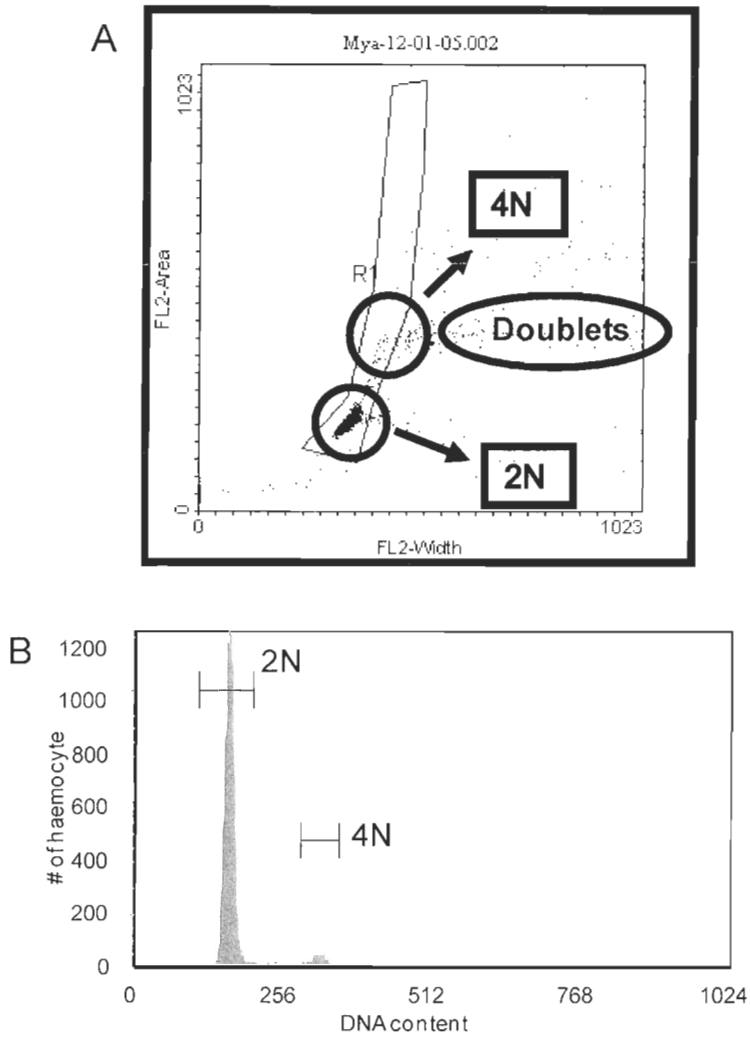


Figure 7. Flow cytometry analysis of diploid (G0/G1) and neoplastic tetraploid (G2/M) cells from a single clam sampled at North River, PEI, Canada. A) Cytogram representing diploid (2N) and tetraploid (4N) single cells on a width vs area FL2. B) Histogram of propidium iodide fluorescence. Markers 2N represent diploid peak and 4N tetraploid peak

1.2.3.4 Statistical analysis

The data for each population was shown by the number of clams in relation with different group levels 0–5%, 5–15%, 15–50% and >50% of tetraploid haemocytes. To analyze the tetraploid status among the clams of each site, multivariate analysis of variance (MANOVA) was applied using the software SigmaStat (San Jose, CA, USA). Dependent factors were the 4 groups of tetraploid cells and the independent factor was the population. To analyze mortality of each population, analysis of variance was made. The data for both analysis were arcsine square root transformed to achieve homogeneity of variances as described in Sokal and Rohlf (1995). Homogeneity was confirmed by the Levene test and the observation of standard residual plots.

1.2.4 RESULTS

During this 62-day experiment, mortalities occurred in clams from each site. There was no significant difference between mortality among population (P=0.954, F=0.162, d.f.=4). The mortalities were lower in the control but not at a statistical level with an average of 9.6 ± 4 %.

For the treated clams, each location displayed a similar pattern of tetraploidy and no transmission was observed (Figure 8). We used the tetraploid level obtained for each clam to classify them into 4 groups of HN development based on the percentage of tetraploid haemocytes: negative group 0-5%, undetermined group 5-15%, intermediate group 15-50% and positive group >50% of 4N haemocytes. There was no significant difference in the tetraploid status among the clams of each site (P=0.436, F=1.033, d.f.=5). At the end of the experiment, the majority of clam belongs to the negative group with less than 5% of 4N haemocytes. Two individuals from Havres-aux-Maisons Lagoon and Barasway Bay, belong to the undetermined group with values between 5 and 15% of 4N haemocytes (5.2 and 6.2 for Havres-aux-Maisons Lagoon, 5.8 and 7.2 for Barasway Bay). For the Anse Saint-Etienne stock, 8 clams were classified undetermined with values varying between 5.5 and 9.4 %. Metis Bay and Kouchibouguac National Park showed a majority of negative clams with few undetermined organisms between 5-15% of 4N haemocytes (1 and 5 clams

respectively). However, the higher levels of 4N cells (intermediate level of HN development) in this study was observed in clams originating from these sites (15.9% and 16.6% respectively).

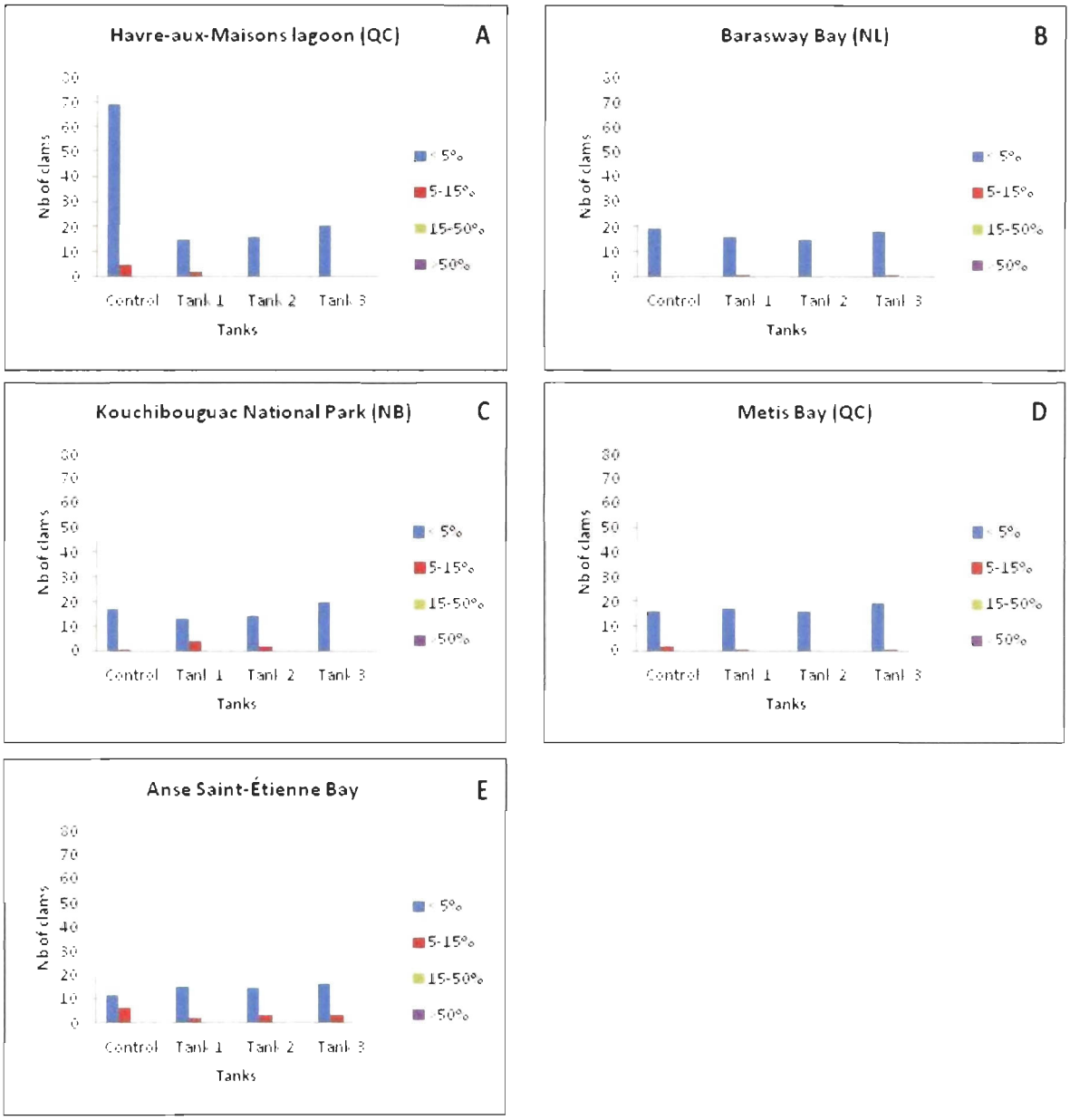


Figure 8. Number of clams in relation with tetraploid group (0-5%, 5-15%, 15-50% and >50%) for 5 populations from East coast of Canada

respectively). However, the higher levels of 4N cells (intermediate level of HN development) in this study was observed in clams originating from these sites (15.9% and 16.6% respectively).

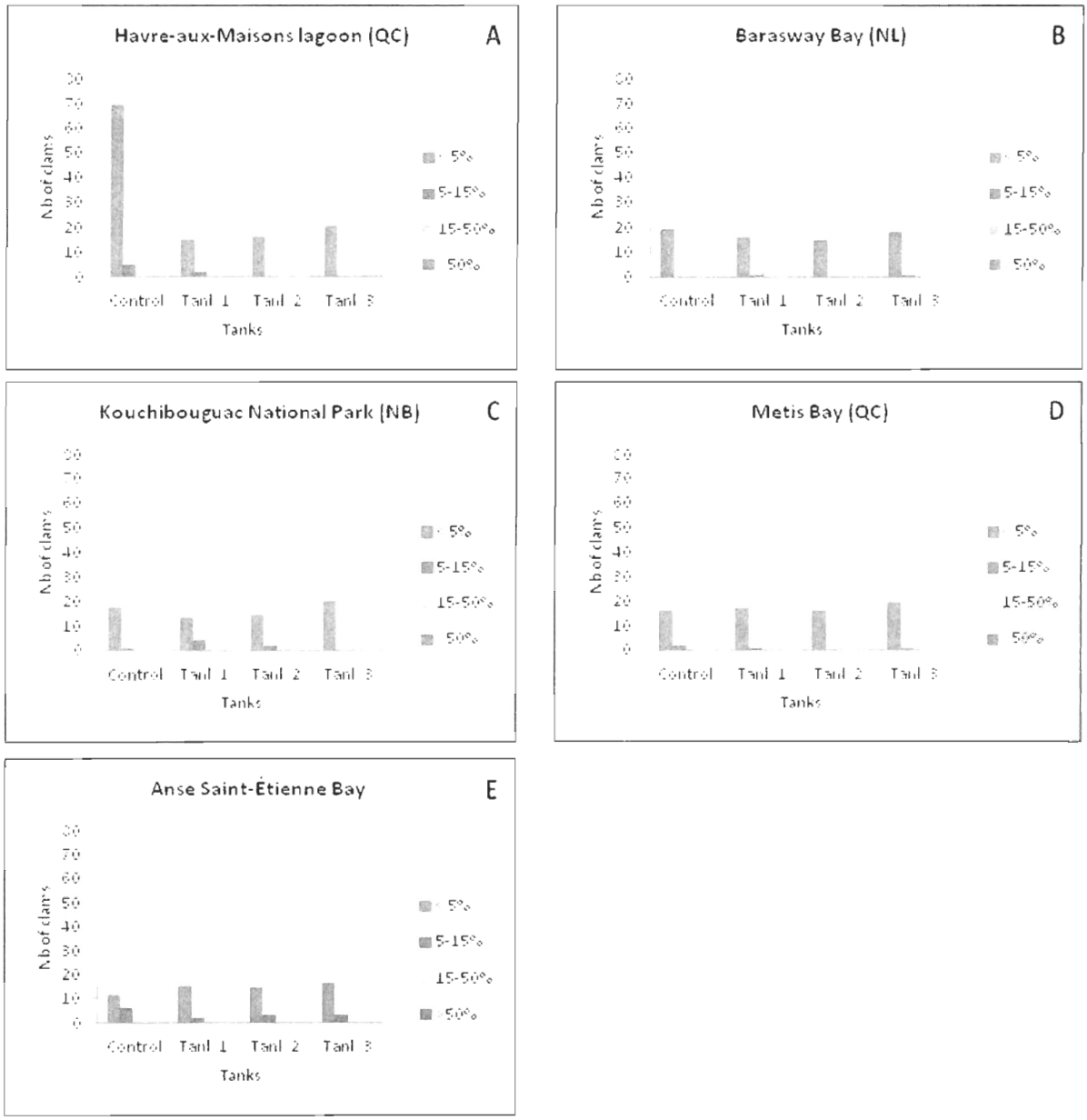


Figure 8. Number of clams in relation with tetraploid group (0-5%, 5-15%, 15-50% and >50%) for 5 populations from East coast of Canada

1.2.5 DISCUSSION

In this experiment, there was no significant difference between mortality among population and the control. The mortalities obtained were lower than the mortalities obtained by MacCallum *et al.*, (2003) in their study of transmissibility of haemic neoplasia in soft-shell clams. Three trials were analyzed by MacCallum *et al.*, (2003) with an averaged mortality of 58 ± 24 % for the treated clams and 47 ± 19 % for the control clams.

For the treated clams, there was no significant difference in the tetraploid status of each site. Delaporte *et al.*, (2008) assessed soft-shell clams in populations from Eastern Canada using the same flow cytometry methods (FCM) and found only one clam presenting abnormal content (23.7%) of 4N haemocytes in Magdalen Island. In Barasway Bay, the results obtained by Delaporte *et al.*, (2008) showed that the third of the clam population presented between 6-10% of 4N cells. For Anse Saint-Etienne, the values were slightly higher than those observed in Delaporte *et al.*, (2008), where clams had less than 5% of 4N cells. Delaporte *et al.*, (2008) found that the majority of the clams for Metis Bay and Kouchibouguac National Park were negative but 1 clam showed 38.5% of 4N cells for Metis Bay and 1 clam showed 8% of 4N cells for Kouchibouguac National Park. For the control, the majority of the clams were negative with less than 5% of neoplastic haemocytes, but for each population we found some clams ranging between 5-15% of 4N cells.

For the clams found in the indetermined group (between 5-15% of 4N cells), caution has to be taken because the percentages of tetraploid cells observed could be due to a physiological status (Elston *et al.*, 1992). Moreover, the normal percentage of haemocytes in each phase of the cell cycle for a negative clam remains unknown because the hematopoietic process in mollusks is unidentified. Recently, experimental threshold was proposed by FCM to define positive and negative clams. Indeed, Delaporte *et al.*, (2008) suggested that clams with less than 5% of neoplastic cells by FCM can be considered negative. This threshold is closed to the one proposed by Reno *et al.*, (1994). Delaporte *et al.*, (2008) recommended also a threshold for positive clams. Soft-shell clams with more

than 20% of 4N cells were confirmed to be positive to HN. This data can be related to the results obtained by Siah *et al.*, (2008a). In their study, Siah *et al.*, (2008a) showed that some clams between 15-50% of 4N cells displayed a significantly high level of p53, p73 and mortalin gene expression (gene involved in HN).

After a cohabitation of 62 days, none of the HN-negative clams developed the disease based on the confirmed level of 20%. Few clams showed a tetraploidy in the intermediate group but none clearly developed HN. Few studies examined transmission by cohabitation with soft-shell clams (Appeldoorn *et al.*, 1984; House, 1997; McGladdery *et al.*, 2001a; MacCallum *et al.*, 2003). The first study was made by Appeldoorn *et al.*, in 1984. In this study, negative and HN-positive clams were held together in a flow-through tank with sediments from a polluted area, a clean area, a mixture of the two, or no sediment for a period of 6 months. Their results showed a prevalence ranging from 20 to 72% within 2 to 4 months. The most severely affected groups were those held in the mixture of the sediments and the animals without sediment. In this experiment, a flow through system was used that permitted an impact by environmental condition like anthropogenic substances on the occurrence of the disease. In our study, all environmental conditions were controlled. Our approach reduced the possible impact of anthropogenic substances so that we could be confident that if clams exhibited haemic neoplasia, that an infectious agent would be involved in the transmission.

In the experiment of House (1997), clams from Alsea Bay, Oregon (USA) were pre-screened by haematocytology and immunocytochemistry using a monoclonal antibody specific to HN cells in *M. arenaria*. Healthy clams were divided in 10 groups and were placed with positive clams (with an average of 90% of neoplastic cells) for 14 months. At the end, transmission of HN occurred in 7 groups and positive clams had more than 50% of neoplastic cells. House (1997) suggested that the transmission was successful because the water in the flow-through tank was from the bay beside the laboratory where HN is enzootic. Before the experiment, the water was not treated to reduce potential contamination with waterborne agent.

McGladdery *et al.*, (2001a) showed that after 36 days of experiment, 20% of clams had HN using histology. The clams were pre-screened by haematocytology and were from Miscouche and Darnley Bassin (PEI). For this experiment, HN-positive clams had more than 1% of neoplastic haemocytes and were categorized according to morphology. Positive clams were classified into 3 groups: light (1-10%), moderate (11-50%) and heavy (51-100%) (McGladdery *et al.*, 2001a).

MacCallum *et al.*, (2003) made 3 trials for a proximity experiment. For the first trial, after 103 days of exposure, the results showed by histology that none of the clams had developed neoplastic haemocytes. The second trial lasted 93 days and the results showed that 17% (12 clams) of clams presented neoplastic haemocytes but no significant difference ($p=0.25$) could be detected between treated and control clams. Finally, the third trial, the proximity experiment lasted for 106 days. At the end, 8 clams had between 8-24% of neoplastic cells. A slightly significant difference ($p=0.054$) between the treated and the control groups indicated that HN had infected HN-negative clams from Newfoundland (Barasway Bay). All the clams from the control group were negative. The results obtained by MacCallum et al. (2003) showed that some HN-negative clams turned HN-positive from two of the three proximity experiments but not at a statistically significant level. At the beginning of the experiment, the clams were pre-screened by haematocytology for HN and at the end they were screened by histology. The diagnostic was based on morphological features and the clams were classified positive when the percentage of enlarged haemocytes and diffused nuclei was greater than 0.1%. Clams were classified negative when no haemocytes with enlarged nuclei were found (MacCallum *et al.*, 2003).

Transmission of HN by cohabitation was also observed in *M. edulis* (Elston *et al.*, 1988) and *M. trossulus* (Brooks & Elston, 1991). Elston *et al.*, (1988) and Kent *et al.*, (1991) suggested that the development of HN in negative control mussels was the result of the water supply being contaminated with an agent from HN infected mussels. More studies, however, have shown that HN was successfully induced by inoculation of whole haemocytes (McLaughlin *et al.*, 1992; McLaughlin, 1994; McGladdery *et al.*, 2001a; MacCallum *et al.*, 2003; Walker *et al.*, 2009). Positive transmission was also reported in

cockles, *C. edule*, by injection of whole cells and cell-free filtrate (Collins & Mulcahy, 2003) and in *M. edulis* (Elston *et al.*, 1988) and *M. trossulus* (Kent *et al.*, 1991) by injection of haemolymph from a positive organism (Twoney & Mulcahy, 1988). In none of the HN positive transmission studies did the experiment reveal whether it was a real transmission or a transplantation of neoplastic cells.

HN diagnosis is usually based on morphological characteristics with haematocytology and histopatology (Brousseau, 1987; Elston *et al.*, 1988; Brousseau & Baglivo, 1991a; McGladdery *et al.*, 2001a; Villalba *et al.*, 2001; Dungan *et al.*, 2002). Although histopathology gives information on which tissues are more affected, those techniques analyze a limited number of cells per slide (Delaporte *et al.*, 2008). In the present study, we used FCM, a more powerful and accurate diagnosis tool which allows the analysis of a large number of cells in few seconds giving multi-parametric data for each cell. In the study of Delaporte *et al.*, (2008), each result was confirmed by haematocytology based on Farley *et al.*, (1986) criteria. The results were generally the same except for some clams. The differences in diagnostic were due to the fact that the sensitivity varies between methods. Delaporte *et al.*, (2008) suggest that a new threshold have to be established for the HN diagnostic. For instance, Reno *et al.*, (1994) suggest that negative clams should have less than 6% of 4N haemocytes and based on morphological characteristics some of those clams were classified as lightly positive (McGladdery *et al.*, 2001a). Delaporte *et al.*, (2008) showed that over 20% of 4N cells are required to clearly confirm that clams are positive to HN. Indeed, FCM is more accurate because it is based on the cell cycle analysis, while haematocytology is based on morphological characteristics. For both methods, distinction between mitosis in normal haemocytes and neoplastic cells is difficult especially for intermediate groups. In fact, the percentages of healthy haemocytes in S and G2/M phases are unknown because the haematopoiesis site in mollusks has not been identified yet.

1.2.5.1 Potential infectious agent

The fact that results in literature showed that haemic neoplasia can be transmitted between individuals suggests the implication of an infectious agent. House (1997), showed that HN can be transmitted to soft shell clams by ingestion of neoplastic cells from clams with high levels of HN to healthy clams, and cohabitation of healthy animals with diseased individuals in 9 months. The positive results suggested that clams with high levels of HN are releasing an infectious agent, neoplastic cells or smaller particles into the water (House, 1997). This may occur in the late stages of the disease or when the animal is dead.

It has been demonstrated by Sunila and Farley (1989) that the HN cells can survive at least 48 hours in seawater. Appeldoorn *et al.*, (1984) found that neoplastic cells have a diameter of 12 microns. These cell sizes are in the size range that bivalves can filter and ingest (Langdon & Siegfried, 1984). The ingestion of HN cells may provide a possible route of entry into the animal for the causative agent of HN (House, 1997).

The massive mortality of soft-shell clams in PEI during the summer 1999, suggested a sudden introduction of a pathogen viral agent in a naïve population (Farley *et al.*, 1986; McGladdery *et al.*, 2001a). Results of some experiments could be related to a viral implication in the development of HN. Indeed, Brown (1980) reported the presence of an oncornavirus and reverse transcriptase activity in HN-positive clams. Futhermore, Oprandy *et al.*, (1981) observed a virus with physical and morphological characteristics of a type B retrovirus in HN-positive clams. In addition, Oprandy & Chang (1983) induced HN and viral production in healthy clams after an injection of 5-bromodeoxyuridine (5-BrdUrd), a technique shown to induce expression of retroviruses in some cultured mammalian cells (Lowry *et al.*, 1971). Those results seem to demonstrate that HN in soft-shell clams is caused by a retroviral agent; however, negative results were obtained when other authors tried to repeat the experiments (Elston *et al.*, 1992; House *et al.*, 1998; Barber, 2004).

Indeed, AboElkhair *et al.*, (2009a) did not find a structure similar to retroviral particles in samples of HN-positive clam haemolymph from North River, PEI. This result is in agreement with studies which failed to show the existence of a retrovirus in *M. arenaria* (Farley, 1976; Appeldoorn *et al.*, 1984; House *et al.*, 1998) *C. edule* (Auffret & Poder,

1986), *M. edulis* (Rasmussen, 1986) and *M. trossulus* (Mix *et al.*, 1979; Elston *et al.*, 1988). However, AboElkhair *et al.*, (2009b) found a positive correlation between reverse transcriptase (RT) activity and tetraploidy in soft-shell clams. The fact that they found RT activity without retroviral particles suggested that the RT activity might be due to the expression of endogenous sources.

Some evidence was obtained on a viral aetiology in HN. Even if the presence of a virus is a good candidate, the nature of the pathogen agent remains unclear (Elston *et al.*, 1992; Barber, 2004). It is suggested that the infectious agent activity and the HN development can be modified by others factors like pollution by anthropogenic substances.

We suggest that the absence of HN transmission, in our study, could be related to controlled environmental conditions, reducing the potential impact of anthropogenic substances. Indeed, the time required for developing detectable levels of HN and prevalence levels in a population could depend on factors including the level of exposure, density of clams and environmental conditions (House, 1997). Furthermore, the fact that some species are more prone to develop HN suggests a genetic basis to a HN development (Barber, 2004). Only little information can be obtained on this criterion. Between 1964 and 1973, Frierman & Andrews (1976), found a high prevalence of HN in laboratory-produced strains of oysters, *Crassostrea virginica* (31/369 = 8.4%) compared to wild oysters (39/51 000 = 0.08%). The result suggested that the laboratory-produced oysters are more prone than the wild oysters and could be related to inbreeding promoting the loss of genetic variability or diversity.

1.2.6 Conclusion

In this study, no transmission of haemic neoplasia was found after 62 days of cohabitation with positive clams from North River, PEI. Our diagnostic technique was based on flow cytometry, a powerful and accurate method, which uses the ploidy status and analyzes several cells in a few seconds. The majority of experiments which found transmission used morphological characteristics and analyzed a limited number of cells per slide. For those experiments, a clam was considered positive with more than 1% of

neoplastic cells while in our experiment, a positive clam was a clam with more than 20% of 4N haemocytes.

The experiment was made under laboratory-controlled conditions reducing the possible impact of environmental factors. The fact that no transmission was obtained suggests that a potential virus alone cannot explain the development of HN. This disease could be a multifactorial aetiology involving a viral agent, environmental conditions and a specific host.

The study of HN development by transplanting soft shell clams from different HN-negative populations in an HN-positive environment can be an example of a strategy that would provide information on the impact of environmental conditions.

1.2.7 ACKNOWLEDGEMENTS

The authors wish to thank Allan Anderson, Garth Arsenault, Lise Chevarie, Iften Redjah and Jean-François Mallette for the sampling of the clams. This work was supported by AquaNet, NSERC, The Canada Research Chairs (CRC) Program, The Atlantic Canada Opportunities Agency (ACOA), and The Canada Foundation for Innovation (CFI).

CHAPITRE 2
UN LIEN POTENTIEL ENTRE L'EXPOSITION ÀUX FONGICIDES CHLOROTHALONIL ET MANCOZÈBE ET LE DÉVELOPPEMENT DE LA NÉOPLASIE HÉMIQUE : UNE ÉTUDE EN LABORATOIRE

2.1 RÉSUMÉ

L'origine de la néoplasie hémique est inconnue, mais plusieurs facteurs ont été suggérés comme un agent viral, une prédisposition génétique et la pollution. Le but de cette étude est de déterminer si une exposition chronique des myes en laboratoire aux deux fongicides (chlorothalonil et mancozèbe) les plus utilisés par les producteurs de pommes de terre de l'Île-du-Prince-Édouard peut induire la néoplasie hémique chez des myes négatives provenant d'un environnement positif. Des expériences d'exposition à court-terme ont également été réalisées. Les myes provenant d'un site positif (North River, IPE) et d'un site exempt (Îles-de-la-Madeleine, Québec) ont été récoltées. Le pourcentage d'hémocyte tétraploïde a été évalué par cytométrie en flux pour chaque mye. La bioaccumulation des pesticides dans les tissus a été quantifiée par chromatographie en phase gazeuse couplée à la spectrométrie de masse pour le chlorothalonil et par plasma par couplage inductif couplé à la spectrométrie de masse pour le mancozèbe. L'exposition à long terme des myes négatives de North River au fongicide Bravo 500® n'a pas induit un pourcentage élevé de cellules tétraploïdes et les analyses de la glande digestive et du manteau n'ont pas révélé de niveau détectable de chlorothalonil. Pour le Manzate 200 DF®, quelques myes ont révélé un niveau élevé de cellules tétraploïdes mais aucune différence n'a été observée entre les myes traitées et les myes contrôles. L'analyse du manganèse pour la glande digestive et le manteau n'a pas révélé de différence significative ($p=0.05$). Pour l'exposition à court terme, l'analyse du chlorothalonil a démontré que l'ingrédient actif était distribué entre trois composés chlorés: 99.5% de chlorothalonil, 0.4% de pentachlorothalonil et 0.1% de trichlorothalonil. Les résultats ont montré que l'accumulation du chlorothalonil est

rapide et est déjà présente après 4 heures d'exposition. L'accumulation la plus importante est détectée après 48 heures d'exposition dans le manteau où la concentration atteint 59,2 $\mu g\ g^{-1}$ de poids sec. L'accumulation diminue jusqu'à un niveau indétectable après 3 jours d'exposition. Après 48 heures, une baisse de la concentration a été notée jusqu'à un niveau indétectable. L'accumulation la plus importante du mancozèbe se trouve dans le manteau après 24 heures d'exposition (48.8 $\mu g\ g^{-1}$). Étant donné l'augmentation de l'accumulation du manganèse (ingrédient actif du mancozèbe) la source de celui-ci ne peut que provenir du mancozèbe. Selon ces résultats, l'accumulation des fongicides semble être transitoire. Le chlorothalonil et le mancozèbe sont des composés connus comme promoteurs de stress oxydatif et pourraient induire des dysfonctions au niveau cellulaire. Des études sur l'effet de ces fongicides sur la protéine p53 sont un exemple de stratégie qui pourrait fournir de l'information sur le développement de la néoplasie hémique.

Ce deuxième article, intitulé « *Potential link between exposure to fungicides chlorothalonil and mancozeb and haemic neoplasia development in soft-shell clam* Mya arenaria », fut corédigé par moi-même ainsi que par le Dr Richard St-Louis, la Dre Maryse Delaporte, mon collègue Mohammed Abo El Khair, Mme Patricia McKenna, le Dr Réjean Tremblay, le Dr T. Jeffrey Davidson, le Dr Émilien Pelletier et le Dr Franck C.J. Berthe. Il fut accepté pour publication dans sa version finale en 2009 par les éditeurs de la revue *Marine Pollution Bulletin*. En tant que premier auteur, ma contribution à ce travail fut l'essentiel de la recherche sur l'association entre la néoplasie hémique et la contamination anthropique, le développement de la méthodologie, l'exécution de l'expérience et la rédaction de l'article. Le Dr Richard St-Louis, second auteur, a contribué à la recherche sur l'association entre la néoplasie hémique et la contamination anthropique, au développement de la méthodologie, à la rédaction de l'article ainsi qu'à la révision de l'article. Dre Maryse Delaporte a contribué au développement de la méthodologie. Mohammed Abo El Khair et Patty McKenna ont contribué à l'exécution de l'expérience. Les Drs Réjean Tremblay, T. Jeffrey Davidson, Émilien Pelletier et Franck C.J. Berthe ont fourni l'idée originale et ont contribué à la rédaction ainsi qu'à la révision de l'article. Une version abrégée de cet article a été présentée à la réunion annuelle du *Réseau Aquaculture Québec* à l'automne 2009.

2.2 POTENTIAL LINK BETWEEN EXPOSURE TO FUNGICIDES CHLOROTHALONIL AND MANCOZEB AND HAEMIC NEOPLASIA DEVELOPMENT IN THE SOFT SHELL CLAM *MYA ARENARIA*: A LABORATORY EXPERIMENT

2.2.1 ABSTRACT

The aetiology of haemic neoplasia (HN) is unknown so far but many causative factors are suggested such as viral, pollution and genetics. The aim of this study was to determine if, under chronic exposure, two major pesticides (chlorothalonil and mancozeb) which are used in potato production could induce HN in soft-shell clams (*Mya arenaria*). Short-term experiments with acute exposure were also performed. Clams were collected from an epizootic site (North River, PEI) and from a site free of the disease (Magdalen Islands, Quebec). The tetraploid level of haemocytes was assessed by flow cytometry for each clam to determine the HN status. The bioaccumulation of pesticides in tissues was quantified by gas chromatography/mass spectrometry (GC/MS) for chlorothalonil while mancozeb and manganese were quantified by inductively coupled plasma-mass spectrometer (ICP/MS). Long term exposure to fungicide Bravo 500® did not induce high tetraploid levels on negative clam from North River and the analysis of the digestive gland and the mantle did not reveal any detectable level of chlorothalonil. In the Manzate 200 DF®, some clams revealed high level of tetraploid cells but no difference were observed between the treatments and the control. The analysis of the digestive gland and the mantle for manganese did not highlight any significant difference in tissue concentration ($p=0.05$). For the acute exposure, chlorothalonil analysis showed that the active ingredient is distributed between three chlorinated compounds: 99.5% for chlorothalonil isomers, 0.4% for pentachlorothalonil and 0.1% for trichlorothalonil isomers. For a 72 hour experiment, the accumulation was within 4 hours, the higher tissue concentration of chlorothalonil was 59.2 $\mu g\ g^{-1}$ in the mantle after 48 hours, following by a decrease to an undetectable level at the end. For the manganese, the accumulation was detected after 4 hours; the higher tissue concentration was 48.8 $\mu g\ g^{-1}$ in the mantle after 24 hours and, over the following 48 hours, the accumulation decreased until the end of the trial. Based on the data, the accumulation of these fungicides seems to be transitory. Chlorothalonil and mancozeb are both oxidative-stress promoters and could have induced cell dysfunction while in the tissue. Study on the effect of these fungicides on the p53

protein system is an example of strategy that would provide information on cellular events promoting neoplasia.

Keywords: *Mya arenaria*, soft-shell clam, fungicides, chlorothalonil, mancozeb, manganese haemic neoplasia, acute exposure, chronic exposure

2.2.2 INTRODUCTION

Potato production in the province of Prince Edward Island (PEI, Canada) is the most important in the Atlantic region of Canada (White *et al.*, 2006), and is heavily dependent on pesticides, with up to 15 applications per growing season (Mutch *et al.*, 2002). Thus, PEI is the province with the most intensive use of pesticides in Canada (Dunn, 2004), with total pesticide sales in 2000 of over one million kg (Reeves, 2001). Several pesticide monitoring programs were initiated and between 1994 to 1999, 169 different types of pesticides were detected in water samples (Mutch *et al.*, 2002; White, 2004). Only chlorothalonil was detected in excess of the Freshwater Aquatic Life Guidelines (FWALG) (CCME, 1999) with observed values of 1.34 $\mu g\ l^{-1}$ compared to its FWALG value of 0.18 $\mu g\ l^{-1}$ (Mutch *et al.*, 2002). Between 1994 and 2002, 16 fish mortality events have been related to pesticide runoff events in PEI and 26 others have been suspected (White, 2004; Gormley *et al.*, 2005). Different classes of pesticides were involved in these fish kills: azinphos-methyl, an organophosphate insecticide; chlorothalonil a chlorinated benzonitrile fungicide and mancozeb a zinc salt of manganese dithiocarbamate fungicide.

In the province of Prince Edward Island, the active ingredients chlorothalonil and mancozeb account for 74% (602 446 kg) of yearly sales of pesticides (Reeves, 2001). These fungicides are used weekly during the potato growing season to control fungal diseases. The nonsystemic fungicide chlorothalonil (tetrachloroisophtalonitrile) is toxic to fish, aquatic invertebrates and marine organisms with a LC_{50} of 0.1 mg/l for rainbow trout, 5.9 mg/l for blue mussel and 35 mg/l for soft-shell clam (Ernst *et al.*, 1991). *In vitro*, chlorothalonil negatively affects tunicate haemocyte functionality (Cima *et al.*, 2008) and suppresses oyster hemocyte reactive oxygen species production (Baier-Anderson & Anderson, 2000). Mancozeb is a fungicide belonging to the ethylenedithiocarbamates family, with zinc and manganese as co-metals. It is moderately toxic to fish and aquatic organisms with a LC_{50} observed for trout of 2.2 mg/l (Tomlin, 2000). However, mancozeb is a suspected carcinogenetic agent in mammals (Belpogi *et al.*, 2002) and induces DNA damage in cells exposed *in vitro* through oxidative mechanisms (Calviello *et al.*, 2006; Domico *et al.*, 2007).

The contaminated river waters which affect fish upstream, invariably flow out into the sea surrounding the Island, and expose the marine invertebrates inhabiting the sediment at the mouth of the impacted rivers to the same pesticides. However, no contaminant related to agriculture, including chlorothalonil, has been detected in soft-shell clams (*Mya arenaria*) sampled in 2004 in

areas known for pesticides applications in PEI (unpublished data). Among the possible explanation to these negative results, considering chlorothalonil as an example, is the efficient degradation of the pesticide in the water by hydrolysis and/or photolysis (Putman *et al.*, 2003; Kwon & Armbrust, 2006; Chaves *et al.*, 2008) or rapid elimination after pesticide uptake by the bivalves as observed in blue mussel (Ernst *et al.*, 1991). Nevertheless, exposure to any transitory chemical, parent compound or toxic degradation product of the pesticide could induce subtle adverse effect in the organism. Thus, observation of haemic neoplasia disease (HN) in molluscs around the Island (Delaporte *et al.*, 2008) prompted the investigation of a potential link with pesticides. In fact, the occurrence of HN appears to be slightly correlated to potato acreages in PEI (MacCallum *et al.*, 2003). In the summer of 1999, mortalities of soft-shell clams (*M. arenaria*) (> 90%) caused by, or associated with, HN occurred on experimental aquaculture site and natural populations of Darnley Basin (McGladdery *et al.*, 2001b). HN is a disseminated tumour of clam haemocytes transforming bivalve blood cells from a functional to a non-functional state, leading to tissue necrosis and eventually death of the clam (Kent *et al.*, 1989; Leavitt *et al.*, 1990; Dugan *et al.*, 2002).

The aim of this study was to determine if, under laboratory conditions with chronic exposure, these two major pesticides used in potato production could induce HN in HN-negative soft-shell clams (*M. arenaria*) inhabiting an HN-positive environment. Short-term experiments with acute exposure were also performed to provide quantitative measurement of pesticide uptake by the bivalves and to document tissue persistence of the active ingredients.

2.2.3 Materials and Methods

2.2.3.1 Soft-shell clams

Soft-shell clams for the chronic exposure experiment were collected during June 2005 in North River (46°15'01''N, 63°10'42''O) near Charlottetown (Prince Edward Island, south of St. Lawrence Gulf, Canada). This site was chosen for the high prevalence of HN (Delaporte *et al.*, 2008). The clams for the acute exposure experiment were also collected during June 2005 in the Havres-aux-Maisons lagoon (47°26'19''N, 61°47'34''O) in Magdalen Islands (Gulf of St. Lawrence, Québec, Canada). This site was chosen for the absence of HN (Delaporte *et al.*, 2008). Organisms were transferred to a wet laboratory at University of Prince Edward Island and maintained in aquaria (one aquarium for each population) with flowing sea water (20±2°C and

29±1) two weeks before the beginning of the experiment. During this time, the clams (5 cm) were fed three times a week with a microalgae paste (Innovative Aquaculture Products®) at 3% of clams dry mass. All clams were placed inside of foam to mimic the pressure of the sediment. Inside the foam, a stainless steel nut was place to maintain the clam at the bottom of the tank. For all clams used, the absence of haemic neoplasia was determined by collection of hemolymph and flow cytometry analyses. For the experiments, we used 220 HN-negatives clams (60 clams from Magdalen Islands and 160 clams from North River).

2.2.3.2 Collection of hemolymph and flow cytometry detection of haemic neoplasia

Hemolymph was collected from clams via the adductor muscle sinus, using a 3ml plastic syringe fitted with a 25-gauge needle. An aliquot of 500 µl of hemolymph was prepared according to da Silva et al. (2005), fixed in absolute ethanol (1:5) and stored at -20°C until the analysis. Fixed hemolymph samples were centrifuged to pellet the cells at 400 g for 10 min. Cells were re-suspended, re-hydrated for 30 min and washed twice with saline PBS (0.01 M) before being filtered through a 80 µm nylon mesh while transferring to a flow cytometer tube. Samples were then treated with DNase-Free RNase A (0.005%) and stained with propidium iodide (PI, 0.005%) for 1 h. PI fluorescence, which is related to the DNA content of each cell, was detected on the FL2 detector (orange light, at 550-600 nm) of a FACSCalibur flow cytometer (Becton Dickinson). For each sample, more than 3000 particles were counted at a low flow rate (15 µl min^{-1}).

On a cytogram representing the width and area of PI fluorescence signal, distinction of single normal diploid cells (G0/G1) and neoplastic tetraploid cells (G2/M) from doublets of normal cells were distinguished (Figure 9A). After gating on the single cell region (R1), PI fluorescence of single cells was plotted on a FL2 area histogram on which a specific marker was placed to calculate the number of diploid and tetraploid cells in this region R1 (Figure 9B). Thereafter, as doublets are part of the normal cell population, the percentage of HN cells was calculated as the ratio of number of tetraploid cells to the total of normal cells (single + doublets of cells) multiplied by 100 (Delaporte *et al.*, 2008).

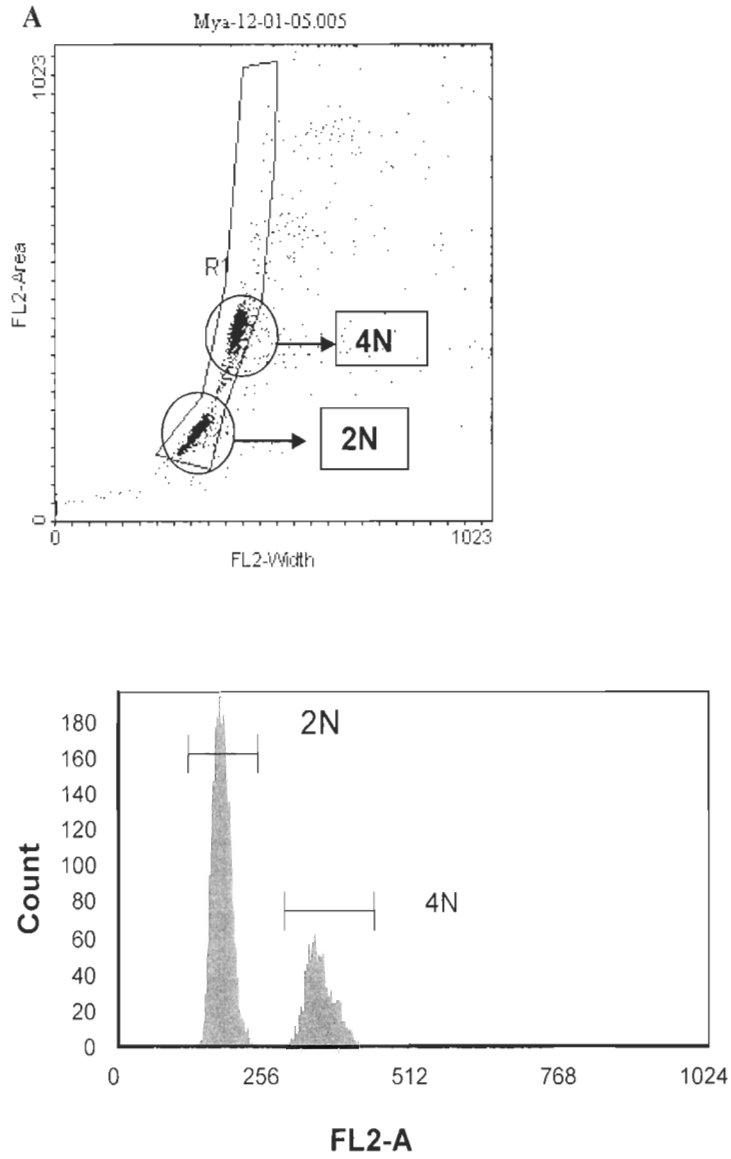


Figure 9. Flow cytometry analysis of diploid (G0/G1) and neoplastic tetraploid (G2/M) cells from a single clam sampled at North River, PEI, Canada. A- Cytogram representing diploid (2N) and tetraploid (4N) single cells on a width vs area FL2. B- Histogram of propidium iodide fluorescence. Markers 2N represent diploid peak and 4N tetraploid peak

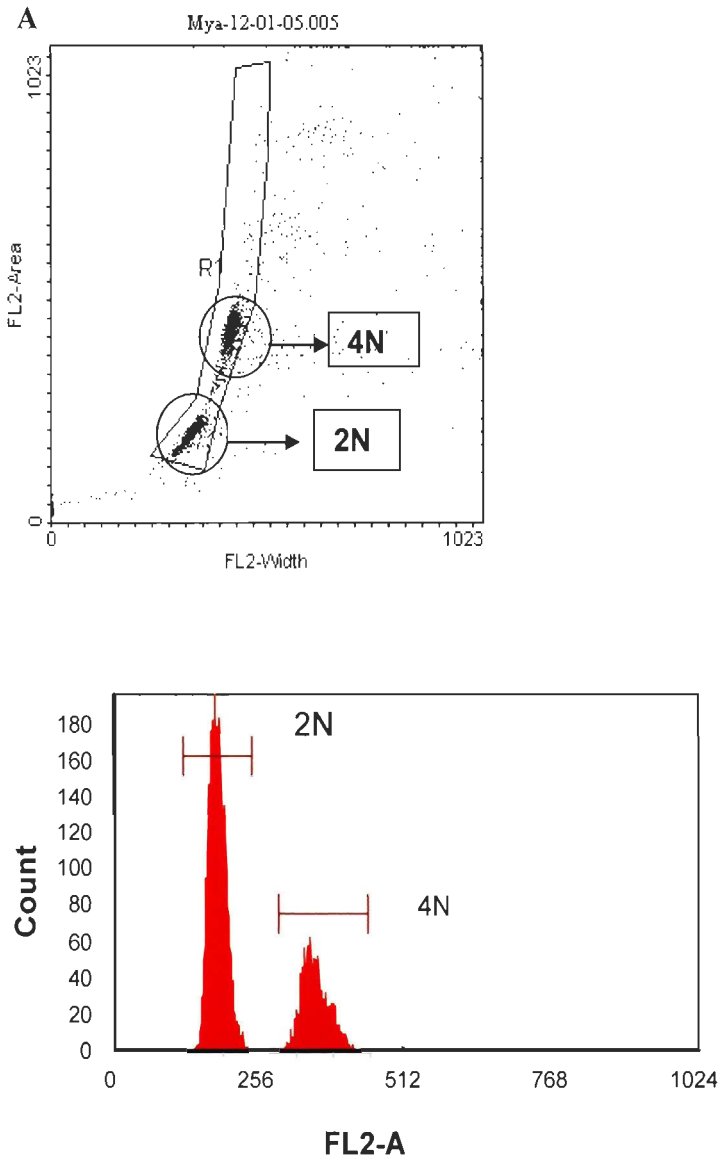


Figure 9. Flow cytometry analysis of diploid (G0/G1) and neoplastic tetraploid (G2/M) cells from a single clam sampled at North River, PEI, Canada. A- Cytogram representing diploid (2N) and tetraploid (4N) single cells on a width vs area FL2. B- Histogram of propidium iodide fluorescence. Markers 2N represent diploid peak and 4N tetraploid peak

2.2.3.3 Chemicals analyses

Tribromoanisole (CAS 607-99-8) that was used as an internal standard and analytical grade chlorothalonil from Riedel-deHaën (CAS 1897-45-6) were purchased from Sigma-Aldrich Canada. Manganese standard solutions were prepared from the multi-element standard solution IV from Fluka (Sigma-Aldrich Canada). Technical formulation of the fungicides Bravo 500® (40% chlorothalonil) and Manzate 200 DF® (75% mancozeb, thus 15% manganese) were obtained from a local supplier. All solvents used were of HPLC grade. High purity OmniTrace nitric acid was purchased from EM Science, Canada. Hydrogen peroxide at 30% was purchased from EMD, USA. High quality water was obtained from a Nanopure UV deionization system, Barnstead/Thermolyne Co. (USA). Helium and nitrogen gas were of HP+ grade.

2.2.3.4 Acute exposure experiment (72 hours)

This short term experiment was conducted to measure the uptake of chlorothalonil and mancozeb from the technical formulation, after exposure to individual pesticide and to a mix of both chemicals, in the soft-shell clams tissues for a period of 72 hours. In June 2005, three shelves at Atlantic Veterinary College (AVC) University of Prince Edward Island (UPEI) were equipped with air supply manifolds to fill individually 60 1.5 L glass jar. These jars were washed with methanol and seawater and then filled with 1l of aerated artificial seawater (20°C and 29 psu). Air flow was put through each air line and the jars were left for one week in order to wash the system. Two days before the beginning of the experiment, one soft-shell clam from Magdalen Islands was added to each jar for a total of 60 clams. The experimental set up was left for two days for the acclimatization of the clam. The experiment began on June 17th and finished on June 20th 2005 without clams being fed or changing water. For the experiment, 20 clams were exposed to each fungicide and 20 clams were exposed to both contaminants for a total of 60 clams. Treated clams received an exposition dose of 2000 $\mu g\ l^{-1}$ of the fungicide (active ingredient alone, or 1000 $\mu g\ l^{-1}$ of each active ingredient in the mixture) diluted with a commercial formulation of seawater solution; the concentration was based on the active ingredient content of the commercial formulation. To measure the uptake of the chemicals, 4 clams were collected (3 clams exposed to the chemical were pooled and the control clam) after 4, 8, 24, 48 and 72 hours.

Water from each jar and the lyophilised mantle and digestive gland of each clam was kept at -20°C until the chemical analysis was done.

2.2.3.5 Chronic exposure experiment (35 days)

This long term experiment was setup to determine if chemicals used in potato production could induce HN in HN-negative soft-shell clams from PEI. The experiment was conducted at AVC at the University of Prince Edward Island between July 27th and September 8th with HN-negative clam from PEI. One week before the beginning of the experiment, 160 glass jars of 1.5 l were washed with methanol and sea water and placed into 3 shelves to verify the stability of the system. Each jar was filled with 1L of aerated artificial seawater (20°C and 29 psu) and air flow was put through each air line. Two days before the beginning of the experiment, one HN-negative soft-shell clam from PEI was added to each jar. The experimental set up was left for two days for the acclimatization of the clam. Treated clams were exposed to fungicides for 35 days, 80 clams were exposed to the commercial formulation of chlorothalonil and 80 clams were exposed to the commercial formulation of mancozeb. Pesticide formulation was dissolved in filtered seawater at an appropriate concentration before dilution in exposure media. For each fungicide, four treatments (20 clams by treatment) were applied randomly: 0 (control), 1, 10 and 100 $\mu g\ l^{-1}$ of active ingredient.

Once a week during the experiment, the jars, the air line and air stone were washed, rinsed with methanol and sea water, then clams were fed with a microalgal paste (Innovative Aquaculture Products® with a quantity of 1 ml per 1 liter) and the new water was contaminated by adding an appropriate volume of the formulated grade pesticide dissolved in seawater. All control clams were fed on the same schedule except they received just sea water. During cleaning, clams were kept on moist paper towel for a maximum of 5 minutes, to avoid any additional stress and to check mortality. A few minutes after reinstallation in the jars, the clams reopened their siphons. At the end of the chronic exposure, hemolymph puncture was obtained for the pathology analysis with the flow cytometer. The organisms were not pooled for chemical analysis; each clam was dissected, the mantle and the digestive gland lyophilised and preserved separately at -20°C until analysis.

2.2.3.6 Treatment of water samples

Liquid-liquid extraction with dichloromethane was used to recover chlorothalonil from the water. A 200 ml aliquot of water was sampled from each exposure vessel; 30 ml of dichloromethane was directly added to the Nalgen bottle. Samples were kept at 4°C until analysis. Before analysis, the dichloromethane fraction was recovered from the water with a 250 ml glass funnel. A second volume of 5 ml of dichloromethane was added to the water sample and after vigorous agitation; the organic fraction was collected and combined to the first. The organic extract was cleaned on a sodium sulphate/florisil column and concentrated to 0.2 ml under a gentle stream of N_2 at room temperature.

Manganese from dissolved mancozeb was analysed directly in a portion of the extracted water diluted to 1:10 with ultrapure water and acidified at 1% with high purity concentrated nitric acid.

2.2.3.7 Treatment of tissue samples

Extraction of chlorothalonil was carried out on 100 mg of lyophilized clam tissue using a Dionex ASE^{TM} 200 Accelerated Solvent Extractor with extraction cell of 11 ml. Cells were filled with diatomaceous earth dispersant (Dionex ASE PREP DE). The experimental conditions were hexane:acetone (50:50) for the extraction solvent at a temperature of 40°C and a pressure of 1500 psi. The samples were extracted by using one cycle of 10 min at the reported conditions. The organic extract was cleaned on a sodium sulphate/florisil column and concentrated to 0.2 ml under a gentle stream of N_2 at room temperature.

Mancozeb (ethylenebis dithiocarbamic acid manganese zinc complex) was not quantified directly; instead the samples were treated for the analysis of manganese. About 40 mg of lyophilized clam tissue was digested in a Teflon tube with a solution of 1 ml of concentrated nitric acid and 1 ml of hydrogen peroxide at 30% for two hours in a water bath at 50°C. After digestion was completed, the concentrated solution was transferred to a Nalgen volumetric flask and diluted to the 50 ml mark with ultrapure water.

2.2.3.8 Analysis of chlorothalonil by Gas Chromatography/Mass Spectrometry (GC-MS)

Chemical analysis was performed at the Laboratoire de Chimie Marine et Spectrométrie de Masse at the Institut des Sciences de la Mer de Rimouski (ISMER). Organic extracts were analysed on a Polaris Q ion trap coupled to a Trace GC (ThermoFinnigan) equipped with a RTX-5MS ® fused silica capillary column (30 m x 0.32 mm i.d; Restek®) with high purity helium as a carrier gas. Tribromoanisole was used as an internal standard. Detection and quantification of chlorothalonil were performed in MSMS scan mode with the following positive ions as precursor: m/z = 266 for chlorothalonil, m/z = 275 for pentachlorobenzonitrile, m/z = 230 for trichlorothalonil and m/z = 329 for tribromoanisole. Control of the system, acquisition and data processing were conducted with the Xcalibur® software (revision 1.3). A seven point's calibration curve was applied with a concentration range from 0.015 μg ml^{-1} to 0.97 μg ml^{-1}. Based on the lowest concentration used in the calibration curve and the final volume of the organic extract, the limit of quantification of chlorothalonil in water was 15 ng l^{-1} and 30 ng g^{-1} (dry weight) in tissues. Relative standard deviation on the analysis of chlorothalonil in water (n = 3) and tissues (n = 4) was 12% for both.

2.2.3.9 Analysis of manganese by Inductively Coupled Plasma-Mass Spectrometer

Chemical analysis was performed at the Laboratoire de Chimie Marine et Spectrométrie de Masse at the Institut des Sciences de la Mer de Rimouski (ISMER). Manganese in aqueous solution (acidified seawater or digested tissues) was analysed by inductively coupled plasma linked to a quadrupole mass spectrometer (ICP-MS, Agilent 7500c) equipped with a micro flow nebuliser. The metal quantification was performed in normal mode with a seven point's calibration plot with a concentration range from 0.125 ng ml^{-1} to 100 ng ml^{-1}. Performance of the method was assessed by the analysis of the certified reference material DOLT-2 (National Research Council of Canada) and procedural blank. Control of the system, acquisition and data processing were conducted with the Agilent 7500 ICP-MS ChemStation® software (revision C). Based on the lowest concentration used in the calibration curve and the final volume of dilution, the limit of quantification of manganese in water was 1.25 μg l^{-1} and 150 ng g^{-1} (dry weight) in tissues. Relative standard deviation on the analysis of manganese in water (n = 3) was 3%. The measured value of manganese in the reference material DOLT-2 (n = 6) was 6.19 ± 0.78 μg g^{-1}

which is within the certified value of 6.88 ± 0.56. The relative standard deviation on the analysis of manganese in tissues by our protocol was 12%.

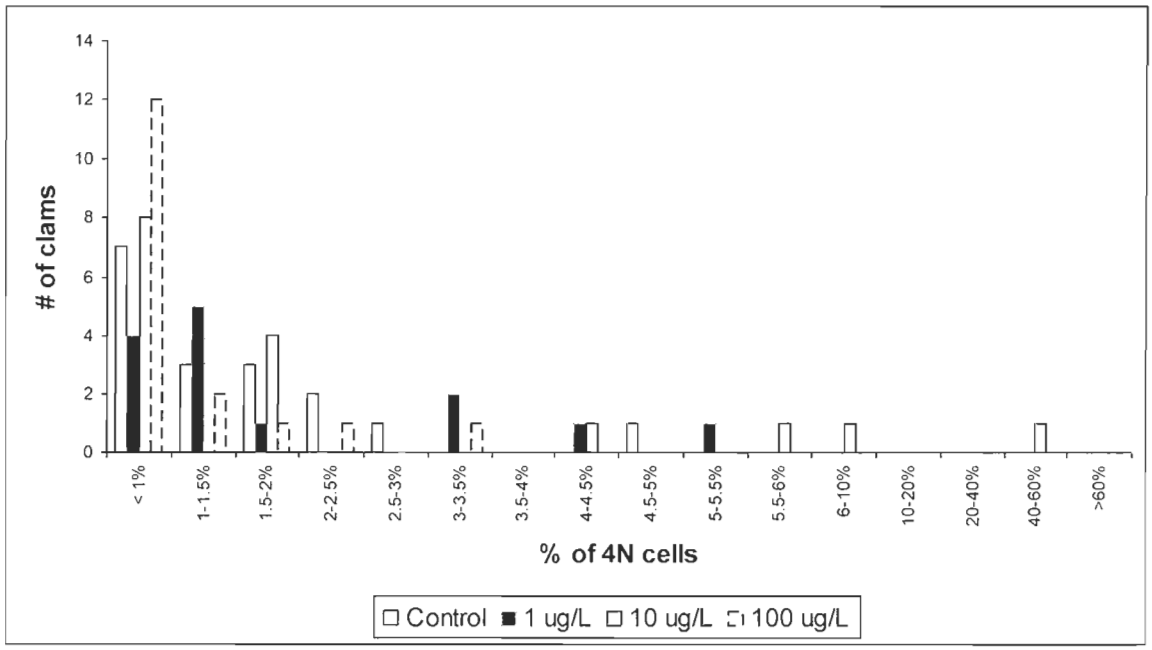
2.2.4 Results

2.2.4.1 Chronic exposure experiment

The experiments had limited impact on the mortality. For the two fungicides, the mortality was generally higher in the first week with an average of ±10%. At the end of experiment mean mortality observed was 20%. The highest percentage of mortality rate (35%) for Bravo 500® (chlorothalonil) was observed at the 1 μg l^{-1} concentration at 35% and for Manzate 200DF (mancozeb) it was observed at the 10 μg l^{-1} concentration and the mortality was 30%.

Long term exposure to the fungicide Bravo 500®, in general, did not induce high tetraploid level (neoplasia) on negative clams from North River, except for one clam which had 58% 4N cells (Figure 10A). The analysis of the digestive gland and the mantle did not reveal any detectable level of chlorothalonil (results not shown).

In the Manzate 200 DF® exposure experiments, some clams from the control and higher contaminant concentrations (100 ug l^{-1}) revealed a positive state of haemic neoplasia (high level of tetraploid cells) at the end of exposure (Figure 10B) but no difference was observed between the treatments and the control. The analysis of the digestive gland and the mantle for manganese did not highlight any significant difference (two-tailed test, p=0.05) in tissue concentration between the exposed and control animals (Table 1).



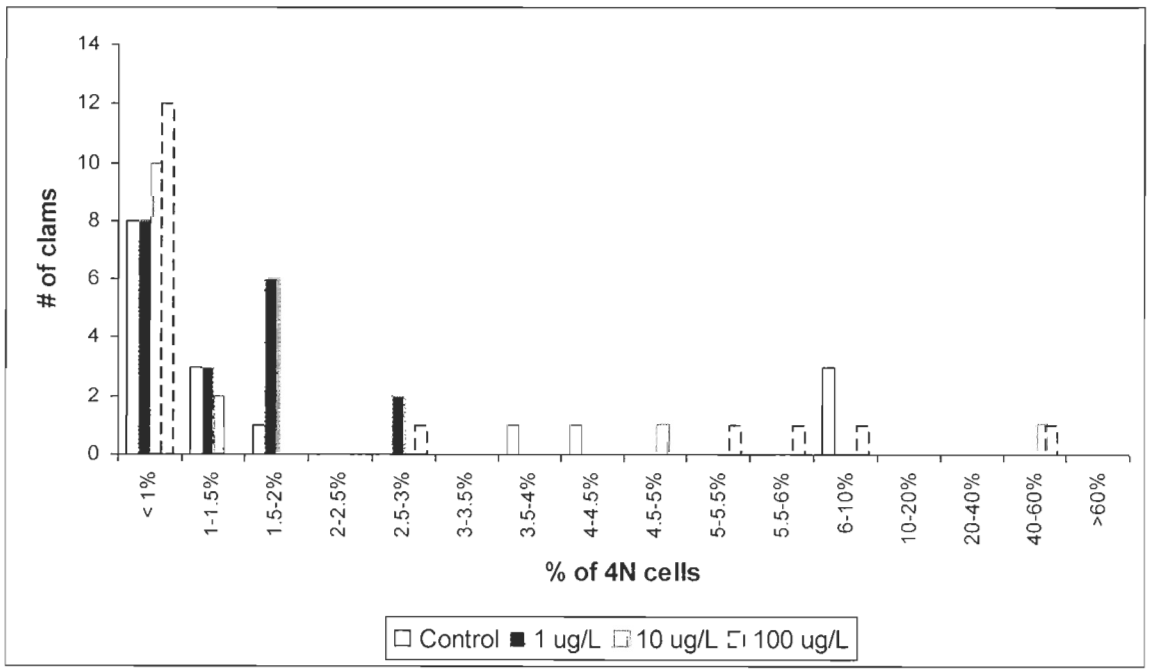


Figure 10. Observation of haemic neoplasia level (tetraploid level) in soft-shell clams of North River (PEI). A- Clams exposed to Bravo 500® for 35 days N=80. B- Clams exposed to Manzate 200 DF® for 35 days N=80

2.2.4.2 Acute exposure experiment

2.2.4.2.1 Pesticides in water

In the short term experiments three exposure regimes were used: Bravo 500® alone (chlorothalonil at 2 mg l^{-1}), Manzate 200 DF® alone (mancozeb at 2 mg l^{-1} with a content of 15 % of manganese), and a mix of both fungicides at 1 mg l^{-1} of each active ingredient. During these three day experiments, all 60 clams survived. The first water samples were collected 4 h after the introduction of the chemicals. At this time, the aqueous concentration of chlorothalonil in both experiments was already decreased to less than 20% of the calculated level at the beginning of exposure (Figure 11A). After 24 h, the aqueous concentration of chlorothalonil with and without mancozeb followed the same trend and reached a steady level at about 5 % of initial concentration. Thus, a rapid decrease in the aqueous concentration of the organic fungicide occurred in the first 4 h; fast uptake by the bivalve and adsorption to the glass wall due to low solubility are probable contributing factors to this observation.

Table 1. Manganese tissues concentration in clams exposed to Manzate 200 DF pesticide for 35 days. Concentrations are express as $\mu g.g^{-1}$ dry weight

Tissues/treatment	N[a]	Range	Mean ± sd	Comparison with control (P = 0.05)
Hepatopancreas				
Control	8	4.0 – 38.9	15.3 ± 13.6	-
1 $\mu g\ l^{-1}$	7	3.4 – 97.6	26.0 ± 37.4	NS
10 $\mu g\ l^{-1}$	10	2.8 – 124.8	18.0 ± 37.6	NS
100 $\mu g\ l^{-1}$	6	4.6 – 17.9	11.6 ± 7.0	NS
Mantle				
Control	8	19.9 – 571.8	226.2 ± 219.9	-
1 $\mu g\ l^{-1}$	6	24.9 –1100.0	430.5 ± 500.3	NS
10 $\mu g\ l^{-1}$	7	18.4 – 933.2	373.2 ± 414.9	NS
100 $\mu g\ l^{-1}$	4	7.9 – 104.6	55.0 ± 40.2	NS

Note[a]: number of individual clam analyzed.

2.2.4.2 Acute exposure experiment

2.2.4.2.1 Pesticides in water

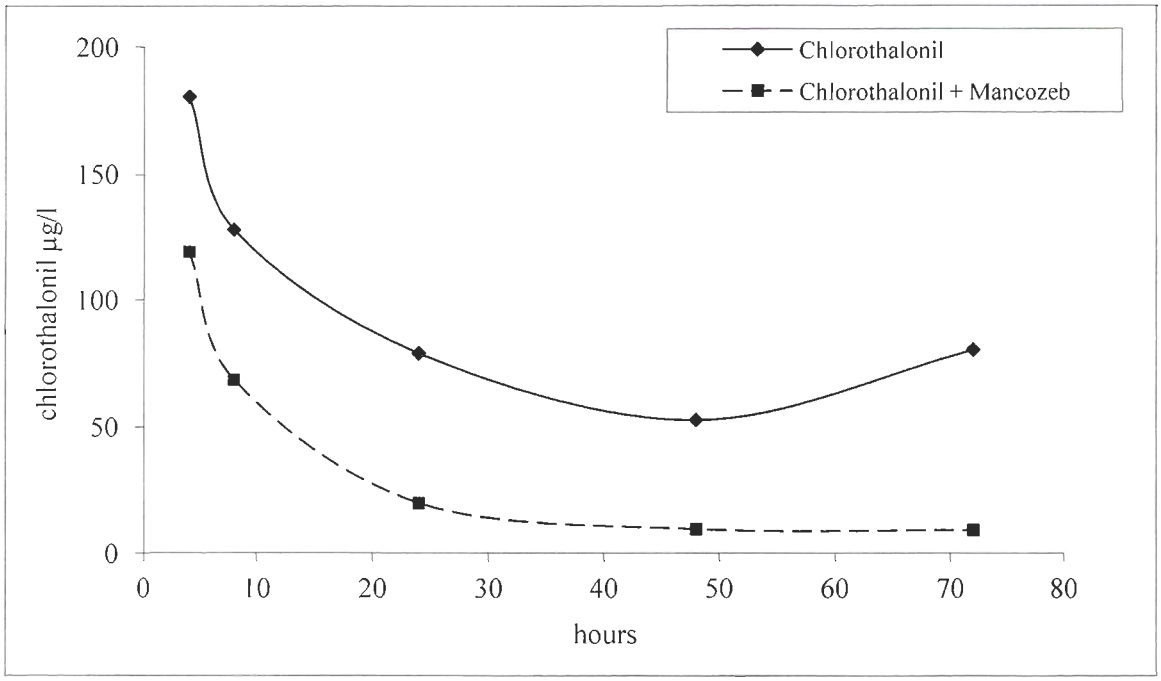
In the short term experiments three exposure regimes were used: Bravo 500® alone (chlorothalonil at 2 mg l^{-1}), Manzate 200 DF® alone (mancozeb at 2 mg l^{-1} with a content of 15 % of manganese), and a mix of both fungicides at 1 mg l^{-1} of each active ingredient. During these three day experiments, all 60 clams survived. The first water samples were collected 4 h after the introduction of the chemicals. At this time, the aqueous concentration of chlorothalonil in both experiments was already decreased to less than 20% of the calculated level at the beginning of exposure (Figure 11A). After 24 h, the aqueous concentration of chlorothalonil with and without mancozeb followed the same trend and reached a steady level at about 5 % of initial concentration. Thus, a rapid decrease in the aqueous concentration of the organic fungicide occurred in the first 4 h; fast uptake by the bivalve and adsorption to the glass wall due to low solubility are probable contributing factors to this observation.

Table 1. Manganese tissues concentration in clams exposed to Manzate 200 DF pesticide for 35 days. Concentrations are express as $\mu g.g^{-1}$ dry weight

Tissues/treatment	N[a]	Range	Mean ± sd	Comparison with control (P = 0.05)
Hepatopancreas				
Control	8	4.0 – 38.9	15.3 ± 13.6	-
1 $\mu g\ l^{-1}$	7	3.4 – 97.6	26.0 ± 37.4	NS
10 $\mu g\ l^{-1}$	10	2.8 – 124.8	18.0 ± 37.6	NS
100 $\mu g\ l^{-1}$	6	4.6 – 17.9	11.6 ± 7.0	NS
Mantle				
Control	8	19.9 – 571.8	226.2 ± 219.9	-
1 $\mu g\ l^{-1}$	6	24.9 –1100.0	430.5 ± 500.3	NS
10 $\mu g\ l^{-1}$	7	18.4 – 933.2	373.2 ± 414.9	NS
100 $\mu g\ l^{-1}$	4	7.9 – 104.6	55.0 ± 40.2	NS

Note[a]: number of individual clam analyzed.

The change in manganese concentration in water with time showed a rapid decrease from the initial level as was for chlorothalonil (Figure 11B). If we suppose that manganese remained associated with the dithiocarbamate structure then the aqueous concentration of manganese allowed the calculation of mancozeb in water. After 4 h following the introduction of Manzate 200 DF, the water concentration of mancozeb dropped to less than 10 % of the initial dose with or without Bravo 500®. However, after 8 h, a 50% increase of the mancozeb water concentration was observed under both conditions. Afterward, for both exposure regimes, the water concentration of mancozeb decreased steadily until the end of experiment to less than 5 % of the initial concentration. From this data, it seems that in the first 8 h of exposure, uptake of mancozeb occurred; followed by a release of manganese, as a free ion or associated with organic molecules.



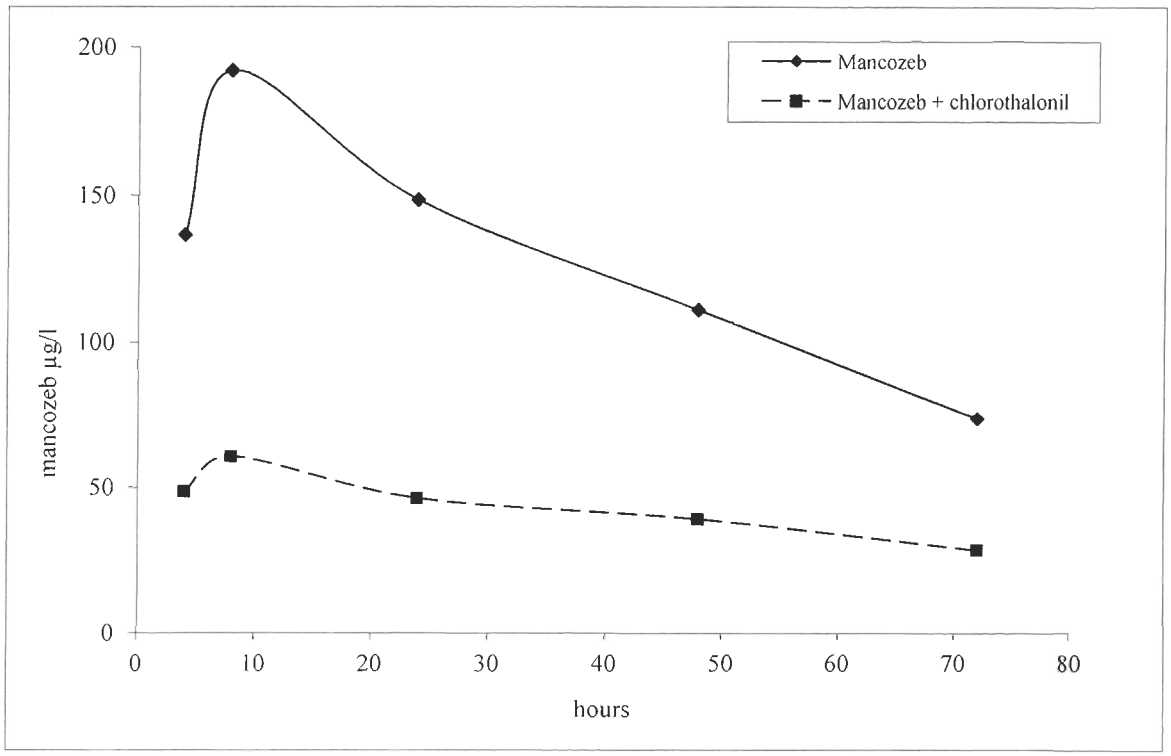


Figure 11. Evolution of fungicides concentrations in water with time; A- chlorothalonil, B- mancozeb. Concentrations are expressed as $\mu g\ l^{-1}$

2.2.4.2.2 Pesticides in tissue

GC-MS analysis of Bravo 500® (Figure 12) showed that about 0.5 % of the active ingredient is distributed between four chlorinated compounds: an isomer of chlorothalonil eluting at a retention time of 10.68 min (mass spectrum and chemical structure I), a pentachlorobenzonitrile with five chlorine atoms with a retention time of 10.59 min (pentachlorothalonil, mass spectrum and chemical structure II) and two isomers of benzodinitrile substituted with three chlorine atoms (trichlorothalonil, mass spectrum and chemical structure III) eluting at a retention time of 9.83 and 10.02 min respectively. Based on the intensity of the signal for each chlorinated compound in the commercial formulation, the relative contribution to the active ingredient was: chlorothalonil isomers 99.5 %, pentachlorothalonil 0.4 % and the trichlorothalonil isomers 0.1%. The hydrophobicity of these molecules depends on the number of chlorine atom on the benzene ring; thus the increasing order of hydrophobicity should be

trichlorothalonil, chlorothalonil and pentachlorothalonil which translate in calculated logP value of 3.78, 4.33 and 4.86 respectively (calculated with the LogP calculator module from ChemOffice Ultra, version 8, CambridgeSoft, 2004). Compound with the highest LogP value should be more prone to bioaccumulation in lipid-rich tissues.

In the bivalves exposed to Bravo 500® alone, chlorothalonil and its co-products were already measurable in tissue after 4 h of exposure (Figure 13). At this time, the level of the main active ingredient, chlorothalonil, was at least 10 times higher in the mantle than in the digestive gland (Figure 13A). Whereas the chlorothalonil level remained low and constant in the digestive gland, large variations were observed in the mantle. In this tissue, the highest level was measured at 48 h with a value of 59 $\mu g\ g^{-1}$. After 72 hours, the tissue concentration in the mantle decreased to the level measured in the digestive tissue (around 3 $\mu g\ g^{-1}$). For trichlorothalonil (Figure 13B), the tissue concentration increased with time in both mantle and digestive gland up to a maximum value observed at 48 and 24 h respectively, followed by a progressive elimination process. The highest trichlorothalonil level was measured in the digestive gland with a concentration of 8.2 $\mu g\ g^{-1}$, whereas for chlorothalonil and pentachlorothalonil the highest level was observed in the mantle. For pentachlorothalonil, accumulation increased in the mantle up until 48 h of exposure but no elimination was apparent after 72 h (Figure 13C) with a steady concentration of about 2 $\mu g\ g^{-1}$. In the hepatopancreas, the highest level was measured after 4 h and afterward the pentachlorothalonil concentration decreased to an undetectable level between 24 and 48 h of exposure.

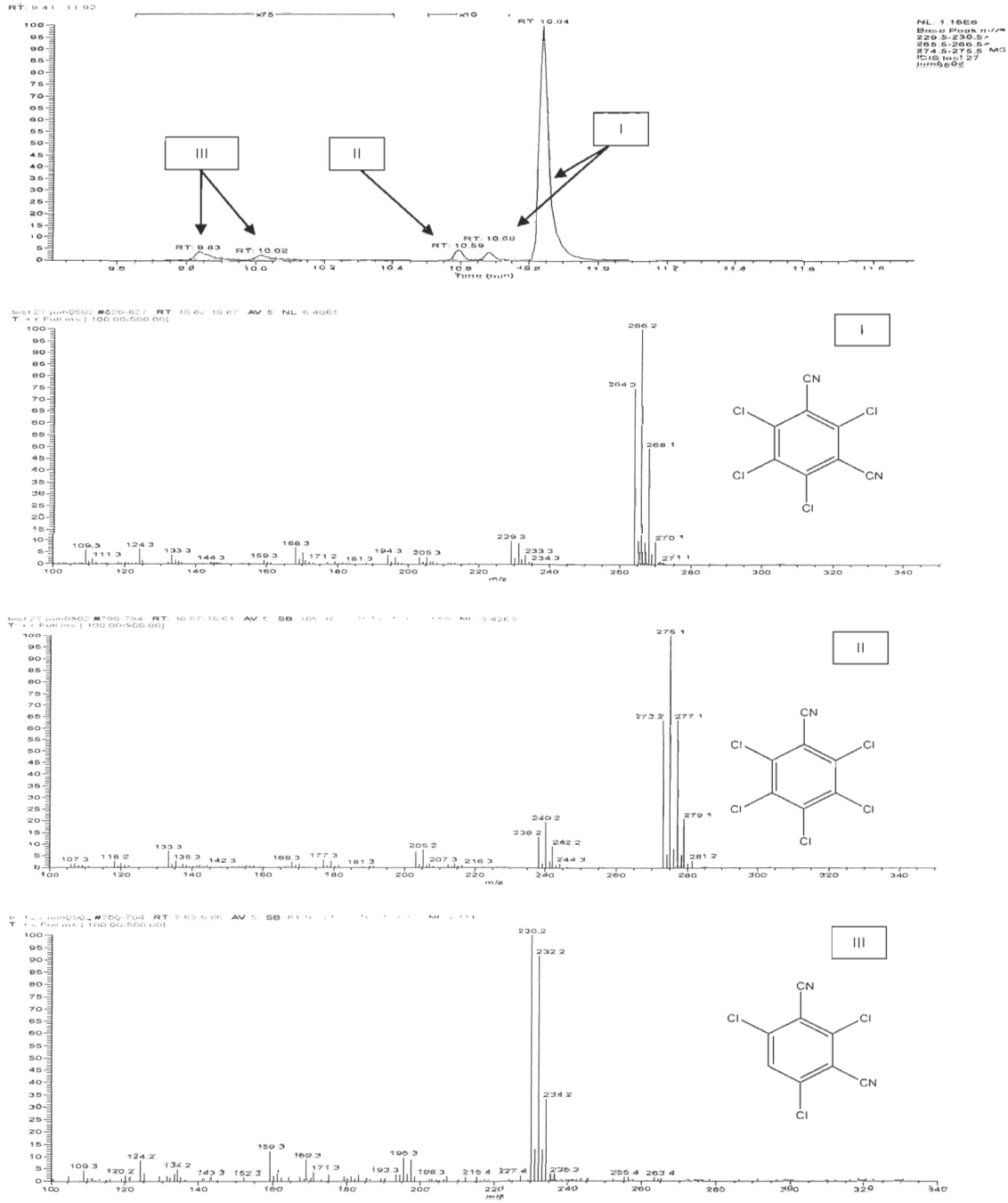


Figure 12. GC-MS analysis of Bravo 500® with detection of positive ions in full scan mode. Upper drawing is the chromatogram from a 30 µg ml^{-1} solution of Bravo 500® diluted in dichloromethane. I: mass spectrum of chlorothalonil with M^+ = 266; II: mass spectrum of pentachlorobenzonitrile with M^+ = 275; III: mass spectrum of trichlorobenzodinitrile with M^+ = 230

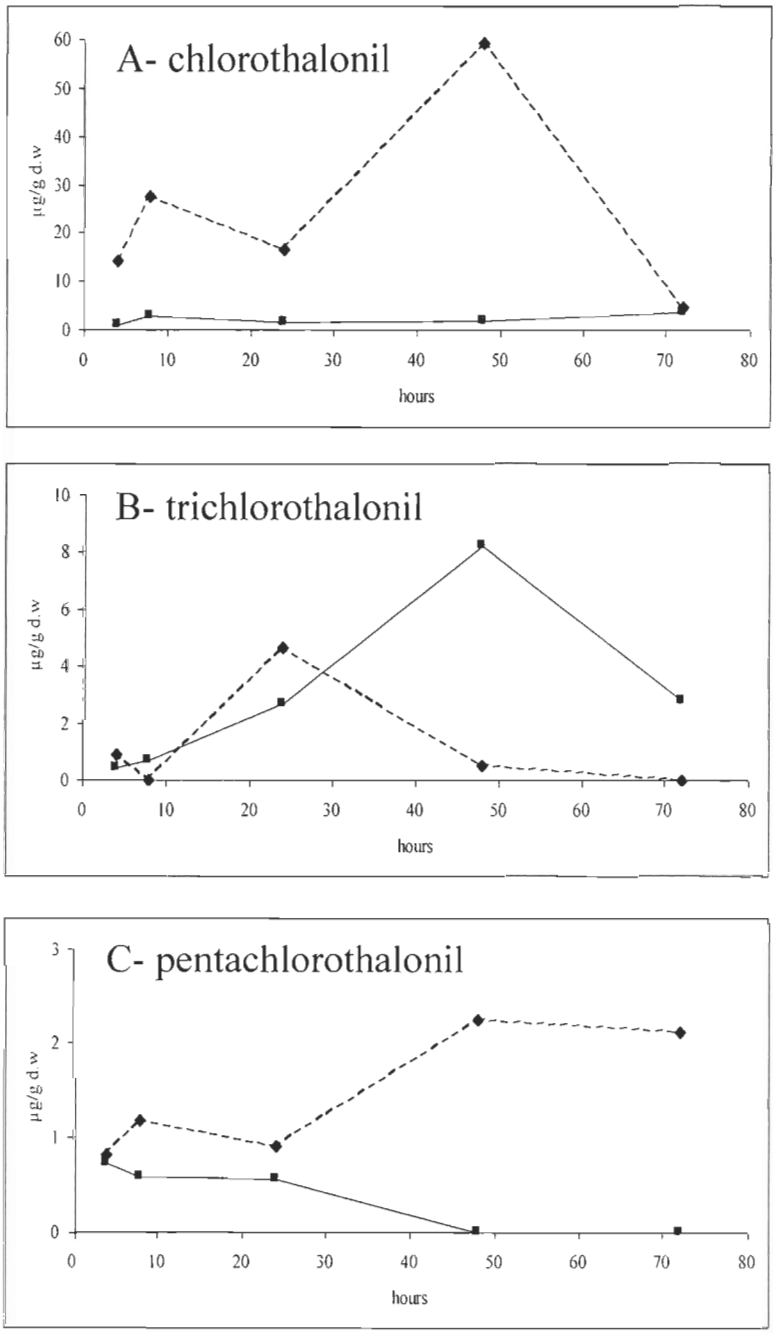


Figure 13. 72 hours accumulation of chlorothalonil and co-products by soft-shell clams exposed to Bravo 500® alone. Concentrations are expressed as µg g^{-1} dry weight

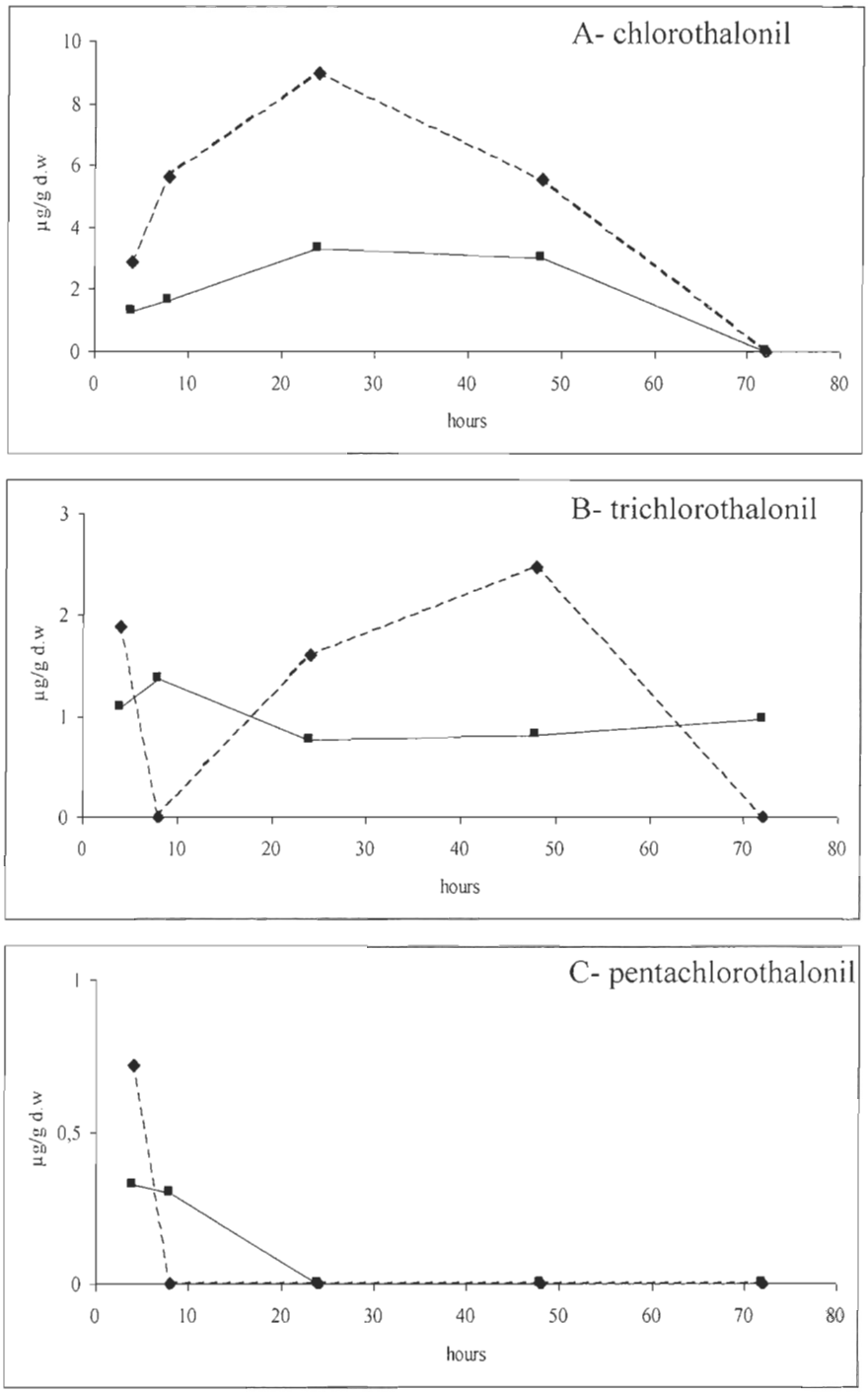
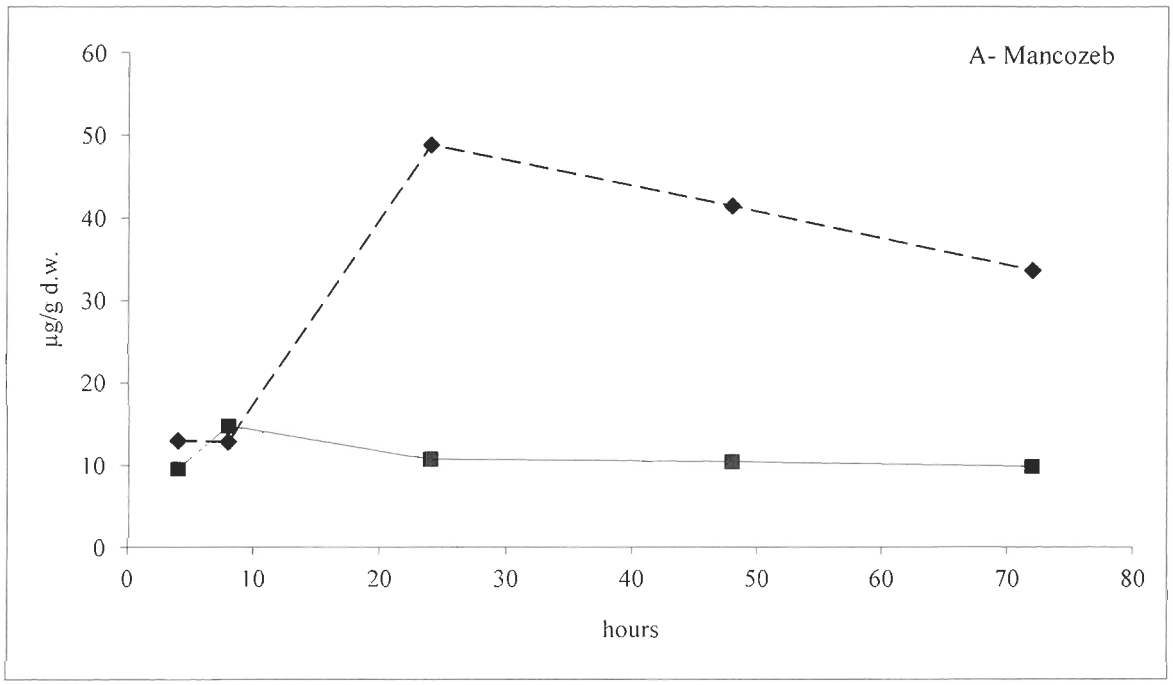


Figure 14. 72 hours accumulation of chlorothalonil and coproducts by soft-shell clams exposed to Bravo 500® and Manzate 200 DF®. Concentrations are expressed as µg g^{-1} dry weight

When the organisms were exposed to the mixture of fungicides, chlorothalonil and mancozeb, chlorothalonil and its co-products were also detectable after 4 h (Figure 14). At this time, tissue levels of chemicals were similar to those observed under exposure to chlorothalonil alone, except for chlorothalonil in the mantle for which tissue concentration was five times less than the value observed at 4 h of exposure to chlorothalonil alone (2.9 $\mu g\ g^{-1}$ *vs* 14.2 $\mu g\ g^{-1}$, Figure 14A and 13A). The presence of mancozeb seems to have affected the uptake of chlorothalonil and its co-products beyond eight hours of exposure; the main difference being the lower maximum value measured for each chemical in both tissues (Figure 14A-C). For example, the highest level of chlorothalonil was 9 $\mu g\ g^{-1}$ in the mantle at 24 h (Figure 14A) compared to 59 $\mu g\ g^{-1}$ at 48 h (Figure 13A). Pentachlorothalonil was undetectable after 8 h (Figure 14C) and trichlorothalonil was the only chlorinated species still measurable at 72 h, in the hepatopancreas (Figure 14B). Reported in Table 2 are the tissue concentrations of chlorothalonil and its co-products at the highest level of chlorothalonil in the mantle. After 48 h of exposure to Bravo 500® alone, the active ingredient accumulated in the digestive gland of the bivalves consisted of chlorothalonil and trichlorothalonil for a total concentration of 10 $\mu g\ g^{-1}$. Although the trichlorothalonil isomers accounted for only 0.1 % of the fungicide in the commercial formulation they contribute to 80 % of the accumulated chlorinated compounds. In the mantle, the three compounds were present after 48 h for a total concentration of 61.9 $\mu g\ g^{-1}$ and chlorothalonil represented 95.6 % of the accumulated fungicides whereas the proportion of trichlorothalonil was less than 1 %. Pentachlorothalonil contributed to 3.6 % of the pesticide measured in the mantle tissue, a five-fold increase compared to its proportion in the commercial formulation. Under co-exposure with Manzate 200 DF, chlorothalonil reached its highest level in the mantle after 24 h but with a lower total concentration of chlorinated fungicides of 10.6 $\mu g\ g^{-1}$, with a corresponding concentration in the digestive gland of 4.1 $\mu g\ g^{-1}$. In both tissues, chlorothalonil accounted between 80% and 85 % of the active ingredient with trichlorothalonil isomers as the only co-products.

Manganese is an essential metal to animals and it is normally measurable in the tissue. For clarity, we omitted the manganese levels from the control animals in Figure 15A and 15B; there was no difference in the concentrations of manganese in the hepatopancreas between the control and the exposed clams ($p = 0.05$; $n = 5$ for each group). The manganese concentration in the

mantle of clams exposed to Manzate 200DF® alone reached its highest level after 24 h (Figure 15A) at a concentration of 48.8 μg g^{-1} and then decreased to a concentration of 33.6 μg g^{-1} at 72 h, a concentration still twice that of the control (16.5 ± 5.7 μg g^{-1}, n = 3 from 24 to 72 h). However, this is not observed in the tissue of clams exposed to both fungicides; manganese level in the mantle showed a slow increase with time and was different from the control only after 72 h (Figure 15B) with a manganese concentration of 35.4 μg g^{-1} compared to the mean manganese concentration in the mantle of 15.5 ± 5.5 μg g^{-1} (n = 5) in the control bivalves.



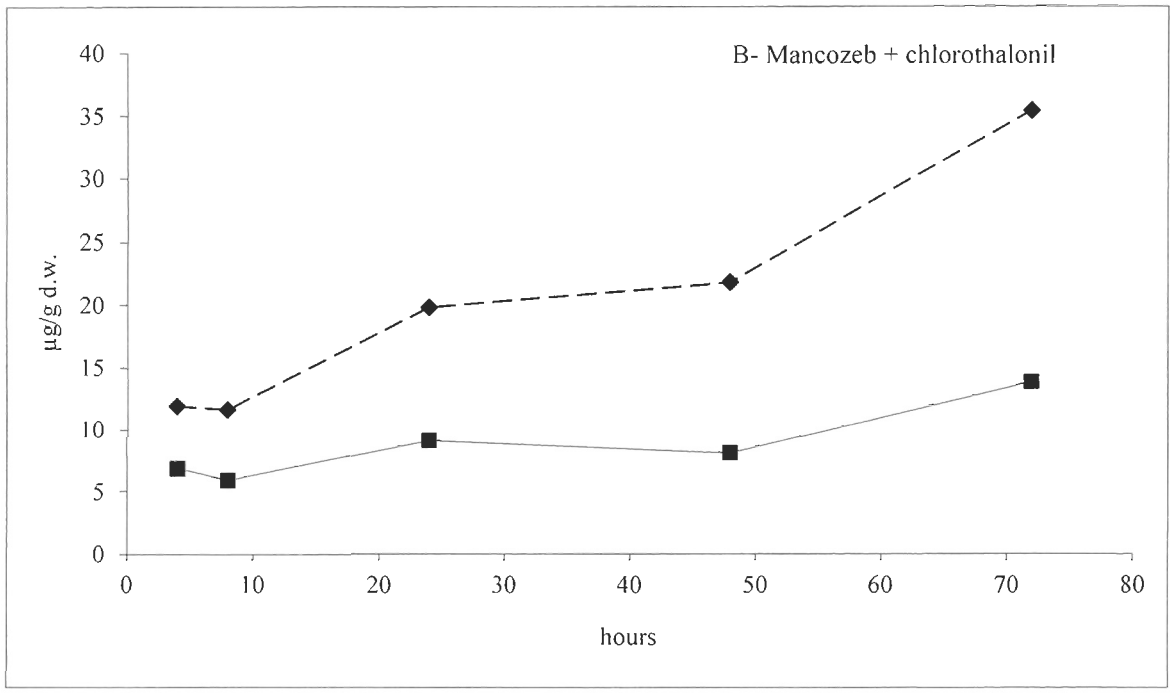


Figure 15. 72 hours accumulations of manganese by soft-shell clams in exposed to A- Manzate 200 DF® alone and B- to Bravo 500® and Manzate 200 DF®. Concentrations are expressed as µg g^{-1} dry weight

2.2.5 Discussion

The causes of HN are unknown although many causative factors have been suggested: retrovirus agent (Romalde *et al.*, 2007), biotoxins from harmful algae (Landsberg, 1996), and pollution with anthropogenic substances like polychlorinated biphenyls and ethyl methanesulfonate (Reinisch *et al.*, 1984; Farley *et al.*, 1991; Craig *et al.*, 1993; Harper *et al.*, 1994; Gardner, 1994; Dopp *et al.*, 1996; Strandberg *et al.*, 1999; Krishnakumar *et al.*, 1999), petroleum compounds (Yevich & Barszcz, 1976; Brown *et al.*, 1977; Harshbarger *et al.*, 1979; Appeldoorn *et al.*, 1984), polycyclic aromatic hydrocarbons (Mix, 1986; Volety *et al.*, 1997), heavy metals and domestic sewage (Brown *et al.*, 1977; Oliver *et al.*, 1998) and herbicides (van Beneden, 1994). The aetiology of haemic neoplasia in soft-shell clams probably consists of many factors: host (genetic, physiology, immunology), pathologic agents (virus, biotoxins) and the environment (pollution, oxygen, salinity, temperature). In our study, we tested the hypothesis that contamination of the marine environment has a causative factor due to the high correlation already established between chemical pollution and neoplasia in bivalves on the east coast of

North America (Elston & Moore, 1992; Harper *et al.*, 1994) and because of the high percentage of HN in *M. arenaria* found around the province of Prince Edward Island (MacCallum *et al.*, 2003; Delaporte *et al.*, 2008) where agriculture relies heavily on pesticides. However, HN-negative soft-shell clams collected in an HN-positive environment did not develop haemic neoplasia after 35 days of chronic exposure to chlorothalonil or mancozeb, the two most used fungicides on PEI, added as commercial formulation (Bravo 500® and Manzate 200DF®, respectively) in seawater, under laboratory conditions (Figure 10). A similar result with no HN induction by chemicals was also reported by Krishnakumar *et al.*, (1999) when they exposed the common mussel *Mytilus edulis* to polyaromatic hydrocarbons or polychlorinated biphenyls for up to 180 days. These authors assessed the presence of the disease in mussels by histology and hematocytology whereas the method of HN determination used in these trials was based on flow cytometry. The first method involves visual inspection of the cells and knowledge of the different stages of haemic neoplasia by the observator, thus positive results rely on well developed adverse effects in haemocytes. HN determination by flow cytometry is an automated process based on DNA content of the affected cells, the method is more sensitive and 1000 of cells per sample are assessed. But, with both methods, we still do not know what cellular mechanisms are possibly triggered by chemicals upstream of neoplasia. One such mechanism is the alteration of the phosphoprotein p53 cell repair function in the nucleus by oxidative-stress (Macip *et al.*, 2003). It is well known in humans that p53 is involved in tumorigenesis (Hofseth *et al.*, 2004). Brzuzan et al. 2006, developed a real-time PCR assay to quantify p53 gene in the whitefish (*Coregonus lavaretus*) using benzo [*a*] pyrene (B[*a*]P); a polycyclic aromatic hydrocarbon find to be a putative p53-inducer. The results demonstrate that p53 is moderately inducible by B[*a*]P in the whitefish. In presence of HN, a study by Barker *et al.*, (1997) showed that clams presented mutations in p53 expression. More recently, Walker *et al.*, (2006), revealed that p53 and p73 seems to be confined in the cytoplasm by mortalin in the presence of HN clams. Also, Walker *et al.*, (2006) and Siah *et al.*, (2008a), showed a good correlation between p53 and mortalin gene expression. Results presented by Siah *et al.*, (2008a), showed a relationship between haemocyte ploidy and p53, p73 gene expression patterns in soft-shell clams with HN. The haemocytes of some clams with moderate level of 4N cells (15-50%) have a significantly ($p \leq 0.05$) high level of p53 and p73 in comparison with clams in the others categories. Some studies with humans,

suggest that high levels of p53, p73 and mortalin genes expressions might be used as a checkpoint for haemocytes who become tetraploid (Toledo & Wahl, 2006 in Siah *et al.*, 2008a).

It has been demonstrated that chlorothalonil and mancozeb interact with reactive oxygen species production mechanisms (Bai-Anderson & Anderson, 2000; Calviello *et al.*, 2006; Domico *et al.*, 2007), thus the sequence of cellular events leading to haemic neoplasia could have been triggered in the bivalves but, we suggest that the duration of exposure was too short to observe its pathology. Persistent bioaccumulation of fungicides was not observed at the end of the chronic exposure. No accumulation of chlorothalonil or its co-products were detected in the mantle tissue nor in the digestive gland of exposed bivalves, and no difference in manganese concentration was measurable in tissue between the organisms from treatment with mancozeb and the control (Table 1). It is likely that chemical uptake occurred during the chronic exposure experiments, because chlorothalonil accumulation in mantle tissue and the digestive gland was demonstrated by the short-term experiments with initial nominal concentration of 2000 $\mu g\ l^{-1}$ and 1000 $\mu g\ l^{-1}$ (Figure 13 and 14). The tissue concentration of chlorothalonil of 59.2 $\mu g\ g^{-1}$ dry weight in the mantle of the soft-shell clams after 48 h of exposure to Bravo 500® alone (Figure 13A) is, to our knowledge, the highest level reported in a marine invertebrate under laboratory conditions. Overall, the accumulation was rapid, within 4 h, but transitory as chlorothalonil tissue concentration decreased to low or undetectable level after 72 h although the compound was still present in the water (Figure 11A), indicating a higher rate of elimination/transformation than accumulation. A similar observation was reported for blue mussels *Mytilus edulis* in which chlorothalonil reached its maximum tissue concentration, about 6 $\mu g\ g^{-1}$ dry weight estimated from the reported wet weight concentration, within 24 h and thereafter being almost undetectable from 96 h of exposition to a mean aqueous concentration of 168 $\mu g\ l^{-1}$ (Ernst et al. 1991). In our long-term experiments with chlorothalonil the highest concentration added was 100 $\mu g\ l^{-1}$ of active ingredient; although exposure medium was renewed once a week, the animals were collected seven days following the last renewal and thus had time to eliminate the fungicide or transform it into metabolites not quantifiable by our analytical method.

The analyses of Bravo 500® by GC-MS showed that chlorothalonil constitutes more than 99 % of the active ingredient and that the two other compounds were present as co-products: pentachlorobenzonitrile (0.4 %) with five chlorine atoms and two trichlorobenzodinitrile isomers (0.1%) with three chlorine atoms (Figure 13). Change in the number of chlorine atoms modifies

the chemical properties of the molecule; pentachlorobenzonitrile should be more fat-soluble due to its higher content of chlorine whereas trichlorobenzodinitrile should be more hydrophilic. This could explain why a dramatic change in the proportion of the chlorinated compounds occurred upon uptake by the soft-shell clams and redistribution in tissue (Table 2). At 48 h of exposure to Bravo 500® alone, the proportion of trichlorobenzodinitrile (trichlorothalonil) in the hepatopancreas was 82 % of the tissue content in chlorinated pesticides; this proportion falls to 0.5 % in the mantle. At 72 h, in the mantle, only chlorothalonil and pentachlorothalonil were detected in proportion of 68.5% and 31.5 % respectively. However, distribution of chlorinated compounds between tissues was not constant; the main observations from Figures 13 and 14 are preferential accumulation of chlorothalonil and pentachlorothalonil in the mantle and persistence of trichlorothalonil in the digestive gland at 72 h. At a concentration of 8.2 $\mu g\ g^{-1}$ dry weight (approximately 1.5 $\mu g\ g^{-1}$ wet weight) in the digestive gland, almost all trichlorothalonil introduced initially in the seawater (2 µg calculated from the nominal quantity of chlorothalonil in seawater (1 l) of 2000 µg) was bioaccumulated by the bivalves after 48 h. As trichlorothalonil has the lowest logP value (3.78) of the three molecules, hydrophobicity cannot explain this high uptake; a more active transfer mechanism should be involved. Another possible explanation is the transformation of chlorothalonil by metabolic processes or associated bacteria in the soft-shell clams; dechlorination of chlorothalonil to trichlorothalonil has been reported in soil and attributed to microbial action (Putnam *et al.*, 2003). Our results on chlorothalonil co-products uptake by soft-shell clams indicate that toxic effects of these compounds should also be investigated when organisms are exposed to Bravo 500®. With an estimated annual use of 125 000 kg of chlorothalonil in Atlantic Canada (Brun *et al.*, 2007), 125 kg of trichlorothalonil and 500 kg of pentachlorothalonil are potentially introduced in the environment each year.

Table 2. Chlorothalonil and co-products tissue concentration in clams at the highest level of chlorothalonil in mantle. Concentrations are express as μg g^{-1} dry weight, percentage of total concentration is reported in parenthesis

Tissues/treatment	chlorothalonil	trichlorothalonil	pentachlorothalonil	total
Chlorothalonil 48hrs				
hepatopancreas	1.8 (18)	8.2 (82)	nd	10.0
mantle	59.2 (95.6)	0.5 (0.8)	2.2 (3.6)	61.9
Chlorothalonil + mancozeb, 24hrs				
hepatopancreas	3.3 (80.5)	0.8 (19.5)	nd	4.1
mantle	9.0 (84.9)	1.6 (15.1)	nd	10.6

Since the manganese content of the mantle tissue increased under exposure to Manzate 200DF (Figure 15A), the source of this manganese could only be from uptake of the active ingredient mancozeb. We do not know if the "extra" manganese in the mantle was sequestrated as the free ion or still associate to the dithiocarbamate moiety; mancozeb is a known oxidative-stress promoter (Calviello *et al.*, 2006; Domico *et al.*, 2007) and evidence of DNA damage from specific biomarkers has been reported for *M. arenaria* with a high manganese content collected from the field (Blaise *et al.*, 2002). Whatever the chemical form of manganese in the tissue, potential toxic effect on cellular integrity from reactive oxygen species could occur from an increased load of this metal.

The uptake of the two main active ingredients from the water was probably due to passive diffusion through semi-permeable membranes, as found in gills or gastrointestinal tract (Rand & Petrocelli, 1985). The soft-shell clam can filter 40-50 l of water per day. The water is pumped into the clam through the incurrent siphon and moves in the water tubes for the oxygenation of the animal (Potts, 1996). The water and waste products are collected and discharged through the anus into the posterior mantle cavity (Potts, 1996; MacCallum *et al.*, 2003). If the chemicals followed the same path in the bivalve, their higher concentration in the mantle could be due to discharge into the mantle cavity.

Table 2. Chlorothalonil and co-products tissue concentration in clams at the highest level of chlorothalonil in mantle. Concentrations are express as $\mu g\ g^{-1}$ dry weight, percentage of total concentration is reported in parenthesis

Tissues/treatment	chlorothalonil	trichlorothalonil	pentachlorothalonil	total
Chlorothalonil 48hrs				
hepatopancreas	1.8 (18)	8.2 (82)	nd	10.0
mantle	59.2 (95.6)	0.5 (0.8)	2.2 (3.6)	61.9
Chlorothalonil + mancozeb, 24hrs				
hepatopancreas	3.3 (80.5)	0.8 (19.5)	nd	4.1
mantle	9.0 (84.9)	1.6 (15.1)	nd	10.6

Since the manganese content of the mantle tissue increased under exposure to Manzate 200DF (Figure 15A), the source of this manganese could only be from uptake of the active ingredient mancozeb. We do not know if the "extra" manganese in the mantle was sequestrated as the free ion or still associate to the dithiocarbamate moiety; mancozeb is a known oxidative-stress promoter (Calviello *et al.*, 2006; Domico *et al.*, 2007) and evidence of DNA damage from specific biomarkers has been reported for *M. arenaria* with a high manganese content collected from the field (Blaise *et al.*, 2002). Whatever the chemical form of manganese in the tissue, potential toxic effect on cellular integrity from reactive oxygen species could occur from an increased load of this metal.

The uptake of the two main active ingredients from the water was probably due to passive diffusion through semi-permeable membranes, as found in gills or gastrointestinal tract (Rand & Petrocelli, 1985). The soft-shell clam can filter 40-50 l of water per day. The water is pumped into the clam through the incurrent siphon and moves in the water tubes for the oxygenation of the animal (Potts, 1996). The water and waste products are collected and discharged through the anus into the posterior mantle cavity (Potts, 1996; MacCallum *et al.*, 2003). If the chemicals followed the same path in the bivalve, their higher concentration in the mantle could be due to discharge into the mantle cavity.

When clams were exposed to the mixture of fungicides, accumulation of chlorothalonil or manganese in the mantle was lower than the accumulation measured under exposure to one fungicide at a time (Figure 14 and 15B). Although nominal concentration of individual fungicide was half the concentration used in single exposure experiments, accumulation in mantle tissue was lowered by a greater factor. The mantle tissue levels of manganese were similar to control animals up to 72 h and the highest concentration of chlorothalonil in mantle was about five times lower than the maximum concentration reported for chlorothalonil alone. Decreased seawater solubility of the active ingredients arising from mixing of fungicides in seawater is a likely explanation, because water concentrations of mancozeb and chlorothalonil were lowered by a mean factor of three and five respectively (Figure 11). Antagonism mechanisms could exist between chlorothalonil and mancozeb in clam and induce a lower uptake of chemicals; but this has yet to be investigated.

Although haemic neoplasia did not develop to a detectable level in *M. arenaria* under chemical stress by chlorothalonil or mancozeb in chronic exposure experiments, our results from acute exposure experiments showed that both chemicals can bioaccumulate in the mantle to high levels. Elimination process was efficient as the tissue concentrations decreased with time and at a water concentration of 100 $\mu g\ l^{-1}$ or less no persistent accumulation of active ingredient was measured. Even if their accumulation is transitory, chlorothalonil and mancozeb are both oxidative-stress promoter and could have induced cell dysfunction while in the tissue. Study on the effect of these fungicides on the protein p53 system is an example of strategy that would provide information on cellular events promoting neoplasia. This work has also highlighted that co-products of chlorothalonil present in commercial formulation, pentachlorobenzonitrile and trichlorobenzodinitrile, should deserve attention since, upon uptake by the bivalves, their relative proportion in the tissue is very different from the initial fungicide composition; and their toxicity is unknown.

2.2.6 Acknowledgements

The authors wish to thank Laury Gauthier and Nathalie Côté for their technical help with the chemical analysis, Garth Arsenault for his technical support in fungicide experiment and Lise Chevarie for the Magdalen Islands clams. This work was supported by AquaNet, NSERC, the

Canada Research Chairs (CRC) program, the Atlantic Canada Opportunities Agency (ACOA), and the Canada Foundation for Innovation (CFI).

CHAPITRE 3

EFFET D'UN MÉLANGE DE PESTICIDES (CHLOROTHALONIL ET MANCOZÈBE) ET BENZO [*A*] PYRÈNE SUR LE NIVEAU D'EXPRESSION DES GÈNES RECEPTEUR ARYL HYDROCARBONE, P53 ET L'UBIQUITINE CHEZ LES HÉMOCYTES DE LA MYE COMMUNE (*MYA ARENARIA*)

3.1 RÉSUMÉ

L'objectif de cette étude est de déterminer chez les hémocytes de la mye les niveaux d'expression des gènes codant pour la p53, l'ubiquitine et le récepteur aryl hydrocarbone 48 et 72 heures après l'injection d'un mélange de chlorothalonil et mancozèbe et d'un hydrocarbure aromatique polycyclique (benzo [*a*] pyrene). Les niveaux d'expression des gènes AhR, p53 et ubiquitine ont été quantifiés par PCR quantitative en temps réel. Pour une quantification précise des transcrits, quatre gènes de références (facteurs d'élongation et protéines ribosomales) ont été sélectionnés à partir de transcrits déjà séquencés et ceux-ci ont été appliqués au logiciel geNorm. Les résultats ont montré une augmentation significative du récepteur AhR après une exposition de 72 heures ($p \leq 0.05$). Une augmentation de l'expression du gène p53 est notée après 48 heures d'exposition, mais pas de manière significative tandis qu'une diminution significative de son expression a été notée après 72 heures d'exposition. Les résultats de cette étude suggèrent que les niveaux d'expression du gène AhR pourraient être utilisés comme indicateur de l'exposition des hémocytes de mye à un mélange de fongicides (chlorothalonil et mancozèbe) et de HAP (benzo [*a*] pyrene). Cependant, d'autres études devront être réalisées pour comprendre les mécanismes moléculaires impliquant la p53.

Ce troisième article, intitulé « *Effects of pesticide compounds (chlorothalonil and mancozeb) and benzo[a]pyrene mixture on aryl hydrocarbon receptor, p53 and ubiquitin gene expression levels in haemocytes of soft-shell clams (*Mya arenaria*)* », fut corédigé par moi-même

ainsi que par Mme Patricia McKenna, mon collègue Mohammed Abo ElKhair, le Dr Richard St-Louis, le Dr Émilien Pelletier, le Dr T. Jeffrey Davidson, le Dr Réjean Tremblay, le Dr Franck C.J. Berthe et le Dr Ahmed Siah. Il fut accepté pour publication dans sa version finale en 2011 par les éditeurs de la revue *Ecotoxicology*. En tant que premier auteur, ma contribution à ce travail fut l'essentiel de la recherche sur l'expression de certains gènes associées à la néoplasie hémique, le développement de la méthodologie, l'exécution de l'expérience et la rédaction de l'article. Mme Patricia McKenna et Mohammed AboElkhair, second et troisième auteurs, ont aidé à l'exécution de l'expérience. Les Drs Richard St-Louis, Émilien Pelletier, T. Jeffrey Davidson, Réjean Tremblay et Franck C.J. Berthe ont contribué à la rédaction ainsi que la révision. Finalement, Dr Ahmed Siah a fourni l'idée originale et a contribué au développement de la méthodologie, à la rédaction ainsi que la révision de l'article. Une version abrégée de cet article a été présentée à *Physiomar* à l'automne 2008.

3.2 Effects of pesticide compounds (chlorothalonil and mancozeb) and benzo[*a*]pyrene mixture on aryl hydrocarbon receptor, p53 and ubiquitin gene expression levels in haemocyte of soft-shell clams (*Mya arenaria*)

3.2.1 Abstract

The aim of this study is to investigate the effects of the pesticides/polycyclic aromatic hydrocarbon (PAH) mixture on aryl hydrocarbon receptor (AhR), p53 and ubiquitin mRNA level in haemocytes of *Mya arenaria* exposed to a mixture of chlorothalonil, mancozeb and benzo[*a*]pyrene (BaP) for 48 and 72 h. AhR, p53 and ubiquitin gene expression levels were quantified using quantitative real time PCR. For robust and accurate quantification of transcripts, suitable housekeeping genes were selected from four sets of ribosomal and elongation factors transcripts previously sequenced from *Mya arenaria* using geNorm open source software. Quantitative real time PCR data exhibited a significantly high expression of AhR after 72 h of exposure ($p \leq 0.05$). P53 gene expression seems to be up-regulated by the mixture after 48 h, however not significantly; but the level of p53 mRNA is down-regulated by the xenobiotics between 48 and 72 h after exposure. This study postulates that AhR mRNA levels could be used as an indicator of the exposure of clams' haemocytes to a mixture of xenobiotics such as chlorothalonil, mancozeb and BaP. However, further studies have to be pursued in order to unravel the molecular mechanisms involved in the p53 signaling pathway.

Key words: p53; Aryl hydrocarbon receptor; ubiquitin; housekeeping genes; *Mya arenaria*; pesticides; benzo[*a*]pyrene

3.2.2 INTRODUCTION

The soft-shell clam, *Mya arenaria*, found in North River in Prince Edward Island (PEI, Canada) is prone to develop disseminated neoplasia (DN), recognized as a fatal disease (McGladdery *et al.*, 2001b). Even though the cause or causes of DN are still unknown, anthropogenic xenobiotics seem to be among the main factors involved in disease development (Reinisch *et al.*, 1984; Strandberg *et al.*, 1999). Epizootilogical studies strongly suggest that the presence in the environment of high levels of substances used in the agriculture industry in PEI seems to be correlated with DN in clams (MacCallum *et al.*, 2003). Elsewhere, such as in polluted sites from New England, mortality rates of 78% in clam populations were related to DN (van Beneden, 1997) and the prevalence was associated with organic contaminants in New Bedford Harbor, Massachusetts (Reinisch *et al.*, 1984).

Chlorothalonil (tetrachloroisophtalonitrile, a chlorinated benzonitrile pesticide) and mancozeb (a zinc salt of manganese dithiocarbamate pesticide) account for 74% (over 600 metric tons) of yearly pesticides sales in PEI (Reeves, 2001). These compounds are used weekly during the season when control of fungal diseases is needed. Our investigations showed an accumulation of chlorothalonil and mancozeb in *M. arenaria* tissues (Pariseau *et al.*, 2009). Moreover, *in vitro* experiments showed that chlorothalonil negatively affects tunicate haemocyte functions by preventing phagocytosis (Cima *et al.*, 2008) and suppresses oyster haemocyte reactive oxygen species production (Baier-Anderson & Anderson, 2000). Furthermore, mancozeb is suspected to be a carcinogenic agent in mammals (Belpogi *et al.*, 2002) by inducing DNA damage in cells exposed *in vitro* through oxidative mechanisms (Calviello *et al.*, 2006; Domico *et al.*, 2007). In addition to the chlorothalonil and mancozeb, polycyclic aromatic hydrocarbons (PAHs) compounds were also detected in clam tissues sampled in PEI (Pariseau *et al.*, 2009). It is well known that PAHs, and more specifically benzo [a] pyrene (BaP), are carcinogenic compounds (Xu *et al.*, 2009) and act as immunosuppressive xenobiotics towards human lymphocytes and monocytes (Davila *et al.*, 1996; Grevenynghe *et al.*, 2003; Grevenynghe *et al.*, 2004).

DN in the soft-shell clam *M. arenaria* is characterized by an increased number of circulating undifferentiated cells, presenting a nearly equal nucleus-to-cytoplasm ratio, a high frequency of mitotic patterns, as well as a loss of essential functions (McGladdery *et al.*, 2001b). Based on flow cytometry examination, the abnormal circulating haemocytes in neoplastic clams are tetraploid, displaying atypical acrocentric and telocentric chromosomes, which are not present

in normal healthy cells (Reno *et al.*, 1994). DN in the soft-shell clam is usually accepted as a tetraploid disorder (Reno *et al.*, 1994; Delaporte *et al.*, 2008). However, our previous study showed no correlation between xenobiotic exposure and tetraploidy status in haemocytes of soft-shell clams (Pariseau *et al.*, 2009). Therefore, it was postulated that the time of exposure was not sufficient for disease establishment, i.e. high prevalence of organisms with a high level of tetraploid cells, therefore excluding the use of tetraploidy status as an early indicator of cell cycle disruption upon contamination.

The molecular mechanisms regulating the development of DN are still under investigation (Barber, 2004). However, it is well known that the tumor suppressor gene p53 plays a pivotal role in regulating the cell cycle as a transcription factor (reviewed in Toledo & Wahl, 2006). Our previous data on the expression of p53 in connection with the tetraploidy of haemocytes have demonstrated a high expression of these genes in some clams with a tetraploidy status ranging between 15 and 50% (Siah *et al.*, 2008a). This observation concurs with the studies performed in humans suggesting that high levels of p53 gene expression could be used as a checkpoint to identify the stage when haemocytes become neoplastic. Indeed, it was shown that BaP induces abnormal cell proliferation by stimulating p53 gene expression in humans (Binkova *et al.*, 2000; Jiao *et al.*, 2008). Similarly, it was shown that BaP disrupts the p53 gene expression level in tissues of the whitefish (*Coregonus lavaretus*). (Brzuzan *et al.*, 2006). In addition, further studies have unraveled the molecular mechanisms by which xenobiotics induce germ cell neoplasms in soft-shell clams and suggest the involvement of ubiquitin and aryl hydrocarbon receptor (Brown *et al.*, 1995; Rhodes *et al.*, 1997; Butler *et al.*, 2004; Olberding *et al.*, 2004). Aryl hydrocarbon receptor (AhR) is one of the proteins with characteristic motifs also known as basic helix-loop-helix (bHLH) (Butler *et al.*, 2001). In addition to its role as an inducer of transcriptional factors related to contamination, it was shown that AhR is also involved in cell proliferation processes and cancer (Gasiewicz *et al.*, 2008). Interestingly, it was recently demonstrated that activation of AhR gene and protein expressions by xenobiotics mediates an up-regulation of ubiquitin mRNA and protein expressions (Reyes-Hernández *et al.*, 2010). Indeed, it was shown that carcinogenic compounds induce E3 ubiquitin gene expression level in the soft-shell clam, *M. arenaria* (van Beneden *et al.*, 1997; Rhodes *et al.*, 1997).

Deciphering the link between disease development and the exposure to a mixture of xenobiotics represents one of the greatest challenges in ecotoxicogenomics (Snape *et al.*, 2004).

Transcript levels need to be measured in order to assess the effects of contaminants (Van Aggelen *et al.*, 2010). However, post-translational processes, as well as protein levels quantification are also crucial to identify the molecular mechanisms involved in the development of the disease (Gygi *et al.*, 1999). For instance, in human cancer RKO cells, it was shown that p53 transcript levels remain unchanged during 5-Fluorouracile exposures, whereas the p53 protein level increased significantly (Ju *et al.*, 2007).

There is a need to understand the modifications at the gene expression level (transcriptomic), protein level (proteomic) and enzyme activities (metabolic) in order to be able to correlate the relationship between phenotype modification such as DN and genotype alteration (molecular mechanisms) (Snape *et al.*, 2004). This study aims to investigate the effect of a mixture of benzo [*a*] pyrene, chlorothalonil and mancozeb on p53, ubiquitin and aryl hydrocarbon receptor gene expression levels. The resulting data will initiate further investigations at the translational level (protein levels), post-translational processes (protein modifications) and finally at the enzymatic activities in order to unravel the complex interaction between the chemical toxicity and the development of the disease.

3.2.3 MATERIALS AND METHODS

3.2.3.1 Experimental design

Adult soft-shell clams, *Mya arenaria* (5-6 cm in length) were sampled at the Havre-aux-Maisons lagoon (47°26'19''N, 61°47'34''W) (Figure 16) (pristine site) in the Gulf of St Lawrence (Îles-de-la-Madeleine, Quebec, Canada). Back to the laboratory at the Institute of Marine Sciences of Rimouski in Pointe-aux-Pères, the aquaculture section of the University of Quebec in Rimouski (Quebec), clams were kept in two 60-liter tanks for a 2–day acclimatization period prior to the experiment's being initiated. The tanks were filled with sand sediment collected in the same site and filtered with (10 µm) UV treated seawater (10°C), oxygenated with air supply manifolds (28 psu). For each tank, 30% of the water was renewed every day. The clams were fed daily with a mix of live microalgae (*Nannochloropsis occulata*, *Pavlova lutheri* and *Isochrysis galbana*, v/v/v) produced by Nutrocean Inc. (Rimouski, Quebec) at a concentration of 50,000 cells/ml.

Exposed clams (n=18, 9 per tank) received an intramuscular injection (300 µL) of a mix of 0.5 mg of Bravo 500® (13 µg/g) (40% chlorothalonil), 0.5 mg of Manzate 200 DF® (13 µg/g) (75% mancozeb) diluted in DiMethylSulfOxyde (DMSO) and 0.4 mg of BaP (10 µg/g) diluted in ethanol for 0, 48 and 72 hours. Clams (n=6 for each exposure period) were sampled and haemolymph (~ 2 ml) was withdrawn. After the clams had been bled, haemolymph from each individual was centrifuged at 400xg for 15 min at 4°C. Then, haemocytes were collected and stored at -80°C for further gene expression analysis. The effect of ethanol and DMSO was investigated and no effect was observed at the gene expression level (data not shown).

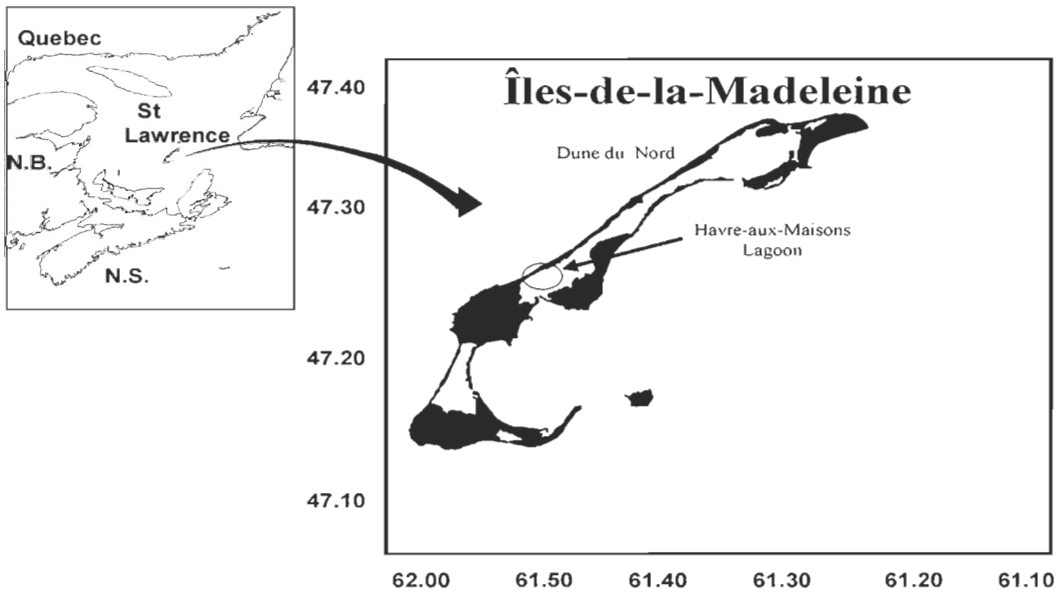


Figure 16. Map of clams sampling at Havre-aux-Maisons lagoon (Magdalen Islands, Quebec, Canada)

3.2.3.2 Total RNA extraction

Total RNA was extracted using 1 ml of TRizol® Reagent™ (Sigma, USA) from haemocytes. Total RNA was treated with DNase I (Sigma, USA). Quality and quantity were evaluated using denaturating gel agarose electrophoresis and Nanodrop (ND 1000, USA) spectrophotometer respectively.

3.2.3.3. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

First strand synthesis was carried out in a 20-µl reaction mixture containing 1 µg of total RNA and the reaction was performed using a SuperScript^{TM} III Platinum Two-Step q RT-PCR kit according to the manufacturer's protocol (Invitrogen, USA). Briefly, an engineered M-MMLV reverse transcriptase and a mix of primers including oligo-$(dT)_{20}$ and random primers were applied to perform the first-strand cDNA to be used for real-time quantitative PCR.

Quantification of AhR, p53 and ubiquitin gene expression in the haemocytes of *M. arenaria* was performed by real-time quantitative PCR using SYBR Green I as a dye. Amplification (25 µL) was performed by RotorGene detection system using 1 µl of cDNA template and SYBR® Green SuperMix (Invitrogen, USA). Platinum® SYBR® Green qPCR SuperMix-UDG is supplied at a 2x concentration and contains Platinum® Taq DNA polymerase, SYBR® Green I dye, Tris-HCl, KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, uracil DNA glycosylase (UDG), and stabilizers. Primer concentrations were 0.4 µM for both forward and reverse primers. Oligonucleotide primer sets used for the amplification of targeted genes are listed in Table 3. Based on the published sequences, the forward and reverse primers were designed in order to obtain a unique amplicon using the Primer 3 software and synthesized by Invitrogen Corp. (Burlington, ON, Canada). The cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles (20 s at 95°C, 20 s at each T_m and 20 s at 72°C). Melting curves were also plotted (60-95°C) in order to ensure that a single PCR product was amplified for each set of primers. A standard curve was established for each experiment using a 10-fold dilution of the cloned amplicons.

3.2.3.4. Selected reference genes

Four reference genes, RpS18, RpL37 (as ribosomal proteins), EF-1α and EF-2α (as elongation factors) were selected as a potential set of housekeeping genes based on the principle that their expressions might not be regulated by the xenobiotics. Based on the expressed sequence tags (EST), clones obtained by subtractive suppressive hybridization previously performed in *M. arenaria* (Siah et al. 2007), the forward and reverse primers were designed in order to obtain a unique amplification product using "Primer 3" software (Table 3).

Table 3. Sequence of primers used to quantify gene expression using real time RT-PCR

Gene name	Primers	Sequence (5'-3')	Tm	Amplicon (bp)
Elongation Factor-1	Forward	GGTGGCTGTTGGTGTCATC		
	Reverse	GGCCTAGGTGTTTTCCATGA	60	158
Elongation Factor-2	Forward	CTACAAGCCTGGCTCAAAGG		
	Reverse	TGACAACTGGGCTGACAGAG	60	218
Ribosomal protein S-18	Forward	AAGATTCCCGACTGGTTCCT		
	Reverse	GCCGGTTGTCTTTGTATGCT	60	189
Ribosomal protein L37	Forward	CCTAAACCTCCTGCTGGACA		
	Reverse	GCGTGCATATCACATTCAG	60	156
Ubiquitin	Forward	TCGCTAAGGAGCTGGACATT		
	Reverse	ACCGTCGCTCCTTGTACATC	60	194
p53	Forward	GGGGACTATGGGTTCGAAAT		
	Reverse	AACGCTTCACAGCCTCTTGT	60	217
AhR	Forward	CGTTGGTTGACCAAGGATCT		
	Reverse	GCCCTTCATTTTGGAGACAA	60	237

3.2.3.5. Data analysis

To determine the expression stability of the chosen housekeeping genes, the geNorm Visual Basic Application (VBA) was used as described by Vandesompele *et al.*, (2002). The gene stability measurement *M* was assessed by determining the average pair-wise variation of a particular gene with all other genes. In order to determine how many reference genes should be used, a normalization factor (NF) was calculated based on the geometric mean of expression levels of the best performing reference genes.

Data were analyzed using the relative expression software tool (qBase version 1.3.4). The basic principle of the model is that a difference (delta) in quantification cycle value between two samples is transformed into relative quantities using the exponential function with the efficiency of the PCR as its basis (Hellemans *et al.*, 2007).

The results of p53, ubiquitin, AhR gene expression levels are presented as mean ± error standard (SE) in relation with different exposure times: 0, 48 and 72 h. The software SigmaStat (San Jose, CA, USA) was used to assess a parametric one-way analysis of variance (ANOVA). When the distribution was not normal, a Kruskal-Wallis one-way ANOVA on ranks was used.

3.2.4 Results

3.2.4.1 Reference gene expression stability analysis

The gene expression stability for the four genes was determined using geNorm software over individual clam haemocytes for the treated clams (n=18) (Figure 17). Pairwise variation of each gene was compared with all other genes as the standard deviation of the logarithmically transformed expression ratios. This enabled to determine the internal gene stability measure, *M*, as the average pairwise variation of a specific gene with all the other genes. According to the *M* value, the ranking from the most to the least stable reference gene, was as follows for the treated clams: Rp S18 and Rp L37 ($M = 1.472$), EF-1α ($M = 1.656$) and EF-2α ($M = 1.769$).

In order to determine the number of housekeeping genes required for accurate normalization, the NF based on the geometric mean of the expression levels of the *n* best housekeeping genes was estimated by adding less stable housekeeping genes according to

Vandesompele *et al.*, (2002). Results showed that two housekeeping genes (Rp S18 and Rp L37, $V_{2/3}$=0.529) are sufficient for normalization. The addition of a third reference gene such as EF-1α has no significant effect ($V_{3/4}$=0.410) (Figure 18).

3.2.4.2 Target gene expression pattern

Using the suitable housekeeping genes (Rp S18 and Rp L37), p53, ubiquitin and AhR gene expression levels were quantified in haemocytes in relation with the different exposure times to a PAH/pesticides mixture. Cloned cDNA specific to each gene was used to generate standard curves using 10-fold serial dilutions. PCR efficiencies were 95%, 98% and 100% for p53, ubiquitin and AhR respectively. After optimization of PCR conditions, melt-curve analysis confirmed gene-specific amplification and no primer-dimers formation.

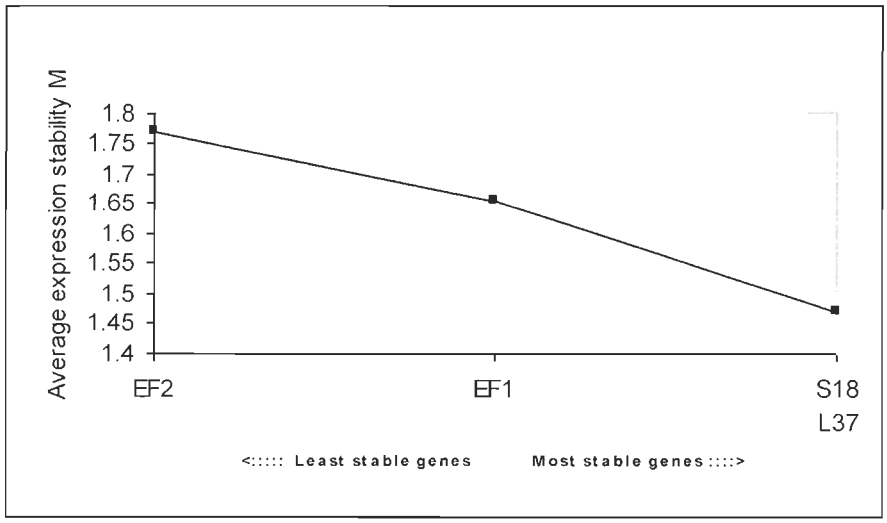


Figure 17. Average expression stability values *M* of four reference genes, Rp L37, Rp S18, EF-1α and EF-2α, ranked from the least stable such as EF-2α to the most stable Rp S18 and Rp L37 for the treated clams

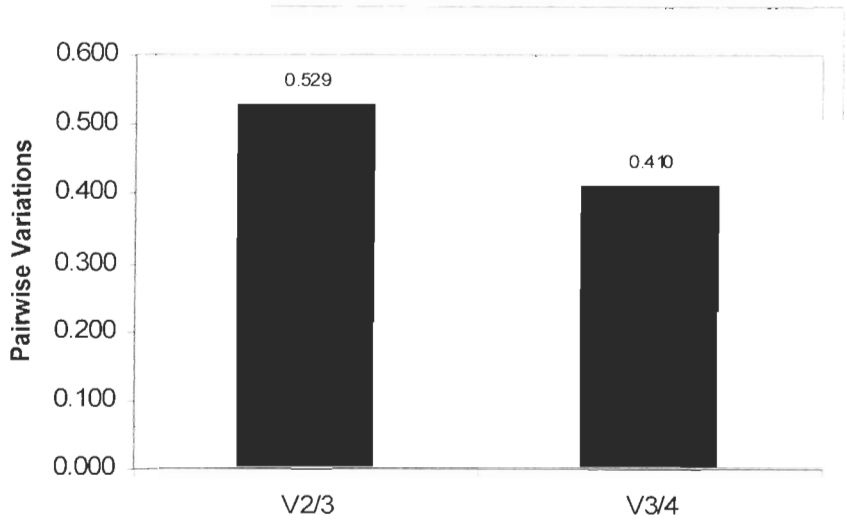


Figure 18. Pairwise variation analysis between NF_n and NF_{n+1} in order to determine the number of housekeeping required for accurate normalization for the treated clams where NF = normalization factor; n = number of housekeeping gene, y = the standard deviation of the logarithmically transformed expression ratios and x = pairwise variation of a specific gene with the other genes

p53 and ubiquitin relative gene expression reached a higher level after 48 h of exposure, followed by a decrease of the expression after 72 h (Figure 19). While this variability was significant for p53 (P=0.006, H=10.2, d.f.=2), it was not for ubiquitin (P=0.082, H=7.6, d.f.=2). P53 gene expression level increased after 48 h and significantly ($p \leq 0.05$) decreased after 72 h of exposure in comparison with the level prior to exposure (T0). However, there was no significant difference for ubiquitin gene expression level during exposure, while it followed the p53 gene expression pattern. The AhR gene expression level showed significant variability with time (P=0.032, F=577, d.f.=2) with higher expression after 72 h of exposure to the mixture comparatively to the control ($p \leq 0.05$; Figure 20). Gene expression levels were also performed

on AhR, p53 as well as ubiquitin in clams exposed to ethanol and DMSO but no regulation was observed thus ensuring that the vectors have no effect on those transcripts (data not shown).

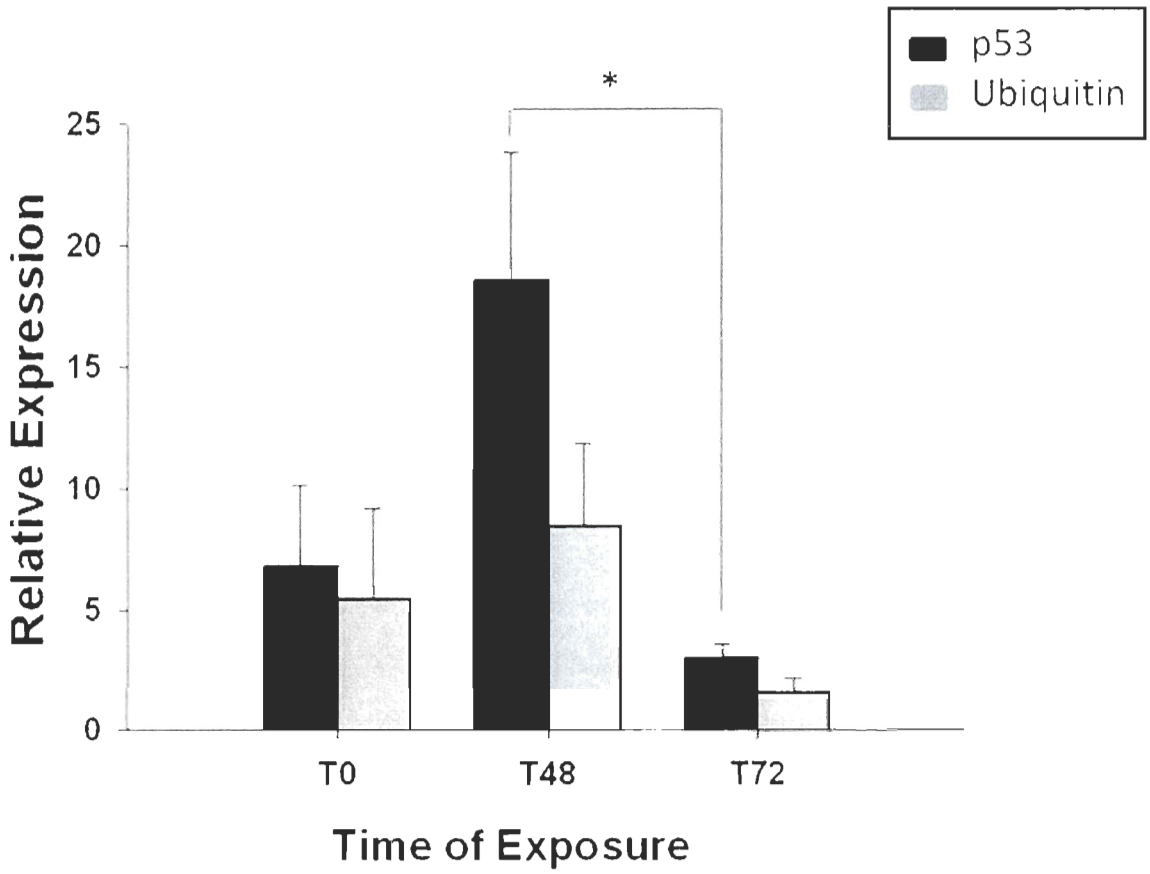


Figure 19. Relative quantification of p53 and ubiquitin to S18 and L37 ratio in haemocytes of *Mya arenaria* control (T0) and exposed to a mixture of chlorothalonil, mancozeb and BaP for 48 (T48) and 72 h (T72). Each value is expressed as the mean (n=4) ± SE (standard error).An asterisk (*) indicates a statistically different values at the level of $p \leq 0.05$

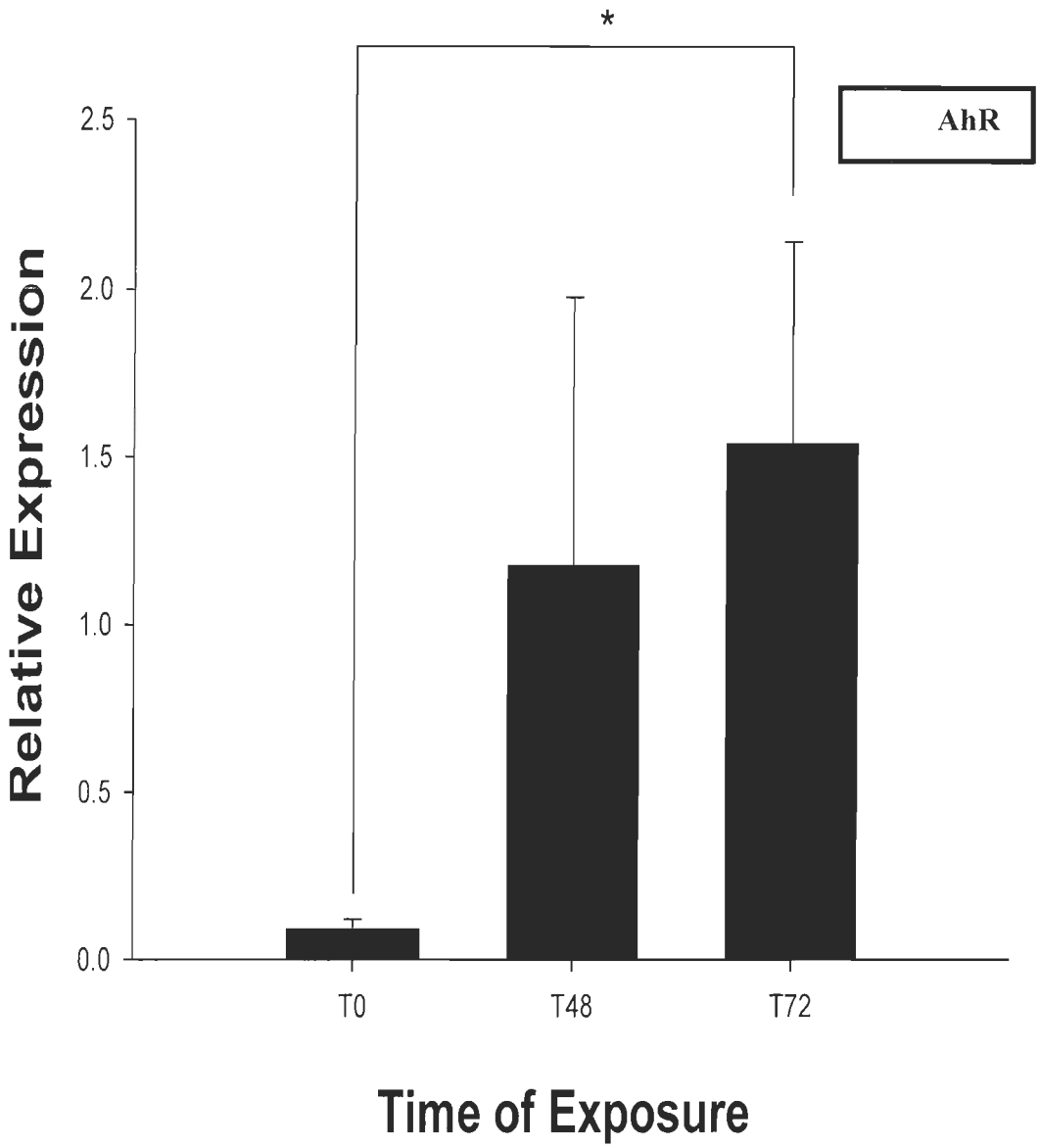


Figure 20. Quantitative real-time RT-PCR of AhR. Relative quantification of AhR to S18 and L37 ratio in haemocytes of *Mya arenaria* control (T0) and exposed to a mixture of chlorothalonil, mancozeb and BaP for 48 (T48) and 72 h (T72). Each value is expressed as the mean (n=4) ± SE (standard error). An asterisk (*) indicates a statistically different values at the level of $p \leq 0.05$

3.2.5 Discussion

In the last decade, ecotoxicogenomic studies have become an emerging new area of research to investigate the molecular mechanisms by which xenobiotics regulate the expression of genes involved in disease development in aquatic organisms (David *et al.*, 2007; Leaver *et al.*, 2010). Our previous studies showed that the expression of p53 gene is higher in clams with 15-50% of tetraploid haemocytes (Siah *et al.*, 2008a).

In vertebrates, it was demonstrated that xenobiotics induce AhR gene and protein expressions, which in turn increase ubiquitin mRNA and protein levels and then result in increased p53 ubiquitination (Reyes-Hernández *et al.*, 2010). An E3 ubiquitin-protein ligase and AhR with sequence similarity to their human homologues were identified and cloned in soft-shell clams *M. arenaria* (Kelley & Van Beneden, 2000; Butler *et al.*, 2001). In this study, p53, ubiquitin and AhR mRNA levels were quantified in clams exposed to a mixture of pesticide compounds (chlorothalonil and mancozeb) and benzo [*a*] pyrene using quantitative real time PCR. For an accurate quantification of transcript levels, recent studies performed in *M. arenaria* have shown the importance of selecting an appropriate set of housekeeping genes to normalize data related to gene expression levels in haemocytes (Araya *et al.*, 2008; Siah et al., 2008b). Four reference genes (ribosomal proteins S18 and L37 and the elongation factors EF1 and EF2) usually used as housekeeping genes were selected from EST clones obtained by subtractive suppressive hybridization performed in *M. arenaria* (Siah *et al.*, 2007). Using geNorm open source program, the stability of gene expression levels related to contamination condition has shown that Rp S18 and Rp L37 could be used as housekeeping genes. Likewise, in our study, ribosomal proteins Rp S18 and Rp L37 were selected among the most stable housekeeping genes in order to compare gene expression levels related to haemocytes ploidy status in *M. arenaria* (Siah *et al.*, 2008b).

Our data have shown that the expression of p53 gene decreased significantly ($p \leq 0.05$) after 3 days in comparison with the level recorded at 2 day. In vertebrates, several studies have investigated the role of p53 in the molecular mechanisms of neoplasia. It has been reported that 50% of human tumorigenesis could be explained by a disruption of p53 gene expression regulation (reviewed in Toledo & Wahl, 2006). Under abnormal conditions of cell cycle proliferation, for instance genotoxic stress, the p53 gene expression is up-regulated in order to stop the disrupted cell cycle progression or to direct the altered cells toward apoptosis (Toledo &

Wahl, 2006). In the literature, it is well documented that p53 is up-regulated by BaP exposure in mammals and whitefish (Binkova *et al.*, 2000; Brzuzan *et al.*, 2006; Jiao *et al.*, 2008).

Regulation of p53 gene expression was investigated in this research by quantifying the gene expression levels of *M. arenaria* AhR and E3 ubiquitin in haemocytes of clams exposed to a mixture of contaminants. Although the expression of ubiquitin seemed to be regulated by the mixture, the kinetic differential expression was not significant (Figure 19). However, a significantly high level of AhR transcript level was observed in haemocytes from clams 3 days after exposure ($p \leq 0.05$) (Figure 20). This result is in accordance with data recorded by Liu *et al.*, (2010) who showed that AhR transcripts in gills and digestive glands of clams, *Ruditapes philippinarum*, were significantly induced by BaP after a 3-day exposure time. Interestingly, it was recently demonstrated that AhR plays a key role in cancer development by acting as an inducer of p53 ubiquitination through activation E3 ubiquitin ligase gene expression (Gasiewicz *et al.*, 2008; Reyes-Hernandez *et al.*, 2010). In this study, the expression of ubiquitin seemed to be regulated by the mixture; however, the kinetic differential expression was not significant (Figure 19). It has been demonstrated that p53 ubiquitination involving E3 ubiquitin ligase plays a pivotal role in the development of tumor cells (Dai *et al.*, 2006; Lee & Gu, 2010). Studies have shown that clams exposed to dioxins revealed an up-regulation of E3 ubiquitin-protein ligase (Rhodes *et al.*, 1997). In vertebrates, Mouse Double-Minute 2 (MDM2) was identified as the main E3 ubiquitin ligase that regulates the p53 ubiquitination through its E3 ubiquitin ligase activity (reviewed in Lee & Gu, 2010). Recently, it was demonstrated that p53 protein from clams has the capacity to interact with MDM2 (Holbrook *et al.*, 2009). However, further investigations are needed to reinforce the postulate of co-regulation of p53 and ubiquitin gene family in relation with carcinogenesis as suggested by Holbrook *et al.*, (2009).

3.2.6 Conclusion

In this study, we have focused on the effect of the mixture on the candidate transcripts in order to respond to the question: Can a mixture of contaminants affect the level of specific transcripts related to DN in soft shell clams *M. arenaria*? The regulation of AhR gene expression by the mixture in clam haemocytes suggests the involvement of these xenobiotics in the haemocyte molecular pathways through AhR. Based on the new discovery of AhR involvement

in oncogenesis (Gasiewicz *et al.*, 2008), this gene could be considered as a new target for a mixture of xenobiotics effects and open a new avenue for ecotoxicological research. However, a combination of xenobiotics and contaminants alone could have different effects on the physiology of bivalve molluscs. It was shown for instance that tributyltin, an antifouling biocide, can antagonize the BaP toxicity in the Arctic charr (*Salvelinus alpinus*) (Ribeiro *et al.*, 2007; Padros *et al.*, 2003), while the pesticides, carbaryl and diquat dibromide, combined with PAH (fluoranthene) had a toxic additive effect on the larval grass shrimp, *Palaemonetes pugio* (Chung *et al.*, 2008). However, in our study, the effect of each contaminant alone and any synergetic, antagonist and/or additional effect of the xenobiotics was not considered. Further investigations are needed to unravel the effects of each contaminant and to highlight the contribution of each class of contaminant to the target gene expressed in *M. arenaria* haemocytes.

3.2.7 Acknowledgements

The authors wish to thank Lise Chevarie for the soft-shell clams, Iften Redjah for the technical support and Karine Paquet for the manuscript review. This work was supported by AquaNet, NSERC, The Canada Research Chairs (CRC) Program, The Atlantic Canada Opportunities Agency (ACOA), and The Canada Foundation for Innovation (CFI).

CHAPITRE 4
DISCUSSION GÉNÉRALE

L'hypothèse générale de ce projet de doctorat est que le développement de la néoplasie hémique est lié à la convergence de 3 variables : l'hôte, l'agent pathogène et l'environnement. Notre objectif est de contribuer à comprendre le développement de la néoplasie hémique en utilisant la mye commune, *Mya arenaria* comme espèce modèle. Plus spécifiquement, 3 objectifs ont été visés : 1) vérifier l'hypothèse de l'implication d'un agent infectieux en présence de néoplasie hémique en déterminant si, en mésocosme, la néoplasie hémique apparaît comme une maladie transmissible d'un organisme malade vers un organisme sain en utilisant pour la première fois un outil de diagnostic précis et puissant, la cytométrie en flux, 2) vérifier l'hypothèse d'une contamination environnementale en présence de néoplasie hémique en déterminant si une exposition chronique en laboratoire des myes avec deux fongicides (chlorothalonil et mancozèbe) peut induire la néoplasie hémique chez des myes négatives provenant d'un environnement positif et 3) étudier l'expression de certains gènes comme indicateurs de néoplasie hémique en déterminant chez les hémocytes de la mye les niveaux d'expression de gènes liés au développement de la néoplasie (p53, l'ubiquitine et le récepteur aryl hydrocarbone après l'injection d'un mélange de fongicides).

Ces trois chapitres font l'objet de trois articles scientifiques dont deux sont publiés et un troisième est en préparation. De plus, j'ai participé comme coauteur à plusieurs autres articles scientifiques sur ce sujet (voir en annexe), qui seront considérés ici.

Pour ce projet, nous avons choisi la mye commune, car c'est une espèce particulièrement susceptible à cette maladie (Brousseau, 1987; Brousseau & Baglivo, 1991b) offrant un modèle idéal pour étudier les différents facteurs influençant le

développement de la néoplasie (Appeldoorn *et al.*, 1980; Synard, 2007; Walker *et al.*, 2009). En effet, la mye commune est un organisme vivant dans les sédiments et est suspensivore ce qui l'expose en permanence aux composés présents dans la colonne d'eau ainsi que dans les sédiments (Walker *et al.*, 2009). De plus, de toutes les espèces pouvant développer la néoplasie hémique, la mye commune est l'organisme chez lequel le plus grand nombre d'études a été effectué pour tenter d'expliquer le développement de cette maladie (Walker *et al.*, 2009). Finalement, la mye commune est une espèce idéale, car, de par son anatomie, il est facile d'effectuer une ponction d'hémolymphe pour l'étude des hémocytes (Walker *et al.*, 2009).

L'origine et le mécanisme de la néoplasie hémique sont encore mal connus, mais il semble y avoir une transmission entre les individus atteints et ceux qui sont en bonne santé (Oprandy *et al.*, 1981; McGladdery *et al.*, 2001b; Dungan *et al.*, 2002). L'hypothèse de base pour tenter d'expliquer une possible transmission entre les individus est l'implication d'un agent infectieux.

Dans un premier temps, nous avons évalué si la néoplasie hémique pouvait être transmise entre des myes positives à la néoplasie et des populations caractérisées par différents niveaux de tétraploïdies (Delaporte *et al.*, 2008). Pour cette expérience d'une durée de 62 jours, la majorité des myes des différentes populations étaient négatives avec moins de 5% de cellules tétraploïdes. Aucune transmission n'a donc pu être observée. Entre chaque population, aucune différence significative n'a été obtenue en termes de tétraploïdie et il n'y avait pas de différence significative observée entre les myes contrôles et les myes traitées.

Pour cette expérience, la technique de diagnostic utilisée était la cytométrie en flux. À notre connaissance, c'était la première fois que cette technique était utilisée pour caractériser la néoplasie hémique dans une étude de transmission. Généralement, le diagnostic est basé sur les caractéristiques morphologiques des cellules (hémocytes) avec l'hématocytologie et l'histopathologie (Brousseau, 1987; Elston *et al.*, 1988; Brousseau & Baglivo, 1991b; McGladdery *et al.*, 2001a; Villalba *et al.*, 2001; Dungan *et al.*, 2002). Une étude réalisée par Delaporte *et al.*, (2008) sur la distribution de la néoplasie hémique dans

l'est du Canada utilisait la cytométrie en flux comme technique de diagnostic tout en confirmant les résultats par hématocytologie selon les critères de diagnostic de Farley *et al.*, (1986). Les résultats ont généralement été les mêmes pour les deux techniques, à l'exception de quelques individus. La différence de diagnostic est attribuée au fait que la sensibilité n'est pas la même pour les deux méthodes. En effet, selon Delaporte *et al.*, (2008), les myes avec moins de 5% de cellules tétraploïdes sont considérées négatives avec la cytométrie en flux tandis que certaines d'entre elles auraient pu être considérées légèrement positives avec les techniques d'hématocytologie et histopathologie (McGladdery *et al.*, 2001a). La technique de cytométrie en flux s'est révélée très sensible parce qu'elle est basée sur l'analyse du cycle cellulaire tandis que l'hématocytologie et l'histopathologie sont basées sur la caractérisation morphométrique des hémocytes. Pour ces deux méthodes, la distinction entre les hémocytes normaux en mitose et les cellules néoplasiques est difficile à établir surtout pour les groupes intermédiaires, car les résultats peuvent être dus à un processus physiologique normal. En effet, les pourcentages des hémocytes sains en phase S et G2/M ne sont pas connus, car le site d'hématopoïèse chez les mollusques n'a pas été identifié. Le diagnostic de néoplasie hémique basé sur des techniques utilisant les caractéristiques morphologiques des hémocytes peut donc être sous-estimé ou surestimé dépendamment des critères de classification choisis.

Les quelques études qui semblent démontrer la transmission de néoplasie hémique par cohabitation pour la mye commune (Appeldoorn *et al.*, 1984; House, 1997, McGladdery *et al.*, 2001a; McCallum *et al.*, 2003), ont utilisé des techniques de diagnostic considérées comme subjectives et peu sensibles, basées sur l'hématocytologie, l'histopathologie et les caractéristiques morphologiques avec des critères de classification différents pour chacune d'elles. Par exemple, dans l'étude de McGladdery *et al.*, (2001a) une mye était considérée comme étant positive si plus de 1% des cellules étaient néoplasiques. Par contre, dans celle de MacCallum *et al.*, (2003) une mye était positive si plus de 0,1% de cellules étaient néoplasiques. Avec le développement de la cytométrie en flux permettant de revoir les seuils proposés, nous pouvons douter de certains résultats suggérant la transmission de cette maladie par cohabitation. En effet, certains résultats

positifs de ces études auraient probablement été considérés négatifs avec la technique de cytométrie en flux avec un seuil expérimental de 5 % pour les myes négatives et, de ce fait, changer le résultat final à propos de la transmission.

Dans un deuxième temps, des études ont été menées pour tenter d'identifier la présence de particules rétrovirales chez des myes néoplasiques. Pour l'étude d'AboElkhair *et al.*, (2009a), aucune structure similaire à des particules rétrovirales n'a été observée dans des échantillons d'hémolymphe avec une coloration négative provenant de North River (Île-du-Prince-Édouard). L'objectif principal était de rechercher la présence d'un rétrovirus dans plusieurs tissus de myes par quantification de l'activité de l'enzyme transcriptase inverse. De plus, la glande digestive (l'organe avec le plus haut niveau d'activité de cette enzyme) et les hémocytes (les cellules utilisées pour le diagnostic de la néoplasie hémique) ont été analysés par microscopie électronique pour y chercher la présence de rétrovirus. Les résultats montrent qu'il y avait un certain niveau basal d'activité de l'enzyme transcriptase inverse dans tous les organes des myes saines. Ces résultats sont en accord avec les études réalisées sur la tortue (Casey *et al.*, 1997), l'escargot (Raghavan *et al.*, 2003) et l'humain (Molès *et al.*, 2007). Par contre, l'activité de l'enzyme dans les organes de myes malades était significativement plus élevée, un résultat qui pourrait s'expliquer par l'infiltration d'hémocytes dans les organes en présence de néoplasie hémique (AboElkhair *et al.*, 2009a). L'activité la plus importante de la transcriptase inverse a été notée dans la glande digestive à la fois pour les myes saines et les myes malades. Ceci pourrait être dû à la nature proliférative des cellules de la glande digestive. En effet, il y a une association qui a été notée dans la littérature entre l'activité de la transcriptase inverse et le potentiel prolifératif des cellules (Osertag & Kazazian, 2001). De plus, l'activité de la transcriptase inverse pourrait être due à l'exposition de la glande digestive à des polluants. Selon Sciamanna *et al.*, (2005), l'expression des gènes codant pour la transcriptase inverse peut être augmentée par une variété d'agents génotoxiques.

Des particules rétrovirales n'ont pu être mises en évidence par microscopie électronique à la fois dans la glande digestive et les hémocytes. Ces résultats sont similaires aux résultats d'études qui n'ont pu montrer l'existence de particules rétrovirales chez *M.*

arenaria (Farley, 1976; Appeldoorn *et al.*, 1984; House *et al.*, 1998) *C. edule* (Auffret & Poder, 1986), *M. edulis* (Rasmussen, 1986) et *M. trossulus* (Mix *et al.*, 1979; Elston *et al.*, 1988). Toutefois, une corrélation positive entre l'activité de la transcriptase inverse et la tétraploïdie chez la mye commune a été observée (AboElkhair *et al.*, 2009b). Une différence significative a été trouvée dans les concentrations de transcriptase inverse entre les groupes possédant de 10 à 20 % et de 30 à 80% de cellules tétraploïdes. L'augmentation significative de l'activité transcriptase inverse lorsque 30% des cellules sont tétraploïdes suggère un lien avec l'hypothèse du début d'une phase «irréversible» de la progression du processus pathologique. La présence de l'activité de l'enzyme transcriptase inverse sans particules rétrovirales identifiées pourrait indiquer une source endogène de cet enzyme au lieu d'une source exogène provenant d'un rétrovirus.

La présence potentielle d'un agent infectieux seul ne semble pas expliquer le développement de la néoplasie hémique. Cependant son action pourrait être amplifiée par d'autres facteurs tels que la pollution par des substances anthropiques toxiques. Des auteurs ont suggéré que la néoplasie hémique identifiée chez des myes provenant d'un site où il y a eu une mortalité massive à la fin des années 1990 aurait pu être causée par l'utilisation des pesticides par les producteurs de pommes de terre de l'Île du Prince Édouard (MacCallum *et al.*, 2003). L'objectif de notre étude a donc été de déterminer s'il était possible d'induire la maladie chez des myes saines provenant d'un environnement où la néoplasie hémique, est présente soit la baie de North River. Notre hypothèse pour cette étude était que les myes négatives auraient été mises en contact avec un virus provenant d'un environnement positif. Les résultats ont montré que les myes négatives qui ont été échantillonnées dans un environnement positif n'ont pas développé la néoplasie hémique après 35 jours d'exposition chronique au chlorothalonil et au mancozèbe dans l'eau de mer sous des conditions de laboratoire. Un résultat similaire avait été obtenu par Krishnakumar *et al.*, (1999) lorsqu'ils ont exposé *M. edulis* à des hydrocarbures aromatiques polycycliques ou des biphényles polychlorés pour une durée de 180 jours. De plus, nous n'avons détecté aucune trace de chlorothalonil dans la glande digestive et le manteau des myes. Pour le mancozèbe, la glande digestive et le manteau ont été analysés et aucune différence

significative n'a été obtenue entre les concentrations trouvées chez les tissus traitées par rapport aux contrôles. Une étude de l'expression de certains gènes impliqués dans le développement de la néoplasie hémique suite à une exposition au chlorothalonil et au mancozèbe aurait peut-être permis d'obtenir des résultats plus significatifs pour cette expérience.

Pour déterminer si une accumulation de ces fongicides est possible dans les tissus de la mye commune, une expérience à court terme d'une durée de trois jours a été réalisée. Tout d'abord, l'analyse du chlorothalonil a permis d'identifier 3 composés constituant les ingrédients actifs du mélange commercial : soit le chlorothalonil qui constitue 99% de la solution, du pentachlorobenzonitrile (0,4%) avec 5 atomes de chlore et 2 isomères de trichlorobenzodinitrile (0,1%) avec 3 atomes de chlore. Les résultats ont montré que l'accumulation du chlorothalonil est rapide et est déjà présente après 4 heures d'exposition. L'accumulation la plus importante est détectée après 48 heures d'exposition dans le manteau où la concentration atteint 59,2 µg/g de poids sec. L'accumulation diminue jusqu'à un niveau indétectable après 3 jours d'exposition. Des observations similaires ont été rapportées chez *M. edulis* (Ernst *et al.*, 1991). L'accumulation la plus importante du mancozèbe se trouve dans le manteau après 24 heures d'exposition. Étant donné l'augmentation de l'accumulation du manganèse (ingrédient actif du mancozèbe) la source de celui-ci ne peut que provenir du mancozèbe.

Il n'y a donc pas de néoplasie hémique qui a pu être induite par la présence de fongicides même si une forte association entre la présence de néoplasie hémique et la contamination par des substances anthropiques est faite dans la littérature. Le chlorothalonil et le mancozèbe sont des fongicides promoteurs de stress oxydatif (Calviello *et al.*, 2006; Domico *et al.*, 2007). De plus, des dommages à l'ADN par un haut niveau de manganèse ont été rapportés chez *M. arenaria* (Blaise *et al.*, 2002). Ces impacts pourraient induire une dysfonction cellulaire dans les tissus et favoriser le développement de la néoplasie hémique. À cette étape du travail, nous avons décidé d'aborder l'étude de la néoplasie hémique en utilisant non plus la tétraploïdie comme indicateur de la néoplasie, mais plutôt l'expression de certains gènes susceptibles de réagir à une contamination. Nous avons donc

tenté de déterminer l'effet de l'exposition de la mye commune à un mélange de benzo[*a*]pyrène, chlorothalonil et mancozèbe sur le niveau d'expression des gènes codant pour la p53, l'ubiquitine et le récepteur AhR. Ces trois gènes ont été choisis, car le gène p53 est connu pour réguler l'intégrité génomique durant le cycle cellulaire, le récepteur aryl hydrocarbone (AhR) étant impliqué dans la métabolisation des xénobiotiques via le cytochrome p450 (Henklova *et al.*, 2008) et l'ubiquitine étant impliquée dans les mécanismes de dégradation des protéines par le protéasome 26S (Olberding *et al.*, 2004). L'hypothèse de notre étude était qu'un mélange de composés cancérigènes de l'environnement (pesticides et HAP) induirait l'expression de ces gènes chez les hémocytes de *M. arenaria*. Ces composés induiraient l'expression du AhR qui, à son tour devrait métaboliser ces composés en produits cancérigènes immunotoxiques et déclencher l'induction des gènes codant pour la p53 et l'ubiquitine. L'augmentation de l'expression du gène p53 par contamination pourrait être observée avant le développement de la néoplasie hémique.

Les résultats de notre étude ont mis en évidence une augmentation significative de l'expression du gène AhR après 72 heures d'exposition et une diminution significative de celle du gène codant pour la p53 après trois jours d'exposition. De plus, le profil d'expression du gène codant pour l'ubiquitine était similaire à celui du gène codant pour la p53 sans toutefois noter de différences significatives entre les temps d'expositions. Selon la littérature, le gène p53 pourrait être un indicateur de la perturbation du cycle cellulaire et les résultats de notre étude suggèrent que le AhR pourrait être utilisé comme indicateur de l'exposition à un mélange de fongicides et de HAP trouvé dans l'environnement aquatique de l'Île-du-Prince-Édouard.

Deux hypothèses principales impliquant le gène p53 ont été suggérées dans la littérature pour expliquer la présence de la néoplasie hémique chez la mye commune. La première hypothèse suggère une mutation du gène p53 en présence de néoplasie hémique (Barker *et al.*, 1997). Lors de nos travaux en laboratoire, nous avons tenté de détecter une mutation du gène p53 à l'aide des endonucléases de restriction *Hae III* et *Taq I* qui agissent dans la région du gène où la mutation est susceptible de se produire. Nous n'avons détecté

aucune mutation (J. Pariseau, résultats non publiés). De plus, aucune mutation n'a été observée suite au séquençage de quatre échantillons d'hémocytes où un haut niveau d'expression du gène p53 avait été déterminé par RT-PCR quantitative en temps réel. Les résultats obtenus par Barker *et al.*, (1997) sont sujets à plusieurs critiques. Tout d'abord, les mutations trouvées sur la protéine et sur le gène ne sont pas les mêmes et ont été observées chez peu d'organismes. De plus, la mutation du gène sur l'exon 6 est située sur un site non fonctionnel ce qui laisse présager qu'une mutation à cet endroit aurait peu d'impact sur la fonction du gène. Finalement, l'examen du chromatogramme apparaît comme un chevauchement des pics d'acides nucléiques plutôt qu'une réelle mutation ce qui porte à croire à une erreur de lecture plutôt qu'une réelle mutation. Il aurait fallu séquencer plus de quatre échantillons pour pouvoir confirmer ou infirmer l'hypothèse de la mutation du gène p53 dans le développement de la néoplasie hémique. Nos résultats négatifs permettent de questionner la validité de l'hypothèse de la mutation posée par Barker *et al.*, (1997).

La deuxième hypothèse (Walker *et al.*, 2006) suggère que la protéine p53 est séquestrée dans le cytoplasme par la protéine mortaline. Pour appuyer cette hypothèse, l'étude réalisée par Siah *et al.*, (2008a) a montré une forte corrélation ($r^2 = 0,68$, $p < 0,01$) entre l'expression du gène mortaline et l'expression du gène p53. Dans cette expérience, les myes qui avaient un haut niveau d'expression du gène p53 avaient également un haut niveau d'expression du gène codant pour la mortaline (Siah *et al.*, 2008a). De plus, les résultats de Siah *et al.*, (2008a) ont montré que quelques myes avec un pourcentage de cellules tétraploïdes variant entre 15 et 50% avaient un niveau significativement élevé d'expression des gènes p53, p73 (protéine de la même famille que p53) et mortaline en comparaison des myes avec un pourcentage de cellules tétraploïdes inférieur à 15% et supérieur à 50%. D'autres études ont également montré l'existence du complexe p53-mortaline dans les neuroblastomes présents chez la souris et l'humain (Wadhwa *et al.*, 2002; Nikolaev *et al.*, 2003). Les résultats de Siah *et al.*, (2008a) supportent donc l'hypothèse d'un complexe entre les protéines p53 et mortalines dans le cytoplasme en présence de néoplasie hémique.

CHAPITRE 5
CONCLUSION GÉNÉRALE ET PERSPECTIVES : LA TÉTRAPLOÏDIE EST MAUVAISE CONSEILLÈRE

L'hypothèse générale de cette étude était que trois groupes de facteurs (hôte, agent pathogène et environnement) président au développement de la néoplasie hémique chez la mye commune. Les résultats obtenus au cours de cette thèse viennent conforter l'hypothèse d'une étiologie multifactorielle.

En effet, lors de l'essai de transmission expérimentale, l'hypothèse de travail testée était celle de l'implication d'un agent pathogène dans le développement de la maladie. Cette expérience a donc été réalisée en conditions contrôlées afin de limiter l'influence des conditions environnementales. Les résultats ont montré qu'aucune transmission de la maladie n'a pu être obtenue. Si l'on accepte que les conditions expérimentales permettaient cette transmission, il semble qu'un possible agent infectieux ne soit pas une condition suffisante.

Dans une seconde expérience, des myes saines provenant d'un environnement où la maladie est présente ont été mises en contact avec des contaminants. Pour cette expérience, l'hypothèse testée était celle de l'implication d'une contamination dans le développement de la maladie. Cet essai n'a conduit à aucune induction significative de néoplasie. Dans cette expérience, si l'on accepte encore que les conditions expérimentales permettaient l'expression de la maladie, il semble que les polluants testés ne soit pas une condition suffisante.

La présence d'un agent infectieux seul ainsi que la présence seule de contamination ne semblent pas expliquer le développement de la néoplasie hémique chez la mye. Ces

résultats sont cohérents avec notre hypothèse de départ. Toutefois, les conditions expérimentales potentielles permettant une transmission et/ou une expression de la maladie qui ont été utilisées sont de taille et entachées d'une incertitude non négligeable. Il est donc difficile d'accepter une série de résultats négatifs comme une validation de notre hypothèse générale.

Si la tétraploidïe reste une manifestation de la maladie sur laquelle il est possible de s'appuyer pour définir le statut d'une population vis-à-vis de la néoplasie hémique, notre travail a montré que cet indicateur devient limitant dans le modèle expérimental que nous avons utilisé. La tétraploidïe signe une transformation cellulaire qui est précédée d'une cascade d'évènements moléculaires; en tant que tel, son intérêt dans le modèle expérimental est limité.

Dans cette perspective, il nous apparait qu'élucider les mécanismes moléculaires impliqués dans le développement de la néoplasie hémique pourrait contribuer significativement à la compréhension de la maladie. C'est ce que nous avons entrepris avec les mesures de l'expression de gènes cibles (par exemple celui codant pour la p53). En effet, les mesures des niveaux d'expression de la p53 sont en phase avec l'évolution de la maladie à savoir une sur-expression des gènes au cours du développement de la néoplasie. De plus, les niveaux d'expression de certains gènes semblent être corrélés à une contamination multiple et complexe. Ainsi, il apparait clairement que les niveaux d'expressions de gènes ouvrent la voie à une nouvelle lecture de la dynamique de la maladie chez les populations de myes.

Objectivement, le développement d'un modèle d'induction de la néoplasie chez des myes en conditions contrôlées devrait être entrepris. Il n'en reste pas moins vrai que le caractère multifactoriel de la maladie et sa maitrise expérimentale pourrait rester délicate.

À partir de ce modèle, les mécanismes moléculaires impliqués dans le développement de la néoplasie pourraient être étudiés. Les nouvelles technologies de séquençages à haut débit appliquées aux gènes exprimés des hémocytes séparés par cytométrie en flux dans chacune des phases du développement de la maladie permettraient d'identifier les mécanismes moléculaires spécifiques à chaque phase de développement de la néoplasie

hémique. Pratiquement, les gènes pertinents exprimés et présentant des niveaux spécifiques à chaque phase de la maladie pourraient être sélectionnés et utilisés comme bio-marqueurs moléculaires capables d'identifier le statut des populations de myes vis-à-vis la maladie. Ces bio-marqueurs constitueraient des marqueurs précoces de santé, i.e. avant même que la maladie ne se manifeste cliniquement au niveau de la population.

Il semble que ces marqueurs permettraient de revisiter les hypothèses de base de la néoplasie hémique chez les mollusques fondés principalement sur des analyses des phases terminales de la maladie. L'analyse du transcriptome permettrait de mieux comprendre la dynamique de développement de la maladie et donc une analyse étiologique plus fine du modèle.

Pour de nombreuses petites communautés côtières, tant le long du Golfe du Saint-Laurent que sur la côte Est des États-Unis, la myiculture est une activité socio-économique et une meilleure compréhension de la dynamique de cette maladie, et l'utilisation de marqueurs de santé permettront de faciliter le développement de cette industrie.

RÉFÉRENCES BIBLIOGRAPHIQUES

AboElkhair M., Siah A., Clark K.F., McKenna P., Pariseau J., Greenwood S.J., Berthe F.C.J., & A. Cepica. 2009b. Reverse transcriptase activity associated with haemic neoplasia in the soft-shell clam *Mya arenaria*. *Diseases of Aquatic Organisms*, **84**: 57-63.

AboElkhair M., Synard S., Siah A., Pariseau J., Davidson J., Johnson G., Greenwood S.J., Casey J.W., Berthe F.C.J. & A. Cepica. 2009a. Reverse transcriptase activity in tissues of the softshell clam *Mya arenaria* affected with haemic neoplasia. *Journal of Invertebrate Pathology*, **102**: 133-140.

Alberts B., Johnson A., Lewis J., Raff M., Roberts K. & P. Walter. 2004. Biologie moléculaire de la cellule. 4ième Édition. Médecine-Sciences Flammarion, Paris. 1463 pp.

Allam B., Ashton-Alcos, K.A. & S.E. Ford. 2002. Flow cytometric comparison of haemocytes from three species of bivalve molluscs. *Fish and Shellfish Immunology*, **13**: 141-158.

Alonso A., P. Suarez C. Alvarez F.S. Juan & P. Molist. 2001. Structural study of a possible neoplasia detected in *Mytilus galloprovincialis* collected from the Ria of Vigo (NW Spain). *Diseases of Aquatic Organisms*, **47**: 73-79.

Appeldoorn R.S., Brown C.W., Brown R.S., Chang P.W., Cooper K., Lorda E., Saila S.B., Walker H. & R.E. Wolke. 1984. Field and Laboratory studies to define the occurrence of neoplasia in the soft shell clam, *Mya arenaria*, API Publ no. 4345, Graduate School of Oceanography, University of Rhode Island, Kingston.

Appeldoorn R.S., Brown R.S. & K.R. Cooper. 1980. Factors affecting the development of molluscan neoplasia in the soft-shell clam *Mya arenaria*: indications from laboratory and field observations. *Journal of Shellfish Research*, **1**: 108. (Abstract).

Araya M.T., Siah A., Mateo D., Markham F., McKenna P., Johnson G. & F.C.J. Berthe. 2008. Selection and evaluation of housekeeping genes for haemocytes of soft-shell clams (*Mya arenaria*) challenged with *Vibrio splendidus*. *Journal of Invertebrate Pathology*, **99**: 326-331.

Arnold W.S., Bert T.M., Hesselman D.M. & N.J. Blake. 1996. Gonadal neoplasia in hard clams (*Mercenaria* spp.) from the Indian River Lagoon, Florida. *Journal of Shellfish Research*, **15**: 518. (Abstract).

Ashton-Alcox K.A. & S.E. Ford. 1998. Variability in molluscan haemocytes: a flow cytometric study. *Tissue Cell*, **30**: 195-204.

Auffret M. & M. Poder. 1986. Sarcomatous lesion in the cockle *Cerastoderma edule* II. Electron microscopical study. *Aquaculture*, **58**: 9-15.

Baier-Anderson C.B. & R.S. Anderson. 2000. The effects of chlorothalonil on oyster hemocyte activation phagocytosis, reduced pyridine nucleotides, and reactive oxygen species production. *Environmental Research* Section A, **83**: 72-78.

Balouet G., Poder M., Cahour A. & M. Auffret. 1986. Proliferative hemocytic condition in European flat oysters (*Ostrea edulis*) from Breton coasts: a 6-year survey. *Journal of Invertebrate Pathology*, **48**: 208-215.

Barber B.J. 1990. Seasonal prevalence and intensity and disease progression of neoplasia in soft shell clams, *Mya arenaria*, from Shewsbury River, New Jersey. *dans*: (Editeur) F.O. Perkins & T.C. Cheng, Pathology in Marine Science. Academic Press, San Diego, CA, USA pp. 377-386.

Barber B.J. 2004. Neoplastic diseases of commercially important marine bivalves. *Aquatic Living Resources*, **17**: 449-466.

Barker C.M., Calvert R.J., Walker C.W. & C.L. Reinisch. 1997. Detection of mutant p53 in clam leukemia cells. *Experimental Cell Research*, **232**: 240-245.

Beal B.F., Bayer R., Kraus MG. & S.R. Chapman. 1999. A unique shell marker in juvenile, hatchery-reared individuals of the softshell clam, *Mya arenaria* L. *Fishery Bulletin*, **97**: 380-386.

Beal B.F. & M. Kraus. 2002. Interactive effects of initial size, stocking density, and type of predator deterrent netting on survival and growth of cultured juveniles of the soft-shell clam, *Mya arenaria* L., in eastern Maine. *Aquaculture*, **208**: 81-111.

Beal B.F., Lithgow C.D., Shaw D.P., Renshaw S., & D. Ouellette. 1995. Overwintering hatchery-reared individuals of the soft-shell clam, *Mya arenaria* L.: A field test of site, clam size, and intraspecific density. *Aquaculture*, **130**: 145-158.

Beckmann N., Morse M.P. & C.M. Moore. 1992. Comparative Study of Phagocytosis in Normal and Diseased Hemocytes of the Bivalve Mollusc *Mya arenaria*. *Journal of Invertebrate Pathology*, **59**: 124-132.

Belpogi F., Soffritti M., Guarino M., Lambertini L., Cevolani D. & C. Maltoni. 2002. Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats. *Annals of the New York Academy of Sciences*, **982**: 123-136.

Bert T.M., Hesselman D.M., Arnold W.S., Moore W.S., Cruz-Lopez H. & D.C. Marelli. 1993. High frequency of gonadal neoplasia in a hard clam (*Mercenaria* spp.) hybrid zone. *Marine Biology*, **117**: 97-104.

Binková B., Giguère Y., Rössner P.Jr, Dostál M., & R.J. Srám. 2000. The effect of dibenzo[a,l]pyrene and benzo[a]pyrene on human diploid lung fibroblasts: the induction of DNA adducts, expression of p53 and p21(WAF1) proteins and cell cycle distribution. *Mutation Research*, **471**: 57-70.

Blaise C., Gagné F., Pellerin J., Hansen P.-D. & S. Trottier. 2002. Molluscan shellfish biomarker study of the Québec, Canada, Saguenay Flord with the soft-shell clam, *Mya arenaria*. *Environmental Toxicology*, **17**: 170-186.

Böttger S., Jerszyk E., Low B. & C. Walker. 2008. Genotoxic stress-induced expression of p53 and apoptosis in leukemic clam haemocytes with cytoplasmically sequestered p53. *Cancer Research*, **68**: 777-782.

Bourque F., Myrand B., Chevarie L., Longuépée L. & V. Poirier. 2001. Inventaire du gisement de myes du Havre-aux-Basques. Rapport préliminaire. Îles-de-la-Madeleine.

Bower S.M. 2004. Synopsis of infectious diseases and parasites of commercially exploited shellfish: Haemocytic neoplasia of clams. URL: http://www.pac.dfo-mpo.gc.ca/shelldis/pages/hncc_e.htm

Brooks K.M. & R.A. Elston. 1991. Epizootiology of hemic neoplasia in *Mytilus trossulus* in Washington State II. *Journal of Shellfish Research*, **8**: 411. (Abstract).

Brousseau D.J. 1987. Seasonal Aspects of Sarcomatous Neoplasia in *Mya arenaria* (Soft-Shell Clam) from Long Island Sound. *Journal of Invertebrate Pathology*, **50**: 269-276.

Brousseau D.J. & J.A. Baglivo. 1991a. Field and laboratory comparisons of mortality in normal and neoplastic *Mya arenaria*. *Journal of Invertebrate Pathology*, **57**: 59-65.

Brousseau D.J. & J.A. Baglivo, 1991b. Disease progression and mortality in neoplastic *Mya arenaria* in the field. *Marine Biology*, **110**: 249-252.

Brown R.S. 1980. The value of multidisciplinary approach to research on marine pollution effects as evidenced in a three-year study te determine the etiology and pathogenesis of neoplasia in soft-shell clam, *Mya arenaria*). *Rapports et Proces-Verbaux des Réunions (Conseil Permanent International pour l'Exploration de la Mer)*, **179**: 125-128.

Brown D.J., van Beneden R.J. & G.C. Clark. 1995. Identification of two binding proteins for halogenated aromatic hydrocarbons in hard-shell clam, *Mercenaria mercenaria*. *Archives of Biochemistry and Biophysics*, **319**: 217-224.

Brown R.S., Wolke R.E., Brown C.W. & S.B. Saila. 1979. Hydrocarbon pollution and the prevalence of neoplasia in feral New England soft-shell clams (*Mya arenaria*). Animals as monitors of environmental pollutants. *Proceedings of the National Academy of Sciences of the United States of America,*: 41-51.

Brown R.S., Wolke R.E., Saila S. & C. Brown. 1971. A preliminary report on neoplasia in feral populations of soft shell clam, *Mya arenaria*: Prevalence, Histopathology and Diagnosis. *American Petroleum*: 151-159.

Brown R.S., Wolke R.E., Salia S.B. & C.W. Brown. 1977. Prevalence of neoplasia in 10 New England populations of the soft-shell clam (*Mya arenaria*). *Annals of the New York Academy of Sciences*, **298**: 522-534.

Brun G.L., MacDonald R.M., Verge J. & J. Aubé. 2007. Long-term atmospheric deposition of current-use and banned pesticides in Atlantic Canada; 1980-2000. *Chemosphere*, **71**: 314-327.

Brzuzan P., Jurczyk L., Luczynski M.K. & M. Gora. 2006. Real-time PCR analysis of p53 mRNA levels in tissues of withfish (*Coregonus lavaretus*) exposed tp benzo [*a*] pyrene. *Polish Journal of Veterinary Sciences*, **9**: 139-143.

Buckner S.C. 1988. Development of a municipal shellfish hatchery and nursery culture facility as integral components of a public resource management program. *Journal of Shellfish Research,* **7**: 571. (Abstract).

Butler R.A., Kelley M.L., Olberding K.E., Gardner G.R. & R.J. van Beneden. 2004. Aryl hydrocarbon receptor (AhR)-independent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on softshell clam (*Mya arenaria*) reproductive tissue. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, **138**: 375-381.

Butler R.A., Kelley M.L., Powell W.H., Hahn M.E. & R.J. van Beneden. 2001. An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, *Mya arenaria*: evidence that invertebrate AHR homologue lack 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and β-naphtoflavone binding. *Gene*, **278**: 223-234.

Calviello G., Piccioni E., Boninsegna A., Tedesco B., Maggiano N., Serini S., Wolf F.I. & P. Palozza. 2006. DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: involvement of the oxidative mechanism. *Toxicology and Applied Pharmacology*, **211**: 87-96.

Canesi L., Gallo G., Gavioli M. & C. Pruzzo. 2002. Bacteria–hemocyte interactions and phagocytosis in marine bivalves. *Microscopy Research and Technique*, **57**: 469–476.

Casey R.N., Quackenbush S.L., Work T.M., Balazs G.H., Bowser P.R. & J.W. Casey. 1997. Evidence for retrovirus infections in green turtles *Chelonia mydas* from the Hawaiian Islands. *Diseases of Aquatic Organisms*, **31**: 1-7.

CCME 1999. Canadian environmental quality guidelines. Canadian Council of Ministers of Environment. Winnipeg. 13 pp.

Chaves A., Shea D. & D. Danehower 2008. Analysis of chlorothalonil and degradation products in soil and water by GC/MS and LC/MS. *Chemosphere*, **71**: 629-638.

Cheng T.C. 1981. Invertebrate Blood Cell. *In*: Bivlaves. N.A. Ratcliffe & A.F. Rowley Éditeurs. London, Academic Press pp. 233-299.

Chevarie L. & B. Myrand. 2002. Élevage de la palourde japonaise (*Tapes philippinarum*) en Colombie-Britannique et dans l'état de Washington (USA), août 2001. Dans le cadre du programme MIM. Rapport de mission. Îles-de-la-Madeleine.

Chevarie L., Myrand B. & R. Tremblay. 2009. Programme de recherche-développement en myiculture aux Îles-de-la-Madeleine (Programme MIM-II) 2005-2006. MAPAQ, DIT, Compte rendu n° 36, 55 pp.

Chevarie L., Myrand B. & R. Tremblay. En préparation. Programme de recherche-développement en myiculture aux Îles-de-la-Madeleine (Programme MIM-II) 2007-2009.

Chung K.W., Chandler A.R. & P.B. Key. 2008. Toxicity of carbaryl, diquat dibromide, and fluoranthene, individually and in mixture, to larval grass shrimp, *Palaemonetes pugio*. *Journal of Environmental Science and Health*. Part. B, **43**: 293-299.

Cima F., Bragadin M. & L. Ballarin. 2008. Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211TM and chlorothalonil. *Aquatic Toxicology*, **86**, 299–312.

Coe W.R. & H. J. Turner. 1938. Development of the gonads and gametes in the soft-shell clam (*Mya arenaria*). *Journal of Morphology*, **62** (1): 91-111.

Collins C. & M. Mulcahy. 2003. Cell-free transmission of haemic neoplasm in the cockle *Cerasyoderma edule*. *Diseases of Aquatic Organisms*, **54**: 61-67.

Cooper K.R., Brown R.S. & P.W. Chang. 1982. The course and mortality of a hematopoietic in the soft-shell clam, *Mya arenaria*. *Journal of Invertebrate Pathology*, **39**: 149-157.

Couch J.A. & J.C. Harshbarger. 1985. Effects of carcinogenic agents on aquatic animals: An environmental overview. *Environmental Carcinogenesis Reviews*, **3**: 63-105.

Craig A., Sakamoto H. & C. Reinisch. 1993. Prevalence of leukemia in soft-shell clams (*Mya arenaria*) transplanted to New Bedford Harbor. *Marine Environmental Research*, **35**: 199-200.

Dai M-S., Shi D., Jin Y., Sun X-X., Zhang Y., Grossman S.R. & H. Lu. 2006. Regulation of the MDM2-p53 pathway by ribosomal protein L11 involves a post-ubiquitination mechanism. *Journal of Biological Chemistry*, **281**: 24304-24313.

David E., Tanguy A. & D. Moraga. 2007. Peroxiredoxin 6 gene: A new physiological and genetic indocator of multiple environmental stress response in Pacific oyster *Crassostrea gigas*. *Aquatic Toxicology*, **84**: 389-398.

Davila D.R., Romero D.L. & S.W. Burchiel. 1996. Human T cells are highly sensitive to suppression of mitogeneis by polycyclic aromatic hydrocarbons and this effect is differentially reversed by α-Naphthoflavone. *Toxicology and Applied Pharmacology*, **139**: 333-341.

Da Silva P.M., Soudant P., Carballal M.J., Lambert C. & A. Villalba. 2005. Flow Cytometric DNA content analysis of neoplastic cells in haemolymph of the cockle *Cerastoderma edule*. *Diseases of Aquatic Organisms*, **67**: 133-139.

Delaporte M., Synard S., Pariseau J., McKenna P., Tremblay R., Davidson J & F.C.J. Berthe. 2008. Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry. *Journal of Invertebrate Pathology*, **98**: 190-197.

Desrosiers G. & J-C. F. Brêthes. 1984. Étude bionomique de la communauté à *Macoma balthica* de la batture de Rimouski. *Sciences et Techniques de l'Eau*, **17**: 251-258.

Domico L.M., Cooper K.R., Bernard L.P. & G.D. Zeevalk. 2007. Reactive oxygen species generation by the ethylene-bis-dithiocarbamate (EBDC) fungicide mancozeb and its contribution to neuronal toxicity in mesencephalic cells. *Neurotoxicology*, **20**: 1079-1091.

Donehower L.A., Harvey M., Slagle B.L., McArthur M.J., Montgomery C.A. Jr, Butel J.S. & A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**: 215-221.

Dopp E., Barker C.M., Schiffman D. & C.L. Reinisch. 1996. Detection of micronuclei in hemocytes of *Mya arenaria*: association with leukemia and induction with an alkylating agent. *Aquatic Toxicology*, **34**: 31-45.

Dungan C.F., Hamilton R.M., Hudson K.L., McCollough C.B. & K.S. Reece. 2002. Tow epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebeius*. *Diseases of Aquatic Organisms*, **50**: 67-78.

Dunn A.M. 2004. A relative risk ranking of pesticides used in Prince Edward Island. Environment Canada. Environmental Protection Branch. Surveillance Report EPS-5-AR-04-03. Atlantic Region. 41 pp.

Elston R.A., Drum A.S & S.K. Allen Jr. 1990. Progressive development of circulating polyploidy cells in *Mytilus* with haemic neoplasia. *Diseases of Aquatic Organisms*, **8**: 59-59.

Elston R.A., Kent M.L. & A.S. Drum. 1988. Transmission of haemic neoplasia in the bay mussel, *Mytilus edulis*, using whole cells and cell homogenate. *Developmental & Comparative Immunology*, **12**: 719-727.

Elston R.A., Moore J.D. & K. Brooks. 1992. Disseminated neoplasia of bivalve molluscs. *Reviews in Aquatic Sciences*, **6**: 405-466.

Emerson C.W. 1990. Influence of sediment disturbance and water flow on the growth of the soft-shell clam, *Mya arenaria* L. *Canadian Journal of Fisheries and Aquatic Sciences*, **47**: 1655-1663.

Ernst W., Doe K., Jonah P., Young J., Julien G. & P. Hennigar. 1991. The toxicity of chlorothalonil to aquatic fauna and the impact of its operational use on a pond ecosystem. *Archives of Environmental Contamination and Toxicology*, **21**: 1-9.

Etchian O.A., Pellerin J., Audet C. & M. Mathieu. 2004. Sexual maturation and related changes in aspartate transcarbamylase activity of gonad tissues in the soft shell clam (*Mya arenaria*). *Comparative Biochemistry and Physiology, Part B*, **139**: 287-297.

Evans D.L., Lautenbacher C.C. & W. T. Hogarth. 2002. Molluscan research and management. Charting a couse for the future. Proceeding from the workshop at Charleston, South Carolina, January 2000. National Oceanic and Atmospheric Administration. 156 pp.

Eversole A.G. & P.B. Heffernan. 1995. Gonadal neoplasia in northern *Mercenaria mercenaria* (Linnaeus, 1758) and southern *M. campechiensis* (Gmelin, 1791) quahogs and their hybrids cultured in South Carolina. *Journal of Shellfish Research*, **14**: 33-39.

Farley C.A. 1969a. Probable neoplastic disease of the hematopoietic system in oysters *Crassostrea virginica* and *Crassostrea gigas*. *Journal of the National Cancer Institute*, **31**: 541-555.

Farley C.A. 1969b. Sarcomatoid proliferative disease in a wild population of blue mussels (*Mytilus edulis*). *Journal of the National Cancer Institute*, **43**: 509-516.

Farley C.A. 1976. Proliferative disorders in bivalve mollusks. *Marine Fisheries Review*, **1210**: 30-33.

Farley C.A. 1987. Selected aspects of neoplastic progression in molluscs. *In*: Progressive stages of malignant growth/development, Vol. II part 4: Comparative aspects of tumors progression, Kaiser H (Eds). pp 1-14 Martinus Nijhoff, New York.

Farley C.A. 1989. Selected aspects of neoplastic progression in molluscs. *In*: Cancer growth and progression. H.E. Kaiser Ed. Martinez Nijhoff. New York. **5**: 24-31.

Farley C.A., Otto S.V. & C.L. Reinisch. 1986. New occurrence of epizootic sarcoma in Chesapeake Bay soft shell clams, *Mya arenaria*. *Fishery Bulletin*, **84**: 851-857.

Farley C.A., Plutschak D.L. & R.F. Scott. 1991. Epizootiology and distribution of transmissible sarcoma in Maryland softshell clams, *Mya arenaria*, 1984-1988. *Environmental Health Perspectives*, **90**: 35-41.

Farley C.A. & A.K. Sparks. 1970. Proliferative Diseases of Hemocytes, Endothelial Cells, and Connective Tissue Cells in Mollusks. *Bibliotheca haematologica*, **36**: 610-617.

Flint S.J., Enquist L.W., Racaniello V.R. & A.M. Skalka. 2004. Principles of virology: molecular biology, pathogenesis and control of animal viruses, 2nd edn. ASM Press, Washington, DC, p217-230.

Ford S.E., Barber R.D. & E. Marks. 1997. Disseminated neoplasia in juvenile eastern oysters *Crassostrea virginica*, and its relationship to the reproductive cycle. *Diseases of Aquatic Organisms*, **28**: 73-77.

Ford S.E. & M.R. Tripp. 1996. Diseases and defense mechanisms. *In*: The Eastern Oyester *Crassostrea virginica*. V.S. Kennedy, R.I.E. Newell & A.F. Able Éditeurs. Maryland Sea Grant College, University of Maryland, College Park, MD. pp 581-660.

Frierman E.M. & J.D Andrews. 1976. Occurrence of hematopoietic neoplsms in Virginia oysters (*Crassostrea virginica*). *Journal of the National Cancer Institute*, **56**: 319-322.

Fujiwara T., Bandi M., Nitta M., Ivanova E.V. Bronson R.T. & D. Pellman. 2005. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. *Nature*, 437: 1043-1047.

Gardner G.R., 1994. Molluscan Tumor Pathology: Environmental Pollutants and Carcinogen Exposures. *In*: (Eds) A. Rosenfield, F.G. Kern and B.J. Keller, Invertebrate Neoplasia: Initiation and Promotional Mechanisms. NOAA Technical Memorandum NMFS-NE-107. pp. 8-11.

Gasiewicz T.A., Henry E.C. & L.L. Collins. 2008. Expression and activity of aryl hydrocarbon receptors in development and cancer. *Critical Reviews in Eukaryotic Gene Expression*, **18**: 279-321.

Gelderblom H.R. 2008. Structure and classification of viruses. Disponible. En ligne. URL: http://gsbs.utmb.edu/microbook/ch041.htm

Gormley K.L., Teather K.L. & D.L. Guignion. 2005. Changes in salmonid communities associated with pesticides runoff events. *Ecotoxicology*, **14**: 671-678.

Green M. & D.J. Alderman. 1983. Neoplasia in *Mytilus edulis* L. from United Kingdom waters. *Aquaculture*, **30**: 1-10.

Grevenynghe J.V., Rion S., Le Ferrec E., Le Vee M., Amiot L., Fauchet R. & O. Fardel. 2003. Polycyclic aromatic hydrocarbons inhibit differentiation of human monocytes into macrophages. *Journal of Immunology*, **170**: 2374-2381.

Grevenynghe J.V., Sparfel L., Vee M.L., Gilot D., Drenou B., Fauchet R. & O. Fardel. 2004. Cytochrome P450-dependent toxicity of environmental polycyclic aromatic hydrocarbons towards human macrophages. *Biochemical and Biophysical Research Communications*, **317**: 708-716.

Gygi S., Rochon Y., Franza B.R., & R. Aebersold. 1999. Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology*, **19**: 1720-1730.

Hankinson O. 1995. The aryl hydrocarbon receptor complex. *Annual Review of Pharmacology and Toxicology*, **35**: 307-340.

Hanks R.W. 1963. The soft-shell clam. United States Department of the Interior, Fish and wildlife Service, Bureau of Commercial Fisheries, pp. 1-18.

Harper D.M., Flessas D.A. & C.L. Reinisch. 1994. Specific reactivity of leukemia cells to polyclonal anti-PCB antibodies. *Journal of Invertebrate Pathology*, **64**: 234-237.

Harshbarger J.C., Otto S.V. & S.C. Chang. 1979. Proliferative disorders in *Crassostrea virginica* and *Mya arenaria* from Chesapeake Bay and intranuclear virus-like inclusions in *Mya arenaria* with germinomas from a Maine oil spill site. *Haliotis*, **8**: 243-248.

Hellemans J., Mortier M., De Paepe A., Speleman F. & J. Vandesompele. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biolology*, **8**: R19.

Henklova P., Vrzal R., Ulrichova J. & Z. Dvorak. 2008. Role of mitogen-activated protein kinases in aryl hydrocarbon receptor signaling. *Chemico-Biological Interactions*, **172**: 93-104.

Hine P.M. 1999. The inter-relationship of bivalve haemocytes. *Fish and Shellfish Immunology*, **9**: 367-385.

Hofseth L.J., Hussain S.P. & C.C. Harris. 2004. p53: 25 years after its discovery. *Trends in Pharmacological Sciences*, **25**: 177-181.

Holbrook L.A.C., Butler R.A., Cashon R.E. & R.J. Van Beneden. 2009. Soft-shell clam (*Mya arenaria*) p53: a structural and functional comparaison to human p53. *Gene*, **433**: 81-87.

House M.L. 1997. Transmission of disseminated neoplasia in the soft shell clam, *Mya arenaria*. PhD thesis, Oregon State University, Corvallis.

House M.L., Kim C.H. & P.W. Reno. 1998. Soft- shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. *Diseases of Aquatic Organisms*, **34**: 187-192.

Huffman J.E. & M.R. Tripp. 1982. Cell types and hydrolytic enzymes of soft shell clam *Mya arenaria* haemocytes. *Journal of Invertebrate Pathology*, **40**: 68-74.

Ioannides C. & D.V. Parke. 1993. Induction of cytochrome P4501 as an indicator of potential chemical carcinogenesis. *Drug Metabolism Reviews*, **25**: 485-501.

Jiao S., Liu B., Gao A., Ye M., Jia X., Zhang F., Liu H., Shi X. & C. Huang. 2008. Benzo(a)pyrene-caused increased G_1-S transition requires the activation of c-Jun

through p53-dependent PI-3K/Akt/ERK pathway in human embryo lung fibroblasts. *Toxicology Letters*, **178**: 167-175.

Ju J., Schmitz J.C., Song B., Kudo K. & E. Chu. 2007. Regulation of p53 expression in response to 5-fluorouracil in human cancer RKO cells. *Clinical Cancer Research*, **13**: 4245-4251.

Kelley M.L., Winge P., Heaney J.D., Stephens R.E., Farell J.H., van Beneden R.J., Reinisch C.L., Lesser M.P. & C.W. Walker. 2001. Expression of homologues for p53 and p73 in the softshell clam (*Mya arenaria*), a naturally-occurring model for human cancer. *Oncogene*, **20**: 748-758.

Kent M.L., Elston R.A., Wilkinson M.T. & A.S. Drum. 1989. Impaired Defense Mechanisms in Bay Mussels, *Mytilus edulis*, with Hemic Neoplasia. *Journal of Invertebrate Pathology*, **53**: 378-386.

Kent M.L., Wilkinson M.T., Drum A.S. & R.A. Elston. 1991. Failure of transmission of hemic neoplasia of bay mussels, *Mytilus trossulus*, to other bivalve species. *Journal of Invertebrate Pathology*, **57**: 435-436.

Kotzin B.L. & R.F. Baker. 1972. Selective inhibition of genetic transcription in sea urchin embryos. *Journal of Cell Biology*, **55**: 74-81.

Kraus R.A. 1988. Hard clam culture at Aquaculture Research Corporation. *Journal of Shellfish Research*, **7**: 545. (Abstract).

Krishnakumar P.K., Casillas E., Snider R.G., Kagley A.N. & U. Varanasi. 1999. Environmental Contaminants and the prevalence of hemic neoplasia (Leukemia) in the common mussel (*Mytilus edulis* Complex) from Puget Sound, Washington, U.S.A. *Journal of Invertebrate Pathology*, **73**: 135-146.

Kwon J.-W. & K.L. Armbrust. 2006. Degradation of chlorothalonil in irradiated water/sediment systems. *Journal of Agricultural and Food Chemistry*, **54**: 3651-3657.

Landsberg J.H. 1996. Neoplasia and Biotoxins in Bivalves: Is there a connection? *Journal of Shellfish Research*, **15**: 203-230.

Langdon C.J. & C.A. Siegfried. 1984. Progress in the development of artificial diets for bivalve filter feeders. *Aquaculture*, **39**: 135-153.

Leaver M.J., Diab A., Boukouvala E., Williams T.D., Chipman J.K., Moffat C.F., Robinson C.D., & S.G. George. 2010. Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways. *Aquatic Toxicology*, **96**, 234-245.

Leavitt D.F., McDowell J., Capuzzo R.M., Smolowitz D.L., Miosky D.L., Lancaster B.A. & C.L. Reinisch. 1990. Hematopoietic neoplasia in *Mya arenaria*: prevalence and indices of physiological condition. *Marine Biology*, **105**: 313-321.

Lee J.T. & W. Gu. 2010. The multiple levels of regulation by p53 ubiquitination. *Cell Death and Differentiation*, **17**: 86-92.

Lewis D.E. & R.M. Cerrato. 1997. Growth uncoupling and the relationship between shell growth and metabolism in the soft shell clam *Mya arenaria*. *Marine Ecology Progress Series*, **158**: 177-189.

Liu N., Pan L., Miao J., Xu C. & L. Zhang. 2010. Molecular cloning and sequence analysis and the response of a aryl hydrocarbon receptor homologue gene in the clam *Ruditapes philippinarum* exposed to benzo(*a*)pyrene. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, **152**: 279-287.

Lopez C., Carballal M., Azevedo C. & A. Villalba. 1997. Morphological characterization of the hemocytes of the clam *Ruditapes decussates* (Mollusca Bivalvia). *Journal of Invertebrate Pathology*, **69**: 51-57.

Lowe D.M. & M.N. Moore. 1978. Cytology and quantitative cytochemistry of a proliferative atypical hemocytic condition in *Mytilus edulis* (Bivalvia, Mollusca). *Journal of the National Cancer Institute*, **60**: 1455-1459.

Lowry D.R., Row W.P., Teich N. & J.W. Hartley. 1971. Murine leukemia virus: high-frequency activation in vitro by 5-Iododeoxyuridine and 5-Bromodeoxyuridine. *Science*, **174**: 155-156.

Ma Q. & J.P. Whitlock. 1996. The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Molecular and Cellular Biology*, **16**: 2144-2150.

MacCallum G.S., McGladdery S.E. & J. Davidson. 2003. Factors, Risks and significance of emergent neoplasia in cultured and wild soft-shell clams (*Mya arenaria*) in Atlantic Canada. AQUANET AP-18 report. 32 pp.

Macip S., Igarashi M., Berggren P., Yu J., Lee S.W. & S.A. Aaronson. 2003. Influence of induced reactive oxygen species in p53-mediated cell fate decisions. *Molecular and Cellular Biology*, **23**: 8576-8585.

Maki C.G., Huibregste J.M. & P.M. Howley. 1996. In vivo ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Research*, **56**: 2649-2654.

Malouf R.E. & S.E. Siddall. 1985. The status and potential of public and private culture of the hard clam *Mercenaria mercenaria* (Linne) in New York. *Journal of Shellfish Research*, **5**: 40. (Abstract).

Mann K.K., Matulka R.A., Hahn M.E., Trombino A.F., Lawrence B.P., Kerkvliet N.I. & D.H. Sherr. 1999. The role of polycyclic aromatic hydrocarbon metabolism in dimethylbenz(*a*)anthracene-induced pre-B lymphocyte apoptosis. *Toxicology and Applied Pharmacology*, **161**: 10-22.

Matthiessen G.C. 1960. Intertidal zonation in populations of *Mya arenaria*. *Limnology and Oceanography*, **5**: 381-388.

McGladdery S.E., MacCallum G.S., MacNair N.G. & J.T. Davidson. 2001b. Mass mortalities of soft-shell clams (*Mya arenaria*) in Atlantic Canada associated with unprecedented levels of hemic neoplasia. *Journal of Shellfish Research*, **20**: 549. (Abstract).

McGladdery S.E., Reinisch C.L., MacCallum G.S., Stephens R.E., Walker C.L. & J.T. Davidson. 2001a. Haemic Neoplasia in Soft-shell Clams (*Mya arenaria*): Recent Outbreaks in Atlantic Canada and Discovery of a p53 Gene Homologue Associated with the Condition. *Bulletin of the Aquaculture Association of Canada*, 101: 19-26.

McLaughlin S.M. 1994. Transmission studies of sarcoma in the softshell, *Mya arenaria*. *Dans*: Invertebrate neoplasia: Initiation and promotion mechanisms, Rosenfield A, Kern F.G., Keller B.J. (Eds). U.S. Department of Commerce, Woods Hole, MA, pp. 21-22.

McLaughlin S.M., Farley C.A. & F.M. Hetrick. 1992. Transmission of sarcoma in the soft shell clam, *Mya arenaria*. *In Vivo*, **6**: 367-370.

Miosky D.L., Smolowitz R.M. & C.L. Reinisch. 1989. Leukemia Cell Specific Protein of the Bivalve Mollusc *Mya arenaria*. *Journal of Invertebrate Pathology*, **53**: 32-40.

Mitton J. B. 1995. Enzyme heterozygosity and developmental stability. Acta Theriol, 40: 33-54.

Mix M.C. 1976. A general model for leukocyte cell renewal in bivalve mollusk. *Marine Fisheries Review*, **38**: 239-248.

Mix M.C. 1983. Haemic neoplasms of bay mussels, *Mytilus edulis L.*, from Oregon: occurrence, prevalence, seasonality and histopathological progression. *Journal of Fish Diseases*, **2**: 239-248.

Mix C.M. 1986. Cancerous diseases in aquatic animals and their association with environmental pollutants: a critical literature review. *Marine Environmental Research*, **20**: 1-141.

Mix M.C., Hawkes J.W. & A.K. Sparks. 1979. Observation on the ultrastructure of large cells associated with putative neoplastic disorders of mussels, *Mytilus edulis*, from Yaquina Bay. Oregon. *Journal of Invertebrate Pathology*, **34**: 41-56.

Molès J.P., Tesniere A. & J.J. Guilhou. 2007. Reverse transcriptase activity in human normal and psoriatic skin samples. *British Journal of Dermatology*, **157**: 482-486.

Moore C.A., Beckmann N. & M.P. Morse. 1992. Cytoskeletal structure of diseased and normal hemocytes of *Mya arenaria*. *Journal of Invertebrate Pathology*, **60**: 141-147.

Moore J.D. 2003. Disseminated neoplasia of *Mytilus trossulus*. *Bulletin of the Aquaculture Association of Canada,* 102: 101. (Abstract).

Moore J.D., Elston R.A., Drum A.S. & M.T. Wilkinson. 1991. Alternate pathogenesis of systemic neoplasia in the bivalve mollusk *Mytilus*. *Journal of Invertebrate Pathology*, **58**: 231-243.

Morrison C.M., Moore A.R., Marryatt V.M. & D. J. Scarratt. 1993. Disseminated sarcomas of soft-shell clams, *Mya arenaria* Linnaeus 1758, from sites in Nova Scotia and New-Brunswick. *Journal of Shellfish Research*, **12**: 65-69.

MPO. 1999. Exigences relatives à la qualité de l'habitat : mye. En ligne. Disponible :URL: http://www.maritimes.dfo.ca/science/hab/f/mya_fr.htm

MPO. 2005. Les espèces de mollusques et de crustacés. En ligne. Disponible : URL: http://www.dfo-mpo.gc.ca/aquaculture/shellfish-mollusque/species-especes-fra.htm

MPO 2008. Évaluation des stocks de mye commune des eaux côtières du Québec en 2007. *Secrétariat canadien de consultation scientifique*, *Avis scientifique*, 2007/051.

MPO. 2010. Pêches commerciales. En ligne. Disponible: URL: http://www.dfo-mpo.gc.ca/stats/commercial/sea-maritimes-fra.htm

Mutch J.P., Savard M.A., Julien G.R.J., MacLean B, Raymond B & J. Doull. 2002. Pesticide monitoring and fish kill investigations on Prince Edward Island, 1994-1999 *dans*: Effects of land use practices on fish, shellfish, and their habitats on Prince Edward Island. *Rapport technique canadien des sciences halieutiques et aquatiques* 2408.

Myrand B., Tremblay R. & J.M. Sévigny. 2002. Selection against blue mussels (*Mytilus edulis* L.) homozygotes under various stressful conditions. *Journal of Heredity*, **93**: 238-248.

Nebert D.W., Roe A.L., Dieter M.Z., Solis W.A., Yang Y. & T.P. Dalton. 2000. Role of the aromatic hydrocarbon receptor and *(Ah)* gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochemical Pharmacology*, **59**: 65-85.

Newell C.R. 1991. The soft-shell clam *Mya arenaria* (Linnaeus) in North America. *Dans* Estuarine and Marine bivalve mollusk culture. Boca Raton, Floride, CRC Press.

Newell C.R. & H. Hidu. 1986. Life histories and environmental requirements of coastal fish and invertebrate (North Atlantic). Biological Report, **82** (11.53): 1-17.

Nikolaev A.Y., Li M., Puskas N., Qin J. & W. Gu. 2003. Parc: A cytoplasmic anchor for p53. *Cell*, **112**: 29-40.

Noël D., Boulo V., Chagot D., Mialhe E., Paolucci F., Clavies C., Hervaud E. & R.A. Elston. 1991. Preparation and characterization of monoclonal antibodies against neoplastic hemocytes of *Mytilus edulis* (Bivalvia). *Diseases of Aquatic Organisms*, **10**: 51-88.

Olberding K.E., Kelley M.L., Butler R.A. & R.J. Van Beneden. 2004. A HECT E3 ubiquitin-protein ligase with sequence similarity to E6AP does not target p53 for degradation in softshell clam (*Mya arenaria*). *Mutation Research*, **552**: 61-71.

Oliver L.M., Fisher F.S. & A.K. Volety. 1998. Hemocyte activities and contaminant burdens in oysters (*Crassostrea virginica*) from five estuaries in the southern United States. *Journal of Shellfisheries,* **17**: 335. (Abstract).

Oprandy J.J. & P.W. Chang. 1983. 5-Bromodeoxyuridine Induction of Hematopoietic Neoplasia and retrovirus Activation in the Soft-Shell Clam, *Mya arenaria. Journal of Invertebrate Patholology*, **42**: 196-206.

Oprandy J.J., Chang P.W., Pronovost A.D., Cooper K.R., Browns R.S. & V.J. Yates. 1981. Isolation of a Viral Agent Causing Hematopoietic Neoplasia in the Soft-Shell Clam, *Mya arenaria. Journal of Invertebrate Patholology*, **38**: 45-51.

Osertag E.M. & H.H. Kazazian. 2001. Biology of mammalian Li retrotransposons. Annual Review of Genetics, **35**: 501-538.

Padros J., Pelletier E. & C.O. Ribeiro. 2003. Metabolic interactions between low doses of benzo[a]pyrene and tributyltin in arctic charr (Salvelinus alpinus): a long-term in vivo study. *Toxicology and Applied Pharmacology*, **192**: 45-55.

Page T.J., O'Brien S., Holston K., MacWilliams P.S., Jefcoate C.R. & C.J. Czuprynski. 2003. 7,12-Dimethylben(*a*)anthracene-induced bone marrow toxicity is p53-dependent. *Toxicological Sciences*, **74**: 85-92.

Pariseau J., Saint-Louis R., Delaporte M., AboElkhair M., McKenna P., Tremblay R., Davidson T.J., Pelletier E., & Berthe F.C.J. 2009. Potential link between exposure to fungicides chlorothalonil and mancozeb and haemic neoplasia development in soft-shell clam *Mya arenaria*: A laboratory experiment. *Marine Pollution Bulletin*, **58**: 503-514.

Parker M., Beal B. & S. Fegley. 1998 Creating a sustainable soft-shell clam fishery in Maine: A community clam culture program. Abstracts of the First Annual Northeast Aquaculture Conference and Exposition. 17 pp.

PEI (Department Environment, Energy and Forestry) 2009. Pesticides Sales Data For Prince Edward Island. Disponible en ligne: URL: http://www.gov.pe.ca/envengfor/index.php3?number=1029945&lang=E

Peters E.C. 1988. Recent investigations on the disseminated sarcomas of marine bivalve molluscs. *Disease Processes in Marine Bivalve Molluscs*: 74-92.

Petrovits E.J. 1985. Commercial mariculture of *Mercenaria mercenaria* (Linne) at Aquaculture Research Corporation, Dennis, Massachusetts. *Journal of Shellfish Research*, **5**: 42. (Abstract).

Potts M.S. 1996. Effect of hematopoietic neoplasia on reproduction and population size distribution in the soft-shell clam. *Journal of Shellfish Research*, **15**: 519. (Abstract).

Provencher L. (éd) 2003. Compte-rendu d'un atelier sur la recherche appliquée aux communautés à *Mya-Macoma*, dans l'estuaire et le golfe du Saint-Laurent, tenu le 28 mars 2002. *Rapport manuscrit canadien des sciences halieutiques et aquatiques*, **2657**, 30 p.

Putman R.A., Nelson J.O. & J.M. Clark. 2003. The persistence and degradation of chlorothalonil and chlorpyrifos in a cranberry bog. *Journal of Agricultural and Food Chemistry*, **51**: 170-176.

Raghavan N., Miller A.N., Gardner M., FitzGerald, P.C., Kerlavage A.R., Johnston D.A., Lewis F.A. & M. Knight. 2003. Comparative gene analysis of *Biomphalaria glabrata* hemocytes pre- and post-exposure to miracidia of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology*, **126**: 181-191.

Rand G.M. & S.R. Petrocelli. 1985. Fundamentals of aquatic toxicology: methods and application. Edited by G.M. Rand & S.R. Petrocelli. New York: Hemisphere Publishing Corporation. 666 pp.

Rasmussen L.P.D. 1986. Occurrence, prvalence and seasonality of neoplasia in the marine mussel *Mytilus edulis* from three sites in Denmark. *Marine Biology*, **92**: 59-64.

Reeves D. 2001. Pesticides sales report. Pesticides Regulatory Program. Department of Agriculture and Forestry. Prince Edward Island.

Reinisch C.L., Charles A.M. & A.M. Stone. 1984. Epizootic neoplasia in soft shell clams collected from New Bedford Harbor. *Hazard Waste*, **1**: 73-81.

Reno P.W., House M. & A. Illingworth. 1994. Flow Cytometric and Chromosome Analysis of Softshell Clams, *Mya arenaria*, with Disseminated Neoplasia. *Journal of Invertebrate Patholog*, **64**: 163-172.

Reyes-Hernández O.D., Mejía-García A., Sánchez-Ocampo E.M., Cabañas-Cortés M.A., Ramírez P., Chávez-González L., Gonzalez F.J. & G. Elizondo. 2010. Ube2l3 gene expression is modulated by activation of the aryl hydrocarbon receptor: implications for p53 ubiquitination. *Biochemical Pharmacology*, **80**: 932-40.

Rhodes L.D., Gardner G.R. & R. Van Beneden. 1997. Short-term tissue distribution, depuration and possible gene expression effects of [^{3}H]TCDD exposure in soft-shell clams (*Mya arenaria*). *Environmental Toxicology and Chemistry*, **16**: 1888-1894.

Ribeiro C.A., Padros J., Domingos F.X., Akaishi F.M. & E. Pelletier. 2007. Histopathological evidence of antagonistic effects of tributyltin on benzo[a]pyrene toxicity in the Arctic charr (Salvelinus alpinus). *Science of the Total Environment*, **372**: 549-553.

Riisgard H.U. & D.F. Seerup. 2003. Filtration rates in the soft clam *Mya arenaria*: effects of temperature and body size. *Sarsia*, **88**: 415-428.

Romalde J.L., Vilarino M.L., Beaz R., Rodriguez J.M., Diaz S., Villalba A. & M.J. Carballal. 2007. Evidence of retroviral etiology for disseminated neoplasia in Cockles (*Cerastoderma edule*). *Journal of Invertebrate Pathology*, **94**: 95-101.

Roseberry L. 1988. Étude de la croissance et de la reproduction chez *Mya arenaria* (Bivalvia : Mollusca) dans la zone intertidale de l'estuaire du Saint-Laurent. Mémoire de maîtrise. Université du Québec à Rimouski.

Roseberry L., Vincent B. & C. Lemaire. 1991. Croissance et reproduction de *Mya arenaria* dans la zone intertidale de l'estuaire du Saint-Laurent. *Canadian Journal of Zoology*, **69** : 724-732.

Ruppert E.E. & R.D. Barnes. 1994. Invertebrate Zoology., Sixth Edition, Saunders College Publishing, Harcourt Brace College Publishers. Montréal, Québec, Canada.

Sciamanna L., Landriscina M., Pittoggi C., Quirino M., Mearelli C., Beraldi R., Mattei E., Serafino A., Cassano A., Sinibaldi-Vallebona P., Garaci E., Barone C. & C. Spadafora. 2005. Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. *Oncogene*, **24**: 3923-3931.

Siah A., Delaporte M., Pariseau J., McKenna P. & F.C.J. Berthe. 2008a. Patterns of p53, p73 and mortalin gene expression associated with haemocyte polyploidy in the soft-shell clam, *Mya arenaria. Journal of Invertebrate Pathology*, **98**: 148-152.

Siah A., Dohoo C., McKenna P., Delaporte M. & F.J.C. Berthe F. 2008b. Selecting a set of housekeeping genes for quantitative real-time PCR in normal and tetraploid haemocytes of soft-shell clams, *Mya arenaria. Fish and Shellfish Immunology*, **25**: 202-207.

Siah A., Masson R., Loup B., Bultelle F., Pellerin J., Leboulenger F. & J.M. Danger 2007. Receptor activated C kinase is down-regulated in the male gonad of the marine bivalve mollusc *Mya arenaria* exposed to tributyltin (TBT). *Aquatic Toxicology*, **83**: 295-305.

Sinderman C.J. 1990. Mechanisms of internal defense. *Principle diseases of marine fish and shellfish.* C.J. Sinderman San Diego, Academic Press Inc. 2: 247-300.

Slee E.A., O'Connor D.J. & X. Lu. 2004. To die or not to die: how does p53 decide? *Oncogene*, **23**: 2809-2818.

Smolowitz R.M. 2005. Disseminated *neoplasia* or hemocytic leukemia: Where do those neoplastic cells come from? *Journal of Shellfish Research*, **24**: 675-676.

Smolowitz R.M., Miosky D. & C.L. Reinisch. 1989. Ontogeny of Leukemic Cells of the Soft Shell Clam. *Journal of Invertebrate Pathology*, **53**: 41-51.

Snape J.R., Maund S.J., Pickford D.B. & T.H. Hutchinson. 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology*, **67**, 143-154.

Sokal R.R. & F.J. Rohlf. 1995. Biometry: the principles and practice of statistics in biological research. W. H. Freeman, New York, 887p.

Stephens R. E., Walker C.W. & C.L. Reinisch. 2001. Multiple protein differences distinguish clam leukemia cells from normal hemocytes: evidence for the involvement of p53 homologues. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, **129**: 329-338.

Stickney A.P. 1963. Histology of the reproductive system of the soft-shell clam (*Mya arenaria*). *Biology Bulletin*, **125** (2): 344-351.

Stickney A.P. 1964. Salinity, temperature and food requirments of soft-shelled clam larvae in laboratory culture. *Ecology*, **45**: 283-291.

St-Onge P., Sévigny J-M., Strasser C. & R. Tremblay. In preparation. Strong population differentiation of softshell clams (*Mya arenaria*) sampled across seven biogeographic marine ecoregions as assessed with microsatellite markers.

Strandberg J.D., Rosenfield J., Berzins I.K. & C.L. Reinisch. 1999. Specific localization of polychlorinated biphenyls in clams (*Mya arenaria*) from environmentally impacted sites. *Aquatic Toxicology*, **41**: 343-354.

Sunila I. 1992. Serum-cell interactions in transmission of sarcoma in the soft shell clam, *Mya arenaria* L. *Comparative Biochemistry and Physiology*, **102**: 727-730.

Sunila I & C.A. Farley. 1989. Environmental limits for survival of sarcoma cells from the soft shell clam *Mya arenaria*. *Diseases of Aquatic Organisms*, **7**: 111-115.

Synard S.L. 2007. Morphological classification of soft-shell clam, *Mya arenaria*, hemocytes: phenotypic description of hemic neoplasia. Mémoire de maîtrise. University of Prince Edward Island.

Toledo F., & G.M. Wahl. 2006. Regulating the p53 pathway: *in vitro* hypotheses, *in vivo veritas*. *Nature Reviews Cancer*, **6**: 909-923.

Tomlin C. 2000. The Pesticide Manual — Incorporating the Agrochemicals Handbook.10th Edition, The British Crop Protection Council and The Royal Society of Chemistry. 1250 pp.

Twomey E. & M.F. Mulcahy. 1988. Transmission of sarcoma in the cockle *Cerastoderma edule* (Bivalvia; Mollusca) using cell transplants. *Developmental and Comparative Immunology*, **12**: 195-200.

Van Aggelen G, Ankley G.T, Baldwin WS, Bearden D.W., Benson W.H., Chipman J.K., Collette T.W., Craft J.A., Denslow N.D., Embry M.R., Falciani F., George S.G., Helbing C.C., Hoekstra P.F., Iguchi T., Kagami Y., Katsiadaki I., Kille P., Liu L., Lord P.G., McIntyre T., O'Neill A., Osachoff H., Perkins E.J., Santos E.M., Skirrow R.C., Snape J.R., Tyler C.R., Versteeg D., Viant R., Volz D.C., Williams T.D., & L. Yu. 2010. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: hurdles, achievements, and future outlook. *Environmental Health Perspectives*, **118**: 1-5.

van Beneden R.J. 1994. Molecular analysis of bivalve tumors: models for environmental/genetic interactions. *Environmental Health Perspectives*, **102**: 81-83.

van Beneden R.J. 1997. Environmental effects and aquatic organisms: investigations of molecular mechanism of carcinogenesis. *Environmental Health Perspectives*, **105**: 669-674.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genom Biology*, **3**: research 034.1-0034.11.

Villalba A., Carballal, M.J. & C. Lopez. 2001. Disseminated neoplasia and large foci indicating heavy haemocytic infiltration in cockles *Cerastoderma edule* from Galicia (NW Spain). *Diseases of Aquatic Organisms*, **46**: 213-216.

Volety A.K., Chu F.-L., Constantin G. & R.C. Hale. 1997. Effects of PAHs on the function of hemocytes from eastern oysters *Crassostrea virginica*. *Journal of Shellfish Research*, **16**: 352. (Abstract).

Wadhwa R., Taira K. & S.C. Kaul. 2002. Mortalin: a potential candidate for biotechnology and biomedicine. *Histology and Histopathology*, **17**: 1173-1177.

Walker C., Böttger S. & B. Low. 2006. Mortalin-based cytoplsamic sequestration of p53 in a nonmammalian cancer model. *American Journal of Pathology,* **168**: 1526-1530.

Walker C.W. & S.A. Böttger. 2008. A naturally occurring cancer with molecular connectivity to human diseases. *Cell Cycle*, **7**: 2286-2289.

Walker C.W., Böttger S.A., Mulkern J., Jerszyk E.C., Litvaitis M. & M. Lesser. 2009. Mass culture and characterization of tumor cells from a naturally occurring invertebrate cancer model: applications for human and animal disease and environmental health. *Biological Bulletin*, **216**: 23-39.

Walker H.A., Lorda E. & S.B. Saila. 1981. A comparison of the incidence of five pathological conditions in soft-shell clams, *Mya arenaria*, from environments with various pollution histories. *Marine Environmental Research*, **5**: 109-123.

Watanabe A., Inokuchi K., Yamaguchi H., Mizuki T., Tanosaki S., Shimada T & K. Dan. 2004. Near-triploidy and near-tetraploidy in hematological malignancies and mutation of the p53 gene. *Clinical and Laboratory Haematology*, **24**: 25-30.

Weinberg J.R., Leavitt D.F., Lancaster B.A. & J. McDowell Capuzzo. 1997. Experimental field studies with *Mya arenaria* (Bivalvia) on the induction and effect of hematopoietic neoplasia. *Journal of Invertebrate Pathology*, **69**: 183-194.

White J. 2004. P.E.I. Shellfish Pesticide Survey Summary 2003. CFIA-Charlottetown, Prince Edward Island.

White L., Ernst W.R., Julien G., Garron C. & M. Leger. 2006. Ambient air concentrations of pesticides used in potato cultivation in Prince Edward Island, Canada. *Pest Management Science*, **62**: 126-136.

Xu D., Penning T.M., Blair I.A. & R.G. Harvey. 2009. Synthesis of phenol and quinine metabolites of benzo [*a*] pyrene, a carcinogenic component of tobacco smoke implicated in lung cancer. *Journal of Organic Chemistry*, **74**: 597-604.

Yevich P.P. & C.A. Barszcz. 1976. Gonadal and Hematopoietic Neoplasms in *Mya arenaria*. *Marine Fisheries Review*, **38**: 42-43.

Yevich P.P. & C.A. Barszcz. 1977. Neoplasia in soft-shell clams (*Mya arenaria*) collected from oil-impacted sites. *Annals of the New York Academy of Sciences,* **298**: 409-426.

Zouros E. 1987. On the relation between heterozygosity and heterosis: An evolution of the evidence from marine mollusks. *Isozymes*, **15**: 255-270.

ANNEXE

Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 98 (2008) 190–197
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry

Maryse Delaporte [a,d,*], Stéphanie Synard [a], Julie Pariseau [a,b], Patricia McKenna [a], Réjean Tremblay [b], Jeffery Davidson [c], Franck C.J. Berthe [a,e]

[a] *University of Prince Edward Island, Atlantic Veterinary College, Department of Pathology and Microbiology, Charlottetown, C1A 4P3, Prince Edward Island, Canada*
[b] *Institut des Sciences de la mer, Université du Québec à Rimouski, Rimouski, G5L3A1, Que., Canada*
[c] *University of Prince Edward Island, Atlantic Veterinary College, Department of Health Management, Charlottetown, C1A 4P3, Prince Edward Island, Canada*
[d] *Université Louis Pasteur, Institut de Virologie, 3 rue Koeberlé, 67000 Strasbourg, France*
[e] *Animal Health and Welfare Unit, European Food Safety Agency (EFSA), Largo N. Palli 5/A, I-43100 Parma, Italy*

Received 8 October 2007; accepted 20 December 2007
Available online 28 December 2007

Abstract

Diagnosis of haemic neoplasia (HN) in the soft shell clam, *Mya arenaria*, is often achieved by hematocytology and histology. Since neoplastic cells display tetraploid DNA contents, haemocyte cell cycle analysis was developed for use as a diagnosis tool. The aim of this study was to assess the application of a flow cytometry procedure of cell cycle analysis established for the common cockle, to clams and to evaluate different thresholds of value for the percentage of tetraploid cells for establishing HN disease status of individual clams and clam populations. HN status of six clam populations from eastern Canada was determined. Results of the present study demonstrate a flow cytometry procedure to be useful for HN diagnosis in clams. Individual clams were considered to be affected by HN when presenting at least 20% of haemocytes in S–4N phase; and negative when presenting less that 5% of haemocytes in S–4N phase. As discussed in this paper, intermediate cases represent uncertain diagnoses including either false-negative or false-positive clams, which are difficult to discriminate. At a population level, an additional threshold of 15% for the mean intensity of the disease is proposed, which means having in the population several individual clams presenting more than 20% of their haemocytes in S–4N phase. Based on these thresholds of value, only one population was considered as free of HN disease, and one population was unequivocally affected by HN. For the four other clam populations, further investigations are needed toward development and use of specific and objective biomarkers of HN.

Keywords: Haemic neoplasia; Population assessment; *Mya arenaria*; Flow cytometry; Cell cycle; Threshold; Cut-off values

1. Introduction

Disseminated neoplasias (DN) have been reported in a variety of bivalve molluscs around the world since the initial description by Farley (1969a, 1969b). The condition has been most intensively studied in the soft shell clam, *Mya arenaria*, in which the disease is referred as haemic neoplasia (HN), and is progressive and fatal in most cases. Three comprehensive papers consider the published literature in detail and critically reviews subjects including diagnosis methodology and detection (Peters, 1988; Elston et al., 1992; Barber, 2004).

HN diagnosis is often based on hematocytology, coupled or not with histological tissue examination (Brousseau, 1987; Brousseau and Baglivo, 1991a; da Silva et al., 2005; Dungan et al., 2002; Elston et al., 1988; McGladdery et al., 2001; Villalba et al., 2001). Haemolymph-screening

* Corresponding author. Address: Université Louis Pasteur, Institut de Virologie, 3 rue Koeberlé, 67000 Strasbourg, France. Fax: +33 3 90 24 37 24.
E-mail address: maryse_delaporte@hotmail.com (M. Delaporte).

0022-2011/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2007.12.005

techniques enable distinction of neoplastic cells based on their morphology. According to the literature, neoplastic cells have a large nucleus and a high nucleus:cytoplasm ratio. The intensity of HN is generally rated as the percentage of neoplastic cells in the total number of haemocytes examined per slide (Farley et al., 1986), and classified in 3 to 5 different stages, depending on authors' classification criteria (Brousseau and Baglivo, 1991b; Farley et al., 1986; Leavitt et al., 1990; McGladdery et al., 2001). Brousseau and Baglivo (1991b) separated clams into three groups relative to the intensity of the disease: non-affected, low severity (1–50% neoplastic cells) and high severity HN (>51% neoplastic cells) while McGladdery et al. (2001) ranged intensity of HN disease as light (1–10%), moderate (11–50%) and heavy (51–100% neoplastic cells).

Complementarily, histopathology provides information on disease pathology within tissues and main organs. Mix (1983) assigned 4 stages of disease intensity for mussels, depending on infiltration rate of neoplastic cells throughout the body based on histological examination. Although hematocytology and histopathology are broadly used diagnostic methods, they present at least three disadvantages: the limited number of cells analysed per slide, subjectivity, and high labour cost for processing and analysing samples.

In the 1990s, flow cytometry methods (FCM) became increasingly common for studying haemocyte types and functions in bivalve mollusc species (see Ashton-Alcox et al., 2000 for review) and also ploidy changes in the HN disease (da Silva et al., 2005; Elston et al., 1990; Harper et al., 1994; Moore et al., 1991; Reno et al., 1994). Analysis of cell cycles by FCM has allowed establishing that relative amounts of DNA vary between normal and neoplastic cells, and that these differences are not the same among species. It has been demonstrated that the blue mussel, *Mytilus edulis*, affected by DN contained tetraploid and pentaploid cells with 2.03 to 2.64 times more DNA than normal diploid haemocytes (Elston et al., 1990). In the soft shell clam *M. arenaria*, neoplastic haemocytes had 1.25 to 2.05 time more DNA than normal diploid haemocytes (Reno et al., 1994). More, recently, da Silva et al. (2005) established that the common cockle *Cerastoderma edule* presented hypodiploid, hyperdiploid, triploid, and pentaploid neoplastic haemocytes. FCM has proven to be a more powerful and accurate diagnosis tool than traditional diagnosis methodologies, since it allows screening large numbers of cells in few seconds, and permits simultaneous multi-parametric data acquisitions per cell (size, granularity, and fluorescence intensity).

Although DN status of *M. edulis* and *C. edule* may be obvious when considering animals with pentaploid cells, assessment of HN status of *M. arenaria* clams appears to be more challenging, especially for lightly affected clams. Indeed, distinction of lightly affected clams exhibiting few abnormal tetraploid cells, from negative clams with potentially normal tetraploid circulating cells in mitotic process had never been discussed. This shortcoming may result from the fact that the hematopoiesis process remains unknown in molluscs, and that many authors have worked with heavily affected clams.

The aim of this study was to determine thresholds of value for the percentage of tetraploid cells obtained by FCM for establishing HN disease status of individual clams, and populations. HN assessments of clams from 6 different *M. arenaria* populations from eastern Canada were performed according to the FCM procedure of da Silva et al. (2005) for *C. edule*.

2. Material and methods

2.1. Clam collection

Soft shell clams *M. arenaria* were collected at low tide from the following 6 populations of eastern Canada (60 clams per population), and brought to the Atlantic Veterinary College (Fig. 1): Anse St-Étienne Bay (QC), Métis Bay (QC), Kouchibougouac National Park (NB), Havre-aux-Maisons lagoon (Magdalen Islands, QC), North River (PE), and Barasway Bay (NL). Upon arrival at the laboratory, clam populations were maintained separately in tanks with static seawater at 18 °C and salinity 28‰, for two days to one week before flow cytometry analysis.

2.2. Haemolymph preparation and flow cytometry analysis

Haemolymph was withdrawn from each clam's anterior adductor muscle using a 3-ml syringe fitted with a 25-gauge needle. A volume of 500 µl of haemolymph was fixed in absolute ethanol (1:5), and treated according to da Silva et al. (2005). Briefly, after centrifugation at 400*g* for 10 min, cells were re-suspended and re-hydrated for 30 min in Saline Phosphate Buffer (PBS, 0.01 M), twice washed with saline PBS (0.01 M), and filtered through an 80-µm nylon mesh while transferring to a flow cytometer tube. Samples were then treated with DNase-free RNase A (Sigma R4875, 50 µg ml^{-1}) and stained with propidium iodide (PI, Sigma, P4170, 50 µg ml^{-1}) for 45 min. PI fluorescence, which is related to the DNA content of each cell, was detected on the FL2 detector (orange light, at 550–600 nm) of a FACSCalibur flow cytometer (Becton–Dickinson). For each sample, 10,000 particles were counts at low flow rate (15 µl min^{-1}).

On cytograms plotting the width and area of PI fluorescence signals, regions (or gates) related to normal diploid cells (G0/G1), phase S cells, and tetraploid (G2/M) cells were drawn, and considered to represent the mean fluorescence of each category of cell types (Fig. 2a). PI fluorescence intensities of single cells were also plotted on a FL2-area histogram on which specific markers were placed to estimate the percentage of normal cells, phase S, and tetraploid cells in the analysed cell population (Fig. 2b). Results of haemocyte cell cycle analysis are presented on histogram-area, 3-axis graphs, on which the *X*-axis is divided into discrete categories representing increasing

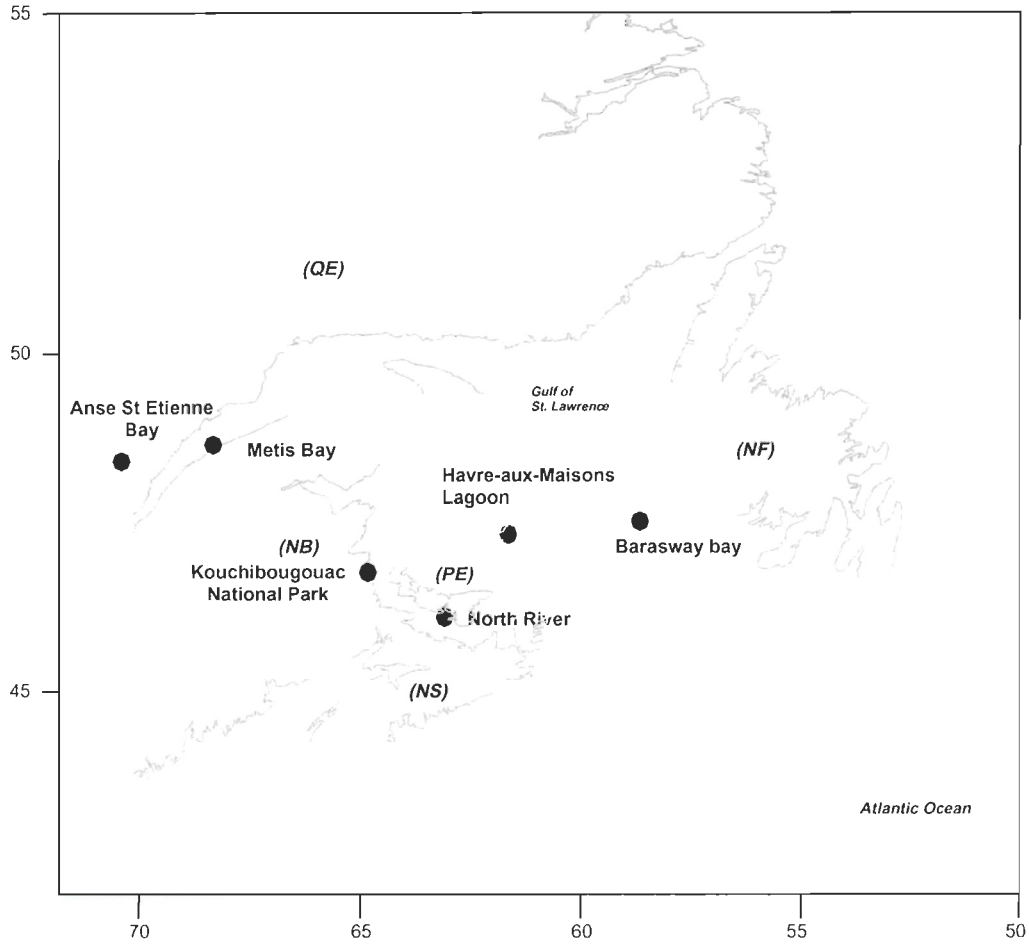


Fig. 1. *Mya arenaria* clam population sampling sites on Atlantic coast of Canada.

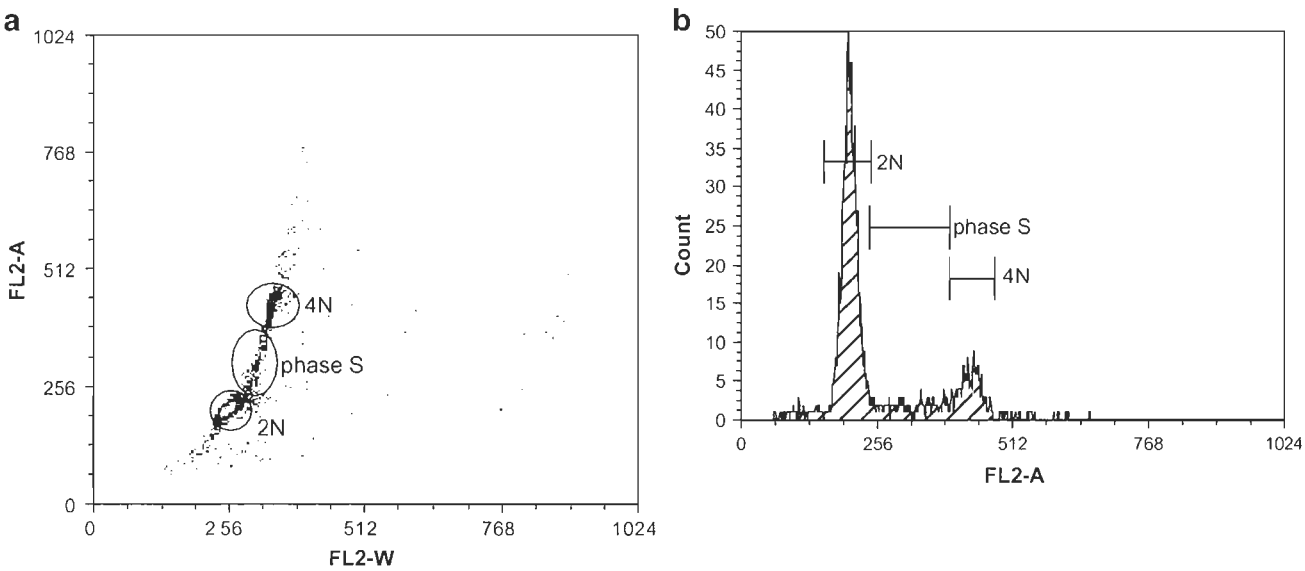


Fig. 2. (a) Gating of normal diploid (G0/G1), phase S and tetraploid (G2/M) single cells on a width vs area FL2 cytogram. (b) Histogram of propidium iodide fluorescence of gated single cells. Markers were placed to estimate the percentage of single cells in each cell cycle phase (G0/G1 (2N), S and G2/M (4N)).

proportions of circulating clam cells in phase S and G2/M stages (phase S–4N), and the *Y*′-axis shows the number of clams per category. Two areas detail the average percentage of cells in the phase S and in the G2/M stage separately

Table 1
Detailed percentages of haemocytes in phase S and 4N stages included in the total percentage of haemocytes in phase S–4N for each *X*-axis category used for the population repartition histogram (mean ± SD)

Site:	Anse St-Étienne Bay (QC)		Kouchibougouac National Park (NB)		Barasway Bay (NF)		Havre-aux-Maisons Lagoon (QC)		Métis Bay (QC)		North River (PE)	
Phase S–4N:	Phase S	4N	Phase S	4N	Phase S	4N	Phase S	4N	Phase S	4N	Phase S	4N
<1%	0.5 ± 0.3	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.5 ± N.A	0.3 ± N.A	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
1–1.5%	0.7 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.5 ± 0.2	1.0 ± 0.1	0.5 ± 0.2	0.8 ± 0.4	0.9 ± 0.4	0.6 ± 0.2	0.6 ± 0.2
1.5–2%	1.0 ± 0.2	0.7 ± 0.3	0.8 ± 0.2	0.9 ± 0.2	1.3 ± 0.2	0.6 ± 0.3	1.2 ± 0.4	0.6 ± 0.4	0.8 ± 0.4	0.9 ± 0.4	1.0 ± 0.4	0.8 ± 0.4
2–2.5%	1.2 ± 0.4	1.0 ± 0.4	1.7 ± 0.5	0.9 ± 1.4	1.3 ± 0.6	0.9 ± 0.6	1.5 ± 0.3	0.8 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	1.4 ± 0.4	0.9 ± 0.3
2.5–3%	1.9 ± 0.2	0.7 ± 0.2	1.5 ± 0.4	1.2 ± 0.4	1.7 ± 0.3	1.0 ± 0.3	2.0 ± 0.6	0.8 ± 0.3	1.3 ± 0.4	1.6 ± 0.4	1.3 ± 0.5	1.4 ± 0.5
3–3.5%	1.9 ± 0.6	1.3 ± 0.3	2.1 ± 0.4	1.2 ± 0.5	2.5 ± 0.5	0.8 ± 0.6	2.0 ± 0.3	1.2 ± 0.5	1.4 ± 0.0	1.6 ± 0.0	1.6 ± 0.5	1.6 ± 0.5
3.5–4%	2.2 ± 0.8	1.6 ± 0.5	2.2 ± 0.4	1.2 ± 0.2	2.4 ± N.A	1.5 ± N.A	2.5 ± 0.6	1.4 ± 0.7			1.7 ± 0.7	2.0 ± 0.8
4–4.5%	2.5 ± 0.3	2.0 ± 0.0					2.0 ± 0.8	2.3 ± 0.8	3.0 ± N.A	1.2 ± N.A	2.6 ± 0.9	1.6 ± 0.8
4.5–5%					3.0 ± 0.2	1.8 ± 0.1	2.6 ± 0.3.	2.1 ± 0.2	2.5 ± N.A	2.3 ± N.A	3.2 ± 0.8	1.3 ± 0.6
5–5.5%					3.1 ± 1.4	2.3 ± 1.4	3.1 ± N.A.	0.8 ± N.A	3.0 ± N.A.	2.2 ± N.A	3.0 ± 0.8.	2.1 ± 0.8
5.5–6%					3.2 ± N.A	2.6 ± N.A	2.7 ± N.A.	3.3 ± N.A	3.5 ± 1.0	2.3 ± 1.0	3.9 ± 0.4	1.8 ± 0.1
6–10%			2.5 ± N.A	6.1 ± N.A	5.8 ± 0.5	2.6 ± 0.8	5.5 ± 2.1	3.4 ± 1.6	3.5 ± 0.7	5.5 ± 0.1	3.9 ± 1.6	3.9 ± 1.8
10–20%					7.3 ± 1.1	3.8 ± 0.3	6.9 ± 1.7	9.8 ± 4.1	8.4 ± 1.8	5.2 ± 2.8	5.2 ± 3.5	8.7 ± 3.4
20–40%									5.2 ± N.A	33.3 ± N.A	10.5 ± 8.8	18.5 ± 8.2
40–60%											20.7 ± 10.9	28.1 ± 15.0
>60%											22.7 ± 21.1	54.0 ± 19.7

according to the *X*-axis scale, with the *Y*-axis showing the percentage of cell types. Numerical means and their SD values are listed in Table 1.

Assessment of HN diagnosis realised by FCM was confirmed by hematocytology according to the diagnostic criteria of Farley et al. (1986), especially for lightly infected clams.

3. Results

3.1. Anse St-Étienne Bay

Cell cycle analysis of haemocytes of clams from Anse St-Étienne Bay showed that clams exhibited a percentage of phase S–4N cells ranging between 0% and 4.5% (Fig. 3). The percentage of cells in phase S varied from 0.5% to 2.5%, and the percentage of 4N cells from 0.2% to 2.0% (Table 1; i.e., ratio 4N/phase S varying from 0.4 to 1.2). By hematocytology and according to McGladdery's classification, all clams were considered as negative (<1% of neoplastic cells; McGladdery et al., 2001).

3.2. Kouchibougouac National Park

Similarly, the majority of clams from Kouchibougouac National Park presented percentages of phase S–4N cells up to 4–4.5% with ratio 4N/phase S varying from 0.6 to 1.3. However, one individual clam had 8.7% of cells in phase S–4N (Fig. 3). This individual clam presented the highest 4N content (6.1%) and a ratio 4N/phase S equalled to 2.5 (Table 1). Interestingly, this clam exhibited a negative profile by hematocytology while some other individuals with 2.1% to 3.5% of phase S–4N cells presented several abnormal neoplastic cells on slides and were considered as lightly affected according to McGladdery et al. (2001).

3.3. Havre-aux-maisons lagoon, Barasway Bay and Metis Bay

Haemocytes of clams from Havre-aux-maisons lagoon, Barasway Bay and Metis Bay had a different pattern than those of the two previous sites (Fig. 3). More individual clams had over 5% of haemocytes in phase S–4N stages. Thus, a third of the clam population from Barasway Bay presented between 6% and 10% of haemocytes in phase S–4N, with an average of 5.8% of cells in phase S and 2.6% in 4N stage (Table 1; ratio 4N/phase S = 0.4). Moreover, clams from Barasway Bay, Havre-aux-maisons lagoon and Metis Bay with more than 4.5%, 3.7% and 6% respectively of haemocytes in phase S–4N were generally diagnosed by hematocytology as lightly affected, except few individual clams, in which no abnormal haemocytes were detected on hematocytology slides.

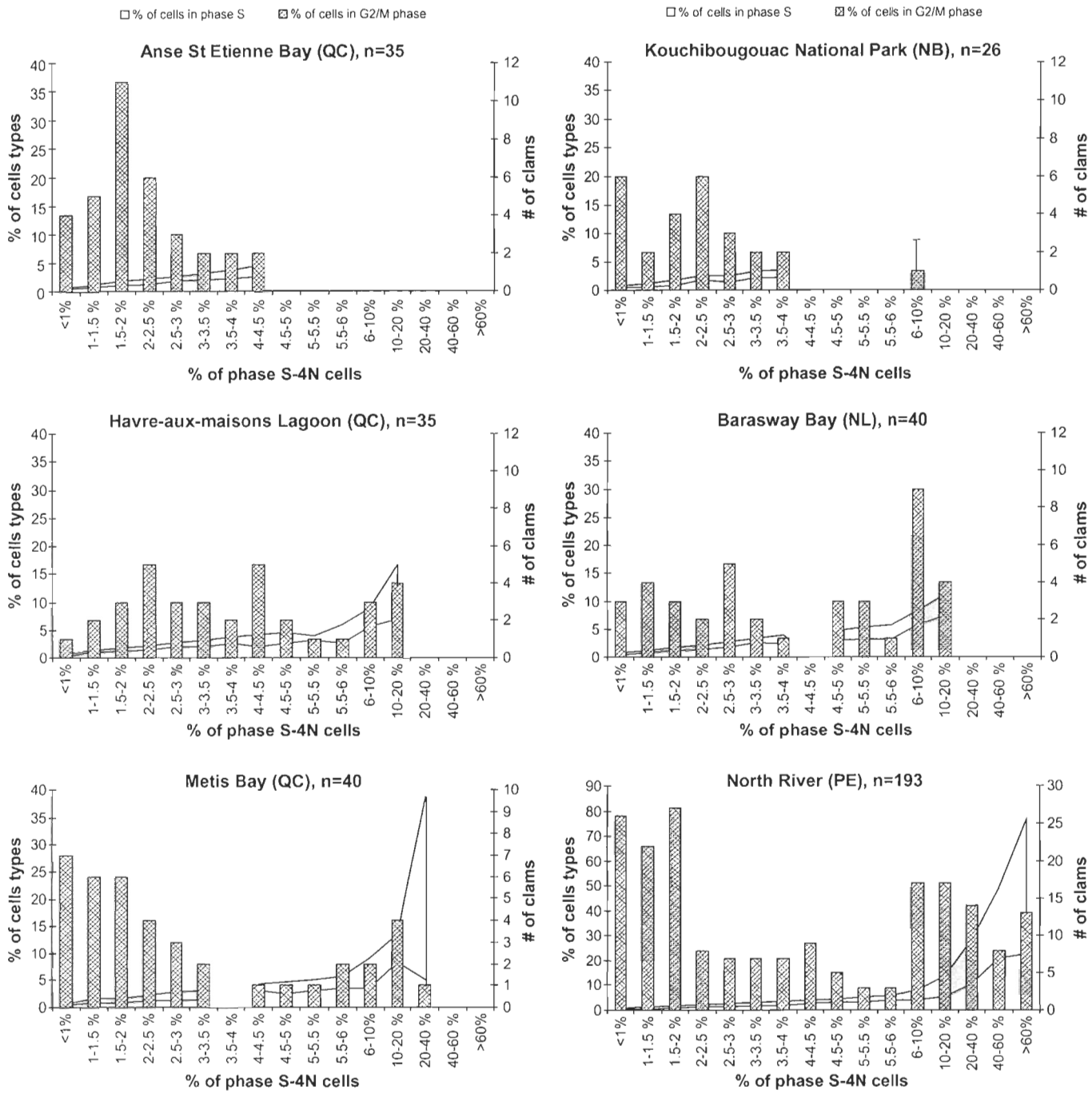


Fig. 3. Representation of haemocyte cell cycle analysis results of each clam population on a histogram-area, 3-axis graphs. *X*-axis are divided into discrete categories representing increasing proportions of circulating clam in phase S and G2/M phases (phase S–4N), *Y*-axis represent the average percentage of cells in the phase S and in the G2/M phases separately, and *Y'*-axis shows the number of clams per category.

4. North River

Clams from North River exhibited percentages of haemocytes in phase S–4N which ranged from 0% up to 92.5% (Fig. 3). Twenty-seven percent of the clam population had over 10% of its haemocytes in phase S–4N, with higher 4N content than the clams belonging to the lowest categories (Table 1; from 8.7% to 54.0% compare to a range of 0.3–3.9%). These clams were confirmed by hematocytology to be lightly to heavily affect by HN.

5. Discussion

Cell cycle analysis of *M. arenaria* haemocytes by FCM according to the procedure described by da Silva et al. (2005) enabled HN diagnosis in six different clam populations from eastern Canada. Although this methodology has been shown to be a powerful tool for accurate diagnosis of HN in the soft shell clam *M. arenaria* (Reno et al., 1994), in the blue mussels *M. edulis* and *M. trossulus* (Elston et al., 1990), and in the common cockle *C. edule*

(da Silva et al., 2005); this report is the first time that flow cytometry data are broadly tested for the diagnosis of HN in multiple large samples. Initially, Reno et al. (1994) presented two histograms obtained by flow cytometry to represent the cell cycle histogram for a negative and heavily affected clam. The authors indicated that HN-negative soft shell clams *M. arenaria* diagnosed by hematocytology could have approximately 4% of haemocytes in phase S (i.e., DNA replication), and fewer than 2% in the G2/M phases of the cell cycle (i.e., around 6% of phase S–4N cells with DNA content higher than diploid DNA content), using FCM. Our results do not contradict those of Reno et al. (1994), but several important points need to be considered with regard to the tetraploid form of HN disease affecting *M. arenaria*.

Based on FCM results of the present study, different arbitrary threshold values are proposed here to define the HN disease status of the individual clam and populations. Thus, we suggest that over 20% of haemocytes in cell cycle processes seemed physiologically abnormal for clams, since haematopoiesis mechanisms remain unknown in molluscs. Moreover, clams with over 20% of phase S–4N haemocytes were systematically confirmed to be affected by HN by hematocytology. On the basis of this arbitrary 20% FCM threshold value, we observed that 38% of all North River clams were highly affected by HN; with clams having high percentages of phase S–4N cells (92.5%, i.e., 81% of 4N cells). This diagnosis corroborated the study of McGladdery et al. (2001) on the presence of HN disease outbreaks in Prince Edward Island (Canada). Magdalen Island and Metis Bay sites each showed only one clam presenting abnormal content of phase S–4N haemocytes (23.7% and 38.5%, respectively).

For the four others populations, caution has to be taken. Indeed, misdiagnosis can be easily formulated for clams presenting intermediate percentages of phase S–4N cells. Individual clams of the different populations presenting between 0% to 6% of phase S–4N cells by FCM could be diagnosed as negative according to Reno et al. (1994), but few abnormal haemocytes (based on morphological characteristics) were reported on slides, which would lead to a status of lightly affected according to McGladdery et al. (2001). This apparent discrepancy is inherent to the respective sensitivities of both methodologies, and highlights requirement of new threshold values for HN diagnosis.

FCM assessment was revealed to be very sensitive because it is based on cell cycle analysis, while hematocytology rests on haemocyte morphological features. However, for both methodologies, distinction of normal mitotic haemocytes from neoplastic haemocytes, and recognition of morphologically transitional abnormal forms of cells (mitotic or neoplastic), is difficult to achieve, especially in the early phase of the disease. Also, some cells may have been assigned to be abnormal while their actual identity remains doubtful. Moreover, until the hematopoiesis process in molluscs is known, the normal percentage of haemocytes in phase S and G2/M stages in a non-diseased clam remains unknown. This may lead to either over- or underestimation of the number of diseased clams in a population, depending on the diagnostic criteria chosen. Also, intermediate cases of HN status in *M. arenaria* represent a grey area including either false-negative or false-positive clams, which are difficult or impossible to identify.

Depending on the purpose of HN assessment, a diagnosis cut-off value for the percentage of cells in S–4N phases may be proposed. From results of the present study, we suggest that populations with clams exhibiting less than 5% of phase S–4N cells by FCM can be considered as negative. Interestingly, this 5% threshold value is closely related to the one arbitrarily proposed by Reno et al. (1994). Indeed, when percentages of cells in phase S–4N stages increased (i.e., early phase of a log-curve of a theoretical distribution), significantly more false-negatives are present in the population; but the population can be considered to be negative. Thus, our Anse St-Étienne Bay population should be considered as the only tested population that was free of HN. As a consequence, the HN status of clams from Kouchibougouac National Park and Barasway Bay remains doubtful using this cut-off value, and highlights that additional diagnostic criteria must be considered.

Comparison of the mean intensity of the HN disease in clam populations, using the threshold of 5% for establishing the HN diagnosis, demonstrated that characteristics of the disease differed among tested populations (test of Kruskal and Wallis, $p < 0.001$). North River presenting highly affected clams, exhibited a mean of intensity of 29.1%. Meanwhile, intensity of HN was significantly lower in clams from Metis Bay and Magdalen Island that were considered as positive for HN with values of 13.5% and 11.6%, respectively (Table 2). Clams from Barasway Bay and Kouchibougouac National Park populations constituted the last clam population cluster with HN intensity of 8.3% and 8.7%, respectively. This demonstrates that those former populations presented a different HN profile compared to North river clams. Interestingly, we noticed that Barasway Bay clam population had a different cell cycle pattern with clams having more cells in phase S than in G2/M phase, suggesting that may be a subjacent physiological process was implicated justifying a high haemocyte turn-over independently of HN and high prevalence. Also, coupling the threshold of value of 5% of S–4N cells with a threshold of HN intensity (15%, which implicate having several individual clams with more than 20% of phase S–4N cells) could be proposed to help to describe the HN status of clam populations. Also, only the North River clam population can be truly considered as affected by HN, and only that from Anse St-Étienne to be free of HN.

For an individual clam assessment, a more restrictive threshold is proposed. Clams with less than 1% of cell in cell cycle process should be considered as free of HN disease. This restrictive value is suggested to avoid as much as possible false-positives, and should be use in specific

Table 2
Estimation of prevalence and intensity of HN disease in different clam populations from eastern Canada

Site	Prevalence	Intensity	Maxima intensity
Anse St-Étienne Bay (QC)	*Negative population*		
Kouchibougouac National Park (NB)	3.8%	8.7% ($n = 1$)	8.7% (*% phase S: 2.4;% of 4N: 6.3*)
Barasway Bay (NF)	42%	8.3% ($n = 17$)	11.8% (*% phase S: 8.6;% of 4N: 3.5*)
Havre-aux-Maisons Lagoon (QC)	25%	11.6% ($n = 9$)	23.7% (*% phase S: 8.7;% of 4N: 15*)
Metis Bay (QC)	25%	13.5% ($n = 9$)	38.5% (*% phase S: 5.2;% of 4N: 33.3*)
North River (PE)	38%	29.1% ($n = 75$)	92.5% (*% phase S: 11.2;% of 4N: 81.3*)

Prevalence and intensity were calculated using the threshold value of 5% for being affected by HN.

studies requiring unaffected clams, such as transmission experiment.

From the richness of cell cycle data, we suggest that the 4N–S phase ratio be also considered to elucidate the health status of clams. Indeed, it has been demonstrated in mammals that the cell cycle process occurs in a few hours (24 h) by using synchronous cell culture, and that the S-phase is the longer phase (10–12 h) in comparison with the G2 phase (3 h); meanwhile mitosis is described to occur very quickly (1 h) (Alberts et al., 2004). Our hypothesis was that a distortion of the 4N–S phase ratio, i.e., an accumulation of tetraploid cells in the circulating haemolymph, may reflect the occurrence of HN disease. This hypothesis was supported for heavily affected clams that have more than 20% of phase S–4N cells. Interestingly, no distortion of the 4N–S phase ratio was generally reported for clams with 0% to 20% of cells in phase S–4N stages. Those clams generally presented higher concentration of phase-S cells than cells in the 4N stage (G2/M phase). Few individual clams presented high 4N cell contents, which was associated with a negative hematocylogy slide reading and vice and versa. Also, since cell cycle analysis presents an instantaneous picture of haemocyte turn-over, which reflects the individual physiological status of clams, the presence of different pattern of percentages of phase S and 4N cells in clam populations having 0% to 20% of cells in phase S–4N stages, does not confirm that clams are free of HN disease; nor do they exclude the possibility of the early phase of HN disease (as for Barasway Bay clams). As a consequence, HN specific biomarkers have to be developed for distinguishing non-diseased clams with haemocytes in proliferative cell cycles due to physiological demands, and lightly affected clams with early stages of neoplastic haemocytes. Utilisation of specific monoclonal antibodies raised against normal and neoplastic haemocytes (Reinisch et al., 1983; Smolowitz et al., 1989) with simultaneous cell cycle analysis would help to bring new information on the course of HN disease.

The protocol of FCM provides a high throughput method for HN status assessment at an individual and population levels. Different arbitrary thresholds of values for the percentages of S–4N cells (5% and 20% for individual assessment) and HN intensity (15% for population assessment) has been proposed hereby, but can vary depending on the objectives of the study. Further research is directed towards development and use of specific biomarkers of HN to reduce ambiguous profiles including either false-negative or false-positive clams.

Acknowledgments

We are grateful to Allan Anderson, Garth Arsenault, Lise Chevarie and Jean-François Mallette for the sampling of the clams on eastern Canada. The authors thank Dr Philippe Soudant (Laboratoire des Sciences de l'Environnement Marin, UMR 6539, UBO, Brest, France) for his help in the adaptation of the FCM procedure of cell cycle analysis for *Mya arenaria*. This research was supported by a AquaNet grant and a Canada Research Chair in Aquatic Health Science.

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2004. Biologie Moléculaire de la Cellule, fourth ed. Médecine-Sciences Flammarion, Paris, pp. 1463.

Ashton-Alcox, K.A., Allam, B., Ford, S.E., 2000. Application of flow cytometry to bivalve pathology. In: Fingerman, N.M., Nagabhushanam, R. (Eds.), Recent Advances in Marine Biotechnology. Immunology and Pathology. Sciences publishers, Enfield, NH, Plymouth, UK, pp. 85–124.

Barber, B.J., 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living Resour. 17, 449–466.

Brousseau, D.J., 1987. Seasonal aspects of sarcomatous neoplasia in *Mya arenaria* (soft-shell clam) from Long Island Sound. J. Invertebr. Pathol. 50, 269–276.

Brousseau, D.J., Baglivo, J.A., 1991a. Disease progression and mortality in neoplastic *Mya arenaria* in the field. Mar. Biol. 110, 249–252.

Brousseau, D.J., Baglivo, J.A., 1991b. Field and laboratory comparisons of mortality in normal and neoplastic *Mya arenaria*. J. Invertebr. Pathol. 57, 59–65.

da Silva, P.M., Soudant, P., Carballal, M.J., Lambert, C., Villalba, A., 2005. Flow cytometric DNA content analysis of neoplastic cells in haemolymph of the cockle *Cerastoderma edule*. Dis. Aquat. Organ. 67, 133–139.

Dungan, C.F., Hamilton, R.M., Hudson, K.L., McCollough, C.B., Reece, K.S., 2002. Two epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebeius*. Dis. Aquat. Organ. 50, 67–78.

Elston, R.A., Drum, A.S., Allen, S.K., 1990. Progressive development of circulating polyploid cells in *Mytilus* with hemic neoplasia. Dis. Aquat. Organ. 8, 51–59.

Elston, R.A., Kent, M.L., Drum, A.S., 1988. Progression, lethality and remission of hemic neoplasia in the bay mussel *Mytilus edulis*. Dis. Aquat. Organ. 4, 135–142.

Elston, R.A., Moore, J.D., Brooks, K., 1992. Disseminated neoplasia of bivalve molluscs. Rev. Aquat. Sci. 6, 405–466.

Farley, C.A., 1969a. Probable neoplastic disease of the hematopoiesis system in oysters, *Crassostrea virginica* and *Crassostrea gigas*. Natl. Cancer Inst. Monogr. 31, 541–555.

Farley, C.A., 1969b. Sarcomatoid proliferative disease in a wild population of blue mussels (*Mytilus edulis*). Natl. Cancer Inst. 43, 509–516.

Farley, C.A., Otto, S.V., Reinisch, C.L., 1986. New occurrence of epizootic sarcoma in Chesapeake Bay Soft shell clam, *Mya arenaria*. Fish Bull. 84, 851–857.

Harper, D.M., Flessas, D.A., Reinisch, C.L., 1994. Specific reactivity of leukemia cells to polyclonal anti-PCB antibodies. J. Invertebr. Pathol. 64, 234–237.

Leavitt, D.F., McDowell Capuzzo, J., Smolowitz, R.M., Miosky, D.L., Lancaster, B.A., Reinisch, C.L., 1990. Hematopoietic neoplasia in *Mya arenaria*: prevalence and indices of physiological condition. Mar. Biol. 105, 313–321.

McGladdery, S.E., Reinisch, C.L., MacCallum, G.S., Stephens, R.E., Walker, C.L., Davidson, J.T., 2001. Haemic neoplasia in soft-shell clams (*Mya arenaria*): Recent outbreaks in Atlantic Canada and discovery of a p53 gene homologue associated with the condition. Can. Bull. Aquat. Assoc. 101, 19–26.

Mix, M.C., 1983. Haemic neoplasms of Bay mussels, *Mytilus edulis* L., from Oregon: occurence, prevalence, seasonality and histopathological progression. J. Fish Dis. 6, 239–248.

Moore, J.D., Elston, R.A., Drum, A.S., Wilkinson, M.T., 1991. Alternate pathogenesis of systemic neoplasia in the bivalve mollusc *Mytilus*. J. Invertebr. Pathol. 58, 231–243.

Peters, E.C., 1988. Recent investigations on the disseminated sarcomas of marine bivalve mollusks. Am. Fish Soc. Spec. Public. 18, 74–92.

Reinisch, C.L., Charles, A.M., Troutner, J., 1983. Unique antigens on neoplastic cells of the soft shell clam *Mya arenaria*. Dev. Comp. Immunol. 7, 33–39.

Reno, P.W., House, M., Illingworth, A., 1994. Flow cytometry and chromosome analysis of Softshell clams, *Mya arenaria*, with disseminated neoplasia. J. Invertebr. Pathol. 64, 163–172.

Smolowitz, R.M., Miosky, D., Reinisch, C.L., 1989. Ontogeny of leukemic cells of the soft shell clam. J. Invertebr. Pathol. 53, 41–51.

Villalba, A., Carballal, M.J., Lopez, C., 2001. Disseminated neoplasia and large foci indicating heavy haemocytic infiltration in cockles *Cerastoderma edule* from Galicia (NW Spain). Dis. Aquat. Organ. 46, 213–216.

Journal of Invertebrate Pathology 102 (2009) 133–140

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/yjipa

Reverse transcriptase activity in tissues of the soft shell clam *Mya arenaria* affected with haemic neoplasia

M. AboElkhair [a], S. Synard [b], A. Siah [a], J. Pariseau [c], J. Davidson [c], G. Johnson [a], S.J. Greenwood [a,d,e], J.W. Casey [f], F.C.J. Berthe [g], A. Cepica [a,*]

[a] Department of Pathology and Microbiology, Atlantic Veterinary College, Charlottetown, PE, Canada C1A 4P3
[b] Department of Fisheries and Aquaculture, Government of Newfoundland and Labrador, St. Albans, Newfoundland, 340, Canada AOH 2E0
[c] Institut des Sciences de la mer, Université du Québec à Rimouski, Rimouski, QC, Canada G5L3A1
[d] Department of Health Management, Atlantic Veterinary College, Charlottetown, PE, Canada C1A 4P3
[e] AVC Lobster Science Centre, Atlantic Veterinary College, Charlottetown, PE, Canada C1A 4P3
[f] Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, USA
[g] European Food Safety Authority (EFSA), Animal Health and Welfare Panel, 43100 Parma, PR, Italy

ARTICLE INFO

Article history:
Received 2 March 2009
Accepted 25 June 2009
Available online 24 July 2009

Keywords:
Mya arenaria
Haemic neoplasia
Reverse transcriptase
Tissues
TaqMan-PERT

ABSTRACT

Since all retroviruses possess reverse transcriptase (RT) enzyme, reverse transcriptase activity has been the main supportive evidence of retroviral etiology of haemic neoplasia (HN) in soft shell clams, *Mya arenaria*. The objective of the present study was to search for a putative retrovirus in various tissues of diseased clams following quantification of RT activity (biochemical indicator of retroviral infection). The clams were assessed by flow cytometry (FCM) for diagnosis of HN. RT activity was quantified by TaqMan-product enhanced reverse transcriptase (TM-PERT) assay in four different organs, gonad, gills, digestive gland, and mantle, at various stages of HN. The digestive gland, the organ with the highest RT activity, and haemocytes, the target cell of HN, were assessed by EM for presence of retroviruses. All organs were assessed by histology. The results of this study demonstrated that although all organs of healthy clams have some background RT activity, the activity observed in most of organs of diseased clams was significantly increased ($p < 0.05$). An association was observed between the degree of neoplastic cell infiltration and the level of RT activity. Digestive gland showed the highest and most consistent RT activity in both healthy and diseased clams. No evidence for the existence of a retrovirus like particle was found by positive staining EM. The presence of RT activity without indications of retroviral particles in digestive gland and haemocytes suggests a probable endogenous source of RT.

1. Introduction

Haemic neoplasia (HN) is a proliferative cell disorder of the circulatory system of the soft shell clam *Mya arenaria*. Severe mortalities of soft shell clams attributed to haemic neoplasia occurred in Prince Edward Island (PEI) in 1999 (McGladdery et al., 2001). The condition has also been reported in a variety of other bivalve species all over the world (Peters, 1988; Elston et al., 1992). The circulating neoplastic cells have large pleomorphic nuclei containing one or more nucleoli, with higher nucleo-cytoplasmic ratios (Peters, 1988; Elston et al., 1992; Barber, 2004). As HN is related to change of ploidy status of circulating haemocytes, flow cytometry methods (FCM) have become an increasingly common tool for the diagnosis of HN in bivalves (Reno et al., 1994; Delaporte et al., 2008). The elucidation of the etiology of HN has been a key issue since the first discovery of this disorder in 1969 (Peters, 1988; Elston et al., 1992). The fact that disease can be transmitted between individuals suggests involvement of an infective agent (Brown, 1980; Appeldoorn et al., 1984). A retroviral etiology has been suggested in *M. arenaria* (Oprandy et al., 1981; Oprandy and Chang, 1983; House et al., 1998) and in *Cerastoderma edule* (Romalde et al., 2007). Since retroviruses possess a reverse transcriptase (RT) enzyme, the presence of RT activity in HN has been considered the main supporting evidence of retroviral etiology of HN (House et al., 1998; Romalde et al., 2007).

Historically, RT has been associated with retroviral genome replication (Baltimore, 1970; Temin and Mizutani, 1970). However, more recently RT has also been associated with a wide range of biological processes, both physiological and pathological (Spadafora, 2004). This is due to the fact that all classes of retroelements, except Alu family, also contain an RT-coding gene. Retroelements have been found to represent about 45% of the human genome

* Corresponding author. Fax: +1 902 56608851.
E-mail address: acepica@upei.ca (A. Cepica).

0022-2011/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2009.06.009

Journal of Invertebrate Pathology 102 (2009) 133–140

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/yjipa

Reverse transcriptase activity in tissues of the soft shell clam *Mya arenaria* affected with haemic neoplasia

M. AboElkhair [a], S. Synard [b], A. Siah [a], J. Pariseau [c], J. Davidson [c], G. Johnson [a], S.J. Greenwood [a,d,e], J.W. Casey [f], F.C.J. Berthe [g], A. Cepica [a,*]

[a] Department of Pathology and Microbiology, Atlantic Veterinary College, Charlottetown, PE, Canada C1A 4P3
[b] Department of Fisheries and Aquaculture, Government of Newfoundland and Labrador, St. Albans, Newfoundland, 340, Canada AOH 2E0
[c] Institut des Sciences de la mer, Université du Québec à Rimouski, Rimouski, QC, Canada G5L3A1
[d] Department of Health Management, Atlantic Veterinary College, Charlottetown, PE, Canada C1A 4P3
[e] AVC Lobster Science Centre, Atlantic Veterinary College, Charlottetown, PE, Canada C1A 4P3
[f] Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, USA
[g] European Food Safety Authority (EFSA), Animal Health and Welfare Panel, 43100 Parma, PR, Italy

ARTICLE INFO

Article history:
Received 2 March 2009
Accepted 25 June 2009
Available online 24 July 2009

Keywords:
Mya arenaria
Haemic neoplasia
Reverse transcriptase
Tissues
TaqMan-PERT

ABSTRACT

Since all retroviruses possess reverse transcriptase (RT) enzyme, reverse transcriptase activity has been the main supportive evidence of retroviral etiology of haemic neoplasia (HN) in soft shell clams, *Mya arenaria*. The objective of the present study was to search for a putative retrovirus in various tissues of diseased clams following quantification of RT activity (biochemical indicator of retroviral infection). The clams were assessed by flow cytometry (FCM) for diagnosis of HN. RT activity was quantified by TaqMan-product enhanced reverse transcriptase (TM-PERT) assay in four different organs, gonad, gills, digestive gland, and mantle, at various stages of HN. The digestive gland, the organ with the highest RT activity, and haemocytes, the target cell of HN, were assessed by EM for presence of retroviruses. All organs were assessed by histology. The results of this study demonstrated that although all organs of healthy clams have some background RT activity, the activity observed in most of organs of diseased clams was significantly increased ($p < 0.05$). An association was observed between the degree of neoplastic cell infiltration and the level of RT activity. Digestive gland showed the highest and most consistent RT activity in both healthy and diseased clams. No evidence for the existence of a retrovirus like particle was found by positive staining EM. The presence of RT activity without indications of retroviral particles in digestive gland and haemocytes suggests a probable endogenous source of RT.

1. Introduction

Haemic neoplasia (HN) is a proliferative cell disorder of the circulatory system of the soft shell clam *Mya arenaria*. Severe mortalities of soft shell clams attributed to haemic neoplasia occurred in Prince Edward Island (PEI) in 1999 (McGladdery et al., 2001). The condition has also been reported in a variety of other bivalve species all over the world (Peters, 1988; Elston et al., 1992). The circulating neoplastic cells have large pleomorphic nuclei containing one or more nucleoli, with higher nucleo-cytoplasmic ratios (Peters, 1988; Elston et al., 1992; Barber, 2004). As HN is related to change of ploidy status of circulating haemocytes, flow cytometry methods (FCM) have become an increasingly common tool for the diagnosis of HN in bivalves (Reno et al., 1994; Delaporte et al., 2008). The elucidation of the etiology of HN has been a key issue since the first discovery of this disorder in 1969 (Peters, 1988; Elston et al., 1992). The fact that disease can be transmitted between individuals suggests involvement of an infective agent (Brown, 1980; Appeldoorn et al., 1984). A retroviral etiology has been suggested in *M. arenaria* (Oprandy et al., 1981; Oprandy and Chang, 1983; House et al., 1998) and in *Cerastoderma edule* (Romalde et al., 2007). Since retroviruses possess a reverse transcriptase (RT) enzyme, the presence of RT activity in HN has been considered the main supporting evidence of retroviral etiology of HN (House et al., 1998; Romalde et al., 2007).

Historically, RT has been associated with retroviral genome replication (Baltimore, 1970; Temin and Mizutani, 1970). However, more recently RT has also been associated with a wide range of biological processes, both physiological and pathological (Spadafora, 2004). This is due to the fact that all classes of retroelements, except Alu family, also contain an RT-coding gene. Retroelements have been found to represent about 45% of the human genome

* Corresponding author. Fax: +1 902 56608851.
E-mail address: acepica@upei.ca (A. Cepica).

0022-2011/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2009.06.009

sequence (Sinibaldi-Vallebona et al., 2006). Mobilization of retroelements within genomes is involved in the genesis of many cellular and physiological functions and disorders (Spadafora, 2004). Product enhanced reverse transcriptase (PERT) assay and its modified version TaqMan-PERT (TM-PERT) have been used as a method for general detection of RT activity of both known and unknown retroviruses (Pyra et al., 1994; Maudru and Peden, 1998; André et al., 2000). However, TM-PERT is also able to detect RT activity of endogenous retroviruses and other retroelements such as retrotransposons (Brorson et al., 2002).

In a previous study (AboElkhair et al., 2009), we have shown that RT activity in cell free haemolymph was positively correlated with the percentage of tetraploid cells circulating in the haemolymph of *M. arenaria*. Moreover, there was no indication for retroviral particles in haemolymph samples analyzed by negative staining electron microscopy. Consequently, we hypothesized that an endogenous source of RT activity, retroelement or endogenous retrovirus, was the likely source in soft shell clams exhibiting HN. In the present study, we quantified RT activity in various tissues of clams at different stages of HN, and we examined the tissues with high levels of RT for the presence of a putative retrovirus by positive staining electron microscopy (EM). The tissues were also assessed by histology for microscopic changes.

2. Materials and methods

2.1. Clams included in the study

Two hundred clams were collected, in October, 2007, from North River (46°15′01″N, 63°10′42″W), Charlottetown, PEI, Canada. The clams were held in tanks at 18 °C at the Atlantic Veterinary College until assessment by flow cytometry.

2.2. Diagnosis of haemic neoplasia

The FCM procedure was performed according to Delaporte et al. (2008). Briefly, haemolymph was withdrawn from the anterior adductor muscle of each clam using a 25-gauge needle fitted with a 3 ml syringe. A sample of 0.5 ml of haemolymph was fixed in 2.5 ml of 95% cold ethanol. After centrifugation at 1200 rpm for 10 min, cell pellets were re-suspended and re-hydrated in phosphate buffered saline (PBS, 0.01 M) for 30 min followed by two washes in PBS (0.01 M). The re-suspended filtered cell pellets were treated with DNase-free RNase A (50 μg ml^{-1}) and stained with Propidium iodide (50 μg ml^{-1}). A specific FL2 detector (orange light, at wavelength 550–600 nm) of a FACSCalibur flow cytometer (BD BioSciences, US) was used to measure fluorescence of PI-stained cells. For each sample, 10,000 particles were counted at a low flow rate (15 μl min^{-1}). For each cell, a single electronic pulse of PI fluorescence was recorded. Each pulse was discriminated by its area, height and width. Based on these data, the tetraploid cells in G2/M phase were distinguished from normal diploid cells (2 N) G0/G1, and from doublets of diploid cells (2 cells have the same DNA quantity/2 N/stuck together) as well by plotting FL2-area vs. FL2-width on cytograms (Fig. 1a). To discriminate single cells from doublets, the R1 region was drawn on dot plot representations, so doublets could be seen on the right of R1 (Fig. 1a). Also, PI fluorescence intensities of single cells were plotted on an FL2-area histogram in order to calculate the percentage of normal cells and tetraploid cells in the tested sample (Fig. 1b). According to Delaporte et al. (2008), clams containing <5% tetraploid cells were considered negative, those with between >5% and <20% tetraploid cells were presumed doubtful, and those with >20% tetraploid cells were presumed positive.

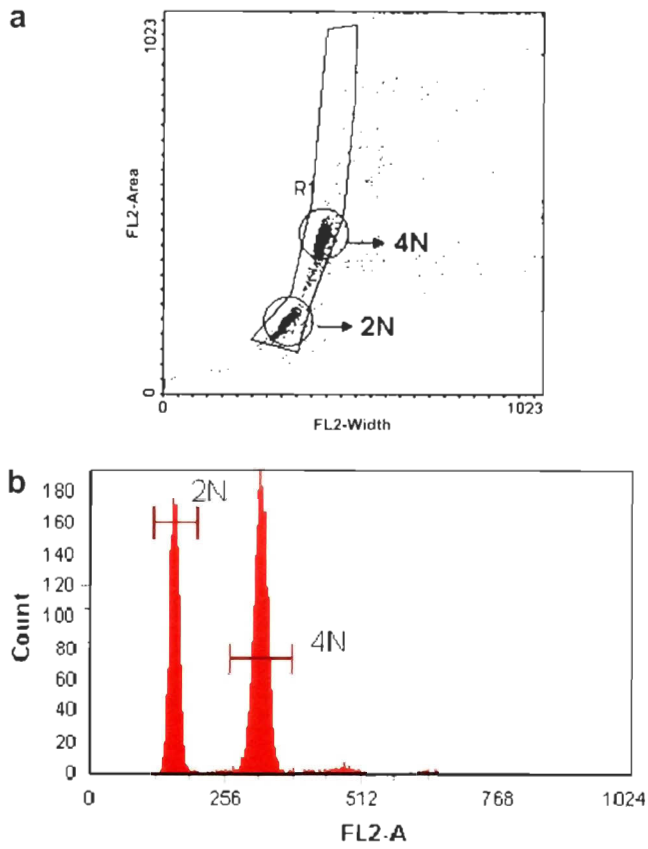


Fig. 1. Analysis of propidium iodide-stained clam haemocyte DNA contents (FL2) by FCM. (a) Diploid and tetraploid single cells are gated in R1 on a width vs. area FL2 cytogram. (b) PI fluorescence of single cells in R1 is plotted on FL2-area histogram. Diploid and tetraploid peaks are delimited by markers, 2N and 4N, respectively.

2.3. Quantification of RT activity

Nineteen animals were selected for this study, and divided into four groups I, II, III, and IV based on their percentages of tetraploidy as listed in Table 1.

Four different tissues (gills, mantle, digestive gland, and gonad) were pooled from individuals of each group into a sample of approximately 250 mg. Pooled tissues were homogenized in 0.85% saline 1:1 (v/w) following Romalde et al. (2007). A protocol adopted from House et al., 1998 was then followed; the homogenized tissues were sonicated in a Fisher sonic dismembrator-300 on ice for five 30 s pulses at 35% of full power with 2 min rests between intervals. The samples were then centrifuged for 5 min at 12,000 rpm at 4 °C. The supernatants were filtered using 0.45 μm syringe filters (VWR International, Canada). The filtrates were stored at −80 °C.

2.4. TaqMan product enhanced reverse transcriptase (TM-PERT) assay

Assay conditions, and primer and probe sequences, were adopted from Maudru and Peden (1998) with some modifications.

Table 1
The number of animals used for the study.

Group	Tetraploidy level (%)	No. of animals
I	<5	3
II	~10 to ~20	6
III	30 to ~50	6
IV	>70	4

sequence (Sinibaldi-Vallebona et al., 2006). Mobilization of retroelements within genomes is involved in the genesis of many cellular and physiological functions and disorders (Spadafora, 2004). Product enhanced reverse transcriptase (PERT) assay and its modified version TaqMan-PERT (TM-PERT) have been used as a method for general detection of RT activity of both known and unknown retroviruses (Pyra et al., 1994; Maudru and Peden, 1998; André et al., 2000). However, TM-PERT is also able to detect RT activity of endogenous retroviruses and other retroelements such as retrotransposons (Brorson et al., 2002).

In a previous study (AboElkhair et al., 2009), we have shown that RT activity in cell free haemolymph was positively correlated with the percentage of tetraploid cells circulating in the haemolymph of *M. arenaria*. Moreover, there was no indication for retroviral particles in haemolymph samples analyzed by negative staining electron microscopy. Consequently, we hypothesized that an endogenous source of RT activity, retroelement or endogenous retrovirus, was the likely source in soft shell clams exhibiting HN. In the present study, we quantified RT activity in various tissues of clams at different stages of HN, and we examined the tissues with high levels of RT for the presence of a putative retrovirus by positive staining electron microscopy (EM). The tissues were also assessed by histology for microscopic changes.

2. Materials and methods

2.1. Clams included in the study

Two hundred clams were collected, in October, 2007, from North River (46°15′01″N, 63°10′42″W), Charlottetown, PEI, Canada. The clams were held in tanks at 18 °C at the Atlantic Veterinary College until assessment by flow cytometry.

2.2. Diagnosis of haemic neoplasia

The FCM procedure was performed according to Delaporte et al. (2008). Briefly, haemolymph was withdrawn from the anterior adductor muscle of each clam using a 25-gauge needle fitted with a 3 ml syringe. A sample of 0.5 ml of haemolymph was fixed in 2.5 ml of 95% cold ethanol. After centrifugation at 1200 rpm for 10 min, cell pellets were re-suspended and re-hydrated in phosphate buffered saline (PBS, 0.01 M) for 30 min followed by two washes in PBS (0.01 M). The re-suspended filtered cell pellets were treated with DNase-free RNase A (50 μg ml^{-1}) and stained with Propidium iodide (50 μg ml^{-1}). A specific FL2 detector (orange light, at wavelength 550–600 nm) of a FACSCalibur flow cytometer (BD BioSciences, US) was used to measure fluorescence of PI-stained cells. For each sample, 10,000 particles were counted at a low flow rate (15 μl min^{-1}). For each cell, a single electronic pulse of PI fluorescence was recorded. Each pulse was discriminated by its area, height and width. Based on these data, the tetraploid cells in G2/M phase were distinguished from normal diploid cells (2 N) G0/G1, and from doublets of diploid cells (2 cells have the same DNA quantity/2 N/stuck together) as well by plotting FL2-area vs. FL2-width on cytograms (Fig. 1a). To discriminate single cells from doublets, the R1 region was drawn on dot plot representations, so doublets could be seen on the right of R1 (Fig. 1a). Also, PI fluorescence intensities of single cells were plotted on an FL2-area histogram in order to calculate the percentage of normal cells and tetraploid cells in the tested sample (Fig. 1b). According to Delaporte et al. (2008), clams containing <5% tetraploid cells were considered negative, those with between >5% and <20% tetraploid cells were presumed doubtful, and those with >20% tetraploid cells were presumed positive.

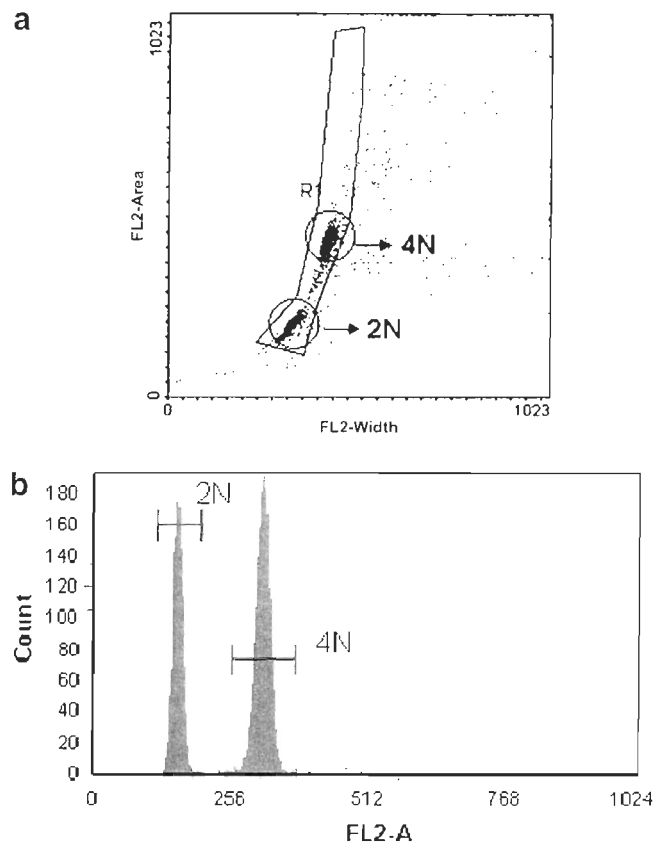


Fig. 1. Analysis of propidium iodide-stained clam haemocyte DNA contents (FL2) by FCM. (a) Diploid and tetraploid single cells are gated in R1 on a width vs. area FL2 cytogram. (b) PI fluorescence of single cells in R1 is plotted on FL2-area histogram. Diploid and tetraploid peaks are delimited by markers, 2N and 4N, respectively.

2.3. Quantification of RT activity

Nineteen animals were selected for this study, and divided into four groups I, II, III, and IV based on their percentages of tetraploidy as listed in Table 1.

Four different tissues (gills, mantle, digestive gland, and gonad) were pooled from individuals of each group into a sample of approximately 250 mg. Pooled tissues were homogenized in 0.85% saline 1:1 (v/w) following Romalde et al. (2007). A protocol adopted from House et al., 1998 was then followed; the homogenized tissues were sonicated in a Fisher sonic dismembrator-300 on ice for five 30 s pulses at 35% of full power with 2 min rests between intervals. The samples were then centrifuged for 5 min at 12,000 rpm at 4 °C. The supernatants were filtered using 0.45 μm syringe filters (VWR International, Canada). The filtrates were stored at −80 °C.

2.4. TaqMan product enhanced reverse transcriptase (TM-PERT) assay

Assay conditions, and primer and probe sequences, were adopted from Maudru and Peden (1998) with some modifications.

Table 1
The number of animals used for the study.

Group	Tetraploidy level (%)	No. of animals
I	<5	3
II	~10 to ~20	6
III	30 to ~50	6
IV	>70	4

The sequences of the primers and probe were primer A: 5′-GCC TTA GCA GTG CCC TGT CT-3′, primer B: 5′-AAC ATG CTC GAG GGC CTT A-3′, probe: 5′-FAM-CCC GTG GGA TGC TCC TAC ATG TC –BHQ1-3′.

Reverse transcription: 0.4 μg of the bacteriophage MS2 genomic RNA template (Roche Applied Science, QC, Canada) was mixed with 5 μM primer A in RNase free water, in a final volume of 2 μl per RT reaction. The mixture was heated at 85 °C for 5 min, annealed at 37 °C for 30 min, and kept at 4 °C for 5 min. Then, 2 μl of clam tissue filtrate was added in the RT reaction mixture containing 50 mM Tris–HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithioerythritol, 10 U RNAsin (Promega Inc. USA), 1 mM dNTPs (Qiagen, ON, Canada), to give a 20 μl final volume. The reaction was then incubated at 37 °C for 1 h, followed by 95 °C for 7 min.

PCR amplification: Five microliters of the synthesized cDNA was added into the PCR reaction mixture to give a total volume of 25 μl. During PCR amplification run, standards were analyzed in duplicates and experimental samples were analyzed in triplicates. The reaction mixture included 1× TaqMan® Universal Master Mix including AmpErase® uracil-N-glycosylase (UNG) (Applied Biosystems, USA), 0.3 μM primer A, 0.3 μM primer B, 0.15 μM probe labeled at 3′ end with FAM and 5′end with Black Hole Quencher (BHQ) 1 (Biosearch Technologies, Inc., USA), and 250 ng of RNase A (Qiagen, ON, Canada). The tubes were placed in the Chromo 4™ system (Bio-RAD, USA), and incubations were controlled by MJ Opticon Monitor version 3.1 with the following thermal cycler conditions: 37 °C for 15 min; 50 °C for 2 min; 95 °C for 10 min; 45 cycles at 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. A standard curve was established by using a 10-fold dilution of M-MLV (Moloney Murine Leukemia Virus) RT (Roche Applied Science, QC, Canada) from 10^2 to 10^{10} picounits (pU). The enzyme was diluted in Buffer A consisting of 50 mM KCl, 20 mM Tris–HCl pH 7.5, 0.2 mM dithiothreitol, 0.25 mM EDTA, 0.025% Triton X-100 (v/v), 50% glycerol (v/v).

2.5. Histopathology

Five clams previously evaluated by FCM were used for histopathology. Two were HN negative and three were HN positive with various degrees of tetraploidy, 20%, 50%, and 80%. The clam soft tissues were fixed in 10% formalin in sea water for 24 h prior to being processed for routine histology. Sections were stained with hematoxylin and eosin according to standard histologic techniques (Sheehan and Hrapchak, 1980), and examined by light microscopy.

2.6. Electron microscopy

2.6.1. Digestive gland

Digestive glands from five clams previously diagnosed by FCM were examined by EM; three glands were from HN-negative clams, one was from a clam with 30% tetraploidy, and one from a clam with 90% tetraploidy. Samples were fixed overnight in 2% glutaraldehyde in sea water (Instant Ocean) at 4 °C. The fixative was removed and samples were washed with sea water for 10 min twice. Post fixation was done in 1% osmium tetroxide in sea water for 1 h at room temperature. Samples were washed consecutively and dehydrated in ascending concentrations of ethanol (50%, 70%, 95% and absolute ethanol). A 10 min propylene oxide treatment was done twice before the infiltration procedure. Infiltration was carried out with a mixture of Epon/Araldite resin and propylene oxide in a ratio of 1:1 followed by 1:3, 1 h each, and a final overnight infiltration step with 100% resin was performed under the vacuum. Tissues were placed in BEEM capsules and blocks were cured overnight in the oven at 60 °C. Semi-thin sections (0.5 μm) were cut from three blocks and stained with 1% toluidine blue in 1% sodium tetra-borate solution, and viewed using light microscopy. Ultrathin sections (80 nm) were cut and recovered using copper super grids, and double stained with uranyl acetate and Sato's lead stain. The sections were examined using a Hitachi H7500 (Nissei-Sangyo, Rexdale, Ontario) transmission electron microscope operated at 80 kV. Images were captured with AMT XR-40 camera.

2.6.2. Haemocytes

Four negative and eight positive clams with various degrees of tetraploidy classified by flow cytometry were analyzed. A volume of 0.5 ml of haemolymph of each clam was mixed with 0.5 ml of double strength (6%) cold glutaraldehyde mixed in seawater and kept at 4 °C overnight. These samples were then centrifuged for 10 min at 1500 rpm, washed in seawater twice and postfixed in 1 ml of 1% osmium tetra-oxide in sea water for 1 h. The fixed samples were centrifuged at 1500 rpm for 10 min. After osmium fixation and centrifugation, the pellets were embedded in 4% agar and cut into small pieces before dehydration, and then processed as previously mentioned.

2.6.3. Data analysis

A linear regression line was calculated and plotted between the logarithms of the RT standards and the corresponding threshold cycle (Ct) values. The logarithms of RT levels in various tissue samples were determined by extrapolating the Ct values from the standard curve. Minitab software version 15 (Minitab Inc., State College, PA, USA) was used for two-way analysis of variance (ANOVA) used to assess multiple comparisons of RT log concentrations among different tissues with various percentages of tetraploidy.

3. Results

The TM-PERT assay optimization enabled detection of up to 10^2 pU of RT 2 μl^{-1} or 5×10^4 pU ml^{-1}, which is equivalent to a 50 retroviral particles ml^{-1} (retrovirus particle contains $\sim 10^3$ RT pU) (Maudru and Peden, 1998). The M-MLV RT standard curve showed a linear relationship between threshold cycle and log concentrations of RT over a wide range of concentrations (10^2–10^{10} pU) (Fig. 2). The numerical means and their standard deviation (SD) values of slopes, *Y*-intercepts, and R^2 of the assays performed for this study were -3.631 ± 0.217, 46.64 ± 3.16, and 0.9965 ± 0.00214, respectively.

3.1. RT activity in tissues

The RT activity of various tissues was quantified in diluted samples. The dilution factor 1:100 showed the highest RT activity compared to the other dilutions of the same sample (except in mantle, 1:10 and 1:100 were similar) (Table 2 and Fig. 3). The assessment of RT activity in all tissues was, therefore, optimized at the dilution 1:100.

All tissues of negative clams (lower than 5% tetraploidy) showed RT activity (Table 3). The two-way ANOVA of the means of the log RT activity indicated that there was a significant effect, of both type of the tissues and the stage of the tetraploidy, on the quantity of RT activity ($p < 0.001$). Compared to normal clams, the RT activity in clams with more than 30% tetraploidy was significantly higher in gills, gonad and digestive gland $p < 0.05$. Gonad showed higher RT activity at >70% tetraploidy compared to normal, but it was not significant (Table 3).

Digestive gland tissue yielded the highest overall RT activity ($9.124 \log \pm 0.209$), followed by gill tissue ($7.706 \log \pm 0.470$) (Table 3). The pattern of RT expression at various stages of the disease in both tissues was similar. The RT activity in both tissues increased with the advancement of the disease. Mantle and gonads showed considerable variability at various stages of the disease

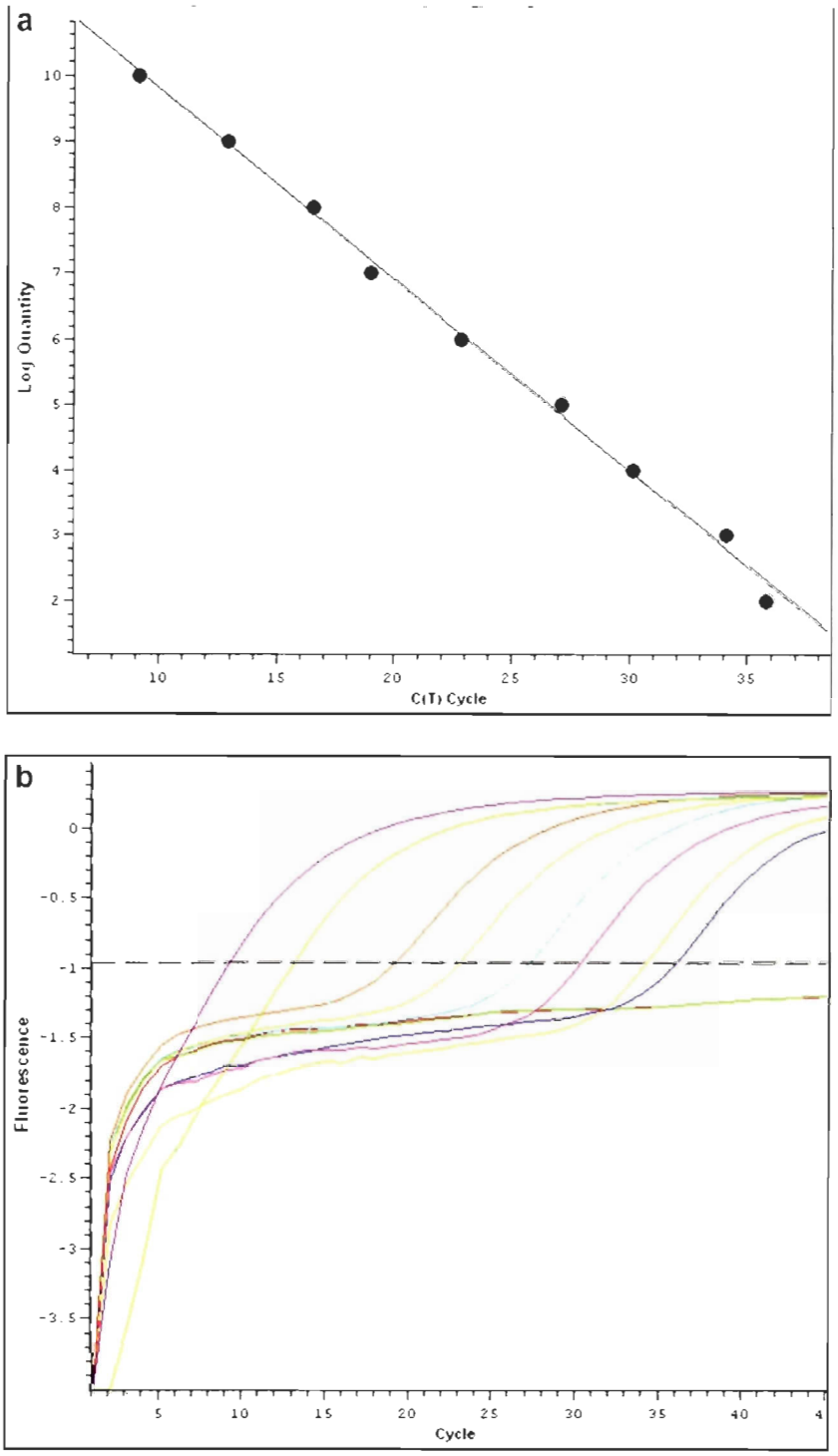


Fig. 2. TM-PERT assay standard curve. (a) Standard curve with the serial dilutions of the M-MLV RT from 10^{10} to 10^{2} pU. The equation for the curve is: $y = -3.4043x + 43.034$ ($R^2 = 0.9989$). (b) Amplification curves of the same serial dilutions of the M-MLV RT from 10^{10} to 10^{2} pU.

Table 2
The effect of tissue dilution on RT activity measurement in various tissues from the soft shell clam, *Mya arenaria*.

Organ	Tissue sample dilution (Ct values)			
	0	1/10	1/100	1/1000
Digestive gland	N/A	31.45	19.75	23.11
Gills	N/A	34.73	22.46	27.11
Mantle	28.64	23.78	23.81	29.95
Gonad	N/A	N/A	23.18	28.63

with the lowest RT activity at the stage of 10–20% tetraploidy (Table 3 and Fig. 4).

3.2. Histopathology

Compared to healthy clams, all diseased clams showed increased level of infiltration of neoplastic haemocytes in various organs particularly digestive gland and gills (Figs. 5b, c and 6b). In early stages of HN, pictures of tissue infiltration by neoplastic

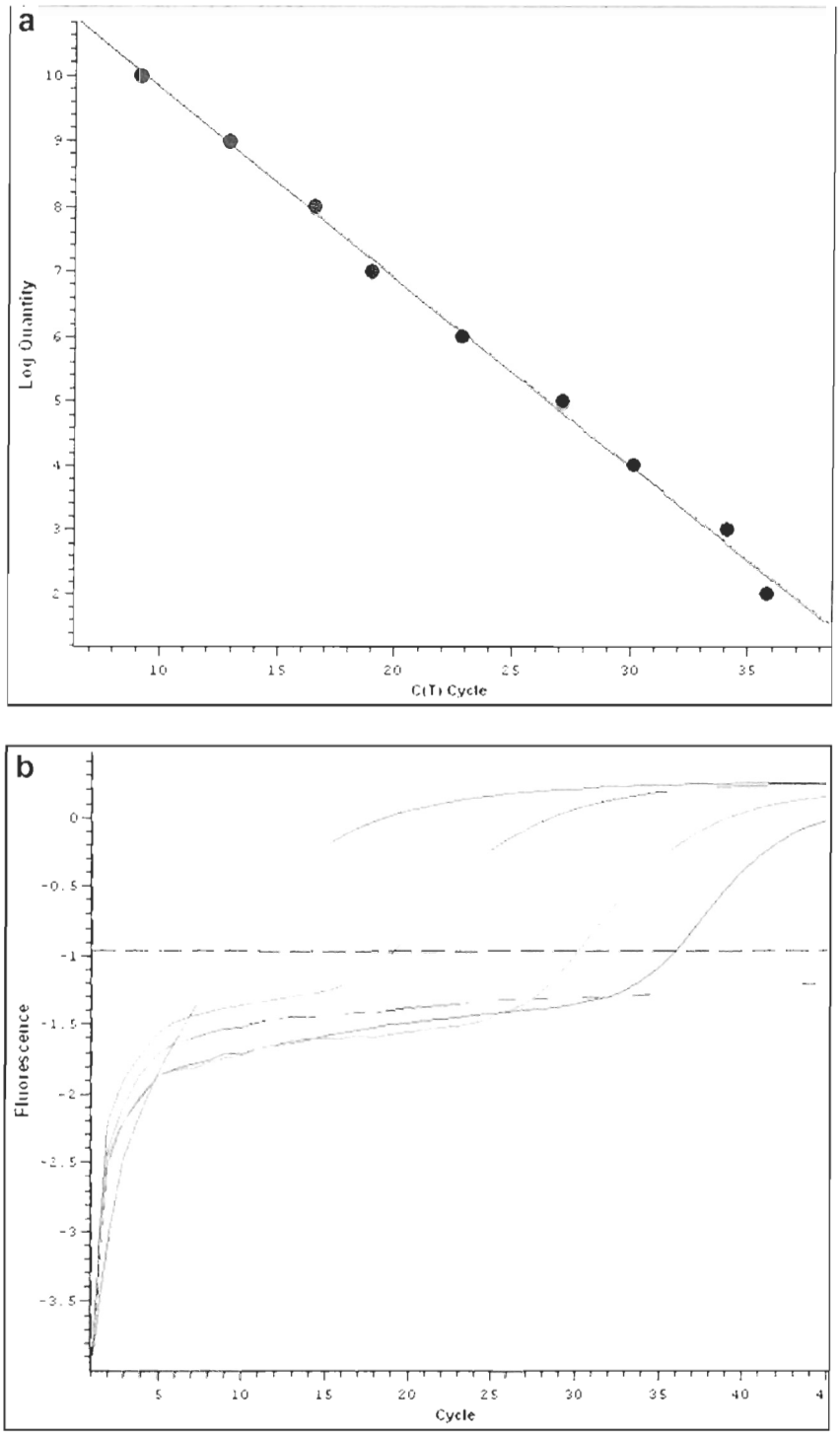


Fig. 2. TM-PERT assay standard curve. (a) Standard curve with the serial dilutions of the M-MLV RT from 10^{10} to 10^{2} pU. The equation for the curve is: $y = -3.4043x + 43.034$ ($R^2 = 0.9989$). (b) Amplification curves of the same serial dilutions of the M-MLV RT from 10^{10} to 10^{2} pU.

Table 2
The effect of tissue dilution on RT activity measurement in various tissues from the soft shell clam, *Mya arenaria*.

Organ	Tissue sample dilution (Ct values)			
	0	1/10	1/100	1/1000
Digestive gland	N/A	31.45	19.75	23.11
Gills	N/A	34.73	22.46	27.11
Mantle	28.64	23.78	23.81	29.95
Gonad	N/A	N/A	23.18	28.63

with the lowest RT activity at the stage of 10–20% tetraploidy (Table 3 and Fig. 4).

3.2. Histopathology

Compared to healthy clams, all diseased clams showed increased level of infiltration of neoplastic haemocytes in various organs particularly digestive gland and gills (Figs. 5b, c and 6b). In early stages of HN, pictures of tissue infiltration by neoplastic

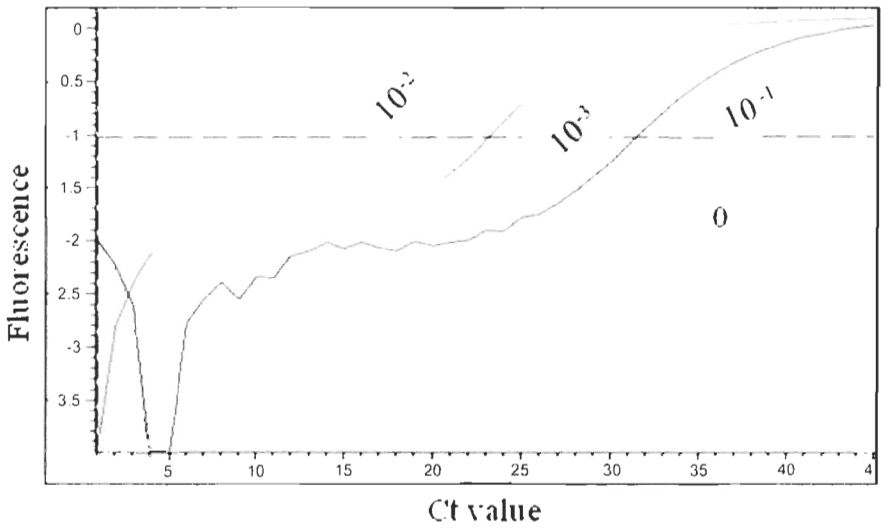


Fig. 3. The effect of tissue dilution on RT activity. Dilution of digestive gland tissue indicated that the optimum dilution factor was (10^{-2}) where RT activity was higher than undiluted (0) and dilutions of 10^{-1} and 10^{-3} as indicated by Ct values.

Table 3
Means ± SD of log RT in various tissues of soft shell clams, *Mya arenaria* at various stages of haemic neoplasia.

Percentage of tetraploidy	RT log pU			
	Gonad	Mantle	Gills	Digestive gland
<5	5.433 ± 0.263	8.573 ± 0.052	6.996 ± 0.221	8.826 ± 0.135
10–20	4.304 ± 0.291	4.696 ± 0.365	7.824 ± 0.201	9.092 ± 0.017
30–50	6.700 ± 0.100	7.3081 ± 0.041	8.155 ± 0.064	9.311 ± 0.0164
>70	5.518 ± 0.090	8.510 ± 0.017	7.850 ± 0.081	9.269 ± 0.042
Overall	5.489 ± 0.903	7.272 ± 1.648	7.706 ± 0.470	9.124 ± 0.209

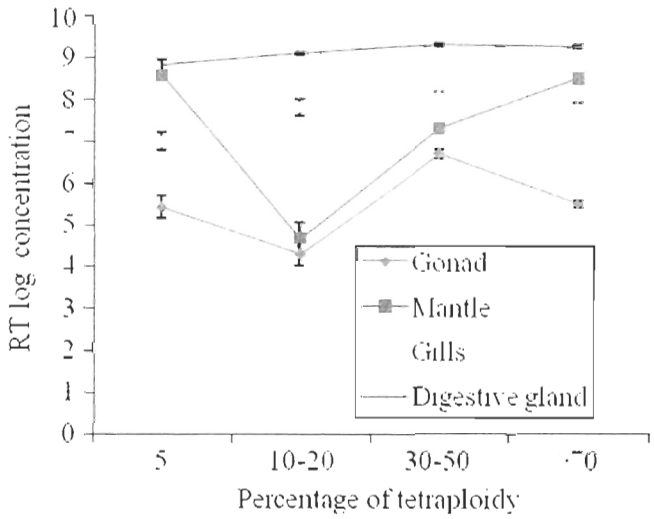


Fig. 4. Comparison of log of RT concentrations at various stages of HN in different organs from soft shell clams. Each value represents the log of RT concentrations ± SD.

haemocytes were observed in the connective tissue surrounding the tubules of the digestive gland (Fig. 5c).

3.3. Electron microscopy

Transmission electron microscopy did not show any indication of a retrovirus-like particles in any of the examined samples at the various stages of HN selected.

4. Discussion

One of the challenging issues that limit exploring a putative retrovirus and its association with HN in soft shell clams is the problem of not knowing how the agent might be spread, and what its tissue tropism, and mode of expression associated with progression of the disease would be. Consequently, the knowledge of the organ and tissue in which the putative transforming process first occurs might provide valuable information about the potential etiology of HN. We previously attempted to identify the stage during HN process at which we could explore the possible retroviral origin based on the RT activity in cell free haemolymph (AboElkhair et al., 2009). In the present study, we attempted to determine the tissue that might be the best target for the search for the putative retrovirus, using RT activity levels as an indicator of viral replication. In view of the fact that no retrovirus isolate or retroviral sequence from HN has previously been made available, there is a strong need to clarify whether an exogenous retrovirus is indeed responsible for HN. Remarkably, in spite of the finding of RT activity in clams (House et al., 1998), and in cockles (Romalde et al., 2007), all previous ultrastructural examinations of neoplastic cells have failed to show any conclusive evidence for the existence of a retrovirus in neoplastic cells of *M. arenaria* (Farley, 1976; Appeldoorn et al., 1984; House et al., 1998), *C. edule* (Auffret and Poder, 1986), *Mytilus edulis* (Rasmussen, 1986), and *Mytilus trossulus* (Mix et al., 1979; Elston et al., 1988). Certainly, finding retroviral particles in neoplastic haemocytes, the target cell of HN, would strengthen the hypothesis of retroviral etiology of HN. In the current study, we extensively searched for retrovirus particles in neoplastic haemocytes from various clams of various degree of HN using positive staining EM. However, we also failed to detect any retrovirus-like particles. Previous reports from *M. arenaria* (Oprandy et al., 1981;

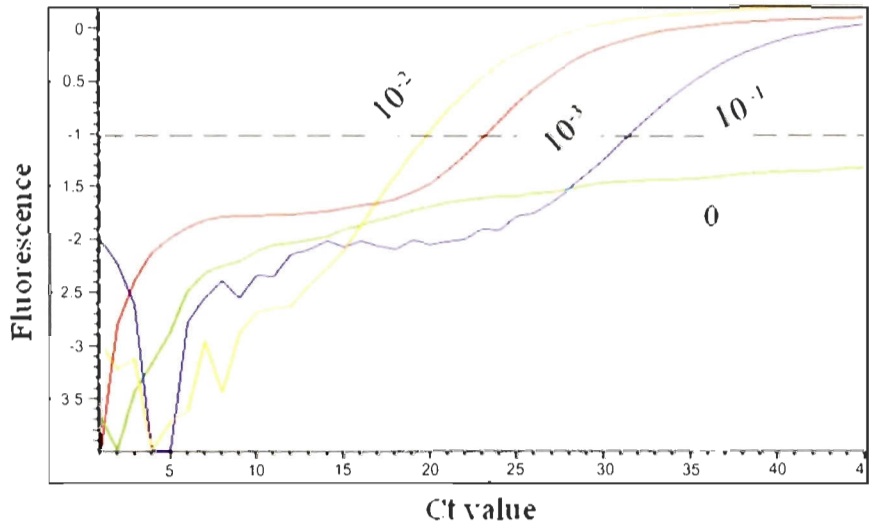


Fig. 3. The effect of tissue dilution on RT activity. Dilution of digestive gland tissue indicated that the optimum dilution factor was (10^{-2}) where RT activity was higher than undiluted (0) and dilutions of 10^{-1} and 10^{-3} as indicated by Ct values.

Table 3
Means ± SD of log RT in various tissues of soft shell clams, *Mya arenaria* at various stages of haemic neoplasia.

Percentage of tetraploidy	RT log pU			
	Gonad	Mantle	Gills	Digestive gland
<5	5.433 ± 0.263	8.573 ± 0.052	6.996 ± 0.221	8.826 ± 0.135
10–20	4.304 ± 0.291	4.696 ± 0.365	7.824 ± 0.201	9.092 ± 0.017
30–50	6.700 ± 0.100	7.3081 ± 0.041	8.155 ± 0.064	9.311 ± 0.0164
>70	5.518 ± 0.090	8.510 ± 0.017	7.850 ± 0.081	9.269 ± 0.042
Overall	5.489 ± 0.903	7.272 ± 1.648	7.706 ± 0.470	9.124 ± 0.209

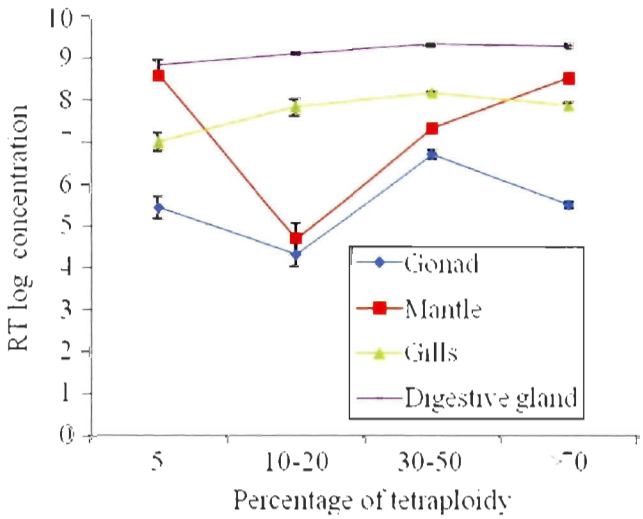


Fig. 4. Comparison of log of RT concentrations at various stages of HN in different organs from soft shell clams. Each value represents the log of RT concentrations ± SD.

haemocytes were observed in the connective tissue surrounding the tubules of the digestive gland (Fig. 5c).

3.3. Electron microscopy

Transmission electron microscopy did not show any indication of a retrovirus-like particles in any of the examined samples at the various stages of HN selected.

4. Discussion

One of the challenging issues that limit exploring a putative retrovirus and its association with HN in soft shell clams is the problem of not knowing how the agent might be spread, and what its tissue tropism, and mode of expression associated with progression of the disease would be. Consequently, the knowledge of the organ and tissue in which the putative transforming process first occurs might provide valuable information about the potential etiology of HN. We previously attempted to identify the stage during HN process at which we could explore the possible retroviral origin based on the RT activity in cell free haemolymph (AboElkhair et al., 2009). In the present study, we attempted to determine the tissue that might be the best target for the search for the putative retrovirus, using RT activity levels as an indicator of viral replication. In view of the fact that no retrovirus isolate or retroviral sequence from HN has previously been made available, there is a strong need to clarify whether an exogenous retrovirus is indeed responsible for HN. Remarkably, in spite of the finding of RT activity in clams (House et al., 1998), and in cockles (Romalde et al., 2007), all previous ultrastructural examinations of neoplastic cells have failed to show any conclusive evidence for the existence of a retrovirus in neoplastic cells of *M. arenaria* (Farley, 1976; Appeldoorn et al., 1984; House et al., 1998), *C. edule* (Auffret and Poder, 1986), *Mytilus edulis* (Rasmussen, 1986), and *Mytilus trossulus* (Mix et al., 1979; Elston et al., 1988). Certainly, finding retroviral particles in neoplastic haemocytes, the target cell of HN, would strengthen the hypothesis of retroviral etiology of HN. In the current study, we extensively searched for retrovirus particles in neoplastic haemocytes from various clams of various degree of HN using positive staining EM. However, we also failed to detect any retrovirus-like particles. Previous reports from *M. arenaria* (Oprandy et al., 1981;

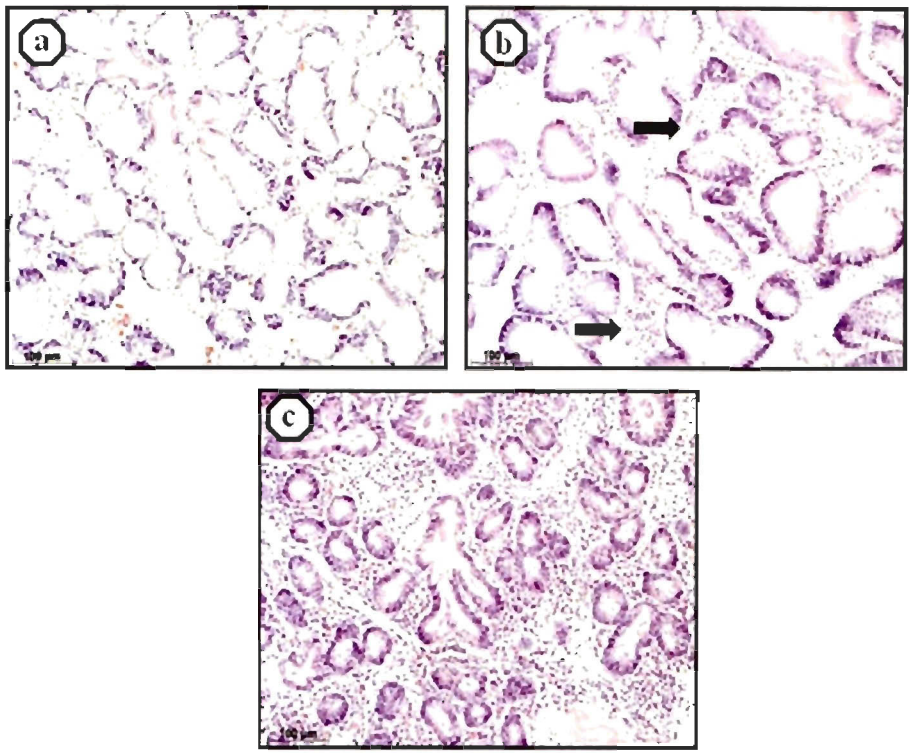


Fig. 5. Photomicrographs of digestive gland at various stages of haemic neoplasia. (a) Normal digestive gland from HN-negative clam. (b) Early infection with multifocal proliferation of enlarged haemocytes (arrows). (c) Advanced haemic neoplasia with massive proliferation of neoplastic haemocytes distorting digestive gland architecture. Scale bar = 100 μm.

Oprandy and Chang, 1983) of retrovirus-like particles observed by EM have been subjected to criticism because of the unconvincing nature of the published evidence and the lack of reproducibility (Elston et al., 1992). Romalde et al. (2007) suggested presence of retrovirus like particle in neoplastic cells of *C. edule*, but this should also be viewed as inconclusive, as the generally required visualization of budding virus particles was not shown.

Our results showed that tissues of HN free clams possessed a background level of RT activity (Table 3). These results are in agreement with Casey et al., 1997 in turtle, Raghavan et al. (2003) in *Biomphalaria* snail, and Molès et al. (2007) in human. Furthermore, nucleotide sequence of RT has even been detected in chickens free of exogenous and endogenous retrovirus (Dunwiddie and Faras, 1985). These results corroborate the prediction of Temin (1971), who proposed that RT activity may be a required cellular activity. Moreover, some evidence has been reported for the association between RT activity and the proliferative potential of the cell (Osertag and Kazazian, 2001). It is interesting in this context that evidence exists from manila clam, *Tapes phillippinarum*, in support of proliferative potential of circulating haemocytes (Matozzo et al., 2008). Therefore, the proliferative potential of haemocytes might have a role in a background level of RT activity.

The increased RT activity in most of the organ tissues of the advanced stages of HN compared to tissues of negative clams might be related to the observed high infiltration by neoplastic haemocytes (Figs. 5 and 6). Although detailed quantification of infiltrating neoplastic cells in tissues was not done, clearly high RT activities were observed in tissues with high infiltrations, compared to normal tissues. The increased levels of RT activity with increased number of neoplastic cells could be explained by the growing body of data that expression of RT-coding genes is active in cells that have high proliferative potential like tumor cells (Spadafora, 2004).

The highest and most consistent RT activity found in the digestive gland tissue of both HN-negative and HN-positive, compared to other organs (Table 2), suggested that digestive gland should be targeted for further investigation of retrovirus replication. However, EM did not show any signs of retroviral particles which could further suggest that detected RT activity is not related to an exogenous retrovirus. There may be several reasons why increased level of RT activity was observed in the absence of retrovirus particles in digestive gland. Firstly, RT may be present due to proliferative nature of the cells of the digestive gland. It was reported that a digestive gland homogenate of *Mytilus galloprovincialis* showed immunoreactivity to the proliferating cell nuclear antigen indicating that the cells of digestive diverticula including haemocytes have the capacity to proliferate (Marigomez et al., 1999; Zaldibar et al., 2004, 2008). Consistent with this, Raghavan et al. (2003) reported greater RT activity in the posterior region of *Biomphalaria* snail, the major site of haemotopoisis, than in the head and foot. Secondly, the RT activity might be due to exposure of digestive gland to pollutants. It was reported that expression of RT-coding genes could be up-regulated by a variety of stimuli that act at genome wide level like genotoxic agents (Sciamanna et al., 2005). Agriculture in Prince Edward Island is dependent on pesticides (Mutch et al., 2002) which might affect clam population in North River. Consistent with this, Brown et al. (2006) reported that gag-pol gene was up-regulated in digestive gland of *M. edulis* following exposure to benzo[*a*]pyrene.

Our result of detection of neoplastic cell infiltration in connective tissue surrounding digestive tubules in early stages of HN is in agreement with previous histologic studies of *M. edulis* (Lowe and Moore, 1978; Mix, 1983; Bower, 1989) reviewed in (Elston et al., 1992). Mix (1983) stated in his classification of the stages of disease severity, based on the occurrence of neoplastic cells in specific location of the body in Oregon mussel, that during the first stage of the disease, small numbers of neoplastic cells occurred in connective tissues surrounding digestive gland. In the next stage of disease, increased infiltration of neoplastic cells occurred into connective tissue away from digestive gland.

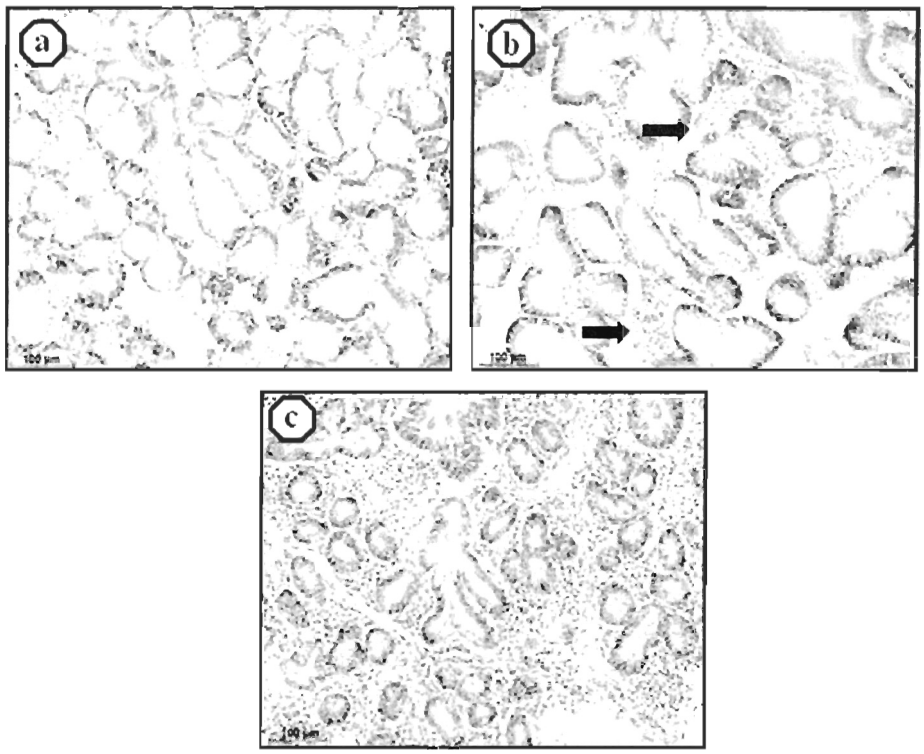


Fig. 5. Photomicrographs of digestive gland at various stages of haemic neoplasia. (a) Normal digestive gland from HN-negative clam. (b) Early infection with multifocal proliferation of enlarged haemocytes (arrows). (c) Advanced haemic neoplasia with massive proliferation of neoplastic haemocytes distorting digestive gland architecture. Scale bar = 100 μm.

Oprandy and Chang, 1983) of retrovirus-like particles observed by EM have been subjected to criticism because of the unconvincing nature of the published evidence and the lack of reproducibility (Elston et al., 1992). Romalde et al. (2007) suggested presence of retrovirus like particle in neoplastic cells of *C. edule*, but this should also be viewed as inconclusive, as the generally required visualization of budding virus particles was not shown.

Our results showed that tissues of HN free clams possessed a background level of RT activity (Table 3). These results are in agreement with Casey et al., 1997 in turtle, Raghavan et al. (2003) in *Biomphalaria* snail, and Molès et al. (2007) in human. Furthermore, nucleotide sequence of RT has even been detected in chickens free of exogenous and endogenous retrovirus (Dunwiddie and Faras, 1985). These results corroborate the prediction of Temin (1971), who proposed that RT activity may be a required cellular activity. Moreover, some evidence has been reported for the association between RT activity and the proliferative potential of the cell (Osertag and Kazazian, 2001). It is interesting in this context that evidence exists from manila clam, *Tapes phillippinarum*, in support of proliferative potential of circulating haemocytes (Matozzo et al., 2008). Therefore, the proliferative potential of haemocytes might have a role in a background level of RT activity.

The increased RT activity in most of the organ tissues of the advanced stages of HN compared to tissues of negative clams might be related to the observed high infiltration by neoplastic haemocytes (Figs. 5 and 6). Although detailed quantification of infiltrating neoplastic cells in tissues was not done, clearly high RT activities were observed in tissues with high infiltrations, compared to normal tissues. The increased levels of RT activity with increased number of neoplastic cells could be explained by the growing body of data that expression of RT-coding genes is active in cells that have high proliferative potential like tumor cells (Spadafora, 2004).

The highest and most consistent RT activity found in the digestive gland tissue of both HN-negative and HN-positive, compared to other organs (Table 2), suggested that digestive gland should be targeted for further investigation of retrovirus replication. However, EM did not show any signs of retroviral particles which could further suggest that detected RT activity is not related to an exogenous retrovirus. There may be several reasons why increased level of RT activity was observed in the absence of retrovirus particles in digestive gland. Firstly, RT may be present due to proliferative nature of the cells of the digestive gland. It was reported that a digestive gland homogenate of *Mytilus galloprovincialis* showed immunoreactivity to the proliferating cell nuclear antigen indicating that the cells of digestive diverticula including haemocytes have the capacity to proliferate (Marigomez et al., 1999; Zaldibar et al., 2004, 2008). Consistent with this, Raghavan et al. (2003) reported greater RT activity in the posterior region of *Biomphalaria* snail, the major site of haemotopoisis, than in the head and foot. Secondly, the RT activity might be due to exposure of digestive gland to pollutants. It was reported that expression of RT-coding genes could be up-regulated by a variety of stimuli that act at genome wide level like genotoxic agents (Sciamanna et al., 2005). Agriculture in Prince Edward Island is dependent on pesticides (Mutch et al., 2002) which might affect clam population in North River. Consistent with this, Brown et al. (2006) reported that gag-pol gene was up-regulated in digestive gland of *M. edulis* following exposure to benzo[*a*]pyrene.

Our result of detection of neoplastic cell infiltration in connective tissue surrounding digestive tubules in early stages of HN is in agreement with previous histologic studies of *M. edulis* (Lowe and Moore, 1978; Mix, 1983; Bower, 1989) reviewed in (Elston et al., 1992). Mix (1983) stated in his classification of the stages of disease severity, based on the occurrence of neoplastic cells in specific location of the body in Oregon mussel, that during the first stage of the disease, small numbers of neoplastic cells occurred in connective tissues surrounding digestive gland. In the next stage of disease, increased infiltration of neoplastic cells occurred into connective tissue away from digestive gland.

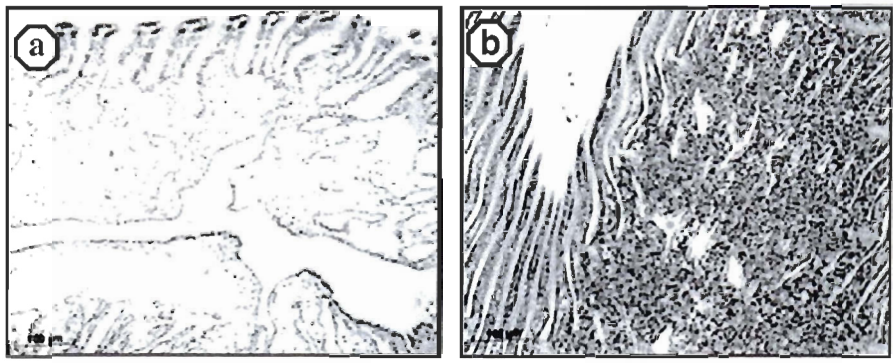


Fig. 6. Photomicrographs of gills at various stages of haemic neoplasia. (a) Normal gill from HN-negative clam. Interstitial area has very few haemocytes. (b) Gills from a clam with advanced HN. The interstitium is filled with neoplastic haemocytes. Scale bar = 100 μm.

In conclusion, detection of RT activity in clam tissues without indication of retroviral particles in both digestive gland and haemocytes suggests that RT activity in HN may be linked to an endogenous source rather than an exogenous retrovirus. However, this requires further investigation. High RT activity in the digestive gland combined with histological observation of neoplastic cells usually first appearing in the digestive gland suggests that the digestive gland might be the place where the transformation process starts. Therefore, we suggest considering digestive gland in further studies of HN of soft shell clams.

Acknowledgments

We thank Dr. H. Stryhn for his help in statistical analysis, C. Jones, P. McKenna, and D. Wadowska for their technical help, and F. Clark and Dr. T. Iwamoto for their valuable scientific discussion. The research was supported by Egyptian Cultural and Educational Bureau in Montréal, QC, Canada.

References

AboElkhair, M., Siah, A., Clark, K.F., McKenna, P., Pariseau, J., Greenwood, S.J., Berthe, F.C.J., Cepica, A., 2009. Reverse transcriptase activity associated with haemic neoplasia in soft shell clam, *Mya arenaria*. Dis. Aquat. Org. 84, 57–63.

André, M., Morgeaux, S., Fuchs, F., 2000. Quantitative detection of RT activity by PERT assay: feasibility and limits to a standardized screening assay for human vaccines. Biologicals 28, 67–80.

Appeldoorn, R., Brown, C.W., Brown, R.S., Chang, P.W., Cooper, K., Lorda, E., Saila, S.B., Walker, H., Wolke, R.E., 1984. Field and Laboratory studies to define the occurrence of neoplasia in the soft shell clam, *Mya arenaria*. API Publ. No. 4345, University of Rhode Island, Kingston.

Auffret, M., Poder, M., 1986. Sarcomatous lesion in the cockle *Cerastoderma edule* II. Electron microscopical study. Aquaculture 58, 9–15.

Baltimore, D., 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature 226, 1209–1211.

Barber, B.J., 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living Resour. 17, 449–466.

Bower, S.M., 1989. The summer mortality syndrome and haemocytic neoplasia in blue mussels (*Mytilus edulis*) from British Columbia. Can. Tech. Rep. Fish. Aquat. Sci. 1703, 65.

Brorson, K., Xu, Y., Swann, P.G., Hamilton, E., Mustafa, M., de Wit, C., Norling, L.A., Stein, K.E., 2002. Evaluation of a quantitative Product-enhanced reverse transcriptase assay to monitor retrovirus in mAb cell-culture. Biologicals 30, 15–26.

Brown, R.S., 1980. The value of the multidisciplinary approach to research on marine pollution effects as evidenced in a three year study to determine the etiology and pathogenesis of neoplasia in the soft shell clam, *Mya arenaria*. Rapp. PV Reun. Cons. Int. Explor. Mer. 179, 125–128.

Brown, M., Davies, I.M., Moffat, C.F., Craft, J.A., 2006. Application of SSH and a macroarray to investigate altered gene expression in *Mytilus edulis* in response to exposure to benzo[*a*]pyrene. Mar. Environ. Res. 62, S128–S135.

Casey, R.N., Quackenbush, S.L., Work, T.M., Balazs, G.H., Bowser, P.R., Casey, J.W., 1997. Evidence for retrovirus infections in green turtles *Chelonia mydas* from the Hawaiian Islands. Dis. Aquat. Org. 31, 1–7.

Delaporte, M., Synard, S., Pariseau, J., McKenna, P., Tremblay, R., Davidson, J., Berthe, F.C.J., 2008. Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry. J. Invertebr. Pathol. 98, 190–197.

Dunwiddie, C., Faras, A.J., 1985. Presence of retrovirus reverse transcriptase-related gene sequences in avian cells lacking endogenous avian leukosis viruses. Proc. Natl. Acad. Sci. USA 82, 5097–5101.

Elston, R.A., Kent, M.L., Drum, A.S., 1988. Transmission of hemic neoplasia in the bay mussel, *Mytilus edulis*, using whole cells and cell homogenate. Dev. Comp. Immunol. 12, 719–727.

Elston, R.A., Moore, J.D., Brooks, K., 1992. Disseminated neoplasia of bivalve molluscs. Rev. Aquat. Sci. 6, 405–466.

Farley, C.A., 1976. Proliferative disorders in bivalve mollusks. Mar. Fish. Rev. 1210, 30–33.

House, M.L., Kim, C.H., Reno, P.W., 1998. Soft shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. Dis. Aquat. Org. 34, 187–192.

Lowe, D.M., Moore, M.N., 1978. Cytology and quantitative cytochemistry of a proliferative atypical hemocytic condition in *Mytilus edulis* (Bivalvia, Mollusca). J. Natl. Cancer. Inst. 60, 145–1459.

Marigomez, I., Lekube, X., Cancio, I., 1999. Immunochemical localization of proliferating cells in mussel digestive gland tissue. Histochem. J. 31, 781–788.

Matozzo, V., Marin, M.G., Cima, F., Ballarin, L., 2008. First evidence of cell division in circulating haemocytes from the Manila clam *Tapes philippinarum*. Cell Biol. Int. 32, 865–868.

Maudru, T., Peden, K., 1998. Adaptation of the fluorogenic 5′-nuclease chemistry to a PCR-based reverse transcriptase assay. Biotechniques 25, 972–975.

McGladdery, S.E., Reinisch, C.L., MacCallum, G.S., Stephens, R.E., Walker, C.L., Davidson, J.T., 2001. Haemic neoplasia in soft-shell clams (*Mya arenaria*): recent outbreaks in Atlantic Canada and discovery of a p53 gene homologue associated with the condition. Can. Bull. Aquat. Assoc. 101, 19–26.

Mix, M.C., 1983. Haemic neoplasms of bay mussels *Mytilus edulis* L., from Oregon: occurrence, prevalence, seasonality and histopathological progression. J. Fish. Dis. 6, 239–248.

Mix, M.C., Hawkes, J.W., Sparks, A.K., 1979. Observation on the ultrastructure of large cells associated with putative neoplastic disorders of mussels, *Mytilus edulis*, from Yaquina Bay, Oregon. J. Invertebr. Pathol. 34, 41–56.

Molès, J.P., Tesniere, A., Guilhou, J.J., 2007. Reverse transcriptase activity in human normal and psoriatic skin samples. Brit. J. Dermatol. 157, 482–486.

Mutch, J.P., Savard, M.A., Julien, G.R.J., MacLean, B., Raymond, B., Doull, J., 2002. Pesticide monitoring and fish kill investigations on Prince Edward Island, 1994–1999. In: Can. Tech. Rep. Fish. Aquat. Sci., Effects of land use practices on fish, shellfish, and their habitats on Prince Edward Island 2408, 94–99.

Oprandy, J.J., Chang, P.W., 1983. 5-bromodeoxyuridine induction of hematopoietic neoplasia and retrovirus activation in soft shell clam, *Mya arenaria*. J. Invertebr. Pathol. 42, 196–206.

Oprandy, J.J., Chang, P.W., Pronovost, A.D., Cooper, K.R., Brown, R.S., Yates, V.J., 1981. Isolation of viral agent causing hematopoietic neoplasia in the soft shell clams, *Mya arenaria*. J. Invertebr. Pathol. 38, 45–51.

Osertag, E.M., Kazazian, H.H., 2001. Biology of mammalian L1 retrotransposons. Annu. Rev. Genet. 35, 501–538.

Peters, E.C., 1988. Recent investigations on the disseminated sarcomas of marine bivalve mollusks. Am. Fish. Soc. Spec. Publ. 18, 74–92.

Pyra, H., Boni, J., Schupbach, J., 1994. Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc. Natl. Acad. Sci. 91, 1544–1548.

Raghavan, N., Miller, A.N., Gardner, M., FitzGerald, P.C., Kerlavage, A.R., Johnston, D.A., Lewis, F.A., Knight, M., 2003. Comparative gene analysis of *Biomphalaria glabrata* hemocytes pre- and post-exposure to miracidia of *Schistosoma mansoni*. Mol. Biochem. Parasitol. 126, 181–191.

Rasmussen, L.P.D., 1986. Occurrence, prevalence and seasonality of neoplasia in the marine mussel *Mytilus edulis* from three sites in Denmark. Mar. Biol. 92, 59–64.

Reno, P.W., House, M., Illingworth, A., 1994. Flow cytometric and chromosome analysis of soft shell clams, *Mya arenaria*, with disseminated neoplasia. J. Invertebr. Pathol. 64, 163–172.

Romalde, J.L., Vilarino, M.L., Beaz, R., Rodriguez, J.M., Diaz, S., Villalba, A., Carballal, M.J., 2007. Evidence of retroviral etiology for disseminated neoplasia in cockles (*Cerastoderma edule*). J. Invertebr. Pathol. 94, 95–101.

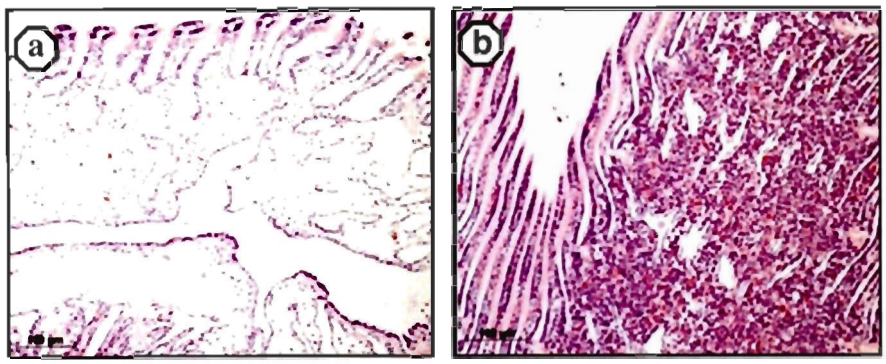


Fig. 6. Photomicrographs of gills at various stages of haemic neoplasia. (a) Normal gill from HN-negative clam. Interstitial area has very few haemocytes. (b) Gills from a clam with advanced HN. The interstitium is filled with neoplastic haemocytes. Scale bar = 100 μm.

In conclusion, detection of RT activity in clam tissues without indication of retroviral particles in both digestive gland and haemocytes suggests that RT activity in HN may be linked to an endogenous source rather than an exogenous retrovirus. However, this requires further investigation. High RT activity in the digestive gland combined with histological observation of neoplastic cells usually first appearing in the digestive gland suggests that the digestive gland might be the place where the transformation process starts. Therefore, we suggest considering digestive gland in further studies of HN of soft shell clams.

Acknowledgments

We thank Dr. H. Stryhn for his help in statistical analysis, C. Jones, P. McKenna, and D. Wadowska for their technical help, and F. Clark and Dr. T. Iwamoto for their valuable scientific discussion. The research was supported by Egyptian Cultural and Educational Bureau in Montréal, QC, Canada.

References

AboElkhair, M., Siah, A., Clark, K.F., McKenna, P., Pariseau, J., Greenwood, S.J., Berthe, F.C.J., Cepica, A., 2009. Reverse transcriptase activity associated with haemic neoplasia in soft shell clam, *Mya arenaria*. Dis. Aquat. Org. 84, 57–63.

André, M., Morgeaux, S., Fuchs, F., 2000. Quantitative detection of RT activity by PERT assay: feasibility and limits to a standardized screening assay for human vaccines. Biologicals 28, 67–80.

Appeldoorn, R., Brown, C.W., Brown, R.S., Chang, P.W., Cooper, K., Lorda, E., Saila, S.B., Walker, H., Wolke, R.E., 1984. Field and Laboratory studies to define the occurrence of neoplasia in the soft shell clam, *Mya arenaria*. API Publ. No. 4345, University of Rhode Island, Kingston.

Auffret, M., Poder, M., 1986. Sarcomatous lesion in the cockle *Cerastoderma edule* II. Electron microscopical study. Aquaculture 58, 9–15.

Baltimore, D., 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature 226, 1209–1211.

Barber, B.J., 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living Resour. 17, 449–466.

Bower, S.M., 1989. The summer mortality syndrome and haemocytic neoplasia in blue mussels (*Mytilus edulis*) from British Columbia. Can. Tech. Rep. Fish. Aquat. Sci. 1703, 65.

Brorson, K., Xu, Y., Swann, P.G., Hamilton, E., Mustafa, M., de Wit, C., Norling, L.A., Stein, K.E., 2002. Evaluation of a quantitative Product-enhanced reverse transcriptase assay to monitor retrovirus in mAb cell-culture. Biologicals 30, 15–26.

Brown, R.S., 1980. The value of the multidisciplinary approach to research on marine pollution effects as evidenced in a three year study to determine the etiology and pathogenesis of neoplasia in the soft shell clam, *Mya arenaria*. Rapp. PV Reun. Cons. Int. Explor. Mer. 179, 125–128.

Brown, M., Davies, I.M., Moffat, C.F., Craft, J.A., 2006. Application of SSH and a macroarray to investigate altered gene expression in *Mytilus edulis* in response to exposure to benzo[*a*]pyrene. Mar. Environ. Res. 62, S128–S135.

Casey, R.N., Quackenbush, S.L., Work, T.M., Balazs, G.H., Bowser, P.R., Casey, J.W., 1997. Evidence for retrovirus infections in green turtles *Chelonia mydas* from the Hawaiian Islands. Dis. Aquat. Org. 31, 1–7.

Delaporte, M., Synard, S., Pariseau, J., McKenna, P., Tremblay, R., Davidson, J., Berthe, F.C.J., 2008. Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry. J. Invertebr. Pathol. 98, 190–197.

Dunwiddie, C., Faras, A.J., 1985. Presence of retrovirus reverse transcriptase-related gene sequences in avian cells lacking endogenous avian leukosis viruses. Proc. Natl. Acad. Sci. USA 82, 5097–5101.

Elston, R.A., Kent, M.L., Drum, A.S., 1988. Transmission of hemic neoplasia in the bay mussel, *Mytilus edulis*, using whole cells and cell homogenate. Dev. Comp. Immunol. 12, 719–727.

Elston, R.A., Moore, J.D., Brooks, K., 1992. Disseminated neoplasia of bivalve molluscs. Rev. Aquat. Sci. 6, 405–466.

Farley, C.A., 1976. Proliferative disorders in bivalve mollusks. Mar. Fish. Rev. 1210, 30–33.

House, M.L., Kim, C.H., Reno, P.W., 1998. Soft shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. Dis. Aquat. Org. 34, 187–192.

Lowe, D.M., Moore, M.N., 1978. Cytology and quantitative cytochemistry of a proliferative atypical hemocytic condition in *Mytilus edulis* (Bivalvia, Mollusca). J. Natl. Cancer. Inst. 60, 145–1459.

Marigomez, I., Lekube, X., Cancio, I., 1999. Immunochemical localization of proliferating cells in mussel digestive gland tissue. Histochem. J. 31, 781–788.

Matozzo, V., Marin, M.G., Cima, F., Ballarin, L., 2008. First evidence of cell division in circulating haemocytes from the Manila clam *Tapes philippinarum*. Cell Biol. Int. 32, 865–868.

Maudru, T., Peden, K., 1998. Adaptation of the fluorogenic 5′-nuclease chemistry to a PCR-based reverse transcriptase assay. Biotechniques 25, 972–975.

McGladdery, S.E., Reinisch, C.L., MacCallum, G.S., Stephens, R.E., Walker, C.L., Davidson, J.T., 2001. Haemic neoplasia in soft-shell clams (*Mya arenaria*): recent outbreaks in Atlantic Canada and discovery of a p53 gene homologue associated with the condition. Can. Bull. Aquat. Assoc. 101, 19–26.

Mix, M.C., 1983. Haemic neoplasms of bay mussels *Mytilus edulis* L., from Oregon: occurrence, prevalence, seasonality and histopathological progression. J. Fish. Dis. 6, 239–248.

Mix, M.C., Hawkes, J.W., Sparks, A.K., 1979. Observation on the ultrastructure of large cells associated with putative neoplastic disorders of mussels, *Mytilus edulis*, from Yaquina Bay, Oregon. J. Invertebr. Pathol. 34, 41–56.

Molès, J.P., Tesniere, A., Guilhou, J.J., 2007. Reverse transcriptase activity in human normal and psoriatic skin samples. Brit. J. Dermatol. 157, 482–486.

Mutch, J.P., Savard, M.A., Julien, G.R.J., MacLean, B., Raymond, B., Doull, J., 2002. Pesticide monitoring and fish kill investigations on Prince Edward Island, 1994–1999. In: Can. Tech. Rep. Fish. Aquat. Sci., Effects of land use practices on fish, shellfish, and their habitats on Prince Edward Island 2408, 94–99.

Oprandy, J.J., Chang, P.W., 1983. 5-bromodeoxyuridine induction of hematopoietic neoplasia and retrovirus activation in soft shell clam, *Mya arenaria*. J. Invertebr. Pathol. 42, 196–206.

Oprandy, J.J., Chang, P.W., Pronovost, A.D., Cooper, K.R., Brown, R.S., Yates, V.J., 1981. Isolation of viral agent causing hematopoietic neoplasia in the soft shell clams, *Mya arenaria*. J. Invertebr. Pathol. 38, 45–51.

Osertag, E.M., Kazazian, H.H., 2001. Biology of mammalian L1 retrotransposons. Annu. Rev. Genet. 35, 501–538.

Peters, E.C., 1988. Recent investigations on the disseminated sarcomas of marine bivalve mollusks. Am. Fish. Soc. Spec. Publ. 18, 74–92.

Pyra, H., Boni, J., Schupbach, J., 1994. Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc. Natl. Acad. Sci. 91, 1544–1548.

Raghavan, N., Miller, A.N., Gardner, M., FitzGerald, P.C., Kerlavage, A.R., Johnston, D.A., Lewis, F.A., Knight, M., 2003. Comparative gene analysis of *Biomphalaria glabrata* hemocytes pre- and post-exposure to miracidia of *Schistosoma mansoni*. Mol. Biochem. Parasitol. 126, 181–191.

Rasmussen, L.P.D., 1986. Occurrence, prevalence and seasonality of neoplasia in the marine mussel *Mytilus edulis* from three sites in Denmark. Mar. Biol. 92, 59–64.

Reno, P.W., House, M., Illingworth, A., 1994. Flow cytometric and chromosome analysis of soft shell clams, *Mya arenaria*, with disseminated neoplasia. J. Invertebr. Pathol. 64, 163–172.

Romalde, J.L., Vilarino, M.L., Beaz, R., Rodriguez, J.M., Diaz, S., Villalba, A., Carballal, M.J., 2007. Evidence of retroviral etiology for disseminated neoplasia in cockles (*Cerastoderma edule*). J. Invertebr. Pathol. 94, 95–101.

Sciamanna, I., Landriscina, M., Pittoggi, C., Quirino, M., Mearelli, C., Beraldi, R., Mattei, E., Serafino, A., Cassano, A., Sinibaldi-Vallebona, P., Garaci, E., Barone, C., Spadafora, C., 2005. Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. Oncogene 24, 3923–3931.

Sheehan, D.Z., Hrapchak, B.B., 1980. Theory and Practice of Histotechnology. Bettelle Press, Columbus, Ohio.

Sinibaldi-Vallebona, P., Lavia, P., Garaci, E., Spadafora, C., 2006. A role of endogenous reverse transcriptase in tumorigenesis and as target in differentiating cancer therapy. Genes Chromosomes Cancer 45, 1–10.

Spadafora, C., 2004. Endogenous reverse transcriptase: a mediator of cell proliferation and differentiation. Cytogenet. Genome Res. 105, 346–350.

Temin, H.M., 1971. The provirus hypothesis speculations on the significance of RNA-directed DNA synthesis from normal development and for carcinogenesis. J. Natl. Cancer. Inst. 46, 56–60.

Temin, H.M., Mizutani, S., 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226, 1211–1213.

Zaldibar, B., Cancio, I., Marigomez, I., 2004. Circatidal variation in epithelial cell proliferation in the mussel digestive gland and stomach. Cell Tissue Res. 318, 395–402.

Zaldibar, B., Cancio, I., Marigomez, I., 2008. Epithelial cell renewal in the digestive gland and stomach of mussels: season, age and tidal regime related variations. Histol. Histopathol. 23, 281–290.

Vol. 84: 57–63, 2009
doi: 10.3354/dao02038

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 9

Reverse transcriptase activity associated with haemic neoplasia in the soft-shell clam *Mya arenaria*

M. AboElkhair[1], A. Siah[1], K. F. Clark[2], P. McKenna[1], J. Pariseau[1,3], S. J. Greenwood[1,2], F. C. J. Berthe[4], A. Cepica[1,*]

[1]Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island C1A 4P3, Canada

[2]AVC Lobster Science Centre, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island C1A 4P3, Canada

[3]Institut des Sciences de la mer, Université du Québec à Rimouski, Rimouski, Québec G5L 3A1, Canada

[4]European Food Safety Authority (EFSA), Animal Health and Welfare Panel, 43100 Parma, PR, Italy

ABSTRACT: Reverse transcriptase (RT) activity has been reported in bivalves affected by haemic neoplasia (HN). Since all retroviruses have RT, detection of RT activity was regarded as evidence for the retroviral etiology of HN. This study investigates the relationship between RT levels and the progress of HN as indicated by percentages of tetraploid cells in soft-shell clams *Mya arenaria*. The percentages of tetraploid cells were estimated by flow cytometry, and the RT levels were quantified using TaqMan product-enhanced RT (TM-PERT) assay. Results demonstrated that the amount of RT was positively correlated with the percentage of tetraploid cells circulating in clam haemolymph (R^2 = 0.974, $p < 0.001$). Compared to HN-negative clams (<5% tetraploid cells), 2 stages with significantly elevated levels of RT activity were observed: the first stage at ~10 to ~20% tetraploid cells, and the second at ~30 to ~80% tetraploid cells ($p < 0.01$). These data support the well established fact from mammalian models that transformed cells express high levels of non-telomeric RT. The observed increase in RT levels at ~30% tetraploidy coincides with previously reported p53 gene expression. Taken together, this could indicate that using RT levels as an indicator of HN, ≥30% tetraploidy is the stage at which the disease process undergoes a change, and perhaps becomes irreversible.

KEY WORDS: *Mya arenaria* · Haemic neoplasia · Reverse transcriptase · Polyploidy · TaqMan · Product-enhanced reverse transcriptase assay · PERT

INTRODUCTION

Haemic neoplasia (HN) is a leukemia-like disease of the soft-shell clam *Mya arenaria*. The condition is often fatal and progressive in most of the locations where the disease has been reported (Barber 2004). In Prince Edward Island (PEI), Canada, massive mortalities of soft-shell clams were linked to high prevalence of HN (McGladdery et al. 2001). Different diagnostic techniques for the detection of HN based on the morphological distinction between normal and neoplastic haemocytes have been employed (Elston et al. 1992, McGladdery et al. 2001, Barber 2004). Neoplastic cells have large pleomorphic nuclei containing one or more nucleoli, with higher nucleo-cytoplasmic ratios. Given the change in ploidy of circulating haemocytes in diseased individuals, DNA content measurement using flow cytometry (FCM) has increasingly become a powerful tool for diagnosis of HN in bivalves (Harper et al. 1994, Reno et al. 1994, da Silva et al. 2005, Delaporte et al. 2008). The ploidy status in HN has been shown to vary among bivalve species. In *Mya arenaria*, HN

*Corresponding author. Email: acepica@upei.ca

affected individuals have been found to show tetraploid cells with 1.25 to 2.05 times more DNA than normal diploid haemocytes (Reno et al. 1994).

Since the initial description of the disease (Farley 1969), its cause has not been clearly defined (Barber 2004). Viral infection, genetic profile, environmental changes and anthropogenic pollution have been proposed as the causative factors (Elston et al. 1992, McGladdery et al. 2001, Barber 2004). Retroviral etiology, in particular, has been suggested by several authors (Oprandy et al. 1981, Oprandy & Chang 1983, Romalde et al. 2007). The main evidence supporting a retroviral etiology of HN has been the detection of reverse transcriptase (RT) activity in individuals affected by this pathological condition in soft-shell clams (Medina et al. 1993, House et al. 1998) and in cockles, *Cerastoderma edule* (Romalde et al. 2007). Reverse transcriptase activity, however, is not exclusive to retroviruses. Other viruses (hepadnaviruses), some bacteria (e.g. strains of Myxobacteria and *Escherichia coli*), and also telomerases (a set of enzymes that lengthen the chromosome end (telomere) by reverse transcription of its own RNA; Flint et al. 2004) have RT activity. Moreover, RT-related sequences are found in cellular genomes generally known as retroelements (Flint et al. 2004). Expression of these endogenous cellular RT-coding genes is repressed in normal terminally differentiated cells, but can be active in tissues with rapidly dividing cells such as germ cells, embryos, and tumors (Spadafora 2004).

The main contention of this study is that the absence of conclusive evidence of an exogenous retrovirus in the presence of RT activity likely means that the RT detected in HN is expressed from sources other than exogenous retrovirus. Thus, quantification of RT activity at different stages of HN development may provide valuable information on possible sources of RT activity. Product-enhanced RT (PERT) assay has been used as a method for general detection of RT activity of both known and unknown retroviruses, which was subsequently modified as TaqMan PERT (TM-PERT). TM-PERT is a quantitative assay in which the resulting cDNA from RT activity is amplified and quantified by fluorogenic 5'-nuclease TaqMan PCR (André et al. 2000, Maudru & Peden 1998). The use of TM-PERT for quantitative detection of RT activity can help to distinguish the background RT signals arising from nonRT sources, such as some thermostable DNA polymerases released from lysed cells. Also, unlike conventional PERT, TM-PERT detects manganese- and magnesium-dependent RT with approximately equal sensitivity (Maudru & Peden 1998). Finally, the TM-PERT method allows quantification of RT activity over a wider range of activities than PERT does (Maudru & Peden 1998).

This study describes the relationship between RT levels and the progress of HN as indicated by the percentage of tetraploidy in circulating haemocytes from soft-shell clams.

MATERIALS AND METHODS

Samples. Two hundred clams were collected from North River (46° 15' 01" N, 63° 10' 42" W), Charlottetown, PEI, Canada in October 2007. Following gradual acclimation from 5 to 18°C over 2 d, the clams were held in tanks at 18°C until use.

Flow cytometry. The FCM procedure was performed according to Delaporte et al. (2008). Briefly, haemolymph was withdrawn from the anterior adductor muscle of each clam using a 25-gauge needle fitted with a 3 ml syringe. A 0.5 ml haemolymph sample was fixed in 2.5 ml of 95% cold ethanol. After centrifugation at 400 × *g* for 10 min, cell pellets were resuspended and rehydrated in phosphate buffered saline (PBS, 0.01 M) for 30 min, followed by 2 washes in PBS (0.01M). The resuspended filtered cell pellets were treated with DNase-free RNase A (Sigma, R4875, 50 µg ml^{-1}) and stained with propidium iodide (PI, Sigma, P4170, 50 µg ml^{-1}). A specific FL2 detector (orange light, at a wavelength of 550 to 600 nm) of a FACSCalibur flow cytometer (BD BioSciences) was used to measure fluorescence of PI-stained cells. For each sample, 10 000 particles were counted at a low flow rate (15 µl min^{-1}). For each cell, a single electronic pulse of PI fluorescence was recorded. Each pulse was discriminated by its area, height and width. Based on these data, the tetraploid cells at the G2/M phase were distinguished from normal diploid cells (2N) at the G0/G1 phase, and from doublets of diploid cells (2 cells with the same DNA quantity (2N) stuck together) by plotting FL2-area vs. FL2-width on cytograms (Fig. 1a). To discriminate single cells from doublets, the R1 region was drawn on data scatter plots so that cell doublets were plotted to the right of R1 (Fig 1a). Also, PI fluorescence intensities of single cells were plotted on an FL2-area histogram in order to calculate the percentage of normal cells and tetraploid cells in the tested sample (Fig. 1b).

Quantification of RT activity. ***Samples:*** After individual assessment of all clams by FCM, clams were assigned to 1 of 7 groups based on the percentages of tetraploid cells in the haemolymph, for TM-PERT analysis (Table 1). Clams with <5% tetraploid cells were considered as HN-negative controls based on Delaporte et al. (2008). Three clams were selected for inclusion in each of the 6 subsequent groups, except the ~70% group, which contained only 2 clams. The detailed composition of the experimental groups and the percentages of tetraploid cells in clams are listed in

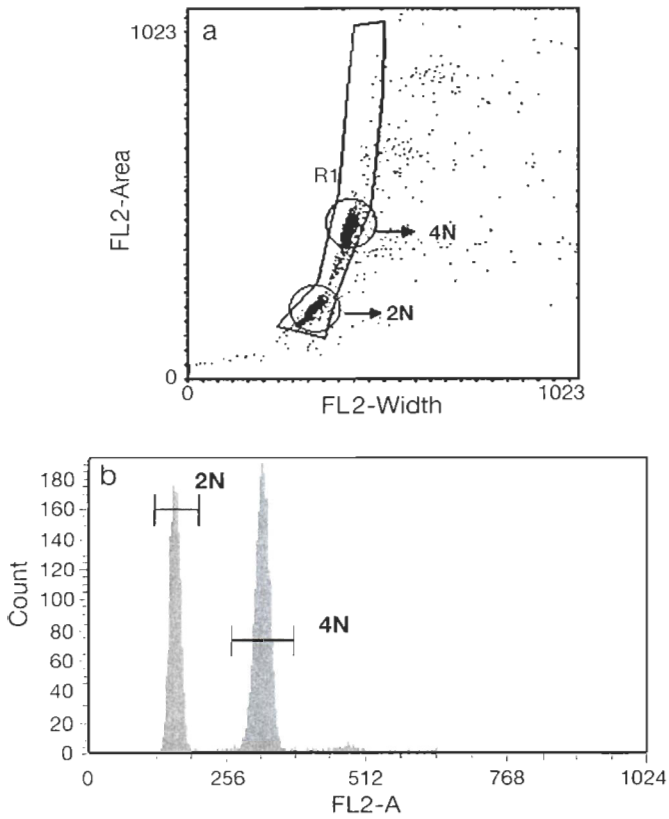


Fig. 1. *Mya arenaria*. Analysis of propidium iodide (PI)-stained clam haemocyte DNA contents (FL2) by flow cytometry. (a) Diploid and tetraploid single cells gated in R1 on an FL2-width versus FL2-area cytogram. (b) PI fluorescence of single cells in R1 plotted on an FL2-area histogram. Diploid (2N) and tetraploid (4N) peaks are delimited by markers. For further definitions see 'Materials and methods'

Table 1. Clams containing between >5% and <20% tetraploid cells were presumed doubtful, and those with >20% tetraploid cells were presumed positive based on Delaporte et al. (2008).

Preparation of samples: From each selected individual, 1 ml of haemolymph was withdrawn from the anterior adductor muscle as described above. The haemolymph samples were centrifuged at 500 × *g* for 15 min, and supernatants were again centrifuged at 13 400 × *g* for 5 min. Supernatants were then passed through 0.22 µm filters (VWR International), aliquoted, and stored at –80°C.

TM-PERT assay: Assay conditions, and primer and probe sequences, were adopted from Maudru & Peden (1998) with some modifications. The respective sequences of primers A and B were 5'-GCC TTA GCA GTG CCC TGT CT-3' and 5'-AAC ATG CTC GAG GGC CTT A-3', while that of the probe was FAM-5'-CCC GTG GGA TGC TCC TAC ATG TC-3'-BHQ1.

For reverse transcription, 0.4 µg of the bacteriophage MS2 genomic RNA template (Roche Applied Science) was mixed with 5 µM of primer A in RNase-free water, in a final volume of 2 µl per RT reaction. The mixture was heated at 85°C for 5 min, annealed at 37°C for 30 min, and kept at 4°C for 5 min. Then, 2 µl of clam haemolymph sample was added to the RT reaction mixture containing 50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithioerythritol, 10 U RNAsin (Promega), and 1 mM dNTPs (Qiagen), to give a 20 µl final volume. The reaction was then incubated at 37°C for 1 h, then at 95°C for 7 min.

Table 1. *Mya arenaria*. Percentages of tetraploidy and group classification for selected individuals assayed using the TaqMan product-enhanced reverse transcriptase (TM-PERT) assay

Groups (% tetraploid cells)	% tetraploidy	No. of animals
Negative group	1.1, 2.0, 2.2	3
~10	11.8, 9.7, 12.0	3
~20	19.3, 21.3, 26.9	3
~30	30.0, 30.0, 30.0	3
~50	55.0, 61.3, 53.8	3
~70	74.8, 74.6	2
~80	80.0, 80.0, 94.0	3

For PCR amplification, 5 µl of the synthesized cDNA was added to the PCR reaction mixture to give a total volume of 25 µl. The reaction mixture included 1× TaqMan Universal Master Mix including AmpErase uracil-N-glycosylase (UNG) (Applied Biosystems), 0.3 µM of primer A, 0.3 µM of primer B, 0.15 µM of probe labeled at the 3' end with FAM and at the 5' end with Black Hole Quencher (BHQ) 1 (Biosearch Technologies), and 250 ng of RNase A (Qiagen). The tubes were placed in the Chromo 4 system (Bio-RAD), and incubations were controlled using MJ Opticon Monitor version 3.1 with the following thermal cycler conditions: 37°C for 15 min; 50°C for 2 min; 95°C for 10 min; 45 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s. A standard curve was established by using a 10-fold dilution of Moloney murine leukemia virus (M-MLV) RT (Roche Applied Science) from 10^2 to 10^{10} picounits (pU). The enzyme was diluted in buffer A consisting of 50 mM KCl, 20 mM Tris-HCl pH 7.5, 0.2 mM dithiothreitol, 0.25 mM EDTA, 0.025% Triton X-100 (v/v), and 50% glycerol (v/v). The amplified PCR products of the serial dilutions were resolved by 1.5% agarose gel electrophoresis in 1× tris-borate-EDTA (TBE) buffer, visualized by staining with ethidium bromide, and photographed using UV illumination.

Electron microscopy. Haemolymph samples from clams (n = 10) previously classified by FCM to be between 8 and 70% tetraploid, were analyzed by electron microscopy (EM) in order to explore the presence of retroviral particles. Haemolymph samples were fil-

tered using a 0.45 µm syringe filter (VWR International), and centrifuged at 100 000 × *g* for 90 min in an SW 60 Ti rotor (Beckman). Each resultant pellet was resuspended in 30 µl TNE buffer (10 mM Tris pH 7.4, 400 mM NaCl and 1 mM EDTA; Oprandy et al. 1981). Volumes of 10 µl were placed on Formvar-coated grids and allowed to dry. One drop of a mixture of phosphotungstic acid and bovine serum albumin was placed on the grid and the excess was blotted off with filter paper. The samples were examined with a Hitachi H7500 electron microscope (Nissei Sangyo) at 80 kV.

Data analysis. A linear regression line between the logarithms of the RT standards and the corresponding threshold cycle (C_T) values was calculated and plotted. The logarithms of RT levels in haemolymph samples were determined by extrapolating the C_T values from the standard curve. One-way ANOVA was performed to assess multiple comparisons of log RT concentrations among different tetraploidy groups using Minitab software version 15 (Minitab). STATA software version 9 (College Station, Texas, USA) was used for exponential regression analysis. The Gompertz model was used to assess correlation between % tetraploidy and log RT concentrations.

RESULTS

The FCM analysis showed that 116 (58%) clams were negative (<5% tetraploid cells), 42 (21%) were diseased (>20% tetraploid cells), 20 contained between 20 to 50% tetraploid cells, and 22 contained >50% tetraploid cells.

TM-PERT assay optimization enabled the detection of up to 10^2 pU of RT 2 μl^{-1} of haemolymph or 5×10^4 pU ml^{-1}, which is equivalent to 50 retroviral particles ml^{-1} (a retrovirus particle contains ~10^3 RT pU) (Maudru &Peden 1998). The M-MLV RT standard curve showed a linear relationship between threshold cycle and the log of RT concentrations over a wide range of concentrations (10^2 to 10^{10} pU) (Fig. 2a). The numerical means and SD values of the slopes, the *y*-intercepts, and the R^2 of the assays performed were −3.631 ± 0.217, 46.64 ± 3.16, and 0.9965 ± 0.00214, respectively. The PCR product target specificity of TM-PERT assay was verified by agarose gel electrophoresis, which revealed a band of the expected size (112 bp). In addition, negative controls with no RT source, which were replaced with either RNase-free water or buffer A, showed no bands (Fig. 2b).

One of the HN-negative haemolymph samples used for quantitative assessment of RT activity exhibited no signal; the other 2 ind. showed C_T values of 34.31 and 39.58, respectively. The logarithms of RT levels in individuals with ~10 to ~80% tetraploid cells ranged from

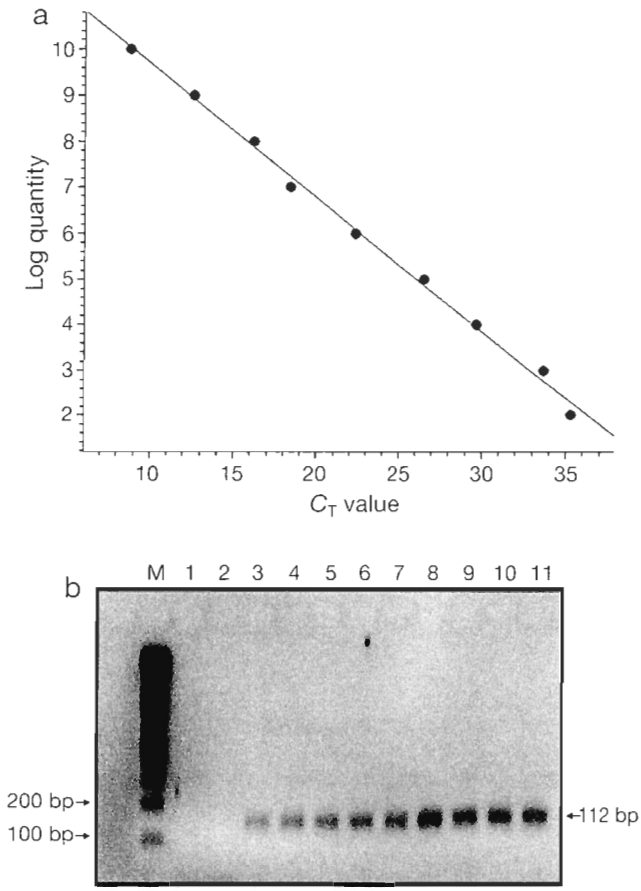


Fig. 2. (a) Standard curve ($y = -3.4043x + 43.034$; $R^2 = 0.9989$) of TaqMan product-enhanced reverse transcriptase (TM-PERT) assay with the serial dilutions of the Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) from 10^{10} to 10^2 pU. (b) Gel electrophoresis of PCR products of TM-PERT assay standard curve reactions. Lanes: 1, negative control (no RT); 2, negative control (buffer A); 3 to 11, 10-fold dilution of M-MLV RT from 10^2 to 10^{10} pU. M: molecular weight marker (100 bp; Roche Applied Science), C_T: threshold cycle number

3.6 to 6.6 pU 2 μl^{-1} (Table 2), which are equivalent to 2×10^6 to 2×10^9 pU ml^{-1} of haemolymph, respectively. Compared to the HN-negative group, a significant increase in RT levels was observed in clams with ~10 and ~20% tetraploidy, with no significant difference in RT levels between the 2 groups. Further significant increase in RT levels was observed in the haemolymph of clams with ~30% tetraploid cells and above ($p < 0.01$) (Table 2). Under the conditions of this study, clams could be classified into 3 distinct groups according to the RT levels: <5% tetraploid cells; ~10 to ~20% tetraploid cells; and ~30 to ~80% tetraploid cells. Overall, there was a significant positive correlation between the percentages of tetraploidy and RT levels ($R^2 = 0.974$, $p < 0.001$) (Fig. 3).

Table 2. *Mya arenaria*. C_T values (mean ± SD) and log of reverse transcriptase (RT) concentrations (mean ± SD) at various percentages of tetraploidy

% tetraploid cells	C_T values	Log RT (pU 2 μl^{-1} haemolymph)
Negative group	0.00 or > 34	1.73 ± 1.64
~10	30.53 ± 2.74	4.38 ± 0.77[a]
~20	30.63 ± 2.02	4.35 ± 0.56[a]
~30	26.32 ± 0.71	5.55 ± 0.20[b]
~50	25.93 ± 0.53	5.66 ± 0.15[b]
~70	24.75 ± 0.96	5.99 ± 0.27[b]
~80	23.85 ± 1.05	6.25 ± 0.29[b]

[a]Statistically different from the negative group ($p < 0.01$)
[b]Statistically different from the negative, the ~10%, and the ~20% groups ($p < 0.01$)

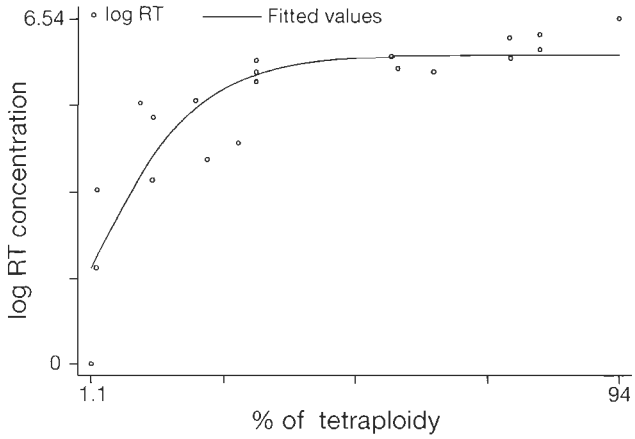


Fig. 3. *Mya arenaria*. Exponential regression between log RT (reverse transcriptase) concentration and percentage of tetraploidy of circulating haemocytes. The scatterplot represents the correlation between % tetraploid cells and log RT concentration. $R^2 = 0.974$, $p < 0.001$, $n = 20$. The equation is $y = b_1 \times \exp\{-\exp[-b_2 \times (x - b_3)]\} = 5.86 \times \exp\{-\exp[-0.99 \times (x - 2.61)]\}$

Electron microscopic examination of haemolymph samples using negative staining did not show retrovirus-like particles.

DISCUSSION

The FCM analysis of the clam population studied showed that 21% of tested clams were affected, which confirms previous reports by Delaporte et al. (2008) and Siah et al. (2008) suggesting that HN is widely spread among North River clams.

The PERT assay has been used as a method to quantify known and detect unknown retroviruses, with recognized limitations in terms of possible detection of RT-like activities of nonretroviral origin (Maudru & Peden 1997, André et al. 2000, Brorson et al. 2002). Apart from retroviruses, there are several other possible sources of RT activity. At the level of the assay itself, there are some reagents that may produce RT-like activity such as Taq DNA polymerases or RNase inhibitors (Pyra et al. 1994). At the level of the tested sample, there are some enzymes that may display RT-like activity such as host DNA polymerases, some other nuclear enzymes, and polymerase from mitochondria (Brorson et al. 2002).

In this study, in order to measure introduction of RT activity from reagents, negative controls with water and buffer A were always included. As a further control to measure possible contribution of sample-unrelated RT activity arising from PCR reagents, an RNA digestion step with RNase A was included before PCR amplification (Maudru & Peden 1997). In order to avoid false positive results possibly arising from released cellular enzymes, haemocytes were carefully removed from haemolymph samples by low speed centrifugation, and the assays were performed on a cell free haemolymph.

Although it could be argued that telomerase activity could be present since HN is widely believed to involve cell transformation, our assay should not have detected telomerase activity for 2 reasons. Firstly, telomerases are template specific, requiring a specific recognition sequence, TTAGGG. Thus, it is generally believed that they are unlikely to reverse transcribe MS2 phage RNA which lacks this target sequence (Brorson et al. 2002). Secondly, the assay was performed on cell free haemolymph as discussed above. On the other hand, TM-PERT is able to detect RT activity of endogenous retroviruses and other retroelements such as retrotransposons (Brorson et al. 2002).

Our detection of low levels of RT activity in some disease-negative clams is in agreement with findings in other species: e.g. in turtles (Casey et al. 1997), humans (Molès et al. 2007), and cockles (Romalde et al. 2007). The observed RT activity in HN-negative clams could be related to the fact that RT activity is associated with a wide range of biological processes, both physiological and pathological (Spadafora 2004). This is because RT-coding genes are also contained in repeated genomic elements called retroelements, which play an important role in many physiological and pathological cellular processes (Spadafora 2004).

We found a statistically significant difference in RT quantities between groups with ~10 to ~20% tetraploid cells and ~30 to ~80% tetraploid cells ($p < 0.01$) (Table 2). This supports previously published obser-

vations of disease remission in clams (Cooper et al. 1982, Leavitt et al. 1994) and mussels (Elston et al. 1988) in early stages of the disease (~20% neoplastic cells). The increased level of significance at ~30% tetraploidy suggests that this might be the beginning of the irreversible stage in the progression of the pathological process.

The absence of structures similar to retroviral particles in negatively stained samples of clam haemolymph is in disagreement with Oprandy et al. (1981) but in agreement with House et al. (1998) and other electron microscopic studies reviewed by Elston et al. (1992) and Barber (2004). The elevated levels of RT activity at various levels of tetraploidy without indication of retroviral particles in haemolymph samples as evaluated by electron microscopy suggests that the observed RT activity might be due to the expression of endogenous source of RT. Others demonstrated the presence of RT protein in tissues that were not included in retroviral particles, and they suggested endogenous retroelements as the source (Molès et al. 2007).

The positive correlation between levels of tetraploidy and RT activity suggests that the transformed haemocytes might have a role in the increased level of RT activity. Our suggestion is supported by the growing body of data showing that transformed cells of mammalian origin express high levels of endogenous non-telomeric RT, with the source of the RT being endogenous retroelements (Spadafora 2004, Oricchio et al. 2007). Involvement of endogenous retrovirus in HN of *Mya arenaria* was previously suggested by Oprandy & Chang (1983).

Further supporting our contention of an endogenous source of RT is the report that RT-coding genes are generally active in cancer cells (Spadafora 2004). In addition, RT gene activity is up-regulated by a variety of stimuli acting at the genomewide level such as cellular stress, heat shock, genotoxic agents and others (Sciamanna et al. 2005).

In conclusion, we suggest that RT activity associated with HN might be due to activation of endogenous retroelements, but whether this activity is a consequence, or is related to the cause of the disease requires further investigation. What retroelement may be responsible for production of RT activity is presently unclear. On the other hand, whether or not an exogenous retrovirus plays a role in induction of HN cannot be conclusively answered by our current data; however, our results on RT activity and EM analysis of haemolymph, combined with observations by other authors (Elston et al. 1992, House et al. 1998, Molés et al. 2007), suggest that the RT activity is not from an exogenous retrovirus.

Compared to HN-negative animals, the 2 significant increases in RT levels — first at ~10 to ~20% tetraploid cells and second at ~30 to ~80% tetraploid cells — suggest that the stage from ≤10 to ≥30% tetraploid cells should be targeted for further investigations on the mechanism of induction of HN.

Acknowledgements. We thank J. W. Casey for his helpful advice, G. Johnson for his valuable support of this study, and A. Mounchili for his help in statistical analysis. The research was supported by the Egyptian Cultural and Educational Bureau in Montréal, Quebec, Canada.

LITERATURE CITED

André M, Morgeaux S, Fuchs F (2000) Quantitative detection of RT activity by PERT assay: feasibility and limits to a standardized screening assay for human vaccines. Biologicals 28:67–80

Barber BJ (2004) Neoplastic diseases of commercially important marine bivalves. Aquat Living Resour 17:449–466

Brorson K, Xu Y, Swann PG, Hamilton E and others (2002) Evaluation of a quantitative product-enhanced reverse transcriptase assay to monitor retrovirus in mAb cell-culture. Biologicals 30:15–26

Casey RN, Quackenbush SL, Work TM, Balazs GH, Bowser PR, Casey JW (1997) Evidence for retrovirus infections in green turtles *Chelonia mydas* from the Hawaiian Islands. Dis Aquat Org 31:1–7

Cooper KR, Brown RS, Chang PW (1982) The course and mortality of a hematopoietic neoplasm in the soft-shell clam *Mya arenaria*. J Invertebr Pathol 39:149–157

da Silva PM, Soudant P, Carballal MJ, Lambert C, Villalba A (2005) Flow cytometric DNA content analysis of neoplastic cells in haemolymph of the cockle *Cerastoderma edule*. Dis Aquat Org 67:133–139

Delaporte M, Synard S, Pariseau J, McKenna P, Tremblay R, Davidson J, Berthe FCJ (2008) Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry. J Invertebr Pathol 98:190–197

Elston RA, Kent ML, Drum AS (1988) Progression, lethality and remission of hemic neoplasia in the bay mussel *Mytilus edulis*. Dis Aquat Org 4:135–142

Elston RA, Moore JD, Brooks K (1992) Disseminated neoplasia of bivalve molluscs. Rev Aquat Sci 6:405–466

Farley CA (1969) Probable neoplastic disease of the hematopoietic system in oysters *Crassostrea virginica* and *Crassostrea gigas*. Natl Cancer Inst Monogr 31:541–555

Flint SJ, Enquist LW, Racaniello VR, Skalka AM (2004) Principles of virology: molecular biology, pathogenesis and control of animal viruses, 2nd edn. ASM Press, Washington, DC, p 217–230

Harper DM, Flessas DA, Reinisch CL (1994) Specific reactivity of leukemia cells to polyclonal anti-PCB antibodies. J Invertebr Pathol 64:234–237

House ML, Kim CH, Reno PW (1998) Soft-shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. Dis Aquat Org 34:187–192

Leavitt DF, Miosky Dragos D, Lancaster BA, Craig AC, Reinisch CL, Capuzzo JM (1994) Initiation and promotion of hematopoietic neoplasia in soft shells, *Mya arenaria*, exposed to natural sediment. In: Rosenfield A, Kern FG, Keller BJ (eds) Proc Int Workshop on Invertebrate Neoplasia: initiation and promotion mechanisms. June 23, 1992, Washington, DC, p 23–25

Maudru T, Peden K (1997) Elimination of background signals in a modified polymerase chain reaction-based reverse transcriptase assay. J Virol Methods 66:247–261

Maudru T, Peden K (1998) Adaptation of the fluorogenic 5'-nuclease chemistry to a PCR-based reverse transcriptase assay. Biotechniques 25:972–975

McGladdery SE, Reinisch CL, MacCallum GS, Stephens RE, Walker CL, Davidson JT (2001) Haemic neoplasia in soft-shell clams *Mya arenaria*: recent outbreaks in Atlantic Canada and discovery of a p53 gene homologue associated with the condition. Bull Aquac Assoc Can 101:19–26

Medina DJ, Paquette GE, Sadasiv EC, Chang PW (1993) Isolation of infectious particles having reverse transcriptase activity and producing hematopoietic neoplasia in *Mya arenaria*. J Shellfish Res 12:112–113

Molès JP, Tesniere A, Guilhoi JJ (2007) Reverse transcriptase activity in human normal and psoriatic skin samples. Br J Dermatol 157:482–486

Oprandy JJ, Chang PW (1983) 5-bromodeoxyuridine induction of hematopoietic neoplasia and retrovirus activation in soft-shell clam *Mya arenaria*. J Invertebr Pathol 42: 196–206

Oprandy JJ, Chang PW, Pronovost AD, Cooper KR, Brown RS, Yates VJ (1981) Isolation of viral agent causing hematopoietic neoplasia in the soft-shell clams *Mya arenaria*. J Invertebr Pathol 38:45–51

Oricchio E, Sciamanna I, Beraldi R, Tolstonog GV, Schumann GG, Spadafora C (2007) Distinct roles for LINE-1 and HERV-K retroelements in cell proliferation, differentiation and tumor progression. Oncogene 26:4226–4233

Pyra H, Boni J, Schupbach J (1994) Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc Natl Acad Sci USA 91:1544–1548

Reno PW, House M, Illingworth A (1994) Flow cytometric and chromosome analysis of softshell clams *Mya arenaria* with disseminated neoplasia. J Invertebr Pathol 64:163–172

Romalde JL, Vilarino ML, Beaz R, Rodriguez JM, Diaz S, Villalba A, Carballal MJ (2007) Evidence of retroviral etiology for disseminated neoplasia in cockles (*Cerastoderma edule*). J Invertebr Pathol 94:95–101

Sciamanna I, Landriscina M, Pittoggi C, Quirino M and others (2005) Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. Oncogene 24: 3923–3931

Siah A, Delaporte M, Pariseau J, McKenna P, Berthe FCJ (2008) Patterns of p53, p73 and mortalin gene expression associated with haemocyte polyploidy in the soft-shell clam *Mya arenaria*. J Invertebr Pathol 98:148–152

Spadafora C (2004) Endogenous reverse transcriptase: a mediator of cell proliferation and differentiation. Cytogenet Genome Res 105:346–350

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: August 4, 2008; Accepted: January 25, 2009
Proofs received from author(s): February 26, 2009

Journal of Invertebrate Pathology 98 (2008) 148–152

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/yjipa

Patterns of p53, p73 and mortalin gene expression associated with haemocyte polyploidy in the soft-shell clam, *Mya arenaria*

A. Siah [a,*], M. Delaporte [a,d], J. Pariseau [a,b], P. McKenna [a], F.C.J. Berthe [a,c]

[a] University of Prince Edward Island, Atlantic Veterinary College, Department of Pathology and Microbiology, Charlottetown, C1A 4P3, Prince Edward Island, Canada
[b] Institut des sciences de la mer de Rimouski (ISMER), 310, allée des Ursulines, C.P. 3300 Rimouski (Québec) Canada G5L 3A1
[c] Animal Health and Welfare, European Food Safety Authority, EFSA, Largo N. Palli 5/A, I-43100 Parma, Italy
[d] Université Louis Pasteur, Institut de Virologie, 3 rue Koeberlé, 67000 Strasbourg, France

ARTICLE INFO

Article history:
Received 24 September 2007
Accepted 3 March 2008
Available online 13 March 2008

Keywords:
Mya arenaria
Haemocytes
p53
p73
Mortalin
Tetraploidy

ABSTRACT

The molecular mechanisms by which haemocytes of clams are transformed in the course of haemic neoplasia remain by far unknown. The aim of this study was to quantify the expression of p53/p73 and mortalin genes, in relation with the ploidy status of clam haemocytes and to correlate the p53 expression with mortalin expression. For this purpose, soft-shell clams, *Mya arenaria*, were collected from an endemic zone for neoplasia. The ploidy of haemocytes was assessed for each individual clam by flow cytometry using a propidium iodide protocol, while p53/p73 and mortalin gene expressions were quantified by real-time RT-PCR. Results show that haemocytes of some clams with a moderate percentage (15–50%) of tetraploid cells have a significantly high level of p53 and p73 in comparison with clams belonging to categories with low (<15%) or high levels (>50%) of tetraploid cells, where low levels of expression of these genes were observed. Furthermore, mortalin gene expression is strongly correlated ($r^2 = 0.68$, $p < 0.01$) with p53 gene expression level. This reinforces the hypothesis of a cytoplasmic p53 sequestration mechanism in clam haemic neoplasia. Further studies are needed to confirm these preliminary results and further unravel the molecular pathways involved in this process. Our results are believed to provide phenotypic foundation for such studies to be undertaken.

1. Introduction

The soft-shell clam, *Mya arenaria*, represents an important resource for Prince Edward Island (PEI), with clam production accounting for a sixth of the total landed value of all clam species in Atlantic Canada. In 1999, severe mortalities attributed to haemic neoplasia occurred in PEI (McGladdery et al., 2001). Field and laboratory studies had previously established a correlation between high prevalence of haemic neoplasia and mass mortality of clams (Brousseau and Baglivo, 1991). Although the etiology of this disease has still not been clearly established, an association with environmental pollution (Appeldoorn et al., 1984) and/or retroviral infection (House et al., 1998) has been suggested. Barber (2004) in his review of neoplasia in bivalves leaves the door for debate open. On the one hand, clams were diagnosed for haemic neoplasia in New Bedford Harbor (MS), an area where high levels of xenobiotic contaminants such as polychlorinated biphenyls (PCB) and heavy metals (Barker et al., 1997) were reported. On the other hand, affected clams were also reported in unpolluted sites, which would support the viral hypothesis (Oprandy, 1983). The two hypotheses are not necessarily exclusive.

Neoplastic haemocytes are characterized by a proliferative nature and a high nucleo-cytoplasmic ratio (Reno et al., 1994); a disrupted cytoskeleton related to altered actin filament patterns (Moore et al., 1992); and a loss of phagocytic ability (Beckmann et al., 1992). These haemocytes also exhibit atypical morphology such as rounded shape and no, or fewer, filopods (Moore et al., 1992). These morphological characteristics are commonly used to differentiate neoplastic from normal haemocytes using microscopy (Kelley et al., 2001; Walker et al., 2006).

Besides structural and morphological features, neoplastic haemocytes can be characterized by a modified ploidy status (Reno et al., 1994). Protocols in flow cytometry have been proposed towards diagnosis of neoplasia in bivalves according to their ploidy (Delaporte et al., 2008 Da Silva et al., 2005; Reno et al., 1994). Basically, analyzing phases of the haemocyte cell cycle using fluorescent DNA stains enables distinction of the G0/G1, S and G2/M phases and identification of cell populations with an abnormal amount of DNA (Ashton-Alcox and Ford, 1998; Allam et al., 2002). In *M. arenaria*, neoplastic haemocytes have been shown to exhibit higher DNA contents (1.25–2.05 times higher) and mitosis rates than in normal cells (Reno et al., 1994). In addition, chromo-

* Corresponding author. Fax: +1 902 566 0851.
E-mail address: asiah@upei.ca (A. Siah).

0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.03.002

Journal of Invertebrate Pathology 98 (2008) 148–152

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/yjipa

Patterns of p53, p73 and mortalin gene expression associated with haemocyte polyploidy in the soft-shell clam, *Mya arenaria*

A. Siah [a,*], M. Delaporte [a,d], J. Pariseau [a,b], P. McKenna [a], F.C.J. Berthe [a,c]

[a] University of Prince Edward Island, Atlantic Veterinary College, Department of Pathology and Microbiology, Charlottetown, C1A 4P3, Prince Edward Island, Canada
[b] Institut des sciences de la mer de Rimouski (ISMER), 310, allée des Ursulines, C.P. 3300 Rimouski (Québec) Canada G5L 3A1
[c] Animal Health and Welfare, European Food Safety Authority, EFSA, Largo N. Palli 5/A, I-43100 Parma, Italy
[d] Université Louis Pasteur, Institut de Virologie, 3 rue Koeberlé, 67000 Strasbourg, France

ARTICLE INFO

Article history:
Received 24 September 2007
Accepted 3 March 2008
Available online 13 March 2008

Keywords:
Mya arenaria
Haemocytes
p53
p73
Mortalin
Tetraploidy

ABSTRACT

The molecular mechanisms by which haemocytes of clams are transformed in the course of haemic neoplasia remain by far unknown. The aim of this study was to quantify the expression of p53/p73 and mortalin genes, in relation with the ploidy status of clam haemocytes and to correlate the p53 expression with mortalin expression. For this purpose, soft-shell clams, *Mya arenaria*, were collected from an endemic zone for neoplasia. The ploidy of haemocytes was assessed for each individual clam by flow cytometry using a propidium iodide protocol, while p53/p73 and mortalin gene expressions were quantified by real-time RT-PCR. Results show that haemocytes of some clams with a moderate percentage (15–50%) of tetraploid cells have a significantly high level of p53 and p73 in comparison with clams belonging to categories with low (<15%) or high levels (>50%) of tetraploid cells, where low levels of expression of these genes were observed. Furthermore, mortalin gene expression is strongly correlated ($r^2 = 0.68$, $p < 0.01$) with p53 gene expression level. This reinforces the hypothesis of a cytoplasmic p53 sequestration mechanism in clam haemic neoplasia. Further studies are needed to confirm these preliminary results and further unravel the molecular pathways involved in this process. Our results are believed to provide phenotypic foundation for such studies to be undertaken.

1. Introduction

The soft-shell clam, *Mya arenaria*, represents an important resource for Prince Edward Island (PEI), with clam production accounting for a sixth of the total landed value of all clam species in Atlantic Canada. In 1999, severe mortalities attributed to haemic neoplasia occurred in PEI (McGladdery et al., 2001). Field and laboratory studies had previously established a correlation between high prevalence of haemic neoplasia and mass mortality of clams (Brousseau and Baglivo, 1991). Although the etiology of this disease has still not been clearly established, an association with environmental pollution (Appeldoorn et al., 1984) and/or retroviral infection (House et al., 1998) has been suggested. Barber (2004) in his review of neoplasia in bivalves leaves the door for debate open. On the one hand, clams were diagnosed for haemic neoplasia in New Bedford Harbor (MS), an area where high levels of xenobiotic contaminants such as polychlorinated biphenyls (PCB) and heavy metals (Barker et al., 1997) were reported. On the other hand, affected clams were also reported in unpolluted sites, which would support the viral hypothesis (Oprandy, 1983). The two hypotheses are not necessarily exclusive.

Neoplastic haemocytes are characterized by a proliferative nature and a high nucleo-cytoplasmic ratio (Reno et al., 1994); a disrupted cytoskeleton related to altered actin filament patterns (Moore et al., 1992); and a loss of phagocytic ability (Beckmann et al., 1992). These haemocytes also exhibit atypical morphology such as rounded shape and no, or fewer, filopods (Moore et al., 1992). These morphological characteristics are commonly used to differentiate neoplastic from normal haemocytes using microscopy (Kelley et al., 2001; Walker et al., 2006).

Besides structural and morphological features, neoplastic haemocytes can be characterized by a modified ploidy status (Reno et al., 1994). Protocols in flow cytometry have been proposed towards diagnosis of neoplasia in bivalves according to their ploidy (Delaporte et al., 2008 Da Silva et al., 2005; Reno et al., 1994). Basically, analyzing phases of the haemocyte cell cycle using fluorescent DNA stains enables distinction of the G0/G1, S and G2/M phases and identification of cell populations with an abnormal amount of DNA (Ashton-Alcox and Ford, 1998; Allam et al., 2002). In *M. arenaria*, neoplastic haemocytes have been shown to exhibit higher DNA contents (1.25–2.05 times higher) and mitosis rates than in normal cells (Reno et al., 1994). In addition, chromo-

* Corresponding author. Fax: +1 902 566 0851.
E-mail address: asiah@upei.ca (A. Siah).

0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.03.002

some caryotype varied from hypotriploidy to hypertetraploidy ($n = 44–80$) with a modal ploidy number close to tetraploidy ($n = 69$) compared with normal cells ($n = 34$). Haemic neoplasia in the soft-shell clam is usually accepted as a tetraploid disorder.

At the end of the G1 phase during normal mitosis, chromosomes segregate only when an adequate kinetochore-microtubule attachment exists enabling cells to pass the spindle assembly checkpoint (Rieder and Maiato, 2004). However, some cells can become tetraploid after prolonged arrest at the spindle assembly checkpoint that promotes cytokinesis failure (Shi and King, 2005). Following cytokinesis failure, tetraploid cells go through a p53-dependent cell cycle arrest in G1 phase (Stukenberg, 2004).

The tumor suppressor gene p53 plays a pivotal role in regulating the cell cycle by monitoring genomic integrity at the G1 and G2/M cell cycle checkpoints (Harms et al., 2004; Hofseth et al., 2004; Toledo and Wahl, 2006). As a "guardian" of the genome, p53 is normally inactivated, but its expression becomes stimulated when DNA is damaged, thus limiting the proliferation of affected daughter cells (Lane, 1992). It has been reported that 50% of human tumorigenesis could be explained by a p53 gene mutation and the other half is associated with an alteration of its regulation pathway (Toledo and Wahl, 2006). A homolog for human Hsp53 was previously cloned and characterized in *M. arenaria* (Kelley et al., 2001). *M. arenaria* p53 protein (p53) is characterized by a DNA binding domain II–V, a transactivation domain, MDM2 domain and tetramerization domain conserved at 73% with those of the human p53 protein. This suggests that the molecular mechanisms regulating the transcription of *M. arenaria* p53 gene are similar to those involved in the human p53 gene expression (Kelley et al., 2001).

Several studies have attempted to unravel the involvement of p53 and its family proteins such as p73, p63 and p97 in *M. arenaria* haemic neoplasia (Kelley et al., 2001; Stephens et al., 2001; Barker et al., 1997; Walker et al., 2006). Interestingly, affected clams express p73 rather than p53 protein (Kelley et al., 2001). Although p53 protein is expressed at similar levels in both healthy and diseased haemocytes (Kelley et al., 2001), only in leukemic cells does the presence of mortalin prevent p53 from acting as a guardian by complexing and sequestering the protein in the cytoplasm near the centriol (Walker et al., 2006). Mortalin, a member of the hsp70 proteins family, is involved in tumorigenesis by sequestering p53 in the cytoplasm and thus inactivates its transcriptional activation and apoptotic functions (Wadhwa et al., 2002). Recently, it was shown that over-expression of mortalin was sufficient to increase the malignancy of breast carcinoma cells (Wadhwa et al., 2006).

In this study, we have investigated (1) the relationship between haemocyte ploidy and p53, p73 and mortalin gene expression patterns in soft-shell clams with different levels of haemic neoplasia, and (2) the correlation between p53 and mortalin gene expression.

2. Materials and methods

2.1. Sampling

Approximately 5 cm long specimens of *M. arenaria* were collected at low tide at a depth of 15–20 cm using a hand rake from October to November 2006 in North River (46°15′01″N, 63°10′42″W) (Charlottetown, Prince Edward Island, Canada). Clams were washed with seawater and transported to the Atlantic Veterinary College at the University of Prince Edward Island for further analysis.

2.2. Flow cytometry

Flow cytometric (FCM) analysis was used to assess the ploidy status of *M. arenaria* haemocytes according to the methods described by Delaporte et al. (2008). This technique enables the screening of cell populations for DNA content. The protocol is based on the binding properties of propidium iodide (PI) which stains by intercalating into DNA strands. The PI fluorescence intensity is proportional to the DNA content in the cell. Briefly, hemolymph (500 μL) was withdrawn from individual clams using a 3 mL syringe fitted with a 25-gauge needle. Haemocytes were fixed in 2.5 mL of cold absolute ethanol and stored at −20 °C for at least 30 min. Fixed cells were centrifuged (400*g* for 10 min at room temperature), and the supernatant was discarded. Haemocyte pellets were re-suspended in 0.01 M phosphate-buffered saline (PBS) and the cells were allowed to re-hydrate for 30 min at room temperature. After two washes in PBS (400*g* for 10 min at room temperature), cells were re-suspended in 380 μL of PBS solution and transferred to flow cytometer tubes by filtering through an 80 μm nylon mesh. Propidium iodide (50 μg mL^{-1}) and DNAse-Free RNase A (50 μg mL^{-1}) were added to each tube before incubating the mixtures in the dark until optimal PI staining for 30 min. PI fluorescence, which is related to the DNA content of each cell, was detected on the orange photomultiplicator of a FACSCalibur flow cytometer (BD Biosciences) at a wavelength between 550 and 600 nm. For each sample, 10,000 particles were counted at low flow rate (15 μL min^{-1}). For each cell event, a single pulse of PI fluorescence was represented according to its area and width. The pulse width needs to be compared with the pulse area in order to discriminate the cells in the phase G2/M from doublets of G0/G1 cells represented by the same DNA quantity. To gate single haemocytes, PI fluorescence intensities were plotted as a FL2-area *vs* FL2-width dot-plot (Fig. 1a). The region R1 was drawn in order to discriminate single cells from the doublets (Fig. 1a). The single cells gated in R1 were plotted on a FL2-area histogram and used to estimate the percentage of normal and tetraploid haemocytes in the analyzed cell population (Fig. 1b). Results are presented as the percentage of tetraploid haemocytes per clam. Four different classes of clams were distinguished according to the percentage of tetraploid haemocytes in the circulating system.

2.3. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Analysis of p53, p73 and mortalin gene expression in the haemocytes of *M. arenaria* collected from North River (PEI, Canada) was performed by real-time quantitative PCR by SYBR Green I. Hemolymph (2 mL) was withdrawn from individual clams using a 3 mL syringe fitted with a 25-gauge needle. Hemolymph was centrifuged at 400*g* for 15 min at 4 °C in order to isolate haemocytes from serum. Total RNAs were extracted from haemocytes using TRIzol® Reagent™ (Sigma, USA), treated with DNaseI (Sigma) and quality of RNA was analyzed by denaturing agarose gel electrophoresis. The measurement of RNA concentrations was performed with a Nanodrop (ND 1000, USA) spectrophotometer. First strand synthesis was carried out in a 20 μL reaction mixture containing 1 μg of total RNA and reaction was performed using SuperScript™ III Platinum Two-Step qRT-PCR Kit according to the manufacturer's protocol (Invitrogen, USA). Real-time PCR amplifications were then performed by a RotorGene detection system using 1 μL of cDNA template and SYBR® Green Supermix (total reaction volume 25 μL). Primer concentrations were 0.4 μM for both forward and reverse primers. Oligonucleotide primer sets used for the amplification of the targeted genes are listed in Table 1. Based on the published sequences, the forward and reverse primers were designed in order to obtain a unique amplicon using the Primer 3 software and synthesized by Invitrogen Corp. (Burlington, ON, Canada). Real-time PCR was performed in RotorGene system. The thermocycling parameters consisted of a 10 min denaturation at 95 °C, followed by 40 cycles of 95 °C for 20 s, 20 s at the

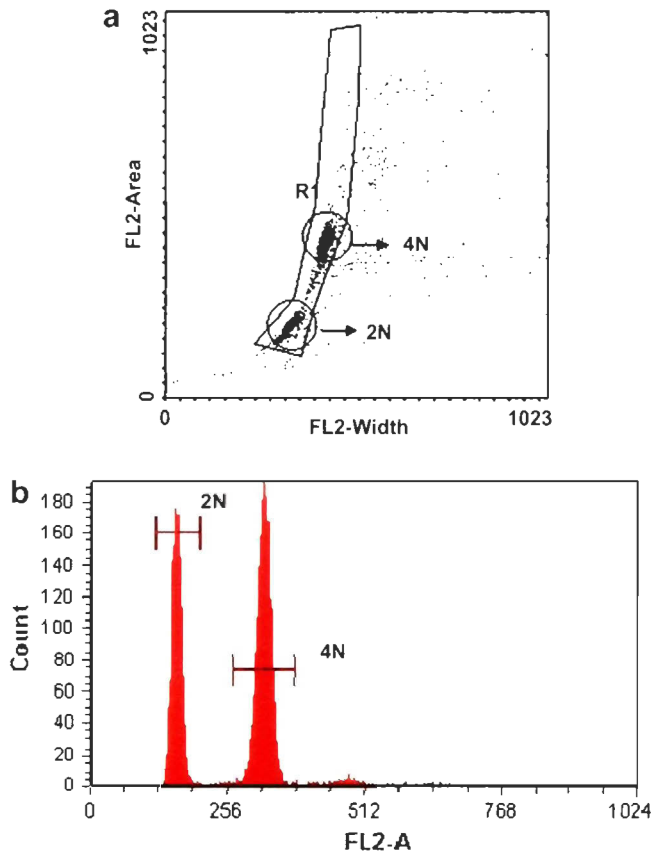


Fig. 1. Flow cytometry analysis of haemocyte cell cycle from a single clam sampled at North River area. (a) Cytogram representing the width and area of PI fluorescence signal. Haemocyte single cells are gated in R1 where diploid (2N chromosomes) and tetraploid (4N chromosomes) haemocytes were identified. (b) Histogram of PI fluorescence intensity signal. Only the PI fluorescence of haemocytes in R1 is plotted. Markers 2N and 4N delimit the diploid and tetraploid peaks, respectively.

Table 1
Sequence size, temperature of melting (T_m) of the various primers used for quantitative real-time RT-PCR

Primer	Sequence (5′–3′)	Size (pb)	T_m
Map53R	TCCCAGTACTTGATTGTCTTT	21	52
Map53F	ACACAAATCGACAGTCAGTGCTCATT	26	53
Map73R	CTTCATTTTCGCGAGGTCAT	20	60
Map73F	GACGAGTTCCGACGTCTCTC	20	60
MortalinR	GCCAGCATCCATTGTTAGGT	20	60
MortalinF	GATACGTTCCTCGGTGGAGA	20	60
18SR	AGACAAATCGCTCCACCAAC	20	60
18SF	AGACTCCGGGAAACCAAAGT	20	60

P53R, p53F are reverse and forward primers, respectively, used for amplification of p53 fragment; p73R, p73F are reverse and forward primers, respectively, used for amplification of p73; MortalinR, MortalinF are reverse and forward primers, respectively, used for amplification of mortalin fragment and 18SR, 18SF are reverse and forward primers, respectively, used for amplification of 18S as housekeeping gene.

melting temperature (TM) for each primer pair (Table 1) and 20 s at 72 °C. Melting curves were also generated (60–95 °C) in order to make sure that a single PCR product was amplified for each set of primers. A standard curve was constructed for each experiment using a 10-fold dilution of the cloned amplicons.

2.4. Data analysis

Data for quantitative real-time RT-PCR were analyzed using the relative expression software tool (qBase version 1.3.4). The basic principle of the model is that a difference (delta) in quantification cycle value between two samples is transformed into relative quantities using the exponential function with the efficiency of the PCR as its base (Hellemans et al., 2007).

The results of p53, p73 and mortalin gene expression levels are presented as means ± error standard (SE) in relation with different group levels 0–5%, 5–15%, 15–50H%, 15–50L% and >50% of tetraploid haemocytes. To assess multiple comparisons, a parametric one-way analysis of variance (ANOVA) was performed. When the distribution was not normal, a Kruskal–Wallis one-way ANOVA on ranks was used.

The software SigmaStat (San Jose, CA, USA) was used to determine the regression analysis, which was carried out to find out the relationship between p53 and mortalin gene expression in clams' haemocytes. A value of $p \leqslant 0.01$ was considered significant.

3. Results and discussion

3.1. Tetraploidy status

Flow cytometric (FCM) analysis was used to diagnose the ploidy status i.e. cell cycle of clams' haemocytes sampled at North River in Prince Edward Island from September to October 2006. This technique enables discrimination between normal diploid (2N) haemocytes (G0/G1 phase) and tetraploid (4N) haemocytes (G2/M). The gate or region R1 of the cytogram containing the majority of cells (Fig. 1a) shows the distribution of single haemocyte cells according to their relative DNA content. Single cells with 2N (G0/G1 phase) and 4N (G2/M) are circled. In order to estimate the percentage of haemocytes in each cell cycle phase accurately, a histogram of PI intensity fluorescence was also drawn on which two different markers were placed (Fig. 1b). This technique enabled us to determine the percentage of tetraploid abnormal haemocytes per clam and then to classify the diagnosed clams in four different categories according to the percentage of tetraploid haemocytes (0–5%, 5–15%, 15–50% and >50% of 4N haemocytes). Our data has shown that 280 out of 366 of total clams sampled (76.5%) exhibited a percentage of haemocytes in phase 4N ranging from 0% to 5% (Fig. 2). For the categories of clams containing 5–15%, 15–50% and more than 50% of tetraploid haemocytes, the percentages were 7.6% (28/366), 10.6% (39/366) and 5.4% (20/366), respectively (Fig. 2).

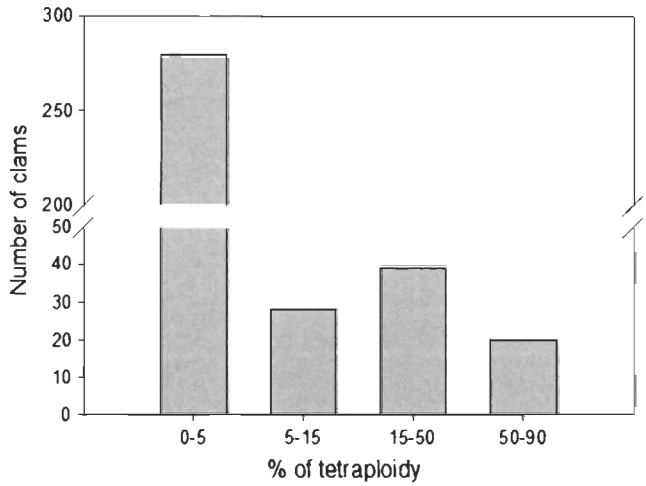


Fig. 2. Bar graph representing the number of clams according to five different categories of haemocyte population containing: 0–5%, 5–15%, 15–50% and more than 50% of tetraploid cells. *X* axis represents the percentage of the five different categories of haemocyte population and *Y* axis is the number of clams.

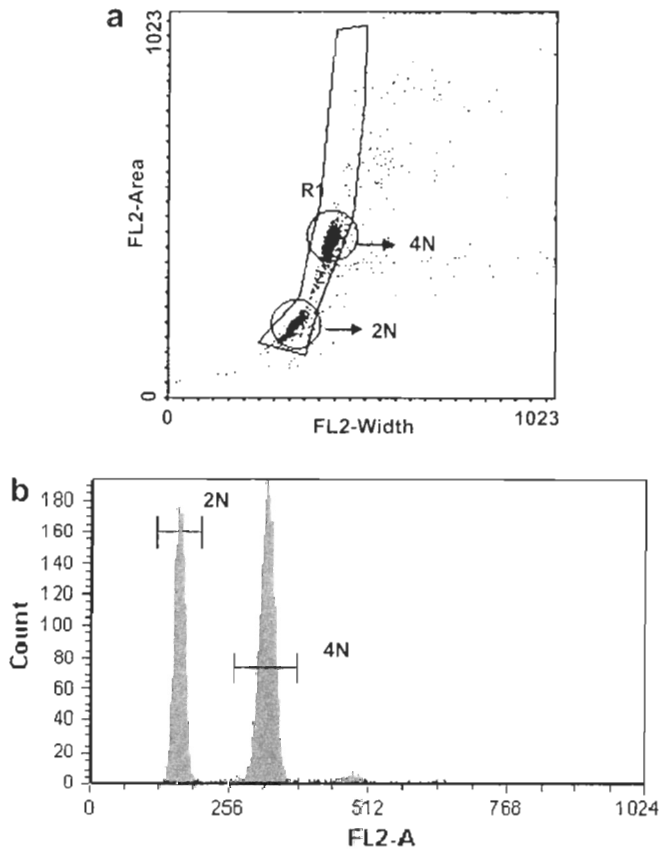


Fig. 1. Flow cytometry analysis of haemocyte cell cycle from a single clam sampled at North River area. (a) Cytogram representing the width and area of PI fluorescence signal. Haemocyte single cells are gated in R1 where diploid (2N chromosomes) and tetraploid (4N chromosomes) haemocytes were identified. (b) Histogram of PI fluorescence intensity signal. Only the PI fluorescence of haemocytes in R1 is plotted. Markers 2N and 4N delimit the diploid and tetraploid peaks, respectively.

Table 1
Sequence size, temperature of melting (T_m) of the various primers used for quantitative real-time RT-PCR

Primer	Sequence (5′–3′)	Size (pb)	T_m
Map53R	TCCCAGTACTTGATTGTCTTT	21	52
Map53F	ACACAAATCGACAGTCAGTGCTCATT	26	53
Map73R	CTTCATTTTCGCGAGGTCAT	20	60
Map73F	GACGAGTTCCGACGTCTCTC	20	60
MortalinR	GCCAGCATCCATTGTTAGGT	20	60
MortalinF	GATACGTTCCTCGGTGGAGA	20	60
18SR	AGACAAATCGCTCCACCAAC	20	60
18SF	AGACTCCGGGAAACCAAAGT	20	60

P53R, p53F are reverse and forward primers, respectively, used for amplification of p53 fragment; p73R, p73F are reverse and forward primers, respectively, used for amplification of p73; MortalinR, MortalinF are reverse and forward primers, respectively, used for amplification of mortalin fragment and 18SR, 18SF are reverse and forward primers, respectively, used for amplification of 18S as housekeeping gene.

melting temperature (TM) for each primer pair (Table 1) and 20 s at 72 °C. Melting curves were also generated (60–95 °C) in order to make sure that a single PCR product was amplified for each set of primers. A standard curve was constructed for each experiment using a 10-fold dilution of the cloned amplicons.

2.4. Data analysis

Data for quantitative real-time RT-PCR were analyzed using the relative expression software tool (qBase version 1.3.4). The basic principle of the model is that a difference (delta) in quantification cycle value between two samples is transformed into relative quantities using the exponential function with the efficiency of the PCR as its base (Hellemans et al., 2007).

The results of p53, p73 and mortalin gene expression levels are presented as means ± error standard (SE) in relation with different group levels 0–5%, 5–15%, 15–50H%, 15–50L% and >50% of tetraploid haemocytes. To assess multiple comparisons, a parametric one-way analysis of variance (ANOVA) was performed. When the distribution was not normal, a Kruskal–Wallis one-way ANOVA on ranks was used.

The software SigmaStat (San Jose, CA, USA) was used to determine the regression analysis, which was carried out to find out the relationship between p53 and mortalin gene expression in clams' haemocytes. A value of $p \leqslant 0.01$ was considered significant.

3. Results and discussion

3.1. Tetraploidy status

Flow cytometric (FCM) analysis was used to diagnose the ploidy status i.e. cell cycle of clams' haemocytes sampled at North River in Prince Edward Island from September to October 2006. This technique enables discrimination between normal diploid (2N) haemocytes (G0/G1 phase) and tetraploid (4N) haemocytes (G2/M). The gate or region R1 of the cytogram containing the majority of cells (Fig. 1a) shows the distribution of single haemocyte cells according to their relative DNA content. Single cells with 2N (G0/G1 phase) and 4N (G2/M) are circled. In order to estimate the percentage of haemocytes in each cell cycle phase accurately, a histogram of PI intensity fluorescence was also drawn on which two different markers were placed (Fig. 1b). This technique enabled us to determine the percentage of tetraploid abnormal haemocytes per clam and then to classify the diagnosed clams in four different categories according to the percentage of tetraploid haemocytes (0–5%, 5–15%, 15–50% and >50% of 4N haemocytes). Our data has shown that 280 out of 366 of total clams sampled (76.5%) exhibited a percentage of haemocytes in phase 4N ranging from 0% to 5% (Fig. 2). For the categories of clams containing 5–15%, 15–50% and more than 50% of tetraploid haemocytes, the percentages were 7.6% (28/366), 10.6% (39/366) and 5.4% (20/366), respectively (Fig. 2).

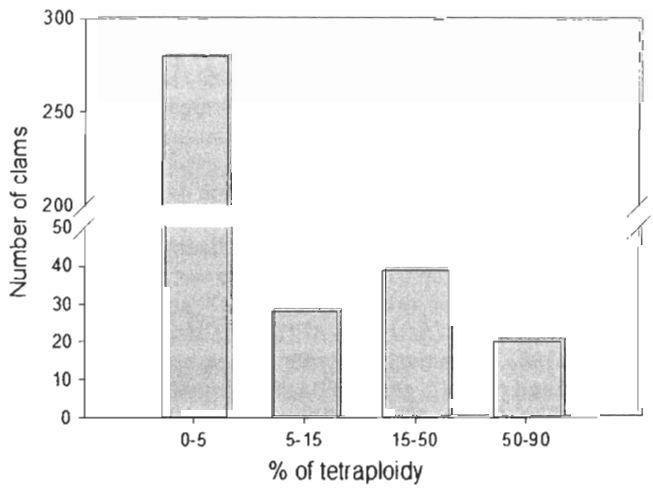


Fig. 2. Bar graph representing the number of clams according to five different categories of haemocyte population containing: 0–5%, 5–15%, 15–50% and more than 50% of tetraploid cells. *X* axis represents the percentage of the five different categories of haemocyte population and *Y* axis is the number of clams.

These results are in accordance with those reported by Delaporte et al. (2008) in clams collected from North River.

A strong body of evidence suggests that FCM constitutes a powerful tool for the analysis of haemocytes' DNA content heterogeneity in a variety of mollusk species. Indeed, studies have been conducted on the American oyster, *Crassostrea virginica* (Ashton-Alcox and Ford, 1998; Allam et al., 2002), the blue mussels *Mytilus edulis* and *Mytilus trossulus* (Elston et al., 1990), the common cockle, *Cerastoderma edule* (Da Silva et al., 2005) and the clams, *Ruditapes philippinarum*, *Mercenaria mercenaria* (Allam et al., 2002), *M. arenaria* (Reno et al., 1994). In order to diagnose haemic neoplasia in clams, Reno et al. (1994) demonstrated using FCM analysis that the proportion of morphologically abnormal neoplastic haemocytes, as determined by phase microscopy, was strongly positively correlated with tetraploid cells. Similar observations had been recorded for blue mussels (Moore et al., 1991) and recently the relation between the percentage of tetraploid cells in the circulating system of clams and haemic neoplasia has been studied (Delaporte et al., 2008). Investigations on haemic neoplasia have suggested that clams or mussels with 10% of tetraploid circulating haemocytes could be considered as diseased (Reno et al., 1994; Moore et al., 1991). More recently, a positive correlation between the percentage of tetraploid cells and haemic neoplasia in clams circulating hemolymph has been reported (Delaporte et al., 2008). Based on the data collected in the present study, this would suggest that approximately 23% of clams sampled from North River in Prince Edward Island are diseased. Our data are fully in accordance with results recorded by Delaporte et al (in press) who observed that 27% of clams sampled from North River in Prince Edward Island had more than 10% of tetraploid haemocytes in their hemolymph. Although tetraploidy represents a valuable indication of the circulating haemocyte physiological status, target gene expression involved in the cell cycle would certainly help in better understanding disease processes involved in clam neoplasia.

3.2. Gene expression pattern

In vertebrates, it is well established that p53 blocks cell cycle progression in polyploid cells (Stukenberg, 2004). Alteration of p53 expression and/or its regulators occurs in 50% of human tumors and p53 mutations occur in the remaining 50% (Vousden and Lu, 2002). P53 and his member p73 were cloned from *M. arenaria* by Kelley et al. (2001). Their nucleic and amino-acid sequences were comparable to those of humans characterized by highly conserved functional regions such as transcriptional activation, DNA binding, MDM2 and tetramerization domains. These features suggest that p53/73's functions could be similar to those of vertebrates (Kelley et al., 2001). In neoplastic clams, p53/73 proteins are absent from the nucleus but sequestered in the cytoplasm (Kelley et al., 2001; Walker et al., 2006). Immunoprecipitation of mortalin and p53 proteins was observed in cytoplasmic compartment of neoplastic clam haemocytes but not in normal cells (Walker et al., 2006). Based on these observations, gene expression of p53/73 and mortalin was evaluated in haemocytes of clams according to their tetraploid status. Primers were designed based on sequences published by Barker et al. (1997) and in the GenBank database for p53, p73 (Accession AF253324) and mortalin (Accession AY326398), respectively. In order to measure gene expression of p53, p73 and mortalin genes in haemocytes with different tetraploid status (0–5%, 5–15%, 15–50% and higher than 50% of tetraploid haemocytes), relative mRNA levels were measured using quantitative real-time RT-PCR. The amplification efficiencies reached 86%, 90% and 94% as measured for p53, p73 and mortalin, respectively, and were 98% for 18S ribosomal RNA, which was used as a reference transcript for normalization. Gene expression of p53, p73 and mortalin pattern was variable in the 15–50% tetraploid group in comparison with the other three groups where very little variation was observed. Thus, in the 15–50% group, we distinguished two categories according to their p53, p73 and mortalin pattern (Fig. 3). In the 15–50% group, interestingly, gene expression of p53, p73 and mortalin was significantly higher ($p \leqslant 0.05$) in some clams (15–50H group containing 38% ($n = 8$) of the total individuals ($n = 21$) of the 15–50% group) compared to other clams from the same group (15–50L ($n = 13$)) (Fig. 3). Furthermore, clams from the 15–50H% group displayed a level of p53 and p73 gene expression significantly higher ($p \leqslant 0.05$) than clams with low levels (0–5%) or high levels (>50%) of tetraploid haemocytes (Fig. 3). For mortalin gene expression pattern, the level recorded in clams from the 15–50H% group is significantly higher ($p \leqslant 0.05$) than the level recorded in clams with high levels (>50%) of tetraploid haemocytes (Fig. 3). In order to determine the relationship between p53 and mortalin gene expression, a significant correlation

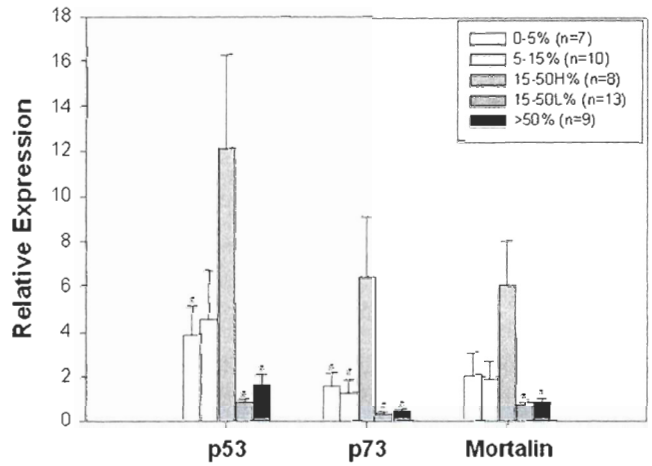


Fig. 3. Quantitative real-time RT-PCR of p53, p73 and mortalin. Relative quantification of p53, p73 and mortalin to 18S ratio in five different groups of haemocyte populations containing: 0–5% ($n = 7$), 5–15% ($n = 10$), 15–50H% ($n = 8$), 15–50L% ($n = 13$) and more than 50% ($n = 9$) of tetraploid cells. Two subgroups represent the group of clams containing haemocytes between 15% and 50% of tetraploid cells: 15–50H% group includes clams with high levels of p53, p73 and mortalin gene expression level and 15–50L% group consists of clams with low levels of p53, p73 and mortalin gene expression. Each value is expressed as the mean ± SE (standard error). An asterisk ( ) indicates that values are statistically different from the 15–50H% groups at the level of $p \leqslant 0.05$.

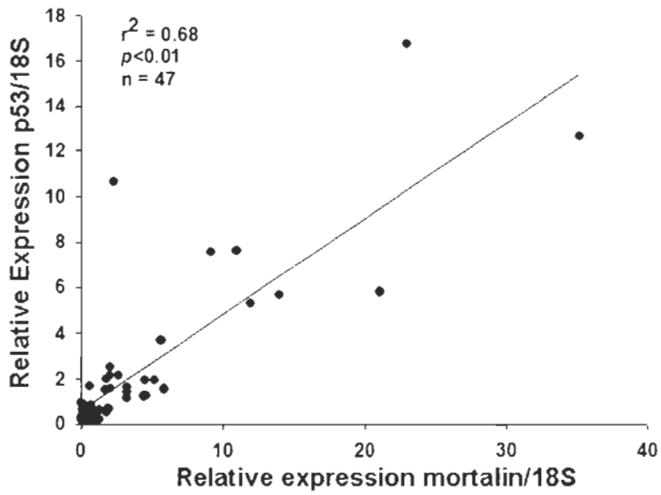


Fig. 4. Correlation between p53 and mortalin gene expression. Curve representing linear correlation between relative quantification of p53 and mortalin to 18S ratio in haemocytes of all clams ($n = 40$) sampled in North River.

($r^2 = 0.68$, $p < 0.01$) was obtained (Fig. 4). Furthermore, mutation of p53 was not observed in clams sampled in the same site (data not shown). These results seem to confirm the hypothesis suggested by Kelley and Walker that sequestration of p53 in the cytoplasm by mortalin could be an explanation for abnormal haemocytes morphology (Kelley et al., 2001; Walker et al., 2006).

4. Conclusion

Our results show that haemocytes of some clams with a moderate percentage (15–50%) of tetraploid cells have a significantly high level of p53 and p73 in comparison with clams belonging to categories with low (<15%) or high levels (>50%) of tetraploid cells, where low levels of expression of these genes were observed. This observation seems to concur with a correlation already established by previous studies in humans, which claim that high levels of these gene expressions might be used as a checkpoint for when haemocytes become tetraploid (for review see Toledo and Wahl, 2006). p53 and mortalin gene expressions are correlated, thus reinforcing the hypothesis that these genes are involved in the development of abnormal haemocytes. In our study, results tend to indicate that the level of expression of selected target genes may be more accurate and specific than the level of ploidy of the haemocytes. Previous studies have shown that p53 and mortalin are involved in the development of abnormal morphological haemocytes displaying rounding, lack of adhesion with high rates of proliferation (Kelley et al., 2001; Walker et al., 2006). It may be speculated that transforming cells are the ones with high expression levels, while transformed cells may not over-express such genes involved in cell transformation (Bode and Dong, 2004). The expression of p53, p73 and mortalin genes could be used as a set of indicators for active transformation of clam haemocytes in the process of haemic neoplasia.

Acknowledgments

The authors express their sincere acknowledgements to Garth Arsenault and Dr. Jeff Davidson for their valuable support to this study. This project was supported by NSERC AquaNet, the Canada Research Chairs (CRC) program, the Atlantic Canada Opportunities Agency (ACOA), and the Canada Foundation for Innovation (CFI).

References

Allam, B., Ashton-Alcox, K.A., Ford, S.E., 2002. Flow cytometric comparison of haemocytes from three species of bivalve mollusks. Fish Shellfish Immunol. 13, 141–158.

Appeldoorn, R., Brown, C.W., Brown, R.S., Chang, P.W., Cooper, K., Lorda, E., Saila, S.B., Walker, H., Wolke, R.E., 1984. Field and laboratory studies to define the occurrence of neoplasia in the soft shell clam, *Mya arenaria*. API Publ. No. 4345, American Petroleum Institute, Washington, DC.

Ashton-Alcox, K.A., Ford, S.E., 1998. Variability in molluscan haemocytes: a flow cytometry study. Tissue Cell 30, 195–204.

Barber, B.J., 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living Resources 17, 449–466.

Barker, C.M., Calvert, R.J., Walker, C.W., Reinisch, C.L., 1997. Detection of mutant p53 in clam leukemia cells. Exp. Cell Res. 232, 240–245.

Beckmann, N., Morse, M.P., Moore, C.M., 1992. Comparative study of phagocytosis in normal and diseased hemocytes of the bivalve mollusc *Mya arenaria*. J. Invertebr. Pathol. 59, 124–132.

Bode, A.M., Dong, Z., 2004. Post-translational modification of p53 in tumorigenesis. Nat. Rev. Cancer 4, 793–805.

Brousseau, D.J., Baglivo, J.A., 1991. Disease progression and mortality in neoplastic *Mya arenaria* in the field. Mar. Biol. 110, 249–252.

Da Silva, P.M., Soudant, P., Carballal, M.J., Lambert, C., Villalba, A., 2005. Flow cytometric DNA content analysis of neoplastic cells in haemolymph of the cockle *Cerastoderma edule*. Dis. Aquat. Org. 67, 133–139.

Delaporte M., Synard S., Pariseau J., McKenna P., Tremblay R., Davidson J. Berthe F.C.J., 2008. Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry. J. Invertebr. Pathol., 98, 190–197.

Elston, R.A., Drum, A.S., Allen, S.K., 1990. Progressive development of circulating polyploid cells in *Mytilus* with haemic neoplasia. Dis. Aquat. Org. 8, 51–59.

Harms, K., Nozell, S., Chen, X., 2004. The common and distinct target genes of the p53 family transcription factors. Cell Mol. Life Sci. 61, 822–842.

Hellemans, J., Mortier, M., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Hofseth, L.J., Hussain, S.P., Harris, C.C., 2004. p53: 25 years after its discovery. Trends Pharmacol. Sci. 25, 177–181.

House, M.L., Kim, C.H., Reno, P.W., 1998. Soft shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. Dis. Aquat. Org. 34, 187–192.

Kelley, M.L., Winge, P., Heaney, J.D., Stephens, R.E., Farell, J.H., Van Beneden, R.J., Reinisch, C.L., Lesser, M.P., Walker, C.W., 2001. Expression of homologues for p53 and p73 in the softshell clam (*Mya arenaria*), a naturally-occurring model for human cancer. Oncogene 20, 748–758.

Lane, D.P., 1992. Cancer: p53, guardian of the genome. Nature 358, 15–16.

McGladdery, S.E., Reinisch, C.L., MacCallum, G.S., Stephens, R.E., Walker, C.L., Davidson, J.T., 2001. Haemic neoplasia in soft-shell clams (*Mya arenaria*): recent outbreaks in Atlantic Canada and discovery of a p53 gene homologue associated with the condition. Can. Bull. Aquat. Assoc. 101, 19–26.

Moore, J.D., Elston, R.A., Drum, A.S., Wilkinson, M.T., 1991. Alternate pathogenesis of systemic neoplasia in the bivalve mollusc Mytilus. J. Invertebr. Pathol. 58, 231–243.

Moore, C.A., Beckmann, N., Morse, M.P., 1992. Cytoskeletal structure of diseased and normal hemocytes of *Mya arenaria*. J. Invertebr. Pathol. 38, 141–147.

Oprandy, J.J., 1983. 5-Bromodeoxyuridine induction of hematopoietic neoplasia and retrovirus activation in the soft-shell clam, *Mya arenaria*. J. Invertebr. Pathol. 42, 196–206.

Reno, P.W., House, M., Illingworth, A., 1994. Flow cytometry and chromosome analysis of Softshell clams, *Mya arenaria*, with disseminated neoplasia. J. Invertebr. Pathol. 64, 163–172.

Rieder, C.L., Maiato, H., 2004. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. Dev. Cell 7, 637–651.

Shi, Q., King, R.W., 2005. Chromosome nondisjunction yields tetraploid rather than aneuploid cells in human cell lines. Nature 437, 1038–1042.

Stukenberg, P.T., 2004. Triggering p53 after cytokinesis failure. J. Cell Biol. 165, 607–608.

Stephens, R.E., Walker, C.W., Reinisch, C.L., 2001. Multiple protein differences distinguish clam leukemia cells from normal hemocytes: evidence for the involvement of p53 homologues. Comp. Biochem. Physiol. C 129, 329–338.

Toledo, F., Wahl, G.M., 2006. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat. Rev. Cancer 6, 909–923.

Vousden, K.H., Lu, X., 2002. Live or let die: the cell's response to p53. Nat. Rev. Cancer 2, 594–604.

Wadhwa, R., Taira, K., Kaul, S.C., 2002. Mortalin: a potential candidate for biotechnology and biomedicine. Histol. Histopathol. 17, 1173–1177.

Wadhwa, R., Takano, S., Kaur, K., Deocaris, C.C., Pereira-Smith, O.M., Reddel, R.R., Kaul, S.C., 2006. Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. Int. J. Cancer 118, 2973–2980.

Walker, C.W., Bottger, S., Low, B., 2006. Mortalin-based cytoplasmic sequestration of p53 in a non mammalian cancer model. Am. J. Pathol. 168, 1526–1530.