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INTRODUCTION GÉNÉRALE

Quelques défis en aquaculture

Face au déclin des principaux stocks mondiaux de poissons marins (*e.g.* anchois du Pérou *Engraulis ringens*, lieu de l'Alaska *Theragra chalcogramma*, merlan bleu *Micromesistius poutassou*, hareng de l'Atlantique *Clupea harengus*, anchois japonais *Engraulis japonicus*), l'aquaculture devra être intensifiée afin de satisfaire la demande alimentaire d'une population humaine sans cesse croissante (6,7 milliards en 2007). Près d'un quart des stocks de ressources marines dont la FAO assure le suivi depuis 1974 serait surexploité, épuisé ou en cours de relèvement après épuisement. La moitié environ (52%) serait pleinement exploitée et le quart restant serait sous-exploité ou modérément exploité. En 2004, l'aquaculture contribuait à hauteur de 43% à la production mondiale de poisson destinée à la consommation humaine (106 millions de tonnes). L'aquaculture mondiale connaît le plus fort taux de croissance annuel (8,8%) entre 1970 et 2004, par comparaison aux captures des pêcheries (1,2%) et aux systèmes terrestres de production animale (2,8%) (FAO, 2006). Entre 2000 et 2004, 25% de la production halieutique mondiale était destinée à l'industrie minotière, c'est-à-dire la fabrication de farine (source de protéine) et d'huile (source de lipide) à partir des poissons fourrages (anchois, capelan, hareng, maquereau, menhaden, sardine, etc.) capturés. Cette farine et cette huile entrent dans la composition des aliments des élevages aquacoles, qui se retrouvent en compétition avec les autres systèmes de production animale (poulet, porc). L'approvisionnement en huile de poissons

sauvages pour l'industrie minotière est limité, si l'on tient compte à la fois du plafonnement de la production mondiale et du fort taux de croissance de l'industrie aquacole en réponse à la pression démographique. Par conséquent, le développement économique de l'aquaculture ne pourra pas compter sur cette ressource d'huile et de farine limitée et onéreuse, il est donc urgent de trouver des alternatives durables et écologiques.

La lutte contre les pathogènes

Dans ce contexte d'accroissement de productivité, l'industrie piscicole marine mondiale est de plus en plus confrontée à des problèmes de fortes mortalités se manifestant au stade larvaire (Skjermo & Vadstein, 1999 ; Diggles *et al.*, 2000 ; Villamil *et al.*, 2003), avec les conséquences économiques défavorables qui en découlent. La plupart des installations d'élevage sont des systèmes intensifs stressants pour les poissons et l'apport important en matière organique contribue à l'affaiblissement de l'état immunitaire des poissons et à l'augmentation des fréquences des maladies dues à des agents pathogènes, tels que des virus, des bactéries ou des parasites proto- et métazoaires (Vadstein *et al.*, 2004 ; Nakase *et al.*, 2007). Différentes solutions visant à inhiber le plus tôt possible l'apparition des bactéries pathogènes chez les larves sont envisagées. L'usage des antibiotiques s'est largement répandu à cet effet, entraînant la sélection de bactéries de plus en plus résistantes, voire multirésistantes et donc plus difficiles à contrôler (Miranda & Zemelman, 2002). Cette résistance bactérienne aux antibiotiques peut être transférée horizontalement vers d'autres espèces de bactéries par l'intermédiaire d'éléments génétiques mobiles, tels que les plasmides et les bactériophages (van Elsas & Bailey,

2002). La vaccination par injection (opération laborieuse, coûteuse et stressante pour les animaux), n'est pas envisageable lorsque les animaux sont de petite taille (Vandenberg, 2004). Il existe alors la vaccination orale, qui malheureusement n'atteint pas souvent le degré d'efficacité d'une vaccination directe (Kondo *et al.*, 2003). De plus, la vaccination orale implique une exposition de l'organisme à des doses importantes d'antigènes (produit coûteux) pour qu'un grand nombre d'entre eux parviennent intacts dans l'intestin après la traversée de la barrière gastrique (Irie *et al.*, 2003 ; Vandenberg, 2004). Cependant, la vaccination ne doit pas être réalisée trop tôt au cours du développement larvaire, lors de la phase de reconnaissance entre le soi et le non-soi par les cellules immunitaires. D'où l'intérêt d'étudier des méthodes alternatives de lutte contre les pathogènes. Des recherches actives sont menées en ce sens autour de plusieurs thèmes, tels que la maturation microbienne de l'eau d'élevage (Skjermo & Vadstein, 1999), les probiotiques (Vine *et al.*, 2006), les peptides antimicrobiens (Patrzykat & Douglas, 2005) et la nutrition (Landolt, 1989), notamment en acides gras polyinsaturés.

Les bactéries opportunistes

L'eau de mer est à la fois un milieu de transport et un milieu de croissance des microorganismes. Les poissons marins partagent donc leur écosystème avec des bactéries potentiellement pathogènes. Or, la composition bactérienne du milieu d'élevage est un facteur important affectant la production d'une écloserie (Eddy & Jones, 2002 ; Hansen & Olafsen, 1999). Un poisson en pisciculture côtoie un milieu bactérien typique d'environ 10^6 bactéries ml^{-1}, dont seulement 0,1 à 1% peuvent être mises en culture sur du milieu non

sélectif (Vadstein *et al.*, 2004). Chez le turbot (*Scophthalmus maximus* L.), la concentration bactérienne dans l'eau d'élevage peut atteindre 10^6-10^7 cellules ml^{-1} après introduction de microalgues et de nourriture (Salvesen *et al.*, 1999). Dans les bassins d'élevage utilisés en production intensive, il peut se produire d'une part une diminution de la qualité de l'eau et, d'autre part, une accumulation de matière organique. L'abondance de substrat est alors propice à la prolifération de bactéries à croissance rapide (stratégie « k »), dites opportunistes (Hansen & Olafsen, 1999 ; Skjermo & Vadstein, 1999). La majorité des bactéries causant des maladies chez les poissons marins sont justement des pathogènes opportunistes présents en grand nombre (Vadstein *et al.*, 2004). Ces bactéries sont présentes normalement dans la microflore de la colonne d'eau, les sédiments (Hansen & Olafsen, 1999) et la microflore indigène des invertébrés participant à l'écosystème larvaire (*e.g.* proies vivantes). Ces pathogènes facultatifs sont capables de tirer avantage d'une faiblesse du poisson qui se développe lorsque les conditions environnementales leur sont favorables, mais défavorables au poisson (Vadstein *et al.*, 2004 ; Olafsen, 2001). La colonisation par ces bactéries opportunistes ou leur ingestion par les larves de poisson peut mener à une faible survie et une faible performance (Muroga, 1987 ; Nicolas, 1989 ; Munro *et al.*, 1994). Certaines bactéries peuvent produire des toxines ou des odeurs répulsives, tel qu'observé par Nicolas (1989) dans un élevage de turbot et entrainer une faible croissance.

Les vecteurs d'infection

Durant la phase d'éclosion, l'épiflore des œufs et la flore bactérienne de l'eau influencent significativement la microflore intestinale des larves en alimentation endogène (présence du sac vitellin) (Campbell & Buswell, 1983 ; Munro *et al.*, 1994 ; Ringø *et al.*, 1996 ; Ringø & Birkbeck, 1999). Cela a été montré chez les poissons plats tels que le turbot (*Scophthalmus maximus* : Nicolas, 1989) et le cardeau d'été (*Paralichthys dentatus* : Eddy & Jones, 2002) ainsi que chez de nombreuses autres espèces (cf. revue de Ringø & Birkbeck, 1999). À la suite de la transition vers l'alimentation exogène (résorption du sac vitellin), ce sont les bactéries associées aux proies vivantes qui vont influencer significativement la composition bactérienne de l'eau d'élevage larvaire, ainsi que celle des larves elles-mêmes (Reitan *et al.*, 1998). L'élevage de larves de poisson marin aux premiers stades implique bien souvent l'arrêt fréquent ou le faible renouvellement d'eau à l'intérieur des bassins, une condition propice à la prolifération des bactéries (Skjermo & Vadstein, 1999). De plus, dans un élevage de proies vivantes, l'apport élevé en substrat et le taux de broutage important entrainent la sélection d'espèces bactériennes à croissance rapide potentiellement pathogènes (Salvesen *et al.*, 1999). En général, on observe une succession de phénotypes bactériens dans l'eau et l'intestin des larves, liée aux types de proies vivantes utilisées (Eddy & Jones, 2002), jusqu'à la stabilisation de la microflore pendant et après la métamorphose (Ringø & Birkbeck, 1999 ; Eddy & Jones, 2002).

Mécanisme d'infection par un pathogène

Pour infecter un poisson, un agent pathogène doit tout d'abord établir un contact physique avec l'hôte et être capable de coloniser ses cellules épithéliales ou son mucus (Figure 1) (Hansen & Olafsen, 1999). Contrairement au poisson adulte, la larve ne possède pas une épaisse enveloppe de mucus, ni même d'écailles et de peau bien développées. Or, ces attributs constituent des barrières physiques externes très efficaces contre l'invasion d'agents pathogènes (Shoemaker *et al.*, 2001). Le mucus agit doublement contre l'invasion de pathogènes, d'une part parce qu'il est produit et éliminé constamment au niveau de la peau, des branchies et de la muqueuse du tractus intestinal et d'autre part, parce qu'il contient des composés antimicrobiens (*e.g.*, lysozyme, peroxydase, catalase, protéase, pleurocidine, etc.) (Alexander & Ingram, 1992 ; Hansen & Olafsen, 1999 ; Ringø & Birkbeck, 1999 ; Douglas *et al.*, 2003 ; Ringø *et al.*, 2007). Chez des ombles chevaliers (*Salvelinus alpinus*) exposés à la bactérie pathogène *Aeromonas salmonicida*, Lødemel *et al.* (2001) ont observé une expulsion de mucus infecté grâce à une augmentation de production de mucus par les cellules caliciformes intestinales. Les branchies et les surfaces intestinales sont des sites importants de fixation et de colonisation des pathogènes, mais l'entrée par la peau est également possible (Hansen & Olafsen, 1999 ; Vadstein, 1997). Ainsi, l'activité de phagocytose chez les poissons est principalement associée aux branchies, à l'intestin et à la peau (Vadstein *et al.*, 2004). Chez le poisson adulte, une bactérie doit tout d'abord passer par l'estomac avant d'entrer dans l'intestin. La sécrétion de substances létales et le faible pH dans cet organe constituent des barrières importantes contre l'invasion des bactéries utilisant cette voie d'infection. Ce n'est pas le cas de la

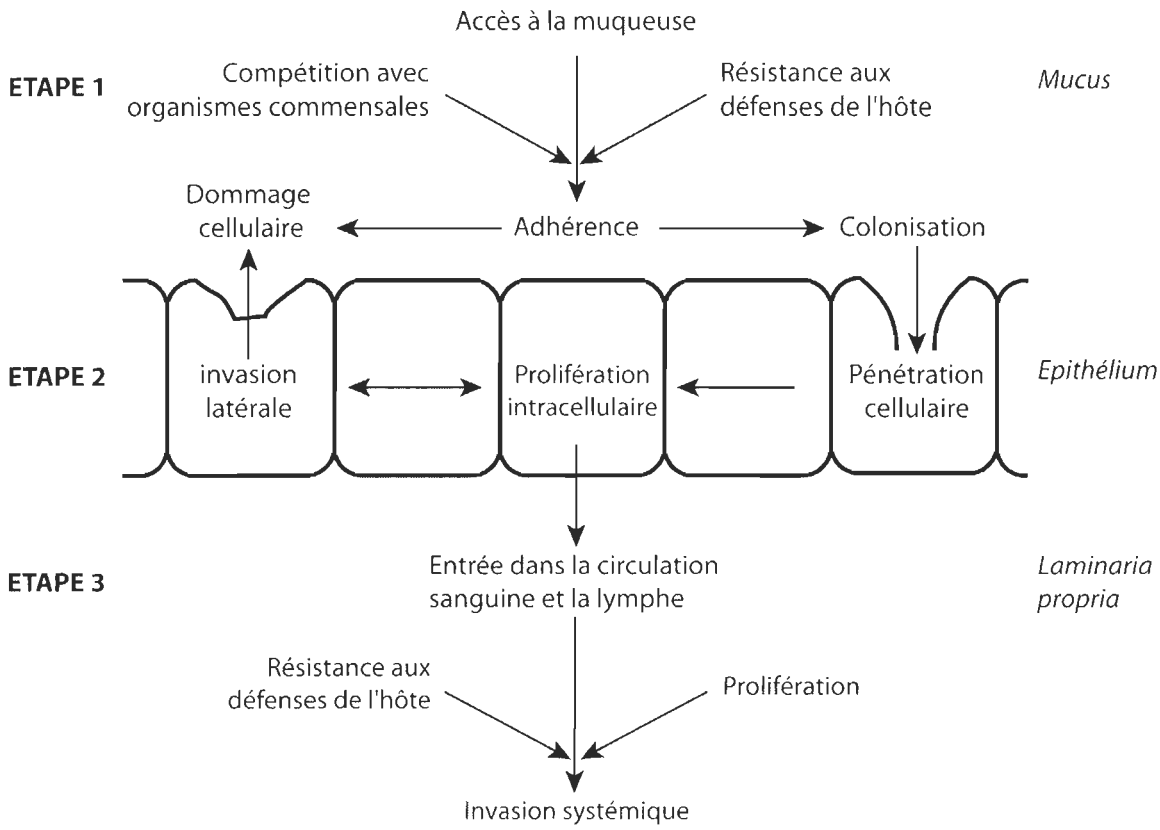

Figure 1 Les trois étapes d'infection par des pathogènes chez le poisson. Modifié de Vadstein *et al.* (2004).

larve dont l'estomac est souvent peu développé (Govoni *et al.*, 1986), d'où un accès plus facile au tissu intestinal par les bactéries potentiellement pathogènes (ligands membranaires des bactéries peu affectés par les enzymes).

Rappel : système immunitaire du poisson

Aux premiers stades du développement, les larves disposeraient d'un système immunitaire spécifique peu développé, caractérisé par une faible différentiation des organes immunocompétents (thymus, rein, rate et foie). Les larves de certaines espèces sont immunisées passivement par le transfert des anticorps maternels, comme chez le tilapia (Sin et *al.*, 1994) et la carpe labéo roho (*labeo rohita*, Ham) (Swain *et al.*, 2006). En dépit de l'acquisition importante de connaissances sur le système immunitaire des poissons adultes, l'ontogénie et le fonctionnement du système immunitaire chez les larves sont relativement peu connus (Ellis, 1999 ; Dalmo *et al.*, 2000). Cependant, ce dernier présente des similitudes avec celui des mammifères. Une partie du système est spécifique et l'autre non spécifique avec une voie humorale et une voie cellulaire (Figure 2). De manière générale, le système immunitaire nonspécifique serait le plus développé chez les larves (Landolt, 1989 ; Ellis, 1999). Les cellules immunitaires nonspécifiques sont représentées par les granulocytes, les macrophages et les monocytes, ainsi que les cellules cytotoxiques nonspécifiques. Les granulocytes (neutrophiles, éosinophiles, basophiles) sont des cellules polymorphonucléaires très mobiles qui contiennent des granules cytoplasmiques constituées de substances (myeloperoxidase, lysosymes) capables de lyser directement des organismes pathogènes. Ces leucocytes voyagent dans le sang et dans le système

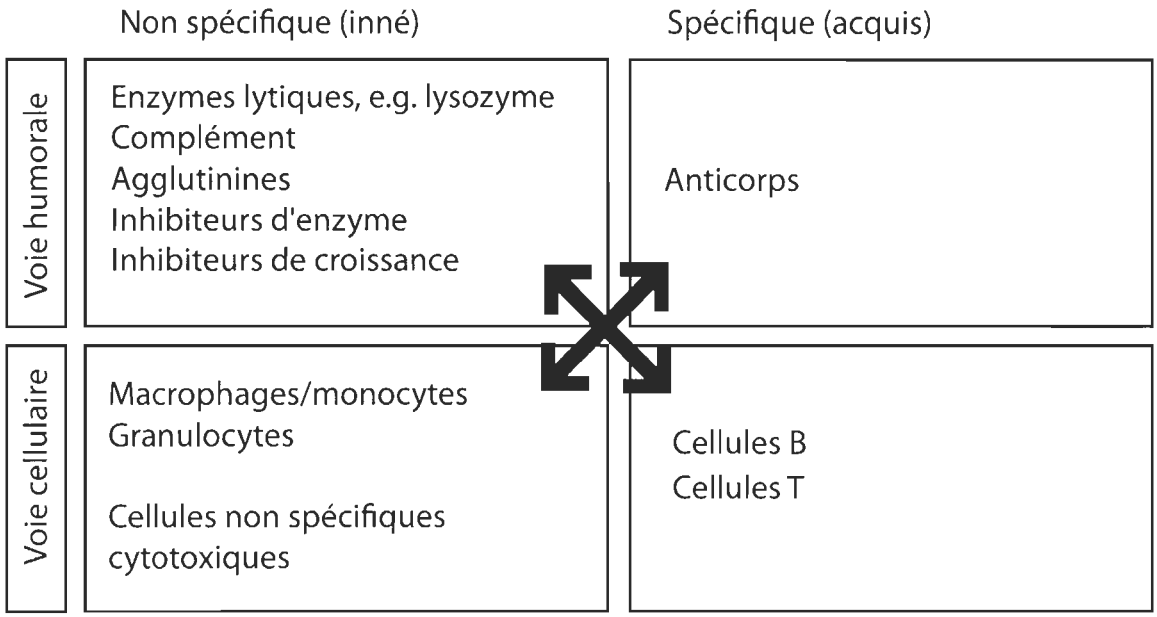

Figure 2 Schéma simplifié des principales composantes du système immunitaire spécifique et nonspécifique du poisson. Modifié de Vadstein *et al.* (2004).

lymphatique jusqu'au site d'infection et jouent un rôle important dans l'inflammation. Les monocytes sont des macrophages immatures qui sont mobiles à travers le système circulatoire et se différencient en macrophages dans les tissus. Les macrophages sont des cellules à forte activité antipathogène (espèce réactives de l'oxygène, oxide nitrique, etc.) et jouent un rôle clef comme cellule présentatrice d'antigène (ou CPA) et comme productrice de cytokine, assurant ainsi la transition entre l'immunité nonspécifique et spécifique. La cytokine est une substance chimiotactique qui permet de guider les autres cellules de défense vers le site d'infection. Elle est aussi responsable de la réaction d'inflammation. La vasodilatation induite permet un afflux sanguin important et accélère du même coup l'afflux des leucocytes. Les cellules cytotoxiques non spécifiques sont similaires aux « cellules tueuses » naturelles chez les mammifères et sont capables de lyser les protozoaires parasites (Shoemaker *et al.*, 2001). Elles se retrouvent dans le sang, les tissus lymphoïdes et l'intestin des poissons.

Les cellules du système immunitaire spécifique sont les lymphocytes B et T. Les lymphocytes B reconnaissent l'antigène et produisent l'anticorps spécifique de cet antigène. Les lymphocytes T reconnaissent l'antigène présenté par les cellules présentatrices d'antigènes et procèdent à la lyse du pathogène reconnu.

Les principales voies d'infection des bactéries

Les branchies, constamment en contact avec l'eau et fortement irriguées par le sang, constituent une voie d'entrée et un habitat naturel des bactéries chez le poisson. Afin d'assurer le processus d'osmorégulation, les larves de poissons marins doivent absorber de l'eau par leurs pseudobranchies (Mangor-Jensen & Adoff, 1987 ; Tytler, 1988 ; Reitan *et al.*, 1998). L'eau ainsi transportée apporte avec elle des bactéries qui s'installent dans le tractus digestif avant le début de l'alimentation active. C'est de cette manière que les larves de morue nouvellement écloses ingèrent des quantités substantielles de bactéries pionnières (Olafsen, 1984 ; Olafsen & Hansen, 1992). Notons également que la flore microbienne des œufs peut affecter l'établissement des espèces microbiennes pionnières chez les larves (Hansen & Olafsen, 1999). C'est le cas dans les incubateurs à œufs où les larves sont en présence d'œufs en éclosion, de débris d'œufs et de substances inorganiques et organiques constituant un substrat propice au développement bactérien.

L'intestin constitue également un habitat naturel pour les bactéries dont certaines sont utiles et nécessaires à la survie du poisson, à l'instar de celles retrouvées chez des amphibiens, des reptiles, des oiseaux et des mammifères herbivores. Ces bactéries peuvent participer à la digestion, comme observé récemment chez des espèces de poissons herbivores de Nouvelles Zélande (Clements *et al.*, 2007 ; Fidopiastis *et al.*, 2006), où ces bactéries agissent comme de véritable symbiontes en convertissant les hydrates de carbone réfractaires d'origine algale en métabolites utilisables par l'hôte, notamment en acides gras à courtes chaînes (Moran *et al.*, 2005). De plus, la flore intestinale peut être une source de

vitamines (métabolites secondaires), *e.g.* la vitamine B12 chez la carpe (*Cyprinus carpio*) et le tilapia (*Tilapia nilotica*) (Sugita *et al.*, 1991a), ou encore la truite arc-en-ciel (*Oncorynchus mykiss*) (Sugita *et al.*, 1991b). Certaines bactéries marines peuvent également produire des acides gras essentiels dont l'acide docosahexaénoïque (DHA) et l'acide eicosapentaénoique (EPA) comme chez le chinchard du Japon (*Trachurus japonicus*) et le hareng du pacifique (*Scomber japonicus*) (Masuda, 2003). Ces bactéries jouent également un rôle très important dans la résistance à la colonisation par leur association étroite avec l'épithélium instestinal. En occupant leurs sites de colonisation, elles forment une barrière protectrice contre l'interaction et la colonisation directe des bactéries pathogènes sur la muqueuse (Ringø & Gatesoupe, 1998 ; Sugita *et al.*, 2002). Ces bactéries autochtones inhibent la croissance des bactéries allochtones en synthétisant des facteurs inhibiteurs (Ringø, 2008) ou en compétitionnant pour le substrat et les éléments essentiels (*e.g.* fer pour certaines espèces de *Vibrio*) (Vine *et al.*, 2006).

Un mécanisme de défense non-immunitaire peut être présent au niveau de l'intestin. En effet, des bactéries ou des antigènes intacts peuvent être capturés par endocytose dans l'épithélium intestinal grâce aux entérocytes. Cela a été observé chez des larves du flétan Atlantique (*Hippoglossus hippoglossus*) au stade du sac vitellin (Dalmo *et al.*, 2000), chez de très jeunes larves de morue (*Gadus morhua* : Olafsen & Hansen, 1992), de même que chez le loup tacheté (*Anarhichas minor* O. : Ringø *et al.*, 2006) et chez beaucoup d'autres espèces de poissons (Ringø *et al.*, 2003). Il pourrait y avoir induction d'une réaction immunitaire par ce biais, *via* la reconnaissance par les CPA. Cependant, nous ignorons

encore si cette reconnaissance par les CPA aboutit à une tolérance par l'hôte ou encore à un déclenchement des défenses immunitaires (Olafsen, 2001). Chez le poisson adulte, une bactérie doit tout d'abord passer par l'estomac avant de pénétrer dans l'intestin. La sécrétion de substances létales et le faible pH dans cet organe constituent des barrières importantes contre l'invasion des bactéries utilisant cette voie d'infection. Ce n'est pas le cas de la larve dont l'estomac est peu développé aux premiers stades du développement, d'où un accès plus facile au tissu intestinal par les bactéries potentiellement pathogènes.

La nutrition et l'immunité

La nutrition est connue depuis longtemps comme étant un des facteurs importants agissant sur la santé et les défenses immunitaires chez les mammifères (Hwang, 1989 ; De Pablo & Cienfuegos, 2000). De nombreuses études ont montré une relation similaire, chez les poisssons (Landolt, 1989 ; Blazer, 1992 ; Lygren & Waagbø, 1999 ; Lin & Shiau, 2003). Plusieurs facteurs nutritionnels influencent indirectement le système immunitaire, tels que les acides gras, les minéraux, les protéines et les vitamines (Landolt, 1989 ; Blazer, 1992). Un excès ou une carence de certains éléments nutritionnels peuvent affecter les réactions immunitaires et donc la résistance aux infections (Waagbø, 1994 ; Wu *et al.*, 2003). De nombreuses recherches ont été menées sur l'apport nutritionnel en lipides et notamment en acides gras polyinsaturés (AGPI), en mettant en évidence l'importance des proportions entre les différents acides gras essentiels et l'équilibre n-3/n-6 (Sargent *et al.*, 1997 chez le poisson ; Calder, 2001 ; Harbige, 2003 chez les mammifères).

La plupart des larves de poissons marins ont un système digestif moins développé que les adultes, d'où une efficacité digestive plus faible. Des expériences d'enrichissement des aliments en phospholipides ont montré que ces derniers améliorent la croissance et le développement des larves (Kanazawa *et al.*, 1985 ; Geurden *et al.*, 1995, Cahu *et al.*, 2003; Kanazawa, 2003). L'efficacité des phospholipides semble diminuer avec l'âge et reflèterait la nature primitive du système digestif des larves de poisson marin. Cette immaturité se caractérise par un tractus digestif faiblement fonctionnel, incluant les sécrétions d'enzymes digestives, jusqu'à ce que la métamorphose soit complète (Gatesoupe *et al.*, 2001 ; Murray *et al.*, 2003a). Les phospholipides apportés par l'alimentation peuvent améliorer la prise de nourriture (Koven *et al.*, 1998), agissant comme émulsifiant dans l'intestin (Koven *et al.*, 1993). Néanmoins, chez les larves, les enzymes digestives sont présentes dès le début de l'ouverture de la bouche. Ces enzymes sont impliquées dans la digestion de protéines et d'hydrates de carbone (lipase/estérase) et ont été détectées dans les larves d'espèces de poissons marins tel que le turbot (*Scophthalmus maximus* : Cousin *et al.*, 1987), la daurade royale (*Sparus auratus* : Izquierdo *et al.*, 2000) et la plie rouge (*Pseudopleuronectes americanus* : Murray *et al.*, 2003a ; Mercier *et al.*, 2004). De plus, au début de l'alimentation exogène, les larves de plusieurs espèces semblent être capables d'absorber les lipides par l'épithélium intestinal (Izquierdo *et al.*, 2000).

Les lipides chez le poisson

Les lipides (du grec *lipos*, graisse) et particulièrement les acides gras qui les composent sont la principale source d'énergie métabolique utilisée pour la croissance et la reproduction des poissons, particulièrement les espèces marines. Chez certaines espèces, la teneur en lipides peut atteindre plus de 20% de leur poids secs, *e.g.* le capelan et le hareng (Sargent *et al.*, 2002). Chez les larves de plie rouge, la teneur en lipides par larve est comprise entre 13 et 33% et fournit deux tiers de l'énergie consommée de l'éclosion jusqu'à l'initiation de l'alimentation exogène (Cetta & Capuzzo, 1982). Les lipides sont des composés insolubles ou peu solubles dans l'eau, mais très solubles dans des solvants organiques non polaires (hexane, chloroforme, etc.) Ils sont classés selon leur polarité : les apolaires ou neutres (triglycérides, TAG ; ester de stérol, ES) et les bipolaires ou polaires pourvus d'une tête polaire fixée sur un squelette carboné hydrophobe (phospholipides, PL). Les triglycérides possèdent un squelette de glycérol sur lequel 2 ou 3 acides gras sont estérifiés (réaction entre un groupement alcool (OH) et un groupement carboxylique (COOH)). Les triglycérides sont des molécules de stockage d'énergie importantes (38 kJ g^{-1} alors que le rendement est de 7 kJ g^{-1} pour les glucides et les protéines), tandis que les phospholipides et les stérols sont des molécules structurales des membranes cellulaires.

Les acides gras sont composés d'un groupement carboxyle (hydrophile) et d'une longue chaine aliphatique (hydrophobe) d'atomes de carbone (C), le plus souvent en nombres pairs, se terminant par un groupement méthyle (CH_3). Les atomes d'hydrogène (H) saturent les liaisons autour des atomes de carbone. Les atomes d'oxygène sont situés à

l'extrémité de la chaîne polaire. Les acides gras se caractérisent par le nombre d'atomes de carbone (C), le nombre (X) et la position (Y) des doubles liaisons (degré d'insaturation) ainsi que par leur géométrie dans l'espace (conformation *cis* ou *trans*). Dans notre étude, les acides gras seront identifiés selon la formulation C:Xn-Y. Le nombre de doubles liaisons (insaturation) d'un acide gras peut atteindre 6, toutes de configuration cis. Cette dernière signifie que la molécule d'acide gras présente une angulation de 30° à chaque double liaison rencontrée. Les propriétés des acides gras sont conditionnées par cette angulation au niveau des doubles liaisons (C=C) qui permet d'obtenir la fluidité membranaire, car les phospholipides sont alors moins compacts. Seuls les acides gras insaturés cis-cis ont une activité biologique alors que les cis-trans ou les trans-trans sont inactifs.

Les acides gras essentiels chez le poisson

Il existe une catégorie d'acides gras polyinsaturés qui ne peuvent pas être synthétisés *de novo* par les poissons marins. Ces acides gras essentiels (AGE) sont répartis en deux familles selon leur précurseurs à plus courte chaine : l'acide linolénique (18:3n-3) et l'acide linoléique (18:2n-6). Les AGE de série n-3 sont l'acide eicosapentaénoïque (20:5n-3, EPA) et l'acide docosahexaénoïque (22:6n-3, DHA). L'acide gras essentiel de série n-6 est l'acide arachidonique (20:4n-6, AA). Ces AGE sont nécessaires pour les processus suivants : la croissance ou la formation *de novo* de la biomasse (la formation de tissus et la différenciation) ; l'activité des membranes et leur métabolisme (transport membranaire, fluidité et modulation des récepteurs membranaires) ; la régulation du

métabolisme et de l'immunité (par les eicosanoïdes) (Watanabe, 1982 ; Sargent *et al.*, 2002 ; Lin & Shiau, 2003 ; Olsen *et al.*, 2004).

Les besoins importants en acides gras hautement insaturés (AGPI de 20 atomes de carbone et plus) sont liés à la synthèse des phospholipides et la formation des membranes cellulaires. Les phospholipides sont synthétisés par des systèmes enzymatiques ayant une plus forte affinité avec les AGPI que pour les autres acides gras. Lors d'une déficience alimentaire en acides gras hautement insaturés n-3, les enzymes peuvent toutefois incorporer des acides gras saturés ou monoinsaturés dans les phospholipides membranaires. Il y a alors diminution de la quantité d'acides gras hautement insaturés de série n-3 dans les membranes, ce qui selon l'espèce peut entraîner progressivement une diminution des capacités physiologiques et de la santé générale de l'organisme (Sargent *et al.*, 2002). Les lipides sont par conséquent une composante importante de l'alimentation, à la fois comme source d'énergie et comme source d'acides gras essentiels pour la structure et la fonction tissulaire.

Tous les poissons marins étudiés jusqu'à maintenant sont incapables d'allonger ou de désaturer efficacement l'acide linolénique en EPA et en DHA (Sargent *et al.*, 1999a). Cette insuffisance métabolique serait due à une déficience de l'une des deux enzymes impliquées dans la voie de conversion de l'acide α-linolénique en EPA, *i.e.* le complexe multienzymatique élongase C_{18} en C_{20} (Tocher *et al.*, 1989) ou le Δ5 acide gras désaturase (Tocher & Ghioni, 1999 ; Tocher *et al.*, 2003) (Figure 3). En milieu naturel marin, le DHA,

l'EPA et l'AA ainsi que leurs précurseurs à plus courtes chaînes sont principalement produits par le phytoplancton, ainsi que par les bactéries, mais en moindre quantité. La plupart des copépodes et les autres organismes du zooplancton satisfont leurs besoins en AGE grâce à ce premier maillon trophique. Les organismes des niveaux trophiques supérieurs comblent ces mêmes besoins en s'alimentant sur ces proies. L'abondance des AGE en milieu marin naturel pourrait expliquer les défaillances de l'équipement enzymatique (élongation et désaturation) chez les poissons marins, les crustacés et les mollusques marins (Masuda, 2003).

L'acide docosahexaénoïque (DHA)

Le DHA ou encore l'acide cervonique est fortement accumulé dans le cerveau (membrane postsynaptique) et la rétine chez les poissons (Tocher & Harvie, 1988 ; Bell *et al.*, 1995a ; Willey *et al.*, 2003). Cet acide gras agit sur le fonctionnement des protéines imbriquées dans les membranes, *i.e.*, la rhodopsine pour la vision et les récepteurs postsynaptiques pour la neurotransmission chez l'être humain (Muskiet *et al.*, 2004). Chez l'être humain, 60% du poids sec du cerveau est constitué de lipides représentés principalement dans les membranes par le DHA (30-50% des acides gras totaux) et l'acide arachidonique (Das, 2006 ; Guesnet *et al.*, 2005). Bell *et al.*, (1995a) ont montré que des larves de hareng, nourries avec une alimentation pauvre en DHA et enrichies en EPA et 18:3n-3, développaient des problèmes de vision et une baisse d'efficacité de capture des

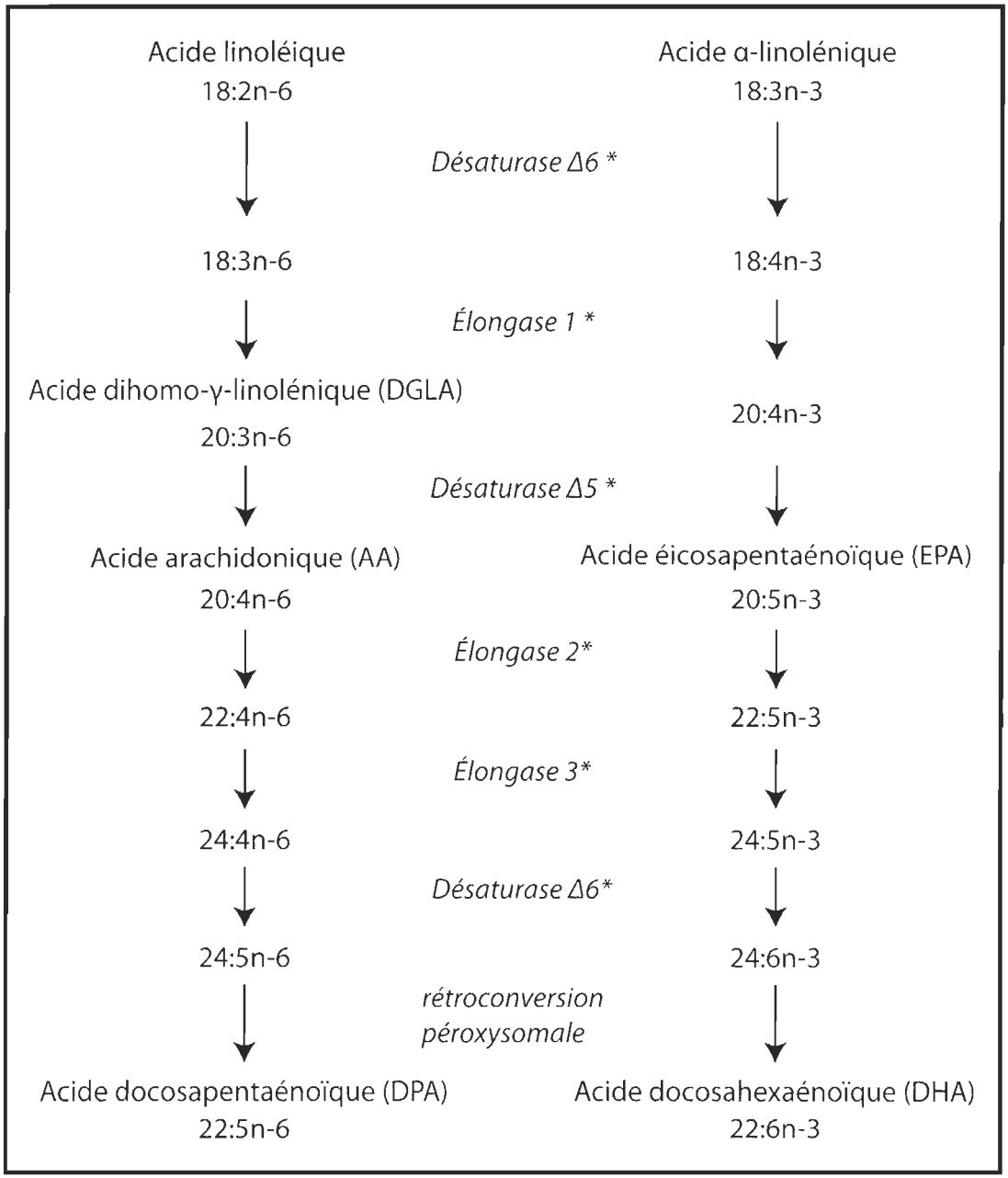

Figure 3 Voies de biosynthèse des acides gras essentiels des séries n-3 et n-6 chez le poisson. * réactions ayant lieu dans les microsomes.

proies à faible intensité lumineuse. Pendant longtemps, les recherches ont porté principalement sur le DHA et l'EPA retrouvés naturellement en fortes concentrations dans les œufs et les larves des poissons marins avec en général un ratio DHA/EPA ≥ 2 (Sargent *et al.*, 2002), au détriment d'un autre acide gras essentiel : l'acide arachidonique (AA, 20:4n-6).

L'acide arachidonique (AA) et l'acide eicosapentaénoïque (EPA)

L'acide arachidonique appartient à la deuxième famille d'acides gras essentiels n-6 et a été moins étudié, car il se retrouve en faible concentration dans les tissus des poissons par rapport au DHA et à l'EPA (Bell & Sargent, 2003). L'acide arachidonique a été identifié comme un facteur contribuant significativement à l'amélioration de la croissance des larves de poissons. Les effets d'un apport en AA alimentaire sur la croissance ont été observés chez des juvéniles de turbot (Castell, 1994 ; Bell *et al.*, 1995b), des larves de daurade royale (*Sparus aurata*) qui développaient alors une meilleure survie (Bessonart *et al.*, 1999) et une plus grande résistance au stress (Koven *et al.*, 2001). Tous ces effets ont également été observés chez des larves de cardeau d'été (*Paralichthys dentatus*) (Willey *et al.*, 2003). Son apport dans l'alimentation des reproducteurs améliore la qualité des œufs et des larves du bar européen (*Dicentrarchus labrax*) (Navas *et al.*, 1997 ; Bruce *et al.*, 1999) et du flétan atlantique (*Hippoglossus hippoglossus*) (Mazorra *et al.*, 2003).

Cependant, la modulation de l'apport en AA dans l'alimentation peut affecter la pigmentation des poissons plats et leur résistance aux facteurs de stress (Estévez &

Kanazawa, 1996 ; Koven *et al.*, 2001). L'acide arachidonique et l'EPA sont des précurseurs d'hormones de régulation à courte durée de vie que sont les eicosanoïdes. Ces derniers sont très importants dans les réactions inflammatoires, le contrôle de la pression sanguine et l'agrégation des plaquettes sanguines. Notons que lors d'un apport insuffisant en AA, l'EPA peut entrer en compétition avec l'AA au niveau des enzymes peu spécifiques (cyclooxygénase et lipooxygénase) produisant ainsi des eicosanoïdes aux effets atténués (Figure 4) (Sargent *et al.*, 2002).

La nutrition en acides gras essentiels et l'immunité chez le poisson

Les acides gras apportés par la nutrition affectent le système immunitaire selon trois mécanismes principaux (Balfry & Higgs, 2001) (Figure 5) : (1) l'influence des acides gras sur la composition des phospholipides des membranes cellulaires, (2) l'influence sur la production d'éicosanoïdes principalement à partir d'acide arachidonique (AA) et d'acide éicosapentaénoïque (EPA), (3) l'induction du signal par l'intermédiaire des interférons. Dans le premier cas, il peut ainsi y avoir un impact indirect sur les interactions des membranes leucocytaires (*e.g.* phagocytose, liaison antigène-anticorps, activation des étapes conduisant à la production de cytokine). Les acides gras polyinsaturés incorporés dans les lipides polaires des membranes cellulaires joueraient alors un rôle dans les fonctions des cellules immunitaires (Wu *et al.*, 2003). Les efforts de recherche sur ces

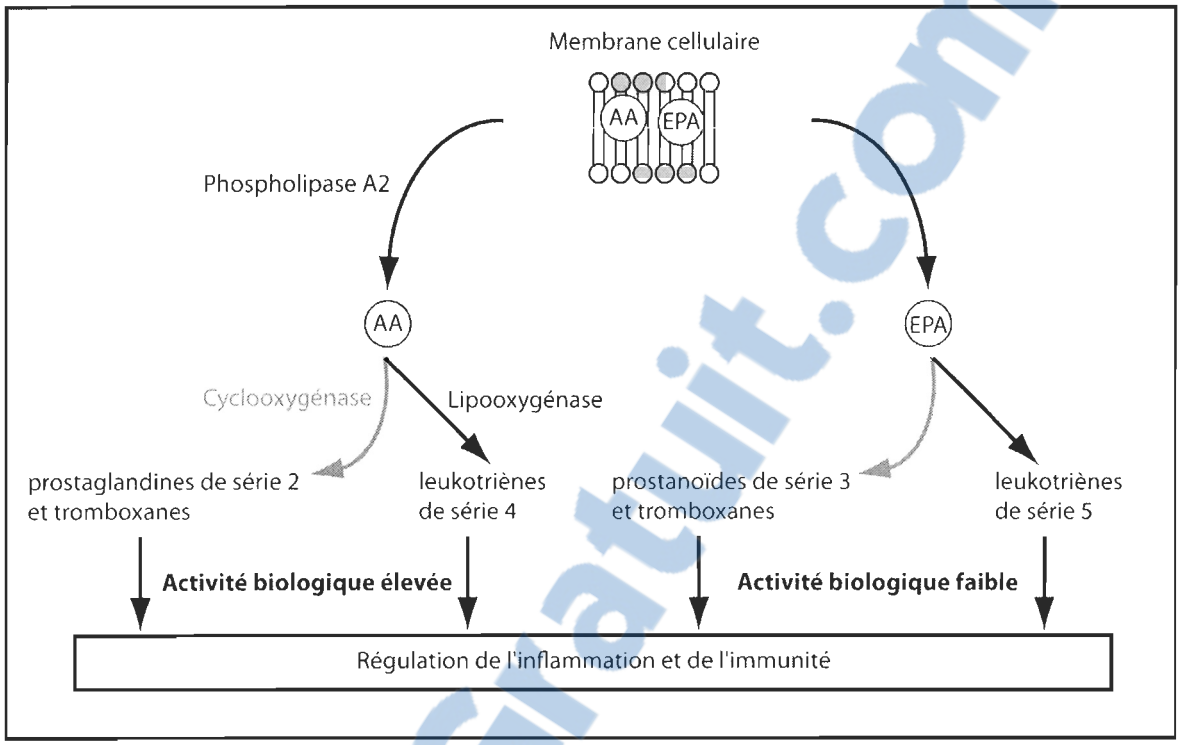

Figure 4 Acide arachidonique (20:4n-6, AA) et acide eicosapentaénoïque (20:5n-3, EPA) compétitionnant pour les mêmes enzymes peu spécifiques (cyclooxygénase ou lipoxygénase) de production des eicosanoïdes. Modifié de Sargent *et al.* (2002).

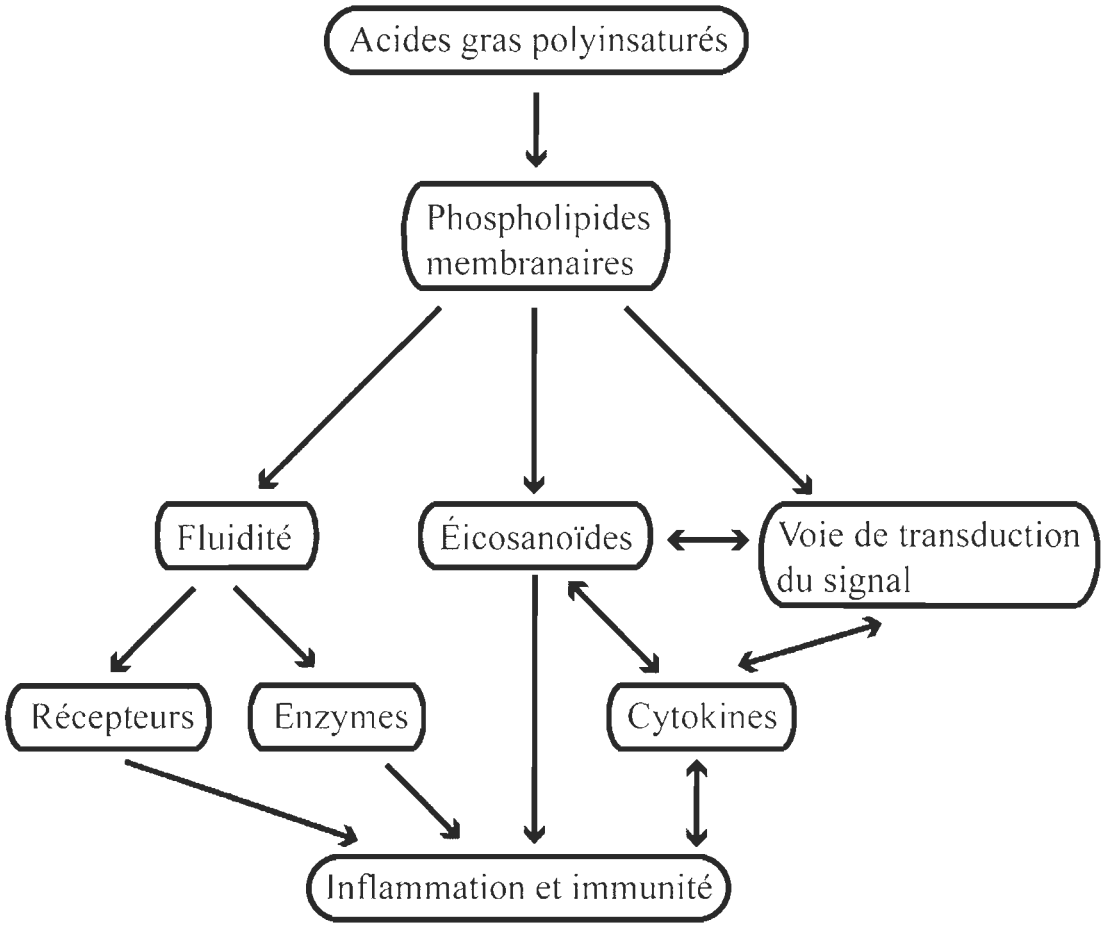

Figure 5 Impact des acides gras polyinsaturés sur l'inflammation et l'immunité chez les mammifères. Modifié de Calder et Grimble (2002).

mécanismes se sont concentrés dès la moitié des années 1990 sur l'optimisation alimentaire des concentrations d'acides gras hautement insaturés de série n-3 dans la nourriture vivante des larves des organismes marins, tout en optimisant les proportions relatives du DHA et de l'EPA (Sargent *et al.*, 1997). Chez le poisson chat (*Ictalurus punctatus*) nourri avec un aliment riche en acides gras n-3, une corrélation positive avec l'activité bactéricide des macrophages a été montrée (Sheldon & Blazer, 1991). Le même traitement imposé à la truite arc-en-ciel (*Onchorhynchus mykiss*) a entraîné une augmentation de sa résistance aux infections bactériennes (Kiron *et al.*, 1995). Il existe une fenêtre optimale d'apport en acides gras dans la nutrition, car l'absorption de trop fortes doses d'AGPI de série n-3 supprime les réactions immunitaires chez le poisson (Blazer, 1992). Chez les mammifères, il doit y avoir un équilibre entre les acides gras en n-3 et n-6 apportés par la nutrition pour assurer l'équilibre du système immunitaire (Harbige, 2003).

Plus récemment (Montero *et al.*, 2004), une déficience en acides gras essentiels (AGE) dans l'alimentation de juvéniles de daurade royale (*Sparus aurata*) entraînait une diminution de l'activité des neutrophiles et du nombre de lymphocytes circulants. Chez les mammifères, une déficience en AGE affecte sévèrement la croissance et le développement des tissus lymphoïdes et entraîne la perte de l'intégrité structurale et donc fonctionnelle de certains leucocytes (cellules T4, monocytes, macrophages et neutrophiles), ainsi qu'une diminution de la chimiotaxie et de la production d'éicosanoïdes (revue de Harbige, 2003). Les effets de la déficience en AGE sur les leucocytes ont été attribués spécifiquement aux acides gras essentiels de série n-6, l'acide linoléique et l'acide arachidonique (Harbige,

2003). La composition en AA peut atteindre 15 à 20% des acides gras présents dans les phospholipides des lymphocytes B et T et des monocytes chez l'être humain (Harbige, 2003 ; Calder, 2001). Lorsque survient l'entrée d'un pathogène dans un poisson, les leucocytes activés par l'interaction avec le pathogène génèrent des eicosanoïdes. Une fois stimulées, les enzymes phospholipases A_2 localisées dans les membranes des cellules leucocytaires activées métabolisent les phospholipides disponibles pour générer des AGPI non estérifiés tel que l'AA, l'EPA et le DHA (Shoemaker *et al.*, 2001). L'acide arachidonique ainsi libéré peut agir comme second messager, induire la réaction d'inflammation par médiation (Sargent *et al.*, 2002 ; Das, 2006) et/ou être converti en éicosanoïdes.

Les proies vivantes et les microalgues

Plusieurs espèces de poissons marins produisent beaucoup de petits œufs (plusieurs centaines de milliers, pour la femelle plie rouge *Pseudopleuronectes americanus*) qui donnent naissance à des larves très petites à croissance rapide (4 mm en moyenne pour la plie rouge) mais qui sont plus vulnérables aux stress environnementaux. Les larves étant petites, les principales proies vivantes largement utilisées pour leur alimentation initiale sont tout d'abord des rotifères, principalement (*Brachionus plicatilis*), puis des nauplii d'artémies (*Artemia* sp.). Cependant, ces organismes sont naturellement déficients en acides gras hautement insaturés de série n-3 et il est nécessaire de les enrichir pour assurer la survie, la croissance et la métamorphose des larves (Sargent *et al.*, 1999b). L'enrichissement des artémies, notamment en DHA, constitue encore un sérieux défi pour

nourrir les larves de poissons. Leur faible capacité à s'enrichir en DHA s'explique en partie par leur teneur élevée en acides gras de faible valeur nutritionnelle (*e.g.*, 18:1n-9, 18:2n-6, etc.) et en partie par le processus de rétroconversion du DHA en EPA (Navarro *et al.*, 1999). De ce fait, il serait difficile d'enrichir les artémies qui seront utilisées dans l'alimentation les larves de plie rouge que nous proposons d'étudier. Par contre, l'enrichissement des rotifères (*Brachionus plicatilis*) ne pose pas de problème majeur (Dhert *et al.*, 2001). Les principales sources d'acides gras hautement insaturés de série n-3 pour la nourriture vivante sont les huiles de poissons dont la teneur en 20:5n-3 et 22:6n-3 peut varier substantiellement (Sargent *et al.*, 1999b).

Les microalgues sont nécessaires au début de la nutrition larvaire de certaines espèces de poisson d'élevage (eau verte) et servent de nourriture pour les proies vivantes consommées par les larves. Les microalgues contiennent des vitamines (*e.g.* C et E) qui ont des fonctions antioxydantes intéressantes lors de la procédure d'enrichissement des rotifères (Sargent *et al.*, 1997) avec des huiles d'origine animale riches en AGPI. De même, l'acide ascorbique stimule la croissance des rotifères (Brown *et al.*, 1998). L'utilisation des microalgues dans la nutrition des larves de poissons marins peut mener à une modification considérable de leur microflore intestinale (Nicolas 1989 ; Bergh *et al.*, 1994 ; Reitan *et al.*, 1997 ; Olsen *et al.*, 2000). Les microalgues favoriseraient l'établissement d'une microflore bactérienne équilibrée en inhibant le développement de bactéries opportunistes (Skjermo & Vadstein, 1999).

Le remplacement des huiles originaires des poissons fourrages par des huiles végétales terrestres fait l'objet d'une intense recherche. La production d'huile végétale est cent fois plus importante (Bimbo, 1990) et reste moins chère que l'huile extraite des poissons sauvages (Pozernick & Wiegand, 1997 ; Huang *et al.*, 2007). Il persiste néanmoins quelques problèmes liés à l'utilisation de cette huile dans l'alimentation de poissons marins ou d'eau douce, dont un réellement problématique pour la santé humaine, *i.e.* diminution en DHA et EPA dans la chair et le foie (Tocher *et al.*, 2006 ; Murray *et al.*, 2007a). D'autres sources d'huiles riches en acides gras de série n-3 sont explorées : telles que les huiles extraites des microalgues (*e.g. Crypthecodinium cohnii*), les macroalgues des eaux froides du Canada (Colombo *et al.*, 2006), ainsi que les bactéries et protistes marins (Barnathan, 2007). Dans le contexte halieutique actuel, cette démarche permettrait de sortir du cercle « des huiles de poissons sauvages pour nourrir des poissons d'élevage ».

OBJECTIF GÉNÉRAL DE L'ÉTUDE

Notre étude a pour objectif principal de vérifier si il existe un effet dû à l'enrichissement en lipides, notamment en acides gras polyinsaturés, sur la microflore des larves et également celle des bassins d'élevage et de mesurer les effets de cet apport sur la croissance larvaire. Nos travaux s'appuient sur l'étude menée antérieurement par Vaillancourt (2008) sur les effets du ratio DHA/EPA sur la croissance et la survie larvaire des larves de plie rouge. Le modèle larvaire utilisé est celui de la plie rouge pour laquelle une solide expertise a été développée à l'ISMER.

Dans un second volet, nous nous sommes intéressés à la possibilité de remplacer les enrichissements commerciaux, composés d'huiles de poissons sauvages, pour rotifères par des microalgues marines.

OBJECTIFS SPÉCIFIQUES DE L'ÉTUDE

Premier objectif de recherche

Notre premier objectif est d'établir s'il existe un effet des enrichissements commerciaux pour proies vivantes, présentant des profils d'acides gras différents, sur la flore bactérienne des larves de plie rouge et celle du milieu d'élevage.

Notre hypothèse de travail H_0 est : il n'y a pas d'effet des enrichissements commerciaux pour rotifères sur la flore bactérienne des larves de plie rouge et celle du milieu d'élevage.

Deuxième objectif de recherche

Afin d'accroître la qualité nutritionnelle de *Brachionus plicatilis* en palliant au problème de leur déficience en acides gras hautement insaturés, nous avons effectué une deuxième série d'expériences dont le but était d'évaluer le taux de transfert du profil lipidique de quatre espèces de microalgues vers celui des rotifères. L'originalité de ces expériences réside dans le fait que ces microalgues étaient sous forme concentrée et congelée.

L'hypothèse de travail H_0 étant : il n'y a pas de transfert significatif des profils lipidiques des microalgues *Isochrysis galbana*, *Pavlova lutheri*, *Nannochloropsis* sp. et *Chaetoceros muelleri* vers les rotifères *B. plicatilis*.

Troisième objectif de recherche

Notre troisième objectif porte sur l'effet de l'enrichissement en acide arachidonique dans l'alimentation sur le développement bactérien à l'intérieur du milieu d'élevage ainsi qu'à l'intérieur des larves à la fin de la période d'alimentation sur rotifères enrichis.

L'hypothèse de travail H_0 sera : il n'y a pas d'effet d'un enrichissement en acide arachidonique dans l'alimentation sur la colonisation bactérienne du milieu d'élevage et à l'intérieur des larves de plie rouge.

CHAPITRE I. BACTERIAL COLONIZATION OF WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*) LARVAE FED LIVE FEED ENRICHED WITH THREE DIFFERENT COMMERCIAL DIETS

I.0 ABSTRACT

The proliferation of bacteria in intensive aquaculture systems may be responsible for poor growth and mass mortality of marine fish larvae. Essential fatty acids provided in the diet could protect larvae by modulation of the immune response *via* arachidonic acid (AA) and eicosapentaenoic acid (EPA). Winter flounder *Pseudopleuronectes americanus* larvae were fed rotifers *Brachionus plicatilis* enriched with three commercial diets containing different fatty acid profiles. Bacterial colonization on the gills and skin and in the intestinal lumen was evaluated at the end of the rotifer feeding period (day 26) and growth was surveyed until metamorphosis.

At 26 days post-hatching, larvae fed rotifers containing the higher AA content and with a higher docosahexaenoic acid (DHA) to EPA ratio showed better growth and the lowest bacterial colonization of the intestinal lumen compared to larvae fed rotifers with the lowest AA and DHA:EPA levels. AA was selectively incorporated into the polar lipids of larvae fed the rotifers enriched with the three diets. This is the first study in winter flounder larvae to report a link between different commercial rotifer enrichments and bacterial density in the intestinal lumen.

I.1 INTRODUCTION

There is an increasing demand for fry from farmed fish, and most are already produced in intensive rearing installations. However, intensive rearing of marine fish larvae may lead to microbial problems, resulting in poor growth and mass mortality (Muroga, 1987; Nicolas, 1989; Munro *et al.*, 1994). The artificial aquaculture environment is characterized by a decrease in water quality and an accumulation of organic matter that promote proliferation of opportunistic bacteria (Skjermo & Vadstein, 1999; Olafsen, 2001). Pathogens often arise in the early stages of larval rearing because the culture of small and sensitive marine larvae often involves a period with no or low water exchange (e.g., feeding period) (Skjermo & Vadstein, 1999).

The bacterial population in the water significantly affects the bacterial colonizing live prey (Nicolas, 1989) and fish digestive tracts (Munro *et al.*, 1994; Ringø *et al.*, 1996; Ringø & Birkbeck, 1999). This has been observed in flatfish larvae such as turbot *Scophthalmus maximus* (Nicolas, 1989) and summer flounder *Paralichthys dentatus* (Eddy & Jones, 2002). Marine larvae must take in water to maintain their osmotic balance, even before mouth opening (Reitan *et al.*, 1998), so small amounts of bacteria could enter the digestive tract when water is ingested for osmoregulation (Tytler, 1988; Hansen & Olafsen, 1999; Ringø & Birkbeck, 1999); this facilitates the inoculation of opportunistic bacteria in larvae. Thus gills and intestinal surfaces are important sites of bacterial colonization. Nicolas (1989) showed that prior to exogenous feeding, turbot larvae had internal microflora similar

to that of the water. Bacterial invasion through the skin is also possible (Vadstein, 1997). A way to decrease the impact of pathogenic bacteria is to stimulate immune function by nutritional supplements. Dietary lipids and their constituent fatty acids are fundamental for very small and rapidly developing larvae as they supply energy and cell components for structural membranes (Sargent *et al.*, 2002).

Several studies have characterized the bacterial flora associated with cold-water marine fish larvae without taking into account larval diet (review by Hansen & Olafsen, 1999). In addition, little information is available on the impact of commonly used enrichment media on the intestinal microbiota of fish larvae (Korsnes *et al.*, 2006) although a link has been revealed between dietary essential fatty acids and immune function in fish: essential fatty acids and their derivative products, eicosanoids, are highly biologically active and are involved in immunity (Tocher, 2003).

Experiments conducted on channel catfish *Ictalurus punctatus* fed a diet enriched with *n-3* fatty acids showed a positive correlation with macrophage activity while excess levels of highly unsaturated *n-3* fatty acids may not be as effective (Sheldon & Blazer, 1991). Similar positive effects were observed in rainbow trout *Onchorhynchus mykiss* (Kiron *et al.*, 1995). These authors showed that dietary levels of *n-3* polyunsaturated fatty acids (PUFA) affect bacterial infections and leucocyte mobility. Experiments on essential fatty acids (EFA) deficiencies conducted on gilthead sea bream (*Sparus aurata*) juveniles showed a decrease in both neutrophil activity and lymphocyte abundance (Montero *et al.*,

2004). Although the role of dietary lipids in fish immunity is not fully understood, they may modulate the immune response by changing the physical properties of immune cell membranes and cell membrane interactions (*e.g.*, phagocytosis, antigen–antibody connections) through eicosanoid production from arachidonic acid (20:4n-6, AA) and eicosapentaenoic acid (20:5n-3, EPA).

The main objectives of this work were two-fold: to study bacterial colonization of winter flounder *Pseudopleuronectes americanus* larvae fed rotifers enriched with three different diets and to examine the fatty acid response in larval membranes and lipid reserves. Winter flounder larvae were fed rotifers that had been enriched with three different commercial formulations for a period of 22 days to obtain rotifers with different contents of AA and 22:6n-3, docosahexaenoic acid (DHA) to EPA ratio.

I.2 MATERIALS AND METHODS

All experiments were conducted at the *Station aquicole de Pointe-au-Père* (UQAR, 48° 27' N; 68° 32' W, Québec, Canada). Larvae were obtained using a reproduction method described previously (Ben Khemis *et al.*, 2000). Newly hatched (day 0) flounder larvae were reared in six 57-l cylindro-conical polyethylene tanks at a rearing density of 250 larvae l^{-1} from April to August 2004. Tanks were supplied with filtered seawater (10 µm, 8.7 ± 0.5°C, 27 ± 1‰) with aeration; water temperature was maintained at 10°C and the photoperiod at 12 L:12 D. At mouth opening, larvae were fed one of the three following formulations: (1) rotifers (*Brachionus plicatilis*) enriched with Culture Selco 3000 (*SEL*), (2) rotifers enriched with DHA protein Selco (*DPS*) (both from INVE Aquaculture, Grantsville, UT, USA), and (3) rotifers enriched with AlgaMac-2000 (*ALG*) (Aquafauna Bio-Marine, Hawthorne, CA, USA). The compositions of the three media used were available from their manufacturers (*SEL*: crude proteins = 40% dry matter, lipids = 8% dry matter, DHA:EPA = 1; *DPS*: crude proteins = 27% dry matter, lipids = 29% dry matter; DHA:EPA = 2; *ALG*: crude proteins = 20% dry matter, lipids = 38% dry matter, DHA:EPA = 36). The *ALG* diet contains a relatively high level of n-6 DPA (22:5n-6), which is generally retroconverted to AA in rotifers (Koven *et al.*, 2001; Bransden *et al.*, 2004; Park *et al.*, 2006; Parrish *et al.*, 2007). Rotifer lipid and fatty acid compositions were obtained from a previous experiment (Vaillancourt, 2008) conducted in our laboratory using the same enrichment protocol (Table I.1). Rotifers were supplied to flounder larvae in

Table I.1 Lipid classes and selected fatty acid composition (mean ± SD, n = 3) of enriched rotifers. Enrichment diets were Culture Selco (*SEL*), DHA protein Selco (*DPS*) and Algamac 2000 (*ALG*). Data were obtained in our laboratory (Vaillancourt, 2008).

	SEL	*DPS*	*ALG*
Total lipids *	11.9 ± 6.14	21.7 ± 3.87	16.4 ± 7.26
Lipid class **			
Steryl/wax esters	2 ± 3[a]	5 ± 4[ab]	7 ± 4[ab]
Triacylglycerols	31 ± 6	44 ± 15	32 ± 10
Free fatty acids	14 ± 12	6 ± 8	13 ± 10
Sterols	5 ± 5	4 ± 6	4 ± 3
Acetone mobile polar lipids	21 ± 10	14 ± 12	14 ± 7
Phospholipids	13 ± 3[ab]	17 ± 40[b]	8 ± 3[a]
Selected fatty acids***			
∑SFA	22.7 ± 3.8[a]	25.0 ± 0.9[a]	34.7 ± 4.0[b]
∑MUFA	47.6 ± 3.2[c]	32.6 ± 1.6[b]	17.8 ± 4.9[a]
∑PUFA	37.1 ± 0.8	48.3 ± 10.0	59.3 ± 16.9
AA	1.1 ± 1.0	1.9 ± 0.2	3.8 ± 0.5
EPA	11.1 ± 6.7	7.2 ± 0.4	6.8 ± 2.5
DHA	5.8 ± 3.8[ab]	12.9 ± 1.3[bc]	21.1 ± 6.1[c]

* Results expressed in: * mg g^{-1} wet weight, ** % of total lipids and *** % of total fatty acids. Different superscript letters indicate significant differences among diets ($P < 0.05$).

excess and their density was maintained at 15–20 rotifers $larva^{-1}$, according to Laurence (1977). Two larval tanks were used for each type of rotifer enrichment.

At hatching (day 0), yolk-sac larvae were placed in rearing tanks. Tank water was continuously renewed for 4 days without food addition, until mouth opening. After mouth opening, the water supply was stopped each day for 12 hours while a pseudo-green-water preparation (a commercial mixture of *Isochrysis galbana*, *Pavlova lutheri* and *Nannochloropsis oculata*) was added to each tank for larval feeding. At the end of the day, water circulation resumed, allowing complete renewal of the tank water during the night. Larvae were sampled with empty stomachs before mouth opening at 4 days post-hatching (dph) and again at 15 and 26 dph as well as at metamorphosis (38 to 46 dph). On day 26, larvae were first weaned onto larger prey (*Artemia franscicana*) and then fed a formulated diet (Lansy Cw 1/8, INVE Aquaculture). Prey concentration was adjusted twice a day to maintain constant prey levels.

At 26 dph, *i.e.*, at the end of the rotifer feeding period, ten larvae were fixed with 10% formaldehyde ($\geq$ 24 h at ambient temperature) for light microscopy observations. Fixed larvae were then dehydrated in an ascending series of ethanol solutions and embedded in methacrylate resin. Tissues were sectioned (3 µm thickness) with a Supercut Reichert-Jung model 2050 (Cambridge Instruments GmbH, Germany); sections were mounted on glass slides and stained with a Gram staining kit from Sigma (#77730). Sections were examined and photographed under 1000× (Olympus BX41, Japan). The occurrence of bacteria was

determined and quantified in the gut lumen and on gill and fin surfaces (Figure 1). Bacterial density was calculated within intestinal lumen (bacteria mm^{-2}), and bacteria:tissue area proportions were calculated on gills and fins with Image Pro Plus software v5.1 (Media Cybernetics, Canada). Observations were made randomly on 20 larvae for each of the three treatments. Three sections were observed for each individual for each tissue studied.

Water from each tank (9 ml) was sampled before the pseudo-green water was added then fixed in 2% formaldehyde (neutral pH) on 4 (before mouth opening), 15, 26, 38 and 46 dph to determine the total number of bacteria using flow cytometry. Samples were frozen at -80°C until analysis. The total abundance of bacteria was determined using an Epics Altra flow cytometer (Beckman-Coulter) equipped with a 488 nm argon laser operated at 15 mW. A 1 ml subsample was incubated with 0.25 µl of SYBR Green I (Molecular Probes Inc., Oregon, USA) for 30 min at room temperature in the dark. Fluorescent beads (10 µl, 0.96 µm diameter, YG Fluoresbrite, Polysciences, USA) were added to each sample as an internal standard (Lebaron *et al.*, 2002). Total abundances of bacterial sub-populations with high and low nucleic acid contents (HNA and LNA respectively) were graphically discriminated and enumerated (Lebaron *et al.*, 2002) using right angle light scatter (SSC, related to cell size) and the green fluorescence of nucleic acid-bound SYBR Green I measured at 525 ± 5 nm. On day 46, culturable bacteria associated with rearing water were characterized after 24 h of incubation in darkness at 20–25°C. Bacteria were quantified as colony-forming units (CFU) using selective (thiosulfate–citrate–bile salts–sucrose, TCBS)

and non-selective (marine agar) media to estimate *Vibrio* spp. and heterotrophs respectively.

For lipid analysis, larvae were randomly sampled in each tank and individually rinsed three times in Millipore-filtered seawater. They were pooled (60 larvae for 4, 15, and 26 dph and 40 larvae for 38 and 46 dph) to reach a sample dry mass of ~0.8 mg and frozen at -80°C. Lipid extraction (Folch *et al.*, 1957) was performed on ice with dichloromethane:methanol:potassium chloride at a final volume ratio of 2:1:0.6. The homogenates were mixed and centrifuged twice at 2 000 rpm for 2 min to obtain a biphasic system. After each centrifugation, the lipid fraction was removed and transferred to a clean tube. The solvent was evaporated under nitrogen flow and lipids were dissolved in 100 µl of CH_2Cl_2. For lipid class determinations, a 2 µl aliquot of each extract was used. Extracts were spotted onto S-III Chromarods (Iatron Laboratories, Tokyo, Japan); classes were separated using four solvent systems, as described in Parrish (1999), and determined by flame ionization detection using an Iatroscan MK-6s.

Lipids were fractionated into neutral lipids (NL, including triacylglycerol [TAG], free fatty acids and sterols) and polar lipids (PL, including mainly phospholipids and minor amounts of glycolipids) using column chromatography on silica gel hydrated with 6% water. The 100 mg columns were preconditioned with 1 ml of methanol and 1 ml of chloroform. Aliquots of 300 µg of lipids were loaded onto the solid-phase extraction column. Samples were gently drawn into the solid phase under slight vacuum

(~ 1 ml min^{-1}). Columns were washed with 1 ml chloroform:methanol (98:2 v/v) to elute neutral lipids as described by Pernet *et al.* (2006).

For fatty acid analyses, lipid extracts were dried and fatty acid methyl esters (FAMEs) were prepared by transesterification with boron trifluoride in methanol at 100°C (AOCS, 1989). FAME identification was performed using a GC (SRI 8610C) equipped with an FID and a DB-WAX column (30 m × 0.25 mm × 0.25 μm). Direct injections were performed using hydrogen as the carrier gas at 40 PSI for the first four minutes and then at 20 PSI. The thermal gradient started at 58°C for 4 minutes and reached 170°C (20°C min^{-1}), then went from 170 to 180°C (1°C min^{-1}), and finally reached 220°C (2°C min^{-1}) for 5 minutes. Fatty acid methyl esters were identified by their retention times compared to those of a standard (Supelco 37 component FAME Mix, Menhaden Fish Oil, and PUFA-3; Supelco, Bellefonte, PA). Chromatograms were analyzed using Peak Simple version 3.21 software.

Ten larvae from each tank were collected for standard length and maximal width (± 0.1 mm) measurements at 4, 15, 26, 38, and 45 dph. To make measurements easier on day 4 larvae, one drop of 37.6% formalin was added and data were corrected using the Hjörleifsson & Klein-MacPhee (1992) equation developed on winter flounder. Larvae surviving until metamorphosis were estimated in one of the duplicate tanks of either the *ALG* or *DPS* treatment. We encountered a problem of water supersaturated in nitrogen, which prevented us from collecting data in the other tanks.

Statistical analyses were done using STATISTICA software version 6.0 (Statsoft, USA) ($\alpha = 0.05$). Normality of the data was examined using the Kolmogorov-Smirnov test. Significant differences among larviculture treatments were tested using one- or two-way analyses of variance (ANOVAs) for total bacteria in tank water, CFU counts, total length and width, and lipid classes; fatty acids were tested using multiple analysis of variance (MANOVA). Homoscedasticity was tested with the Brown-Forsythe test (Zar, 1999). For subsequent multiple comparisons, Tukey tests or Tukey tests for unequal *n* were performed. The Games and Howell test was used when the homoscedasticity condition was not met.

I.3 RESULTS

At the end of the rotifer feeding period (26 dph), bacterial density in the intestinal lumen was six-fold lower ($0.9 \pm 1.3\ 10^3$ bacteria mm^{-2}) in larvae fed rotifers enriched with the *ALG* diet than in larvae fed rotifers enriched with the *DPS* and *SEL* diets ($5.0 \pm 5.6\ 10^3$ bacteria mm^{-2}). We observed no difference in the bacterial colonization of the gills ($3.0 \pm 4.6\%$) and fins ($23.4 \pm 13.4\%$) among the three larval treatments.

Bacterial cell abundance in the tank water, which was determined by flow cytometry, was constant during the experiment (4 to 46 dph) and similar among the three larviculture treatments, with an average of $4.8\ 10^5$ bacteria ml^{-1}. Two bacterial populations were observed, some with high and some with low nucleic acid contents (HNA and LNA respectively). The HNA and LNA cell abundance and % HNA (average of 71.5%) remained constant during the experiment.

At metamorphosis (46 dph), we observed similar mean counts of *Vibrio* (49.7 ± 21.2 CFU ml^{-1}) and culturable bacteria (551.1 ± 412 CFU ml^{-1}) in the rearing water among feeding treatments. Although the ANOVA indicated a significant treatment effect for the number of heterotrophic bacteria in tank water at 46 dph, no significant difference was detected with the post-hoc test (Games and Howell). The *Vibrio* index, calculated as the geometric mean of colony-forming units (CFU) on selective medium (TCBS) divided

by the geometric mean of CFU on marine agar, was 0.10 ± 0.01, 0.07 ± 0.00 and 0.07 for tank water in the *SEL*, *DPS* and *ALG* treatments respectively.

At 26 dph, the relative proportions of triacylglycerol (TAG) were low and similar in larvae from the three dietary treatments (Table I.2). At 46 dph, the TAG proportion was higher in larvae previously fed rotifers enriched with the *ALG* diet compared to larvae from the *SEL* treatment.

From day 15 to 26, we observed accumulations of AA in the NL that were twice as high in larvae fed rotifers enriched with the *ALG* diet compared to larvae fed the other two diets (Figure I.1A). The PL fraction of AA was similar among treatments (Figure I.1B). The relative proportion of AA in the PL fraction dropped by 2.5 times between day 26 and day 46 and was highest in larvae fed the *ALG* diet. The same pattern was observed in NL at metamorphosis. From 4 to 26 dph, the EPA proportion in NL decreased slightly and was generally higher in larvae fed rotifers enriched with *DPS* than in larvae that had the *SEL* diet (Figure I.1C). From 4 to 26 dph, EPA levels in PL were similar to those in NL, with the lowest EPA level observed at 26 dph in PL of larvae fed the *ALG* diet (Figure I.1D). At 38 and 46 dph, the EPA content in NL decreased to around 2% of the TFA and to an average of 6% of the TFA in PL. Before mouth opening (4 dph), the DHA proportions in NL and PL were similar among treatments (Figure I.1E and F). The highest DHA level was observed in larvae fed *ALG* at 15 and at 26 dph in both NL and PL. In larvae fed rotifers

Table I.2 Lipid class contents presented as % weight of total lipids (mean ± SD) of winter flounder larvae fed with rotifers raised on one of three media treatments (AlgaMac 2000 [*ALG*], DHA protein Selco [*DPS*] and Selco 3000 [*SEL*]). Different letters indicate significant differences among treatments for a given sampling day ($P < 0.05$). dph: days post-hatching.

dph	Treatment	Triacylglycerol	Sterol	Phospholipid
	ALG	0.9 ± 1.0	7.6 ± 3.9	87.7 ± 2.3
	DPS	2.1 ± 2.5	7.0 ± 3.3	85.5 ± 3.4
4	*SEL*	3.6 ± 1.3	11.1 ± 10.2	82.1 ± 13.0
	ALG	1.4 ± 1.7	8.6 ± 5.4	86.2 ± 7.0
	DPS	1.9 ± 1.3	8.3 ± 3.0	88.6 ± 4.9
15	*SEL*	0.9 ± 1.0	8.2 ± 3.6	88.7 ± 5.2
	ALG	8.7 ± 5.1	8.5 ± 2.8	80.9 ± 7.0
	DPS	5.8 ± 2.8	8.1 ± 2.9	84.6 ± 5.3
26	*SEL*	3.2 ± 0.8	8.9 ± 3.4	87.0 ± 4.5
	ALG	19.0 ± 4.5	9.7 ± 2.8	65.8 ± 11.4
	DPS	15.1 ± 3.5	9.7 ± 2.0	66.3 ± 11.4
38	*SEL*	15.1 ± 3.5	9.9 ± 3.1	70.3 ± 8.9
	ALG	24.5 ± 4.1[b]	9.6 ± 2.3	62.5 ± 10.7
	DPS	21.9 ± 4.1[b]	11.4 ± 2.2	62.6 ± 9.3
46	*SEL*	13.6 ± 4.0[a]	10.5 ± 3.2	74.0 ± 8.2

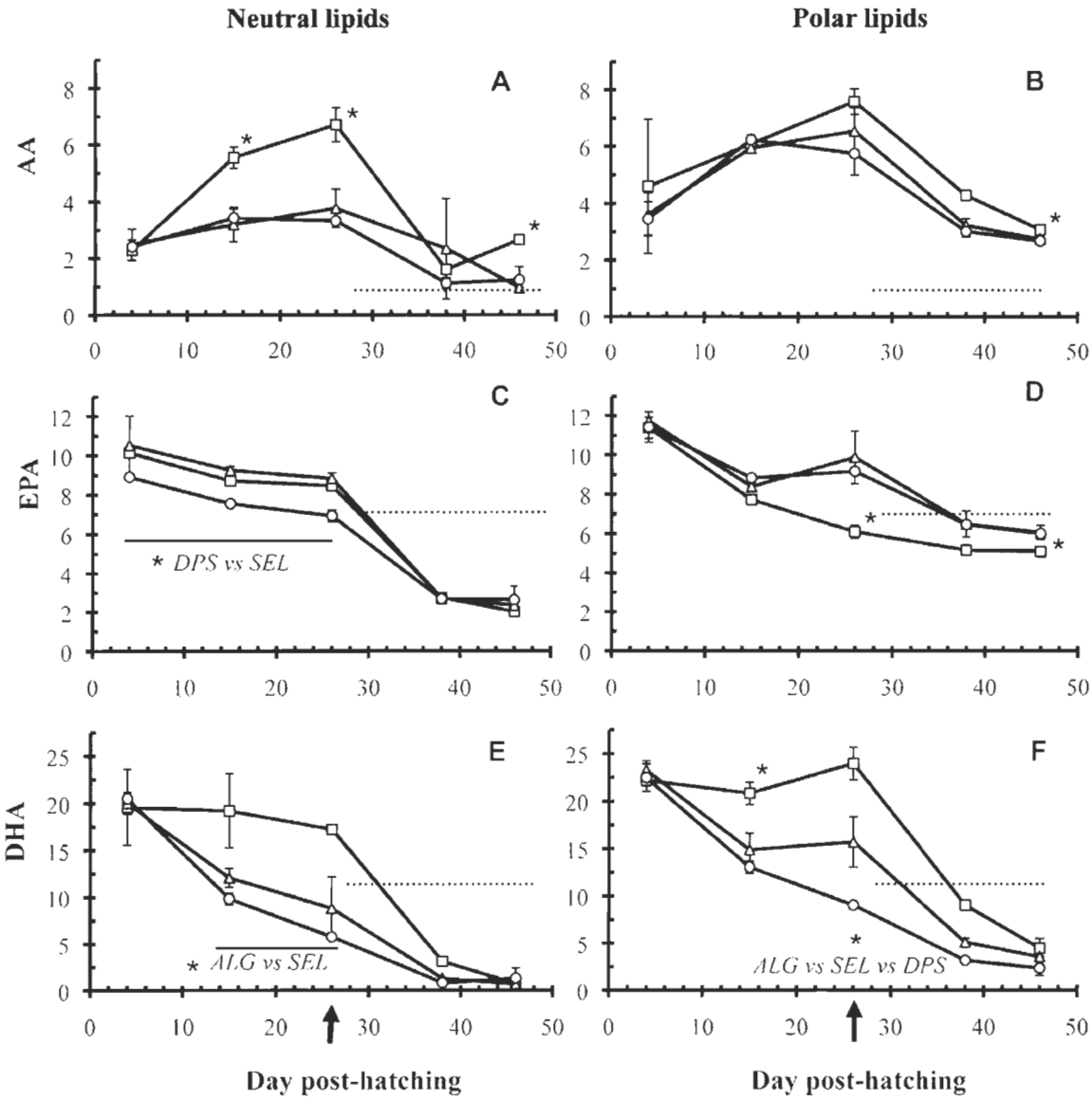

Figure I.1 Essential fatty acids (% of TFA ± SD) in neutral and polar lipids in winter flounder larvae fed three rotifer enrichments (□ AlgaMac 2000 [*ALG*], △ DHA protein Selco [*DPS*], ○ Selco 3000 [*SELJ*) until 26 dph then weaned onto non-enriched *Artemia* and a dry diet (Lansy Cw). Asterisks indicate significant differences in fatty acid content in the indicated treatment on a given sampling day. Arrows on the x-axes indicate the switch between rotifers and co-feeding and the horizontal dashed lines indicate the relative fatty acid level in the dry diet.

enriched with *ALG*, we observed accumulations of 22:5n-6 (n-6 DPA) in NL and PL that represented more than 6.5% of the TFA (Table I.3).

From 15 to 26 dph, PUFA content in both NL and PL was higher in larvae fed the *ALG* diet than in those of the *SEL* treatment (Table I.3) and was higher at 46 dph in NL from larvae fed the *ALG* diet than in those fed the two other diets (Table I.3). From 15 to 26 dph, the relative proportion of monounsaturated fatty acids (MUFA) was the lowest in NL and PL from larvae fed the *ALG* diet. Globally, MUFA content increased in the NL and PL of all larvae up to 26 dph whereas the proportion remained constant in the PL fraction of larvae fed the *ALG* diet. The relative percentage of saturated fatty acids (SFA) in the NL and PL of larvae decreased at 26 dph and was higher in the NL of larvae fed the *ALG* diet than in those fed the *DPS* diet from 15 to 26 dph. After the beginning of co-feeding, the relative proportion of linolenic acid reached 18 to 26% of the TFA at 46 dph in NL and PL respectively (Table I.3).

On day 26, larvae from the *ALG* diet were longer than those from the *SEL* diet (Figure I.2). On day 46, all larvae had reached similar total lengths. Total widths were similar among larviculture treatments (data not shown) on day 26 (data not shown). Larval survival rates from the *ALG* and *DPS* treatments were 11.1 and 16.6% respectively.

Table I.3 Selected fatty acid contents (% of TFA ± SD) in neutral and polar lipids of winter flounder larvae fed one of three rotifer enrichments until 26 days post-hatching (dph): AlgaMac 2000 (*ALG*), DHA protein Selco (*DPS*) and Selco 3000 (*SEL*). Different superscript letters indicate significant differences among treatments for a given sampling day ($P < 0.05$).

		Neutral lipids			Polar lipids		
Fatty acid	dph	*ALG*	*DPS*	*SEL*	*ALG*	*DPS*	*SEL*
16:0	4	19.0 ± 5.0	16.3 ± 0.3	15.8 ± 0.3	24.4 ± 0.6	23.4 ± 0.1	23.3 ± 0.1
	26	14.1 ± 0.1	14.1 ± 2.9	12.2 ± 0.0	21.8 ± 0.1	19.3 ± 0.5	18.0 ± 0.5
	46	9.2 ± 0.1	9.5 ± 0.1	10.1 ± 0.7	16.3 ± 0.4	16.3 ± 0.3	16.1 ± 0.2
18:0	4	8.9 ± 0.6	8.6 ± 0.5	8.0 ± 0.1	8.9 ± 0.7	8.5 ± 0.2	8.7 ± 0.3
	26	5.0 ± 0.9	5.6 ± 0.9	7.2 ± 0.1	7.5 ± 0.6	7.6 ± 0.4	8.7 ± 0.2
	46	5.1 ± 0.3	6.0 ± 0.2	6.8 ± 0.8	6.9 ± 0.3	7.0 ± 0.2	7.2 ± 0.1
16:1n-7	4	1.8 ± 0.2	2.9 ± 1.1	2.5 ± 0.0	1.7 ± 0.3	1.9 ± 0.1	1.9 ± 0.1
	26	10.0 ± 2.6	11.0 ± 3.2	11.0 ± 0.2	4.0 ± 0.3	4.8 ± 0.8	6.0 ± 0.2
	46	3.2 ± 0.1	3.2 ± 0.2	2.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1
18:1n-9	4	11.8 ± 1.9	14.3 ± 1.5	14.1 ± 0.4	10.4 ± 0.3	9.6 ± 0.3	10.2 ± 0.4
	26	4.6 ± 0.2	13.9 ± 0.1	14.6 ± 0.0	5.3 ± 0.1	11.1 ± 0.2	13.2 ± 0.1
	46	19.9 ± 0.3	20.3 ± 0.1	19.4 ± 0.7	15.6 ± 0.8	15.9 ± 0.2	15.9 ± 0.0
18:1n-7	4	2.5 ± 1.1	3.1 ± 0.9	3.3 ± 0.3	3.0 ± 0.1	3.1 ± 0.3	3.0 ± 0.2
	26	5.9 ± 0.8	3.9 ± 0.2	4.8 ± 0.1	5.5 ± 0.0	4.2 ± 0.4	4.8 ± 0.2
	46	8.1 ± 0.3	8.5 ± 0.2	8.1 ± 0.3	6.6 ± 0.0	6.5 ± 0.0	6.3 ± 0.1
24:1n-7	4	4.5 ± 0.4	4.1 ± 1.1	5.8 ± 0.0	1.5 ± 2.1	2.2 ± 0.4	2.7 ± 0.2
	26	2.4 ± 0.2	2.3 ± 1.1	3.6 ± 0.2	1.0 ± 0.1	0.8 ± 0.3	1.1 ± 0.1
	46	0.1 ± 0.1	0.3 ± 0.0	0.9 ± 0.8	0.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.4
18:2n-6	4	3.9 ± 0.2	5.1 ± 1.1	4.0 ± 0.2	3.5 ± 0.3	3.5 ± 0.4	3.5 ± 0.1
cis	26	1.6 ± 0.3	5.6 ± 0.1	5.0 ± 0.0	1.0 ± 0.1	4.4 ± 0.1	5.4 ± 0.1
	46	5.6 ± 0.7	5.6 ± 0.0	5.5 ± 0.0	7.4 ± 0.3	7.8 ± 0.1	7.9 ± 0.3
18 :3n-3	4	0.0 ± 0.0	2.2 ± 0.4	1.3 ± 0.3	0.7 ± 0.0	1.0 ± 0.3	0.8 ± 0.1
	26	0.2 ± 0.2	0.7 ± 0.1	1.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.0	0.6 ± 0.1
	46	25.6 ± 0.2	24.6 ± 0.4	22.2 ± 1.2	18.6 ± 0.0	18.4 ± 0.2	18.0 ± 0.9
22:5n-6	4	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.4	0.2 ± 0.3
	26	7.1 ± 0.6	0.4 ± 0.1	0.2 ± 0.0	6.7 ± 0.3	0.3 ± 0.4	0.4 ± 0.0
	46	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.3	0.2 ± 0.0	0.1 ± 0.1
22:5n-3	4	1.9 ± 0.3	2.1 ± 0.6	1.6 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
	26	3.5 ± 0.2	4.5 ± 0.4	4.7 ± 0.2	2.8 ± 0.4	5.9 ± 0.1	6.4 ± 0.1
	46	0.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.3	0.8 ± 0.0	1.4 ± 0.3	1.5 ± 0.1
SFA	4	34.1 ± 3.9^{b}	27.5 ± 0.2^{a}	27.2 ± 0.4^{ab}	35.2 ± 2.2	33.7 ± 0.0	34.1 ± 0.9
	26	25.1 ± 0.6^{b}	24.2 ± 3.2^{a}	24.0 ± 0.4^{ab}	32.0 ± 0.7	28.9 ± 0.0	29.0 ± 0.5
	46	16.3 ± 0.3	17.2 ± 0.2	19.1 ± 1.4	24.5 ± 0.7	24.2 ± 0.6	24.7 ± 0.4
MUFA	4	23.8 ± 5.3^{a}	27.7 ± 4.7^{b}	30.8 ± 1.0^{b}	19.7 ± 0.8	19.5 ± 0.3	20.9 ± 0.7
	26	27.0 ± 0.7^{a}	39.3 ± 1.6^{b}	44.2 ± 0.5^{b}	17.2 ± 0.2^{a}	24.5 ± 0.7^{b}	29.6 ± 0.4^{c}
	46	36.3 ± 0.0^{a}	37.5 ± 0.2^{a}	36.4 ± 0.7^{a}	27.7 ± 0.7	28.2 ± 0.2	28.6 ± 0.7
PUFA	4	42.1 ± 9.2^{b}	44.8 ± 4.5^{ab}	42.0 ± 0.6^{a}	45.0 ± 1.4	46.8 ± 0.3	44.9 ± 1.6
	26	47.9 ± 0.1^{b}	36.5 ± 4.8^{ab}	31.8 ± 0.9^{a}	50.8 ± 0.9^{b}	46.5 ± 0.7^{ab}	41.4 ± 0.1^{a}
	46	47.4 ± 0.3^{b}	45.3 ± 0.0^{a}	44.5 ± 0.7^{a}	47.9 ± 1.4	47.6 ± 0.3	46.7 ± 1.1

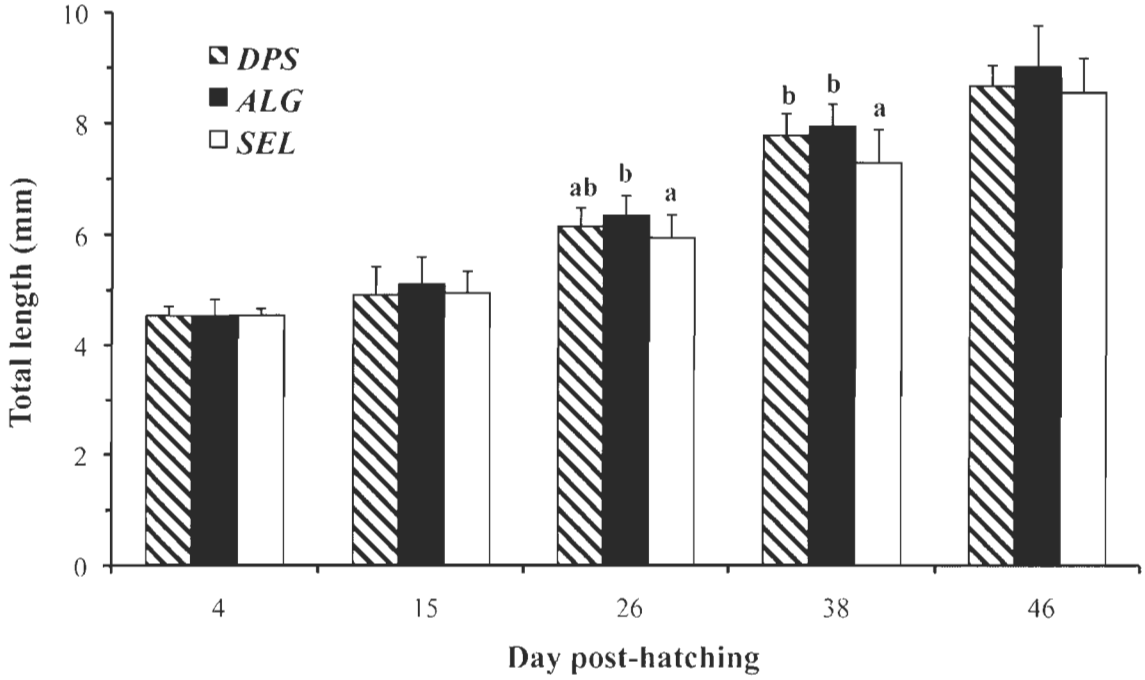

Figure I.2 Total lengths of winter flounder larvae fed three rotifer enrichment media (■ Algamac 2000 [*ALG*]; ▨ DHA protein Selco [*DPS*]; □ Selco 3000 [*SEL*]) until 26 dph and then weaned onto *Artemia* and a dry diet (Lansy Cw). Different letters indicate significant difference among larviculture treatments at a given day post-hatching.

I.4 DISCUSSION

As early as the time of mouth opening, winter flounder larvae have been shown to be equipped with enzymes involved in lipid digestion (Murray *et al.*, 2003), highlighting the importance of dietary lipids for larval growth. Arachidonic acid seems to be an important fatty acid for winter flounder larvae, as they appear to have the ability to selectively incorporate dietary AA into their cellular membranes. Larvae fed rotifers enriched with the *DPS* and *SEL* diets may have encountered AA deficiencies since they retained this fatty acid in their PL, with values similar to larvae with a higher AA content in their NL (*ALG* treatment). AA has been shown to be highly conserved under conditions of dietary deficiency (Bell & Sargent, 2003) in the larvae of several flatfish species such as turbot (Rainuzzo *et al.*, 1994), yellowtail flounder *Limanda ferruginea* (Copeman & Parrish, 2002), and summer flounder *Paralichthys dentatus* (Willey *et al.*, 2003).

Although we observed a higher relative percentage of AA in the NL of larvae fed rotifers containing a higher relative level of AA, no parallel increase was detected in PL. However, studies have shown that an increased AA content in the PL of gilthead sea bream *Sparus auratus* larvae was related to an increase in the dietary AA level (Bell *et al.*, 1995; Bessonart *et al.*, 1999). The same phenomenon was observed for EPA in turbot larvae (Reitan *et al.*, 1994). The fatty acid composition of NL generally reflects that of the diet while the fatty acid composition of PL is more strongly regulated and reflects membrane requirements (Sargent *et al.*, 2002). We suggest that the relative AA level (6.6% of TFA)

reached in the cellular membranes of 26 dph winter flounder larvae could be sufficient to sustain larval development.

The lower bacterial density in the intestinal lumen was related to the dietary treatment that resulted in a higher AA level and higher DHA to EPA ratios in rotifers (*ALG* treatment). High standard deviations observed for bacterial density data in intestinal lumen are indications of great differences among larvae from each treatment. This variability was also observed in intestinal flora of tilapia (*Oreocrhomis mossambicus*), carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) (Asfie *et al.*, 2003). Using four commercial enrichments for rotifers (Algamac 2000, AquaGrow Advantage [Advanced Bionutrition Columbia, USA], Marol E [SINTEF, Trondheim, Norway] and Protein Selco [INVE, Baasrode, Belgium]), Korsnes *et al.* (2006) observed an effect on bacterial colonization in the gastro-intestinal tracts of cod *Gadus morhua* larvae as well as on the bacterial concentration in enrichment cultures.

Direct bacterial counts in larval tank water by flow cytometry were low compared to values obtained in other studies using the plate method, which only takes into account culturable bacteria (corresponding to 0.1–1% of the total bacteria) (Vadstein *et al.*, 2004). Several studies have reported low levels of *Vibrio* in rotifer rearing (Verdonck *et al.*, 1997; Skjermo & Vadstein, 1999). The low levels of *Vibrio* in larval tanks during the rotifer-feeding period could be explained by the use of microalgae, both as food for rotifers and as green water in the larval rearing tank. The addition of algae to the water in fish tanks can

considerably alter the composition of the bacterial flora associated with rearing water, larval skin and gut (Skjermo & Vadstein, 1993) and can prevent *Vibrio* proliferation (Salvesen *et al.*, 1999). *Pavlova lutheri* and *Isochrysis galbana*, which were present in the pseudo-green water used in our experiment, have been shown to increase the diversification of bacterial communities (Støttrup & McEvoy, 2003). A larger fraction of slow growers and fewer opportunistic (potentially pathogenic) fast growing bacteria were observed compared to larval rearing in clear water. The *Vibrio* indexes of tank water at 46 dph (0.07–0.15) were lower than the value of 0.24 observed by Eddy and Jones (2002) on summer flounder larvae of a similar stage.

The low levels of total culturable heterotrophic bacteria and *Vibrio* that we observed could be due to the fact that the bottom of the larval rearing tank was cleaned at regular intervals from 26 to 46 dph, thus *Vibrio*, which survive and proliferate in fish feces (Olsson *et al.*, 1998), could have been partially eliminated. In addition, the *Artemia* used in this study were non-enriched and harvested in 0.2 µm prefiltred seawater. Ritar *et al.* (2004) observed that the bacteria load associated with *Artemia* enriched with Algamac was six times higher as early as 6 h after enrichment compared to those enriched with oil emulsion and *Chaetoceros muelleri*.

In our study, tank conditions could be inadequate for the growth of bacteria associated with live prey (rotifers and *Artemia*). Thus, winter flounder larvae were probably exposed to low levels of opportunistic bacteria in tank water before and after weaning onto the

Artemia and dry diet. This was shown by the constant abundance of high and low nucleic acid bacteria and the low *Vibrio* index observed at 46 dph. However, bacteria associated with live feed are the major influx to tank water and hence to the intestinal microbiota of larvae (Skjermo & Vadstein, 1993; Munro *et al.*, 1994; Makridis *et al.*, 2000; Jensen *et al.*, 2004). Bacteria associated with individual rotifers have been shown to be active and able to grow (Nicolas, 1989). *Artemia* can have high *Vibrio* levels (Tanasomwang & Muroga, 1988; Tanasomwang & Muroga, 1989; Verschuere, 1997; Olsen *et al.*, 1999; Olsen *et al.*, 2000; Eddy & Jones, 2002), and its use as a feed for marine fish larvae has been shown to coincide with an increase in total bacteria and specific *Vibrio* abundance in the water, as recently observed by Plante *et al.* (2007).

At 26 dph, larvae fed rotifers containing the highest relative AA level and DHA:EPA ratio grew faster than larvae fed *SEL*-enriched rotifers. Recently, Garcia *et al.* (2008) observed a higher specific growth rate in cod larvae fed *ALG*-enriched *Artemia* compared to those fed AquaGrow Advantage. Studies have demonstrated the ability of AA to enhance larval fish growth in the presence of high dietary DHA to EPA ratios (Bessonart *et al.*, 1999; Willey *et al.*, 2003). Juvenile turbot fed pure AA and DHA showed higher growth than when fed a diet containing just AA (Castell, 1994).

After the beginning of weaning onto non-enriched *Artemia* and the dry diet, we observed a decrease in the relative proportion of EFAs in all larvae. The decrease in essential fatty acids in both neutral and polar lipids reflects a problem during weaning. The

concomitant increase in linolenic acid in both larval neutral and polar lipids suggests that the flounder larvae could not assimilate the dry diet (which was characterized by low levels of linolenic acid, 3.8% of TFA) but could profit from the non-enriched *Artemia* (which have high levels of linolenic acid).

CHAPITRE II. ESSENTIAL FATTY ACID ENRICHMENT OF CULTURED ROTIFERS (*BRACHIONUS PLICATILIS*, MÜLLER) USING FROZEN-CONCENTRATED MICROALGAE

II.0 ABSTRACT

There is a growing interest in preserving microalgal preparations to maintain constant properties over a long period. The aim is to ensure sufficient delivery of essential fatty acids (and other key nutrients) to mollusc and crustacean larvae and to zooplankton used as live prey in the first feeding of fish larvae. For example, the rotifer *Brachionus plicatilis* has to be enriched with polyunsaturated fatty acids (PUFAs) prior to fish feeding. We used four microalgal species (*Isochrysis galbana* [T-ISO], *Chaetoceros muelleri* [CHGRA], *Pavlova lutheri* [MONO], and *Nannochloropsis* sp.) both as fresh cultures and in a frozen-concentrated form to enrich rotifers. Overall, rotifers had similar relative fatty acid levels when fed the frozen-concentrated or fresh microalgal diets. The levels of 20:4n-6, 22:6n-3, and 20:5n-3 between *B. plicatilis* and the microalgal diets were linearly correlated. The fatty acid 20:4n-6 was the most readily assimilated: the content found in rotifers reached half the level measured in the microalgal diets. Our results indicate that both the fresh and frozen-concentrated forms of the four microalgal species can be used to enrich PUFA levels in rotifers. Further experiments should be conducted to test if assimilation differs when rotifers are enriched with mono- or multispecific microalgal preparations.

II.1 INTRODUCTION

Although there have been encouraging recent studies on the use of artificial formulated diets to feed fish larvae (Cahu & Zambonino Infante, 2001; Cahu *et al.*, 2003), live prey will continue to be an important food source for the first-feeding of early larval stages of different freshwater and marine fish species in aquaculture (Støttrup & McEvoy, 2003). The rotifer *Brachionus plicatilis* (Müller) is an important live prey widely used for the first feeding of several marine fishes. This is mainly due to its small size (130-300 μm) and the availability of large quantities from mass culture. Although rotifers synthesize some n-3 polyunsaturated fatty acids (PUFAs) *de novo,* such as docosahexaenoic acid (DHA, 22:6n-3) (Lubzens *et al.*, 1985), the synthesis rate is not high enough to supply sufficient amounts to marine fish larvae. Rotifers must therefore be enriched with PUFAs.

Growth, survival, and the normal development of visual functions of fish larvae require dietary essential fatty acids (EFAs) for incorporation into membrane phospholipids. These phospholipids generally contain 16:0, 18:1n-9, 20:5n-3, and 22:6n-3 as their principal fatty acids (Sargent *et al.*, 2002). Docosahexaenoic acid is involved in the maintenance of the structural and functional integrity of biological membranes due to its unique configuration, which facilitates rapid configurational changes in membrane proteins that are particularly important in visual and neuromuscular processes (Sargent *et al.*, 2002). Moreover, the intrinsic structure of DHA is resistant to temperature and pressure changes, allowing it to function independently of these environmental variables (Rabinovich &

Ripatti, 1991). In fish phospholipids, DHA is generally present at about twice the level of eicosapentaenoic acid (EPA, 20:5n-3). The other EFAs, arachidonic acid (AA, 20:4n-6) and EPA, are respectively the primary and competitive precursors for the production of eicosanoids, which are a group of highly biologically active hormones. Eicosanoid production is associated with stressful situations, and excess eicosanoid production has been recorded under pathological conditions (Bell & Sargent, 2003). Although marine fish larvae need dietary lipids to obtain the correct balance of DHA, EPA, and AA, they also need phospholipids. This is probably related to the limited ability of fish larvae to synthesize phospholipids *de novo* (Sargent *et al.*, 2002). Phospholipids are the preferred vehicle for the delivery of PUFA and constitute around 34% to 43% of the lipid content of rotifers.

Marine microalgae are the primary food source for mollusc and crustacean larvae, including live prey that are used to feed fish larvae. They contain essential nutrients such as vitamins, essential fatty acids, amino acids, and pigments that may be transferred to the higher trophic level. However, most microalgal species are rich in one or two EFAs, and the levels may vary between species (Reitan *et al.*, 1994; Fábregas *et al.*, 1998). Several studies have shown that culture conditions such as light intensity (Thompson *et al.*, 1990; Brown *et al.*, 1997), temperature (James *et al.*, 1989; Thompson *et al.*, 1992), pH (Guckert & Cooksey, 1990), culture media (Wikfors *et al.*, 1984), and harvest stage (Brown *et al.*, 1997; Pernet *et al.*, 2003) may influence EFA levels. Microalgae are commonly added to larval tanks as "pseudo-green" water to improve survival, growth, and the food conversion

index in larval fish (Støttrup & McEvoy, 2003). The culture of marine microalgae is costly (Palmtag *et al.*, 2006) and may reach approximately 30% to 40% of hatchery costs (Borowitzka, 1997). Thus, there is a growing need to develop preservation methods for microalgae that will stabilize the nutritional value over a long period and that will provide appropriate levels of EFAs to meet the nutritional requirements of the consumers in the next trophic level (Dhert *et al.*, 2001).

Rotifers have been successfully reared with various forms of microalgae such as frozen (Lubzens *et al.*, 1995), dried (Robert & Trintignac, 1997), frozen and dried (Yúfera and Navarro, 1995; Navarro & Sarasquete, 1998), and concentrated (Maruyama *et al.*, 1997; Yoshimura *et al.*, 1997; Palmtag *et al.*, 2006). However, concentrated-frozen microalgae have been identified as good source for rotifer enrichment (Lubzens *et al.*, 1995), providing high quality algal biomass to sustain hatcheries (Cañavate & Fernández-Díaz, 2001).

Traditionally, microalgal species used to feed zooplankton contain high levels of EPA (*e.g.*, *Nannochloropsis* sp.), DHA (*e.g.*, *Isochrysis galbana* Haines, clone T-ISO), or both (*e.g.*, *Pavlova lutheri* Droop) (Brown *et al.* 1997). The aim of this study was to test whether rotifers could be enriched with EFA from four concentrated-frozen microalgae species (*Nannochloropsis* sp., *Isochrysis galbana*, *Pavlova lutheri*, and *Chaetoceros muelleri* Johansen) and how this enrichment compares with the use of the fresh forms. Surprisingly, very few studies have been proposed to examine fatty acid enrichment from fresh and

concentrated-frozen forms of the same microalgal species to rotifers. We discuss the microalgal diet composition for rotifer enrichment with respect to larval fish nutrition.

II.2 MATERIALS AND METHODS

Experiments were conducted at the marine station of the Institut des sciences de la mer de Rimouski (Québec, Canada). Marine microalgae cultures were obtained from Nutrocean Inc. (Rimouski, Québec, Canada). Culturing was conducted in natural filtered seawater enriched with f/2 medium (Guillard, 1975) and harvested in a semi-continuous system in closed photobioreactors under the following conditions: pH between 7.5 and 8.5, temperature between 20 and 23°C, salinity from 28-30‰, continuous illumination (4.4 to 5.8 W m^{-2}), bubbling air at 10 ml l^{-1} of culture min^{-1} and CO_2 (1% in air). Microalgae strains (the flagellates *Isochrysis galbana* T-ISO and *Pavlova lutheri* MONO, the diatom *Chaetoceros muelleri* CHGRA, and the Chlorophyceae *Nannochloropsis* sp. NASP) were obtained from the Center for Culture of Marine Phytoplankton (CCMP) at the Bigelow Laboratory for Ocean Sciences (Maine, USA). The diatom culture was supplemented with a silicate solution to fulfil the requirement for siliceous frustule formation (Lombardi & Wangersky, 1991). Microalgae were first added to 250 ml of autoclaved culture media in Erlenmeyer flasks. After about 1 week of growth, the 250-ml flasks were used to inoculate 20-l carboys that were in turn used to inoculate the 260-l photobioreactors.

The microalga paste was prepared by tangential filtration followed by a centrifugation (5000-6000 g for 20 minutes). The paste was then mixed with a cryoprotective agent (glycerol; 10% of the paste's wet weight) and stored at -20°C in 500-g jars. A ration of 1 g

of frozen-concentrated microalgae for 10^6 rotifers was used. The frozen microalgae used were either 11 days (T-ISO and MONO) or 2 months (NASP and CHGRA) old.

Rotifers were cultured in 18-l tanks at 20-25°C in a greenhouse using filtered (0.2 μm) seawater (27‰), natural illumination, and aeration. For each microalgal species, we tested the enrichment of rotifers with the two microalgae forms (fresh, frozen-concentrated) simultaneously in triplicate, but each microalgal species was tested one after the other. Sexual reproduction (egg production) was present in all rotifer cultures and the average rotifer population on day 0 was 76,000 ± 30,420 l^{-1}. Fresh microalgae were added once a day, with the total amount of cells provided being adjusted based on rotifer numbers in the culture tanks (1 l per million rotifers). The concentrations of fresh microalgae solutions (in cells l^{-1}) were 3.9×10^9 (CHGRA), 10.3×10^9 (T-ISO), 8.3×10^9 (MONO), and 20×10^9 (NASP). Frozen-concentrated microalgae were given three times per day (8:00, 11:00, and 16:00). The total daily ration was 1 g per day and concentrations of frozen microalgae (in cells g^{-1}) were 3.9×10^9 for CHGRA, 13.3×10^9 for T-ISO, 16.0×10^9 for MONO, and 36.0×10^9 for NASP. After 72h of enrichment, two aliquots of the frozen and fresh forms of the microalgae as well as two rotifer samples from each replicate were taken for lipid analysis.

Living microalgae (5 ml from fresh culture) were filtered onto pre-combusted (450°C) GF/F filters (25 mm) and stored in 1 ml chloroform in amber glass vials with Teflon-lined caps at -80°C until lipid extraction. Frozen microalgae were first diluted in 1 l

of filtered (0.2 μm) sea water prior to filtration as described above. Filters were rinsed with ammonium formate (3%) for dry weight determination. The dry weight was determined after drying known volumes of the frozen-concentrated suspension and fresh cultures at 70°C for 24 h (Lubzens *et al.* 1995).

Two samples of 20 000 rotifers (Lubzens *et al.* 1995) were collected from each triplicate tank and pre-rinsed with filtered sea water (0.2 μm) on a 50 μm net. The samples were filtered as previously described above for microalgae and immediately frozen at -80°C until lipid analyses. Subsamples were used for dry weight determination.

Rotifer lipid analyses were performed at the Institut de Recherche sur les Zones Côtières (IRZC, New Brunswick, Canada) on one sample per replicate for a total of 24 samples. Microalgae lipid analyses were carried out on two samples for each form (fresh, frozen-concentrated) of each microalga for a total of 16 samples. Lipid extraction from rotifers and microalgae was carried out on ice according to Folch *et al.* (1957), but dichloromethane was substituted for chloroform (Chen *et al.*, 1981) for a final dichloromethane:methanol volume ratio of 2:1. Rotifers and microalgae were homogenized in 3 ml of the dichloromethane:methanol mixture (2:1 v/v). The grinder was rinsed twice with 3 ml of dichloromethane:methanol (2:1; v/v), and 2.25 ml KCl (0.88%) were added for a final CH_2Cl_2–MeOH–KCl ratio of 2:1:0.6; v/v/v (Folch *et al.*, 1957). The homogenates were mixed and centrifuged at 432 g for 2 min to obtain a biphasic system. The bottom

lipid fraction was removed and transferred to a clean tube with a Pasteur pipette. The solvent was evaporated under nitrogen flow and lipids were dissolved in 100 µl CH_2Cl_2.

For lipid class determinations, one 2-µl aliquot from each extract was used. Extracts were spotted onto S-III Chromarods (Iatron Laboratories, Tokyo, Japan). Lipid classes were separated using four solvent systems as described in Parrish (1987) and were determined using the flame ionization detector of the Iatroscan MK-6s. The following lipid classes were obtained in this study: hydrocarbon (HC), ketone (KT), steryl and wax esters (SE-WE), triacylglycerol (TAG), free fatty acids (FFA), sterol (ST), diacylglycerol (DAG), acetone-mobile polar lipids (AMPL), and phospholipids (PL).

Fatty acid methyl esters (FAMEs) were prepared by transesterification with boron trifluoride in methanol at 100°C (AOCS, 1989). FAMEs were run on an SRI 8610C gas chromatograph (SRI, Torrance, CA, USA) equipped with a DB-wax fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness; Agilent, Bellfonte, PA, USA). Hydrogen was used as the carrier gas (flow velocity: 80 $cm \cdot s^{-1}$ or 2.1 ml min^{-1} at 145°C). FAMEs were directly introduced into a glass liner (uniliner, drilled, 4 mm, 6.3 × 78.5 mm; Restek, Bellfonte, PA, USA) maintained at 300°C with an internal pressure of 40 psi for 4 min as described in Pernet *et al.* (2006), using splitless injection. The temperature was initially held at 58°C for 4 min at 40 psi followed by a series of temperature ramps: 20°C min^{-1} to 170°C at 20 psi, 1°C min^{-1} to 180°C, and finally 2°C min^{-1} to 220°C, where it was held for 5 min. The FID system was maintained at 260°C. FAMEs were identified by

comparison of retention times with known standards (a 37-component FAME mix and menhaden oil) and further quantified using tricosanoic acid (23:0) as an internal standard (Supelco, Bellfonte, PA, USA). Menhaden oil and the 37-component FAME mix were injected after every eight samples to compare the relative proportion of each fatty acid to expected values.

All statistical analyses were done with STATISTICA software version 6.0. Normality of the data was examined using the Kolmogorov-Smirnov test. Homoscedasticity was tested with the Brown-Forsythe test (Zar, 1999). Two-way ANOVAs were used to test for significant differences among treatments in lipid classes and fatty acid composition of microalgae and rotifers. For subsequent multiple comparisons, Tukey tests or Tukey tests for unequal n were performed when appropriate. In absence of homoscedasticity, we used Games and Howell tests (Sokal & Rohlf, 1995). For all statistical tests, $\alpha = 0.05$.

II.3 RESULTS

Lipid classes and fatty acid composition of microalgae

Comparisons between lipid class contents in both forms of each microalga species are shown in Figure II.1. In general, no differences in the proportions of lipid classes concentrations were detected between the fresh and frozen-concentrated forms of MONO, T-ISO, and CHGRA. In NASP, the TAG and AMPL levels were higher in the frozen-concentrated form while the amount of PL was 2.2 times higher in the fresh form (Figure II.1).

Lipid contents were similar between the four microalgae except for FFA, which were higher in both the fresh and frozen forms of CHGRA: they made up respectively 25.7% and 17.0% of the total lipid classes compared to percentages lower than 5% in the other species.

The highest percentage of SFA was observed in the frozen-concentrated form of CHGRA (Table II.1); this amount was 1.5 times higher than that in the fresh form. This high percentage was due to an increase in 14:0 (60%) and 16:0 (39%). A 48% increase in 16:0 was also responsible for the increase (1.3 times) in SFA observed in the frozen form of NASP compared to the fresh one. No difference in SFA was observed between the fresh and frozen forms of MONO and T-ISO.

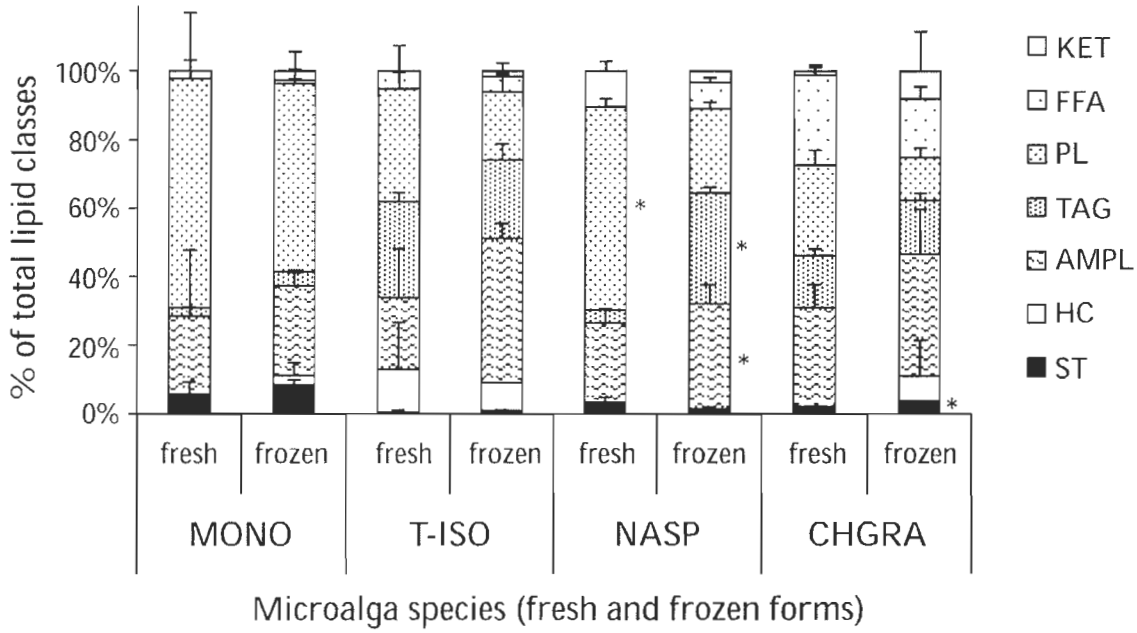

Figure II.1 Mean proportions of lipid classes ± SD (% weight of total lipids) contained in fresh and frozen-concentrated microalgae. Asterisks indicate significant difference between the two forms. KET, ketone; FFA, free fatty acids; PL, phospholipids; TAG, triacylglycerol; AMPL, acetone-mobile polar lipids; HC, hydrocarbon; and ST, sterol. Microalga species are: *Pavlova lutheri* (MONO), *Isochrysis galbana* (T-ISO), *Nannochloropsis* sp. (NASP), and *Chaetoceros muelleri* (CHGRA).

Table II.1 Fatty acid composition of microalgae (% of total fatty acids) from fresh culture and frozen forms.

	MONO		CHGRA		T-ISO		NASP		
Fatty acids	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	M X F
14:0	11.2 ± 0.46	10.6 ± 0.59	8.6 ± 0.16	13.8 ± 0.05	14.2 ± 0.44	19.2 ± 0.22	5.6 ± 0.20	5.5 ± 0.18	$P < 0.001$
16:0	12.5 ± 0.47	16.2 ± 0.65	14.6 ± 0.44	20.3 ± 0.14	14.1 ± 0.87	14.3 ± 0.25	17.1 ± 0.37	25.3 ± 0.58	$P < 0.001$
17:0	0.0 ± 0.00	0.0 ± 0.00	0.1 ± 0.11	0.3 ± 0.40	1.5 ± 0.05	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.00	$P < 0.001$
18:0	1.4 ± 0.00	0.5 ± 0.08	1.4 ± 0.01	2.3 ± 0.17	2.0 ± 1.38	0.7 ± 0.05	1.4 ± 0.47	1.6 ± 0.09	$P > 0.05$
ΣSFA	**25.1 ± 1.0^{bcd}**	**27.5 ± 1.4^{abc}**	**26.1 ± 0.4^{bd}**	**38.0 ± 0.6^{a}**	**32.4 ± 2.9^{abc}**	**34.5 ± 0.1^{ab}**	**24.8 ± 0.2^{cd}**	**33.2 ± 0.6^{ac}**	***P* < 0.01**
16:1n-7	8.7 ± 0.37	16.9 ± 0.08	31.6 ± 0.77	30.4 ± 0.08	4.3 ± 0.12	4.5 ± 0.17	21.8 ± 1.19	18.6 ± 0.48	$P < 0.001$
18:1n-9	1.1 ± 0.10	1.1 ± 0.06	0.9 ± 0.10	1.7 ± 0.03	17.2 ± 0.89	18.4 ± 0.23	5.1 ± 0.37	9.4 ± 0.08	$P < 0.001$
18:1n-7	2.4 ± 0.07	2.2 ± 0.04	1.4 ± 0.00	1.0 ± 0.08	2.1 ± 0.14	2.4 ± 0.18	0.8 ± 0.15	1.2 ± 0.04	$P < 0.01$
20:1n-5	2.0 ± 0.02	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	$P < 0.01$
ΣMUFA	**15.3 ± 0.1^{b}**	**22.7 ± 1.9^{ab}**	**35.4 ± 0.8^{a}**	**35.1 ± 0.4^{a}**	**25.4 ± 1.4^{ab}**	**26.4 ± 0.0^{a}**	**30.8 ± 0.6^{a}**	**31.6 ± 0.3^{a}**	***P* < 0.01**
16:2n-6	1.0 ± 0.18	0.5 ± 0.65	0.1 ± 0.12	1.1 ± 0.94	0.0 ± 0.00	0.4 ± 0.05	0.0 ± 0.00	0.5 ± 0.02	$P > 0.05$
16:2n-4	1.0 ± 0.43	1.0 ± 0.10	2.9 ± 0.10	2.1 ± 0.20	1.3 ± 0.10	1.3 ± 0.06	0.0 ± 0.00	0.2 ± 0.00	$P < 0.05$
16:3n-4	0.7 ± 0.04	0.7 ± 0.01	0.3 ± 0.21	0.4 ± 0.03	0.0 ± 0.00	0.1 ± 0.18	1.1 ± 0.68	0.1 ± 0.13	$P < 0.001$
16:4n-3	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.5 ± 2.19	$P > 0.05$
18:2n-6	0.0 ± 0.00	0.6 ± 0.26	0.6 ± 0.04	0.7 ± 0.12	3.6 ± 1.16	3.7 ± 0.03	4.0 ± 0.20	4.0 ± 0.15	$P < 0.001$
18:3n-6	0.0 ± 0.00	0.3 ± 0.00	1.8 ± 0.01	0.9 ± 0.09	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.2 ± 0.05	$P < 0.001$
18:3n-3	0.7 ± 0.32	0.9 ± 0.29	0.0 ± 0.00	0.0 ± 0.00	3.9 ± 0.14	5.3 ± 0.12	1.1 ± 0.92	7.5 ± 0.15	$P < 0.001$
18:4n-3	12.0 ± 0.55	7.9 ± 0.05	0.6 ± 0.02	1.1 ± 0.07	15.3 ± 0.29	12.9 ± 0.02	0.0 ± 0.00	1.1 ± 0.04	$P < 0.001$
20:4n-6	**0.3 ± 0.14^{c}**	**0.2 ± 0.06^{c}**	**9.6 ± 0.01^{a}**	**3.4 ± 0.02^{b}**	**0.2 ± 0.05^{c}**	**0.1 ± 0.00^{c}**	**6.8 ± 0.86^{abc}**	**2.6 ± 0.09^{b}**	***P* < 0.001**
20:3n-3	0.2 ± 0.00	0.0 ± 0.00	0.3 ± 0.48	0.0 ± 0.00	1.2 ± 0.21	1.1 ± 0.02	0.3 ± 0.35	0.2 ± 0.14	M, $P < 0.001$
20:5n-3	**25.6 ± 1.00^{abd}**	**22.9 ± 0.41^{ac}**	**14.2 ± 0.04^{cd}**	**9.7 ± 0.10^{be}**	**0.7 ± 0.09^{f}**	**0.8 ± 0.58^{ef}**	**27.3 ± 0.50^{a}**	**12.5 ± 0.15^{d}**	***P* < 0.001**
22:4n-6	0.9 ± 0.12	1.0 ± 0.07	0.0 ± 0.00	0.0 ± 0.00	1.7 ± 0.37	1.4 ± 0.09	0.0 ± 0.00	0.1 ± 0.16	M, $P < 0.001$
22:6n-3	**13.0 ± 0.57^{abcd}**	**12.9 ± 0.18^{a}**	**1.8 ± 0.01^{c}**	**1.0 ± 0.06^{cd}**	**12.8 ± 0.59^{abcd}**	**10.2 ± 0.13^{b}**	**0.2 ± 0.07^{e}**	**0.7 ± 0.04^{de}**	***P* < 0.001**
ΣPUFA	**59.6 ± 1.1^{ab}**	**49.7 ± 0.5^{c}**	**38.5 ± 1.2^{cde}**	**26.9 ± 1.0^{f}**	**42.2 ± 1.5^{cde}**	**39.1 ± 0.1^{bf}**	**44.4 ± 0.7^{cd}**	**35.2 ± 0.9^{ef}**	***P* < 0.01**
Σn-3	55.0 ± 0.01	45.2 ± 0.55	17.1 ± 0.39	14.1 ± 0.22	34.9 ± 2.12	32.0 ± 0.52	31.1 ± 0.34	24.7 ± 1.60	$P < 0.01$
Σn-6	2.8 ± 1.04	2.7 ± 0.20	12.7 ± 0.68	7.0 ± 0.85	6.1 ± 0.70	5.5 ± 0.06	11.8 ± 0.39	8.5 ± 0.42	$P < 0.001$
DHA/EPA	0.5 ± 0.04	0.6 ± 0.00	0.1 ± 0.00	0.1 ± 0.01	18.7 ± 3.31	16.2 ± 11.48	0.0 ± 0.00	0.1 ± 0.00	M, $P < 0.001$
Σ others*	0.1 ± 0.06	0.1 ± 0.09	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.14	0.1 ± 0.03	

Bold rows represent variables for which the results of two-way ANOVA analysis are discussed (factors: microalgae, form). As factor interactions ($M \pm F$) were significant, the eight groups were compared with a *posteriori* tests ($\alpha = 0.05$). Different letters indicate the presence of significant difference among groups. *Include minor components (< 0.1% of total fatty acids). *Abbreviations*: MONO, *Pavlova lutheri*; T-ISO, *Isochrysis galbana*; NASP, *Nannochloropsis* sp.; CHGRA, *Chaetoceros muelleri*; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

CHGRA and NASP, followed by T-ISO, had the highest relative content of MUFA. In MONO, the frozen form contained 48% more MUFA than the fresh form due to a doubling in the 16:1n-7 level. The highest PUFA level was observed in fresh MONO, 59.6% of total fatty acids (TFA). In its frozen-concentrated form, a 17% drop in PUFA occurred along with a 34% decrease in the 18:4n-3 level and an 11% decrease in the EPA levels. In the frozen forms of CHGRA and NASP, the decreases in PUFA were related to decreases in AA (65% and 62% of TFA) and EPA (31% and 54%), respectively. The highest content of arachidonic acid was observed in fresh cultures of CHGRA and NASP.

The highest DHA proportions were detected in both forms of MONO as well as in the fresh form of T-ISO (close to 13% of TFA), while the highest EPA proportion was found in fresh NASP followed by fresh MONO (27.3 and 25.6% of TFA, respectively). The proportions of EPA in NASP and CHGRA were respectively 54% and 31% lower in the frozen than in the fresh forms (Table II.1).

Relationship between microalgal diets and the EFA content of rotifers

Highly significant relationships ($P < 0.001$) were observed between EFAs contained in either fresh or frozen-concentrated microalgae and those contained in rotifers fed on these microalgae (Figure II.2). The AA content in rotifers was closest to the diet contents and reached half of the relative proportion observed in the microalgal diets (Figure II.2a). The same thing can be observed, but at a lower level, for DHA (Figure II.2b) and EPA

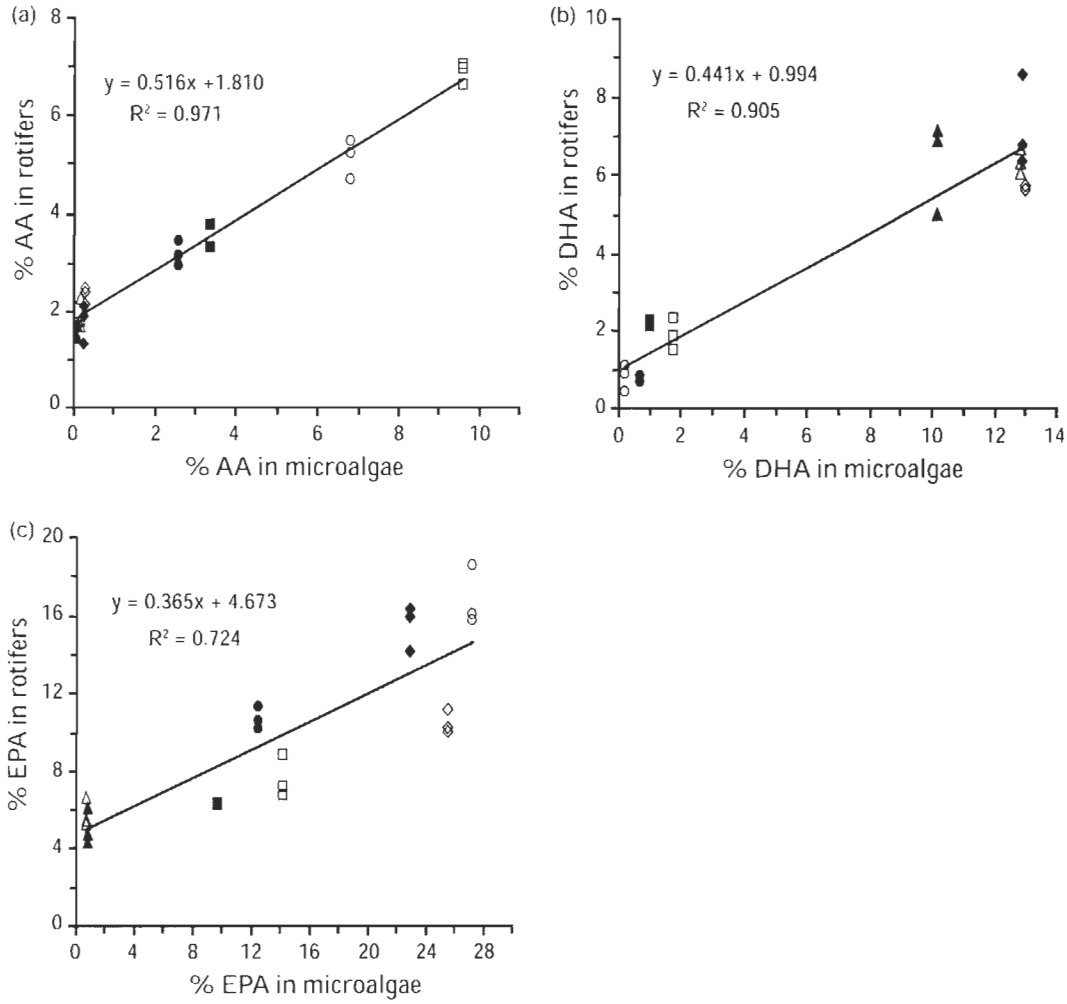

Figure II.2 Regressions between the relative essential fatty acid contents in microalgae and rotifers fed with those microalgae (a, b, and c: $P < 0.001$). Microalgal species were provided either in fresh or frozen-concentrated form. Fresh (open symbols) and frozen (filled symbols) forms of *Pavlova lutheri* (◊, ♦), *Chaetoceros muelleri* (□, ■), and *Nannochloropsis* sp. (○, ●).

(Figure II.2C). The pattern of relative EFA levels (Table II.2) observed in rotifers generally reflects the relative EFA levels contained in each microalgal species, in either the fresh or frozen form (Table II.1). In rotifers fed the frozen-concentrated microalgae, those fed with CHGRA and NASP showed the highest AA levels, 3.5% and 3.2% of TFA respectively (Table II.2 and Figure II.2A). The highest proportions of EPA and DHA in rotifers fed frozen microalgae occurred with the MONO diet, followed by the NASP (for EPA) and MONO and T-ISO (for DHA) diets.

Lipid class and fatty acid composition of rotifers

No differences in TAG, FFA, and ST levels were detected between the rotifers fed with either form of the four microalgal diets (Table II.2). The mean proportions of TAG, FFA, and ST in rotifers represented respectively 19%, 5%, and 5% of the total lipid classes. The amount of AMPL was 3.2 times higher in rotifers fed T-ISO (16%) than in rotifers fed other microalgae (around 5% of the total lipid classes). The proportion of PL was 38% lower in rotifers fed with T-ISO.

The SFA contents of rotifers fed the four microalgal diets were similar (total mean of 25% TFA) despite small but significant differences (Table II.2). Rotifers fed frozen T-ISO showed an SFA level 1.2 times lower than those fed fresh T-ISO concomitant with a slight decrease in 14:0 and 16:0. The MUFA proportion was higher in rotifers fed CHGRA (41%) than in those fed with other microalgae (around 30%).

Table II.2 Lipid composition (lipid classes: % of total lipids; fatty acids: % of total fatty acids) of rotifers enriched with either fresh or frozen-concentrated microalgae.

	MONO		CHGRA		T-ISO		NASP		
Lipid classes	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	M X F
TAG	**8.3 ± 2.9**	**15.5 ± 5.1**	**27.2 ± 6.6**	**13.3 ± 0.6**	**27.0 ± 10.6**	**26.3 ± 15.5**	**11.8 ± 4.7**	**22.8 ± 10.4**	***P* > 0.05**
FFA	**6.3 ± 2.1**	**8.4 ± 5.8**	**0.6 ± 1.0**	**6.7 ± 3.9**	**6.3 ± 7.6**	**6.0 ± 5.3**	**5.7 ± 0.4**	**3.1 ± 3.6**	***P* > 0.05**
ST	**4.2 ± 1.3**	**3.0 ± 3.1**	**6.1 ± 1.3**	**8.3 ± 0.6**	**5.2 ± 4.6**	**5.6 ± 5.0**	**0.0 ± 0.0**	**4.2 ± 3.8**	***P* > 0.05**
AMPL	**(5.0 ± 2.3**	**7.9 ± 3.1)^{b}**	**(2.2 ± 0.8**	**1.7 ± 0.4)^{c}**	**(14.7 ± 1.3**	**17.2 ± 4.1)^{a}**	**(4.1 ± 2.8**	**5.6 ± 1.4)^{bc}**	**M, *P* < 0.001**
PL	**(76.1 ± 4.4**	**58.8 ± 1.6)^{a}**	**(63.7 ± 5.9**	**69.9 ± 5.6)^{a}**	**(40.3 ± 13.6**	**42.9 ± 7.7)^{b}**	**(70.4 ± 3.4**	**62.3 ± 10.3)^{a}**	**M, *P* < 0.001**
Fatty acids									
14:0	5.7 ± 0.18	6.0 ± 0.30	6.3 ± 0.67	5.2 ± 0.16	7.3 ± 1.00	5.9 ± 1.38	3.9 ± 0.66	4.8 ± 0.54	M, *P* < 0.001
15:0	0.5 ± 0.04	0.3 ± 0.25	1.0 ± 0.10	0.8 ± 0.05	0.7 ± 0.05	0.9 ± 0.12	0.7 ± 0.04	0.7 ± 0.06	*P* < 0.05
16:0	12.2 ± 0.82	13.4 ± 0.81	13.4 ± 1.12	13.2 ± 0.51	15.4 ± 1.01	12.8 ± 0.58	16.7 ± 0.17	16.9 ± 1.37	*P* < 0.05
18:0	4.1 ± 0.07	4.0 ± 0.48	3.5 ± 0.46	3.9 ± 0.34	4.6 ± 0.52	3.8 ± 0.32	3.9 ± 0.29	3.4 ± 0.22	*P* > 0.05
ΣSFA	**(23.3 ± 0.9)^{b}**	**(24.1 ± 1.0)^{b}**	**(24.8 ± 1.3)^{b}**	**(23.7 ± 0.2)^{b}**	**(28.7 ± 2.0)^{a}**	**(24.9 ± 1.0)^{b}**	**(26.1 ± 0.6)^{ab}**	**(26.4 ± 1.1)^{ab}**	***P* < 0.05**
14:1n-5	1.2 ± 0.14	0.5 ± 0.43	0.9 ± 0.17	1.2 ± 0.43	1.4 ± 0.05	2.6 ± 0.19	1.3 ± 0.16	1.1 ± 0.11	*P* < 0.001
16:1n-9	1.2 ± 0.48	0.4 ± 0.14	0.3 ± 0.09	0.6 ± 0.02	0.8 ± 0.08	1.1 ± 0.15	0.4 ± 0.09	0.9 ± 0.02	*P* < 0.001
16:1n-7	11.0 ± 0.51	11.2 ± 0.25	23.8 ± 1.77	19.8 ± 1.15	4.7 ± 0.21	4.4 ± 0.51	12.6 ± 1.08	14.1 ± 1.28	*P* < 0.01
18:1n-9	7.3 ± 1.36	3.5 ± 1.14	3.6 ± 0.57	6.7 ± 1.67	9.7 ± 0.50	9.6 ± 1.29	6.2 ± 1.20	7.9 ± 0.20	*P* < 0.001
18:1n-7	4.3 ± 0.33	6.9 ± 0.56	6.0 ± 0.61	7.4 ± 0.65	2.5 ± 0.42	10.2 ± 2.72	3.8 ± 0.15	5.6 ± 1.42	*P* < 0.001
18:1n-5	0.7 ± 0.11	1.3 ± 0.17	0.5 ± 0.07	1.3 ± 0.30	0.3 ± 0.07	2.1 ± 0.43	0.6 ± 0.11	1.0 ± 0.39	*P* < 0.001
20:1n-9	2.7 ± 0.20	1.4 ± 0.42	1.4 ± 0.27	2.1 ± 0.50	2.7 ± 0.67	2.4 ± 0.78	1.8 ± 0.22	1.8 ± 0.14	*P* < 0.05
20:1n-7	0.9 ± 0.23	1.1 ± 0.08	1.0 ± 0.06	1.3 ± 0.10	0.5 ± 0.01	0.8 ± 0.14	0.8 ± 0.03	0.8 ± 0.05	M, *P* < 0.001
ΣMUFA	**(31.7 ± 1.7)^{de}**	**(28.8 ± 1.2)^{e}**	**(39.9 ± 1.2)^{ab}**	**(43.2 ± 2.0)^{a}**	**(24.4 ± 0.9)^{f}**	**(36.6 ± 0.6)^{bc}**	**(29.7 ± 1.4)^{e}**	**(34.8 ± 1.9)^{cd}**	***P* < 0.001**
16:2n-6	0.5 ± 0.10	0.5 ± 0.03	0.4 ± 0.01	0.5 ± 0.13	0.6 ± 0.10	0.9 ± 0.27	0.3 ± 0.10	0.3 ± 0.07	M, *P* < 0.001
18:2n-9	2.2 ± 0.21	0.0 ± 0.00	1.7 ± 1.47	1.9 ± 2.66	0.0 ± 0.08	0.4 ± 0.69	1.6 ± 0.20	1.4 ± 1.18	*P* > 0.05
18:2n-6	6.2 ± 0.08	2.7 ± 0.80	2.7 ± 0.66	4.5 ± 0.79	6.8 ± 0.30	5.9 ± 0.18	6.4 ± 0.67	5.8 ± 0.42	*P* < 0.001
18:3n-4	0.4 ± 0.05	0.3 ± 0.10	1.5 ± 0.18	0.6 ± 0.13	0.0 ± 0.00	0.0 ± 0.00	0.1 ± 0.06	0.0 ± 0.06	*P* < 0.001

18:3n-3	0.8 ± 0.10	1.3 ± 0.24	0.5 ± 0.21	0.8 ± 0.00	5.7 ± 1.20	3.3 ± 0.11	0.4 ± 0.08	5.5 ± 0.23	$P < 0.001$
18:4n-3	0.9 ± 0.17	2.0 ± 0.14	0.2 ± 0.03	0.2 ± 0.06	5.0 ± 0.42	2.9 ± 1.08	0.2 ± 0.00	0.5 ± 0.03	$P < 0.001$
20:3n-6	1.0 ± 0.01	0.5 ± 0.05	1.2 ± 0.12	1.1 ± 0.01	0.4 ± 0.07	0.3 ± 0.05	1.2 ± 0.10	1.3 ± 0.06	$P < 0.001$
20:4n-6	**(2.3 ± 0.17)^{de}**	**(1.8 ± 0.43)^{e}**	**(6.9 ± 0.22)^{a}**	**(3.5 ± 0.31)^{c}**	**(1.9 ± 0.33)^{e}**	**(1.7 ± 0.20)^{e}**	**(5.1 ± 0.39)^{b}**	**(3.2 ± 0.25)^{cd}**	***P* < 0.001**
20:4n-3	3.3 ± 0.56	5.1 ± 0.27	1.0 ± 0.26	1.5 ± 0.00	6.5 ± 0.05	3.8 ± 0.41	0.9 ± 0.15	2.1 ± 0.03	$P < 0.001$
20:5n-3	**(10.5 ± 0.62)^{b}**	**(15.5 ± 1.16)^{a}**	**(7.6 ± 1.10)^{c}**	**(6.3 ± 0.09)^{c}**	**(5.8 ± 0.73)^{c}**	**(5.1 ± 0.91)^{c}**	**(16.8 ± 1.51)^{a}**	**(10.7 ± 0.55)^{b}**	***P* < 0.001**
22:4n-6	1.1 ± 0.56	0.7 ± 0.12	0.2 ± 0.05	0.4 ± 0.13	0.9 ± 0.14	0.8 ± 0.24	0.5 ± 0.25	0.3 ± 0.11	M, $P <$ 0.01
22:5n-3	5.6 ± 0.47	5.4 ± 0.74	3.8 ± 0.95	4.4 ± 0.97	2.1 ± 0.15	2.1 ± 0.63	6.1 ± 0.59	3.2 ± 0.47	$P < 0.01$
22:6n-3	**(5.7 ± 0.08**	**7.3 ± 1.19)^{a}**	**(1.9 ± 0.41**	**2.2 ± 0.10)^{b}**	**(6.4 ± 0.32**	**6.4 ± 1.16)^{a}**	**(0.8 ± 0.35**	**0.8 ± 0.09)^{c}**	**M, *P* > 0.01**
ΣPUFA	**(42.4 ± 2.7)^{a}**	**(44.7 ± 2.6)^{a}**	**(33.1 ± 1.3)^{bc}**	**(30.1 ± 2.1)^{c}**	**(44.7 ± 1.6)^{a}**	**(36.1 ± 1.6)^{bc}**	**(41.9 ± 0.9)^{a}**	**(36.8 ± 0.6)^{b}**	***P* < 0.001**
Σn-3	27.7 ± 1.4	37.5 ± 3.4	15.7 ± 1.5	16.2 ± 1.1	32.7 ± 1.5	24.5 ± 0.9	25.8 ± 1.6	23.9 ± 1.1	$P < 0.001$
Σn-6	11.6 ± 0.9	6.6 ± 1.2	12.3 ± 0.9	10.4 ± 0.1	11.2 ± 0.5	9.8 ± 0.3	14.0 ± 0.9	11.1 ± 0.7	$P < 0.01$
DHA/EPA	0.5 ± 0.03	0.5 ± 0.06	0.3 ± 0.09	0.4 ± 0.01	1.1 ± 0.17	1.3 ± 0.42	0.0 ± 0.02	0.1 ± 0.01	$P < 0.001$
Unknown	2.5 ± 0.27	2.3 ± 0.41	2.3 ± 0.34	3.0 ± 0.11	2.2 ± 0.32	2.3 ± 0.52	2.3 ± 0.21	2.1 ± 0.26	$P > 0.05$
Σ others*	0.2 ± 0.07	0.1 ± 0.04	0.2 ± 0.08	0.2 ± 0.06	0.2 ± 0.10	0.2 ± 0.13	0.2 ± 0.07	0.2 ± 0.05	

Bold rows represent variables for which the results of two-way ANOVA analysis are discussed (factors: microalgae, form). When factor interactions were significant, the eight groups were compared with a *posteriori* tests ($\alpha = 0.05$), with each compared group appearing in the same set of parentheses. Different letters indicate the presence of significant differences among groups. When significant differences were only found between algal groups independent of whether they were fresh or frozen-concentrated (M, $P < 0.05$), then the a *posteriori* tests were applied among the four data sets representing each algal form (the two groups within a single set of parentheses).

Abbreviations: MONO, *Pavlova lutheri*; T-ISO, *Isochrysis galbana*; NASP, *Nannochloropsis* sp.; CHGRA, *Chaetoceros muelleri*; TAG, triacylglycerol; FFA, free fatty acids; ST, sterol; AMPL, acetone-mobile polar lipids; PL, phospholipids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

MUFA levels were 1.5 and 1.2 times higher in rotifers fed the frozen forms of T-ISO and NASP compared to their respective fresh forms. In rotifers fed T-ISO, this was related to a higher value of 18:1n-7 (4.1 times higher in the frozen form than in the fresh one) (Table II.2).

Rotifers fed either form of MONO and CHGRA showed no difference in their PUFA contents. PUFA levels decreased by a factor of 1.2 in rotifers fed the frozen forms of T-ISO compared to the ones fed the fresh form, mainly due to a decrease in 18:3n-3 and 18:4n-3. In rotifers fed the frozen form of NASP, the decrease in PUFA compared to those fed the fresh form is more related to decreases in the proportions of AA and EPA.

The AA content was the highest in rotifers fed with the fresh culture of CHGRA (6.9%) followed by rotifers fed the fresh culture of NASP (5.1% of TFA). Only rotifers fed on these microalgal diets showed different AA level between the frozen and fresh forms, with decreases of 2 and 1.6 times in rotifers fed the fresh form. The highest EPA proportion was detected in rotifers fed frozen MONO (15.5%) and fresh NASP (16.8% TFA); the form of the microalga diet (fresh, frozen) had no effect on the rotifers' DHA content. The highest DHA levels were observed in rotifers fed the flagellates MONO and T-ISO (6.4% of TFA on average).

II.4 DISCUSSION

This study is one of the few studies reporting the lipid enrichment from different frozen-concentrated microalgae species to rotifers. The EFA rotifer content was highly correlated with the EFA content present in both frozen-concentrated and fresh microalgae species. In general, the fatty acid composition of rotifers reflected the relative levels of the fatty acids present in each preserved microalgal species, and we noted linear relationships between the fatty acid content in microalgae *vs* that in rotifers. One exception was noted for the FFA level in CHGRA: the high level observed could indicate suboptimal rearing conditions of this alga in the photobioreactor or that problem occurred during preservation process (Parrish, 1999; Pernet & Tremblay, 2003).

The total lipid and fatty acid composition of live microalga cultures obtained in the present study are in agreement with previous observations (Brown *et al.*, 1997; Muller-Feuga *et al.*, 2003). Our live NASP contained 14 times more AA and 39 times more EPA than the levels reported by Palmtag *et al.* (2006), which suggests a good optimization of the semi-continuous culture of NASP in our study. The lipid composition of fresh CHGRA compared well with data published by Pernet *et al.* (2003) even though we observed higher AA (9.6% *vs* 2.1%) and EPA (14.1% *vs* 12.5%) levels and a slightly lower DHA level (1.7% *vs* 2.1%.) in the present study. The experimental conditions do not allow us to explain the differences that occurred between fresh and frozen-concentrated microalgae. These could be due to different factors, including differences between algal

cultures, cooling rate during the freeze-concentration process (Molina Grima *et al.*, 1994; Taylor & Fletcher, 1999), nature of the cryoprotectant (Tzovenis *et al.*, 2004), or to other factors such as storage period prior to lipid analysis and so on. Nevertheless, the comparison with previous studies on fresh algae presented above and the similarity of lipid profiles between the fresh and frozen-concentrated forms indicate a relatively comparable food quality between the two forms of algae.

Palmtag *et al.* (2006) provided recent data on rotifers enriched with live and concentrated T-ISO and NASP, but no such data are available for CHGRA and MONO. For T-ISO and NASP, rotifers fed fresh algae in that study and ours showed comparable EFA levels. However, we observed slightly higher DHA levels in the present study. Rotifers fed the fresh NASP culture generally showed twice the levels of AA and EPA compared to levels reported by Palmtag *et al.* (2006), but the proportions are very similar when concentrated products are compared. Our results also compare well with those obtained by Lubzens *et al.* (1995) in rotifers fed frozen-concentrated NASP. Overall, rotifers fed the frozen form of NASP could be a good source of EPA and AA for early feeding of marine fish larvae.

We found few differences in fatty acid levels from rotifers fed the fresh and frozen forms of MONO, and the fatty acid composition of rotifers fed fresh MONO is in agreement with that reported by Nichols *et al.* (1989). Rotifers fed the frozen form of MONO could be a good source of DHA (7.3 ± 1.2% of TFA) and EPA (15.5 ± 1.2% of

TFA) for marine fish larvae. Although live and frozen forms of CHGRA have shown some differences in total lipids and fatty acids, no differences were detected between rotifers fed both forms except for AA, which were higher in rotifers fed the fresh form. Overall, CHGRA remains the best source among the microalgae tested for AA supplementation.

In general, the frozen-concentrated forms of all microalgae used in the present study could be good substitutes for the fresh forms. However, the different microalgae exhibit different lipid compositions that are also present in the frozen form, and this has to be taken into account in the elaboration of diets for rotifer enrichment. Frozen-concentrated microalgae could be used to ensure constant high availability of PUFAs, especially EFAs. During larval growth, there is a rapid proliferation of cellular membranes into which 16:0, 18:1n-9, EPA, and DHA are incorporated as primary structural components. These components enable the fluidity required for the proper functioning of biological membranes (Sargent *et al.*, 2002). As larval stages are characterized by very rapid growth, their inability to synthesize sufficient amounts of PUFA make them more reliant on food sources (Willey *et al.*, 2003). We propose a diet consisting of a mixture of frozen-concentrated MONO (as a good source of EPA and DHA) and either frozen CHGRA (as a good source of AA) or frozen NASP (as a good source of AA and EPA) as enrichment for rotifers used to feed marine fish larvae. Further experiments should be conducted to test if assimilation differs when rotifers are enriched with mono- or multispecific frozen-concentrated microalgal preparations.

CHAPITRE III. EFFECT OF DIETARY ARACHIDONIC ACID ON WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*) LARVAL DEVELOPMENT AND BACTERIAL COLONIZATION OF LARVAL TISSUES AND CULTURE TANKS

III.0 ABSTRACT

Intensive cultivation conditions of marine fish larvae may induce mortality events; these commonly occur because of the development of opportunistic or pathogenic bacteria. The aim of this study was to determine whether arachidonic acid (AA) enrichment could affect larval growth, AA assimilation in larval tissues, and bacterial colonization in both the environment (tank water) and in larval tissues of winter flounder (*Pseudopleuronectes americanus*). Three replicate larval rearing tanks were fed rotifers enriched with AA and a second group of three replicate tanks was fed control rotifers with no AA enrichment. Total bacterial abundance was monitored in rotifer culture water and in larval tanks by flow cytometry whereas *Vibrio* sp. abundance was monitored by culture on specific media. Bacterial colonization of larval tissues (intestinal tract, gills and skin) was examined using photonic microscopy, bacterial community diversity in rotifer cultures and in larval tanks was assessed by molecular tools, and larval fatty acid composition was analyzed separately in the neutral and polar lipid fractions. The relative proportion of AA in enriched rotifers was three-fold higher than in control rotifers. However, dietary AA enrichment did not alter the AA content in larval cell membranes. The enrichment treatment affected the bacterial flora in rotifer and larval rearing waters both quantitatively and qualitatively. Bacterial communities observed in AA enriched larval tanks were different from those found in control tanks and *Vibrio* sp. counts were significantly higher in AA enriched rotifer cultures and larval tanks. No AA effect was observed on bacterial colonization of larval tissues. Though no significant effect of AA enrichment on larval growth was detected, earlier

metamorphosis in smaller AA-enriched larvae was observed which is surprising and need to be investigated more closely.

III.1 INTRODUCTION

Marine fish larvae may be exposed to numerous stress factors in intensive culture conditions; these may cause high vulnerability to microbial infections and lead to mass mortality (Hansen & Olafsen, 1999; Olafsen, 2001; Thomson *et al.*, 2005). Microbial infections are probably due to the proliferation of opportunistic pathogens rather than specific obligate pathogenic bacteria (Olsen *et al.*, 2000). Identifying and preventing potential stress sources are the primary approaches to consider for reducing opportunistic infections. However, monitoring and modifying bacterial communities or improving the efficiency of the larval immune function using dietary supplements can be an effective complementary tool.

Dietary lipids and their constituent fatty acids are required by very small and rapidly developing larvae as they supply energy as well as the cell components of structural membranes (Sargent *et al.*, 2002). Essential fatty acids (EFA), *i.e.*, those that cannot be synthesized *de novo*, and their derivative products, termed eicosanoids, are highly biologically active and are involved in immunity (Tocher, 2003). Arachidonic acid (AA), an n-6 EFA, is one of the main components of some membrane phospholipids (*i.e.*, phosphatidylinositol) and the main precursor of eicosanoids (*i.e.*, series-2 prostanoids and series-4 leukotrienes), which are physiologically more active than those derived from eicosapentaeonic acid or EPA (20:5n-3) (*i.e.*, series-3 prostanoids and series-5 leukotrienes) (Bell and Sargent, 2003; Tocher, 2003). Much evidence indicates that AA is an important

factor regulating stress response and immune responses. For example, AA, fed prior to handling stress has been shown to improve survival (Koven *et al.*, 2001) and growth (Bessonart *et al.*, 1999) in gilthead seabream (*Sparus aurata*) and survival in larval Asian seabass (*Lates calcarifer*) (Dhert *et al.*, 1990). Summer flounder (*Paralichthys dentatus*) larvae receiving AA supplements showed significantly higher growth, survival and salinity stress tolerance than those fed lower levels of AA (Willey *et al.*, 2003). Nevertheless, the contribution of AA to growth and survival may be masked if other EFA levels are sub-optimal (Bessonart *et al.*, 1999; Koven *et al.*, 2001).

AA can also act as a secondary messenger in the inflammation process. In marine larval striped bass (*Morone saxatilis*), AA both increases the cortisol response and promotes higher leucocyte numbers (Harel *et al.*, 2001). In Pacific oyster (*Crassostrea gigas*) supplemented with AA, total hemocyte numbers tend to increase along with the percentage of phagocytic hemocytes and the basal reactive oxygen species production in granulocytes and hyalinocytes (Delaporte *et al.*, 2006).

Dietary lipid enrichments may have important effects on intestinal bacterial flora (Ringø *et al.*, 2002). Recently, Korsnes *et al.* (2006) reported a significant effect of different commercial rotifer enrichments on bacterial concentration in both the rearing water and the gastro-intestinal tract of larval cod (*Gadus morhua*).

Winter flounder *Pseudopleuronectes americanus* is a common inshore marine flatfish of Atlantic Canada, ranging from Labrador to Georgia (USA) (Scott & Scott, 1988). Larval production has been successfully achieved since the 1990s, but the production of plentiful high quality larvae remains a bottleneck, with high mortalities occurring during larval culture. Our objective was to study the effect of an AA enrichment on growth and assimilation in larval tissues as well as bacterial colonization in both the environment (tank water) and in larval tissues. AA enrichment was performed indirectly through an enrichment of the rotifers (*Brachionus plicatilis)* that were given as live prey during the first larval feeding stage.

III.2 MATERIALS AND METHODS

Rotifers: culture and sampling

Experiments were conducted at the Station aquicole de Pointe-au-Père (UQAR-ISMER, 48° 27' N; 68° 32' W, Quebec, Canada) from April to August 2006. Rotifers (*Brachionus plicatilis*) were cultured in 18-l tanks at 20–25°C using filtered (0.2 µm) seawater at a salinity of 27‰ and aeration. Rotifers were fed a mixture of three living microalgal species (*Nannochloropsis occulata*, *Pavlova lutheri* and *Isochrysis galbana,* v/v/v) in combination with a commercial enrichment (Culture Selco Plus, INVE Aquaculture, Belgium). AA-enriched rotifers also received a supplement of arachidonic acid (AA; Sigma-Aldrich #10931-1G) at a ratio of 1 mg 10^{-6} phytoplankton cells according to Seguineau *et al.* (2005). The flagellates *Isochrysis galbana* and *Pavlova lutheri* are good sources of DHA (Kayama *et al.*, 1989); *Pavlova lutheri* contains a high relative proportion of EPA, and the chlorophycean *Nannochloropsis occulata* is a good source of EPA and AA (Kayama *et al.*, 1989). Microalgae were obtained from Nutrocean Inc. (Rimouski, Québec, Canada). Microalgal cultures were carried out in natural filtered seawater enriched with f/2 medium (Guillard, 1975) and harvested in a semi-continuous system in closed photobioreactors under the following conditions: pH 7.5–8.5, temperature 20–23°C, salinity 28–30‰, continuous illumination (4.4–5.8 W m^{-2}), and bubbling air at 10 ml l^{-1} of culture min^{-1} and CO_2 (1% in air). Rotifers were sampled every two weeks during the larval experiment. Two samples of 20 000 rotifers (Lubzens *et al.*, 1995) were collected from each tank and pre-rinsed with filtered seawater (0.2 µm) on a 50 µm net. Samples were

filtered onto pre-combusted (450°C) GF/C filters (25 mm). Following the filtration step, filters were stored at -80°C in 1 ml chloroform in amber glass vials with Teflon-lined caps until lipid extraction. The dry weight was determined after drying at 70°C for 24 h.

Larvae: culture and sampling

Newly hatched flounder (day 0) larvae were reared in triplicate 57 l cylindro-conical polyethylene tanks for each dietary treatment. Flounder larvae were fed rotifers from day 4 to 26 after hatching. From day 26 to metamorphosis, they received a mixed diet: rotifers as described above plus a dry diet (Lansy Cold Water, INVE aquaculture, Dendermonde, Belgium).

Rotifers were given in excess and their density was adjusted three times a day to 5 rotifers ml^{-1}. Larval tanks were supplied with filtered seawater (10 µm, 27 ± 1‰). Larval culture tanks were maintained at 10°C with a photoperiod of 12 L:12 D. Water renewal was stopped for 8.5 hours once the light period began in the morning. At this time, a pseudo-green-water preparation was added to the tanks. This microalgal preparation was a refrigerated (4°C) concentrated liquid mixture of the three species (v/v/v) used for rotifer enrichment that was obtained from Nutrocean Inc. (Rimouski, Canada). Water circulation was resumed to enable complete renewal of tank water during the night. Flounder larvae were sampled just before light-on and the first meal to ensure that stomachs were empty. Samplings were done at mouth opening (day 4), days 15, 26, 32, and 38 (during metamorphosis), and at settlement. Larvae were randomly sampled in each tank and

individually rinsed three times in Millipore-filtered seawater. For each tank, four larval pools (60 larvae from day 4 to 26, 40 larvae for days 32 and 38, and 20 at the juvenile stage) were frozen at -80°C ; two of these were used for dry weight determination for each larval tank.

At the end of the rotifer-feeding period (day 26), ten larvae per larval tank (total of 30 in the AA-enriched treatment and 20 in the control treatment) were fixed with 10% formaldehyde (≥ 24 h at ambient temperature) for light microscopy observations. The number of control larvae was lower as one of the initial three tanks was lost on day 26.

Lipid analysis

Lipid analyses were done at the Coastal Zones Research Institute (IRZC, Shippagan, New Brunswick, Canada). Lipid extraction from rotifers and larvae was carried out on ice according to the modified Folch method (Folch *et al.*, 1957) as described by Parrish (1999). Chloroform was substituted with dichloromethane (Chen *et al.*, 1981) for a final dichloromethane:methanol volume ratio of 2:1. Lipid samples were homogenized in 3 ml of the dichloromethane:methanol mixture (2:1 v/v). The grinder was rinsed twice with 3 ml of dichloromethane:methanol (2:1 v/v), and 2.25 ml KCl (0.88%) were added for a final CH_2Cl_2–MeOH–KCl ratio of 2:1:0.6 v/v/v (Folch *et al.*, 1957). The homogenates were mixed and centrifuged at 432 g for 2 min to obtain a biphasic system. The bottom lipid fraction was removed and transferred to a clean tube with a Pasteur pipette. The solvent was evaporated under nitrogen flow and lipids were resuspended in 100 µl CH_2Cl_2.

Lipid classes were determined on one 2 µl aliquot from each extract. Extracts were spotted onto S-III Chromarods (Iatron Laboratories, Tokyo, Japan). Lipid classes were separated using four solvent systems (Parrish, 1999) and were determined using the flame ionization detector of the Iatroscan MK-6s. The following lipid classes were looked at in this study: triacylglycerols, free fatty acids, sterols, acetone-mobile polar lipids, and phospholipids. They were summed to obtain total lipids, and chromatograms were analyzed using integration software (Peak Simple version 3.2, SRI Inc.).

Lipids extracted from larvae were divided into neutral and polar lipids (NL and PL), using silica gel (100 mg of 6% water mass). Aliquots (200 µl) of lipids (300 µg) were loaded onto the column. NL were eluted in 2 ml chloroform–methanol (98:2) and PL in 5 ml of methanol. Fatty acid methyl esters (FAMEs) were obtained from the constituent fatty acids by acid-catalyzed transesterification with 2% (v/v) H_2SO_4 in methanol at 100°C.

Bacterial analysis

For each feeding treatment, two larval tanks were surveyed for bacterial analysis. For each larval sampling day, 2 × 4 ml of larval rearing water from replicate larval tanks and rotifer tanks were sampled for total bacterial abundance and fixed in 2% formaldehyde at pH 7. Samplings were done in the morning before lights-on and before the addition of microalgae for the pseudo-green-water in larval tanks. Water from rotifer tanks was sampled before the enrichment procedure in the morning. Seawater was sampled before being added to the larval tanks, it is hereafter termed "natural seawater". Samples were

frozen at -80°C until analysis. The total abundance of bacteria was determined using an Epics Altra flow cytometer (Beckman-Coulter) equipped with a 488 nm argon laser operated at 15 mW. A 1 ml subsample was incubated with 0.25 µl of SYBR Green I (Molecular Probes Inc., Oregon) for 30 min at room temperature in the dark. Fluorescent beads (10 µl, 0.96 µm in diameter; YG Fluoresbrite, Polysciences, USA) were added to each sample as an internal standard (Lebaron *et al.*, 2002). The bacterial concentration was calculated by weighing a sample before and after a 3 min analysis with the cytometer to estimate the volume analyzed. This volume was corrected for a dead volume of 50 µl (the water volume taken from the sample tube but not counted when data acquisition is stopped). Total abundance of the different bacterial sub-populations were graphically discriminated and enumerated using right angle light scatter (SSC, related to cell size) and the green fluorescence of nucleic-acid-bound SYBR Green I measured at 525 ± 5 nm. The sub-population with high nucleic acid contents (HNA) was discriminated from the sub-population with low nucleic acid contents (LNA) (Lebaron *et al.*, 2002).

Bacterial diversity was analyzed on larval and rotifer rearing water and on source seawater. Seawater sample aliquots of 20 or 100 ml were filtered in duplicate on 0.2 µm polycarbonate filters (25 mm diameter). Filters were cut and transferred to sterile 1.5 ml tubes containing 840 µl of lysis buffer (40 mM EDTA, 50 mM Tris, pH 8, 0.75 M sucrose) and 50 µl of lysozyme (20 mg ml^{-1}). The samples were incubated at 37°C for 45 min according to the method developed by Ghiglione *et al.* (2005) of Observatoire Océanologique de Banyuls-sur-mer. Before incubation at 55°C for 60 min, 100 µl of

sodium dodecyl sulfate solution (10%) and 10 µl of proteinase K (20 mg ml^{-1}) was added to each sample.

Phenol–chloroform–isoamyl alcohol (25/24/1 ; 900 µl) was added to each sample, which was then centrifuged twice at 13 400 g for 10 min. Between the two centrifugation steps, the resulting aqueous top layer was transferred to a separate sterile tube ; this top layer was extracted with 800 µl of chloroform–isoamyl alcohol (24/1) and centrifuged at 13 400 g for 10 min. The final aqueous layer was placed in a separate tube with one volume of isopropanol (100%) and stored at -20°C for 120 min to enable DNA precipitation. Samples were then centrifuged at 16 100 g for 30 min. The DNA pellet was washed a second time by removal of the top layer and addition of 500 µl of ethanol (70%), and centrifugation at 16 100 g for 20 min. Ethanol was evaporated using a Vacufuge system (Eppendorf) for approximately 20 min and the resultant DNA pellet was resuspended in 100 µl of Tris EDTA buffer 1X pH 8. RNA was eliminated from the sample by a RNaseA treatment. Each sample was diluted (1:100) and placed in a 5 µl quartz microcell (Hellma) to determine optical density at 260, 270 and 280 nm, using a DU 800 (Beckman-Coulter) spectrophotometer. DNA absorption was read at 260 nm and enabled the calculation of the DNA concentration. The ratio 260 nm:280 nm was calculated to check the purity of the DNA solution (Lemarchand *et al.*, 2005). The ratio 260 nm:270 nm was used to check for possible phenol contamination (Planken *et al.*, 2005).

PCR amplification of the 16S rDNA gene was then carried out using an universal set

of primers, (1) 341F-GC (5′-CGC-CCG-CCG-CGC-CCC-GCG-CCC-GTC-CCG-CCG-CCC-CCG-CCC-GCC-TAC-GGG-AGG-CGA-CAG-3′), (2) 907R (5′-CCGTCA-ATT-CMT-TTG-AGT-TT-3′), following the method proposed by Schäfer and Muyzer (2001). Three PCR amplifications were performed on each DNA sample to overcome the effect of PCR biases (Perreault *et al.*, 2007). The final PCR reaction mixture was performed in a total volume of 50 µl. The master mix contained 5 µl of 10X PCR buffer (QIAGEN), 0.5 µl of 2 mM dNTPs stock solution, 1 µl (stock concentration: 50 µM) of each of the forward and reverse primers and 1 unit of HotStar Taq DNA polymerase (QIAGEN). This mixture was completed to 50 µl with 42 µl of sterile distilled water containing 200 ng of previously extracted DNA samples. A touchdown PCR was performed using a Mastercycler epS (Eppendorf) thermal cycler. Amplification reaction conditions started with an initial denaturation step at 94°C for 15 min (HotStar Taq Polymerase activation step) followed by 20 cycles of 1 min at 94°C (denaturing step), 1 min at 65°C (annealing step), and 3 min at 72°C (extension step), in which the annealing temperature was decreased by 0.5°C every cycle until it reached 55°C. Then, 15 cycles of 1 min at 94°C, 1 min at 55°C and 3 min at 72°C were performed before a final extension period of 7 min at 72°C.

An aliquot (5 µl) of each amplification reaction was analyzed on 1% (w/v) agarose gel in 1X TAE buffer stained with ethidium bromide, run at 90 V for 75 min, and visualized under UV light. Gel images were imported into AlphaImager HP (AlphaInnotech). Amplicons were purified with the MinElute (QIAGEN) columns according to the manufacturer's instructions and stored at -20°C until DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE) was performed using a DGGE-4001-Rev-B (C.B.S. Scientific Company, CA, USA) system. Amplicons were run on a 6% polyacrylamide gel with a 20–80% linear gradient of urea and formamide at 100 V and 35 mA for 13 h (Schäfer & Muyzer, 2001). Gels were then stained with SYBR Green I (Molecular Probes, Oregon) for 40 min according to the manufacturer's instructions. Gels were photographed under UV light and bands were discriminated using the AlphaImager HP system. DGGE fingerprints were clustered to visualize bacterial community relationships using the AlphaImager HP software. Similarity matrices were based on Jaccard similarity coefficients. The similarity matrix based on natural seawater and larval tank water samples was further analyzed using non-metric multidimensional scaling (NMDS) (Clarke, 1993; Bourne *et al.*, 2006).

Vibrio sp. colony forming units (CFU) were enumerated in triplicate from day 4 to 26, after 24–48 h of incubation on selective medium (thiosulfate–citrate–bile salts–sucrose, TCBS, Merck KgaA, Germany) at room temperature in darkness.

Histology

Fixed larvae were dehydrated in an ascending series of ethanol solutions and embedded in methacrylate resin. Tissues were sectioned (3 µm thickness) with a Supercut Reichert-Jung model 2050 (Cambridge Instruments GMbH, Germany). They were mounted onto glass slides and stained with Gram Staining kit (Sigma #77730) and photographed at 1000× (Olympus BX41, Japan). The occurrence of bacteria was determined and quantified

in gut lumen, gills, and skin. Bacterial density was randomly calculated within the intestinal lumen (nb. of bacteria mm^{-2}) and the ratio "area occupied by bacteria/total tissue area" was calculated on three histological gill and fin sections for each larva with Image Pro Plus Software (Media Cybernetics, Canada).

Statistical analysis

All statistical analyses were done using STATISTICA software version 6.0 (Statsoft, USA), with $\alpha = 0.05$. Normality of the data was examined using the Kolmogorov-Smirnov test. Homoscedasticity was tested with the Brown-Forsyte test (Zar 1999). Differences between treatments were tested using one- (rotifer diet, D) or two- (rotifer diet and sampling time, T) way analysis of variance. For subsequent multiple comparisons, Tukey tests or Tukey tests for unequal n were performed when appropriate. The square-root transformation was used for data related to *Vibrio* counts in seawater sampled prior to filling larval tanks. The Games & Howell test was used when heteroscedasticity was observed.

III.3 RESULTS

Larval growth

Before exogenous feeding, larvae from the AA-enriched group were slightly longer. Length was similar between the two groups during larval rearing except at settlement, when control larvae were 5% longer (D × T: $p < 0.001$, $F = 5.01$, $df = 5$) (Figure III.1A). Larvae exhibited similar widths during rearing except at settlement, when control larvae were 16% wider than AA-enriched larvae (D × T: $p < 0.001$, $F = 20.75$, $df = 5$) (Figure III.1B). Dry weight significantly increased on day 26 (T: $p < 0.001$, $F = 49.26$, $df = 5$) and stayed roughly constant until settlement (D, D × T: $p > 0.05$) (Figure III.1C). Settlement peaks occurred earlier in flounder larvae fed AA-enriched rotifers (487 ± 28 degree days) than in those fed control rotifers (585 ± 94 degree days).

Lipid analysis

The lipid class composition of rotifers was similar between dietary treatments (Table III.1). In contrast, the fatty acid composition varied as a function of dietary treatment : the level of AA in enriched rotifers was three-fold higher than that of control rotifers while other essential fatty acids such as EPA and DHA remained similar between diets.

Larval lipids were mainly phospholipids (89%) and sterols (9%). Larvae contained small amounts of triacylglycerols (2%). After mouth opening, there were sharp decreases

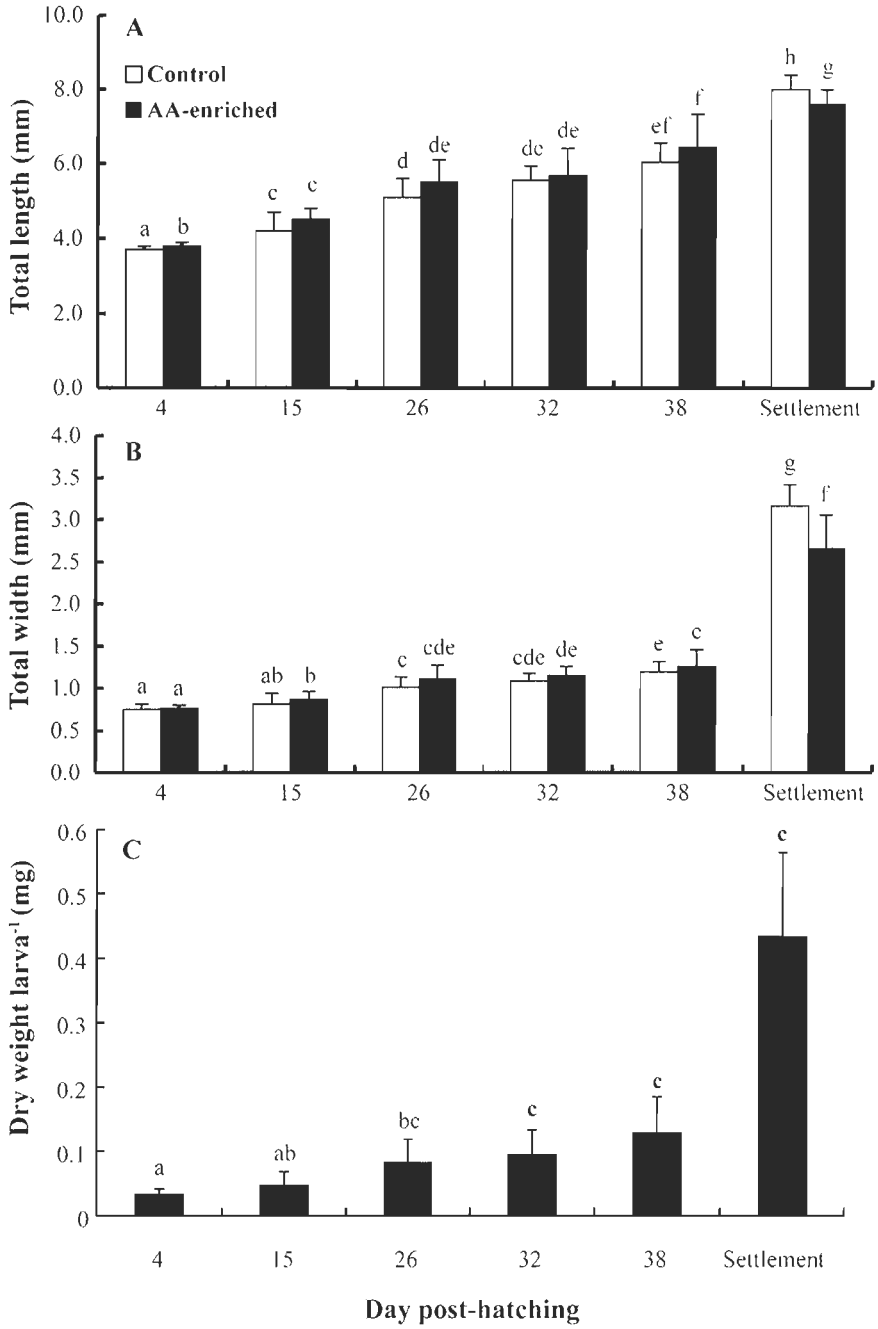

Figure III.1 Mean (± SD) total length (A), width (B) and dry weight (C) of winter flounder larvae fed control and AA-enriched rotifers. Different letters indicate significant differences among sampling times or diets.

Table III.1 Lipid classes and fatty acid composition (mean ± SD) in rotifers fed control and AA-enriched diets. Asterisks indicate a significant difference between the two diets. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

	Control rotifers	AA-enriched rotifers	Summary of ANOVA results
Lipid classes (% weight of total lipids)			
Triacylglycerol	16.0 ± 5.2	17.8 ± 5.6	All NS
Free fatty acids	7.0 ± 2.3	7.4 ± 1.4	T: $p < 0.05$; D × T: $p > 0.05$
Sterol	4.3 ± 0.9	4.1 ± 1.3	All NS
Acetone mobile polar lipids	5.4 ± 2.3	5.5 ± 2.8	All NS
Phospholipids	67.3 ± 7.8	65.2 ± 8.4	All NS
Fatty acid proportions (% TFA) and ratio			
AA	0.9 ± 0.2	3.0 ± 0.9*	D: $p < 0.001$; D × T: $p > 0.05$
EPA	3.7 ± 0.6	3.7 ± 0.9	All NS
DHA	2.7 ± 0.5	2.7 ± 0.6	T: $p < 0.01$; D × T: $p > 0.05$
SFA	21.6 ± 0.9	22.3 ± 1.4*	D, T: $p < 0.01$; D × T: $p > 0.05$
MUFA	55.5 ± 2.7*	53.2 ± 3.5	D, T: $p < 0.01$; D × T: $p > 0.05$
PUFA	22.9 ± 2.4	24.5 ± 2.8*	D, T: $p < 0.01$; D × T: $p > 0.05$
DHA:EPA	0.7 ± 0.1	0.8 ± 0.2	All NS

D: diet; T: sampling time; D × T: interaction between D and T factors; NS: not significant.
* indicates significant dietary effect.

in the proportions of EPA and DHA proportion in NL (Figure III.2C, 2E). These decreases were less pronounced in PL (Figure III.2D, 2F), and the AA proportion in PL increased overtime (Figure III.2B). However, at the end of the rotifer-feeding period, there was no significant difference of AA retention between treatments both in either larval NL (Figure III.2A) or PL. The relative proportion of n-6 DPA (22:5n-6) was very low (<0.6% TFA) in both NL and PL.

Bacterial analysis

In source seawater, total bacterial abundance was similar from day 0 to 15 ($2.76 \pm 0.32\ 10^5$ cells ml^{-1}) and was 1.3-fold higher on day 26 ($3.27 \pm 0.57\ 10^5$ cell ml^{-1}) (D: $p < 0.001$, $F = 11.12$, $df = 3$). *Vibrio* sp. counts were similar from day 0 to 15 (0.6 ± 0.7 CFU ml^{-1}) but slightly higher on day 26 (2.2 ± 0.3 CFU ml^{-1}) (D: $p < 0.001$, $F = 20.35$, $df = 3$).

In rotifer tanks, total bacteria abundance dropped significantly on day 26 (T: $p = 0.001$, $F = 9.32$, $df = 2$) in the two treatments (D, D × T: $p > 0.05$) (Figure III.3A). The *Vibrio* sp. concentration in AA-enriched rotifer tanks increased significantly after 4 days of larval rearing (D × T: $p < 0.001$, $F = 24.44$, $df = 2$) and was higher than in control rotifer tanks on day 15 (Figure III.3B). The similarity matrix of Jaccard coefficient showed different bacterial communities between control and AA-enriched rotifers and between days 4 and 26 of larval rearing (Table III.2).

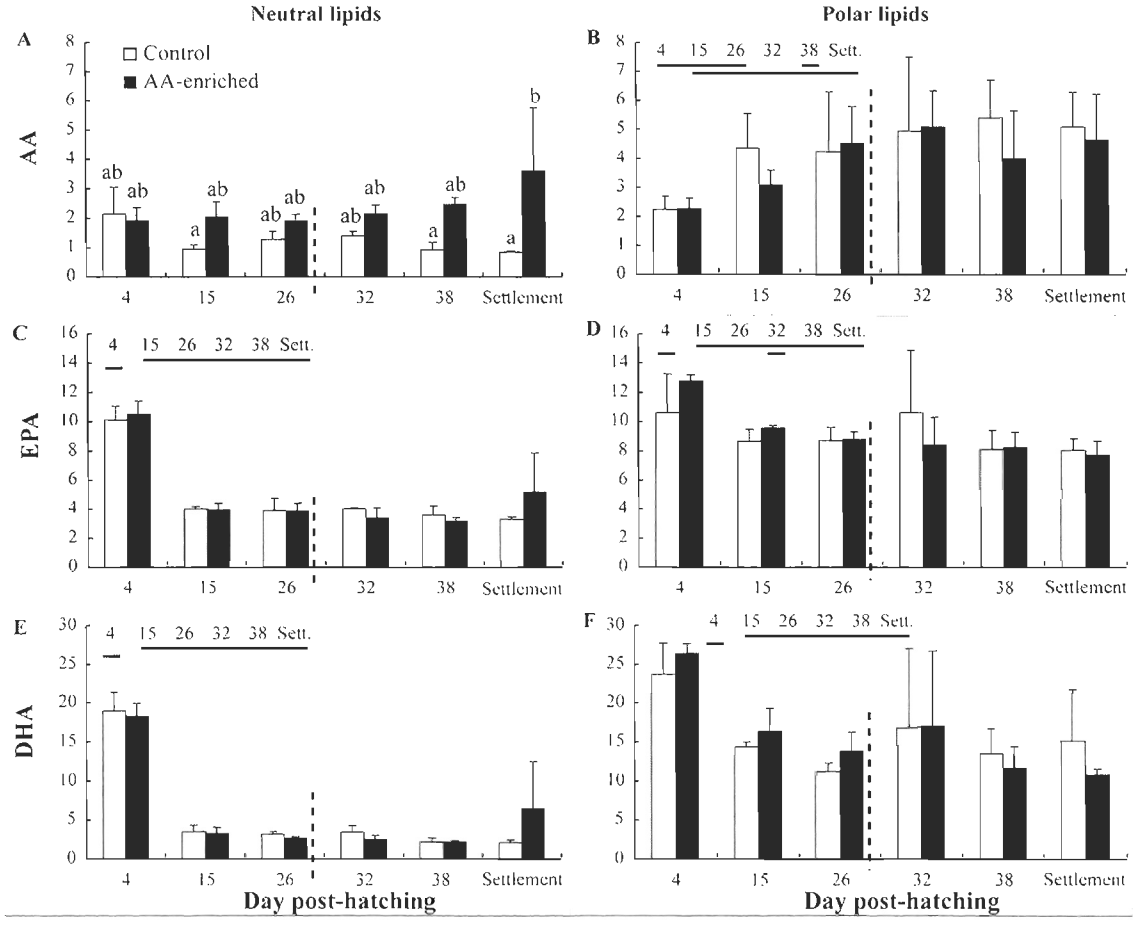

Figure III.2 Essential fatty acids (as percentage of TFA ± SD) in winter flounder larvae fed control or AA-enriched rotifers. Different letters indicate significant differences among sampling times or diets. The results of the *a posteriori* tests are presented: solid lines underlie those days that are not significantly different one from the other.

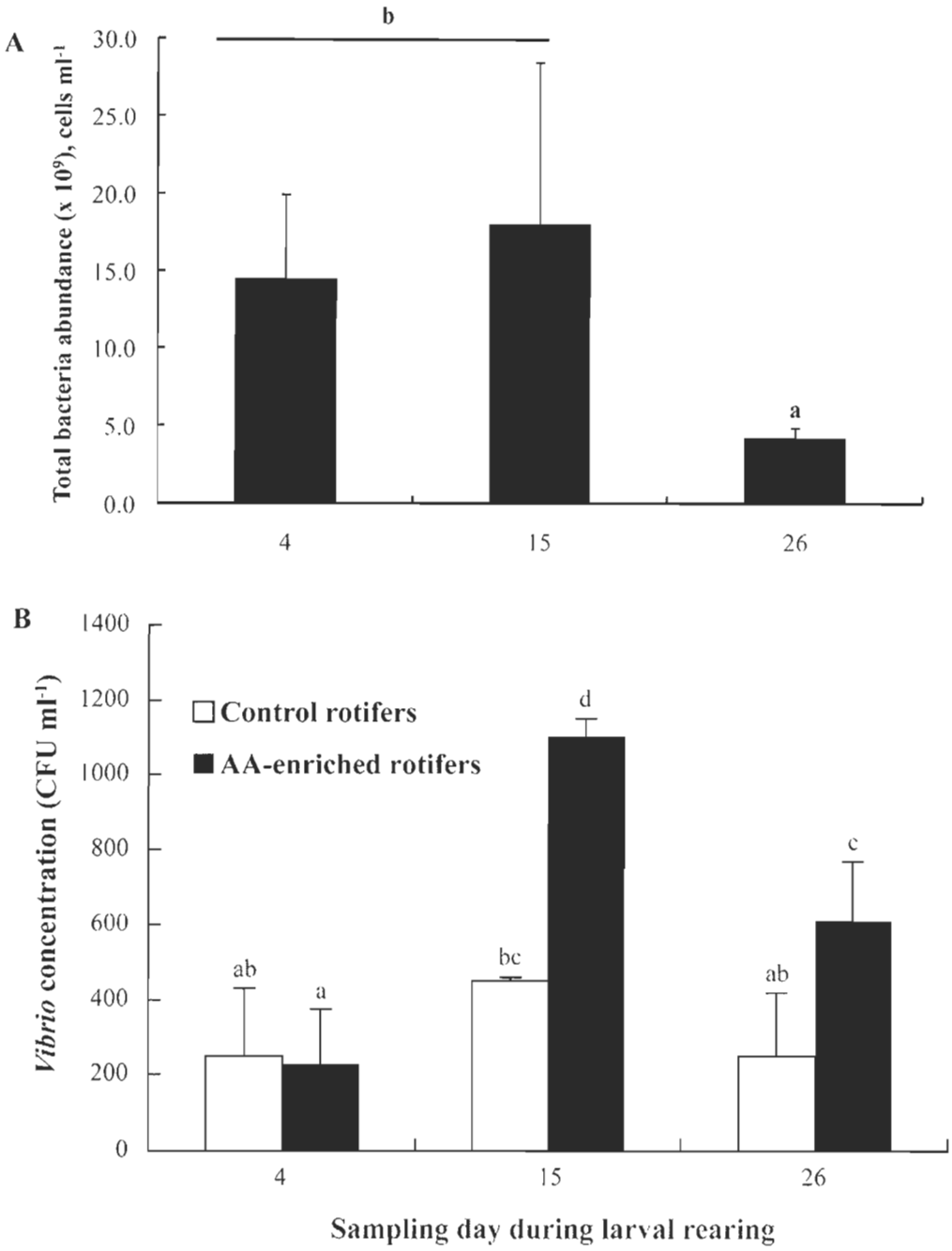

Figure III.3 A) Total bacteria abundance (cells ml^{-1} ± SD) and B) *Vibrio* abundance (CFU ml^{-1} ± SD) rotifer tanks. Different letters indicate significant differences among sampling times or diets.

Table III.2 Similarity matrix of Jaccard coefficients calculated from PCR-DGGE binary data in rotifer tank water. CT: Control rotifers at day (d) 4 and 26. AA: AA-enriched rotifers at day 4 and 26.

	CT-d4	AA-d4	CT-d26	AA-d26
CT-d4	1			
AA -d4	0.17	1		
CT-d26	0.10	0.11	1	
AA-d26	0.15	0.17	0.17	1

Total bacteria abundance in larval tanks varied differently between treatments throughout the experimental period (D × T: $p = 0.012$, $F = 2.92$, $df = 6$) (Figure III.4A) and was highly variable. The lowest abundances were observed on day 38 in controls and on day 4 in the AA-enriched treatment while the highest abundances were observed on day 26 (both treatments) and day 32 (controls only). *Vibrio* sp. concentration peaked on day 26 in the AA-enriched treatment (D × T: $p < 0.001$, $F = 943.77$, $df = 3$) (Figure III.4B).

Bacterial communities in larval and rotifer tank were generally composed of LNA and HNA bacteria cells, except for day 26 when LNA bacteria were absent from the culture water of AA-rotifer tanks. On the same day, a third bacterial sub-population appeared in both larval and rotifer tanks that was identified by the very high fluorescence of the nucleic acid content (VFHNA). Such a VFHNA sub-population was never detected in the natural seawater (sampled prior to use in the larval tanks).

The NMDS analysis of DGGE community profiles indicated differences in bacterial communities in larval tanks between day 4 and 26 (Figure III.5). The similarity of bacterial communities increased on day 26 for all water samples (the two larval groups and natural seawater). However, the similarity of bacterial communities on that same day was stronger between natural seawater and larval control tanks than with AA-enriched larval tanks.

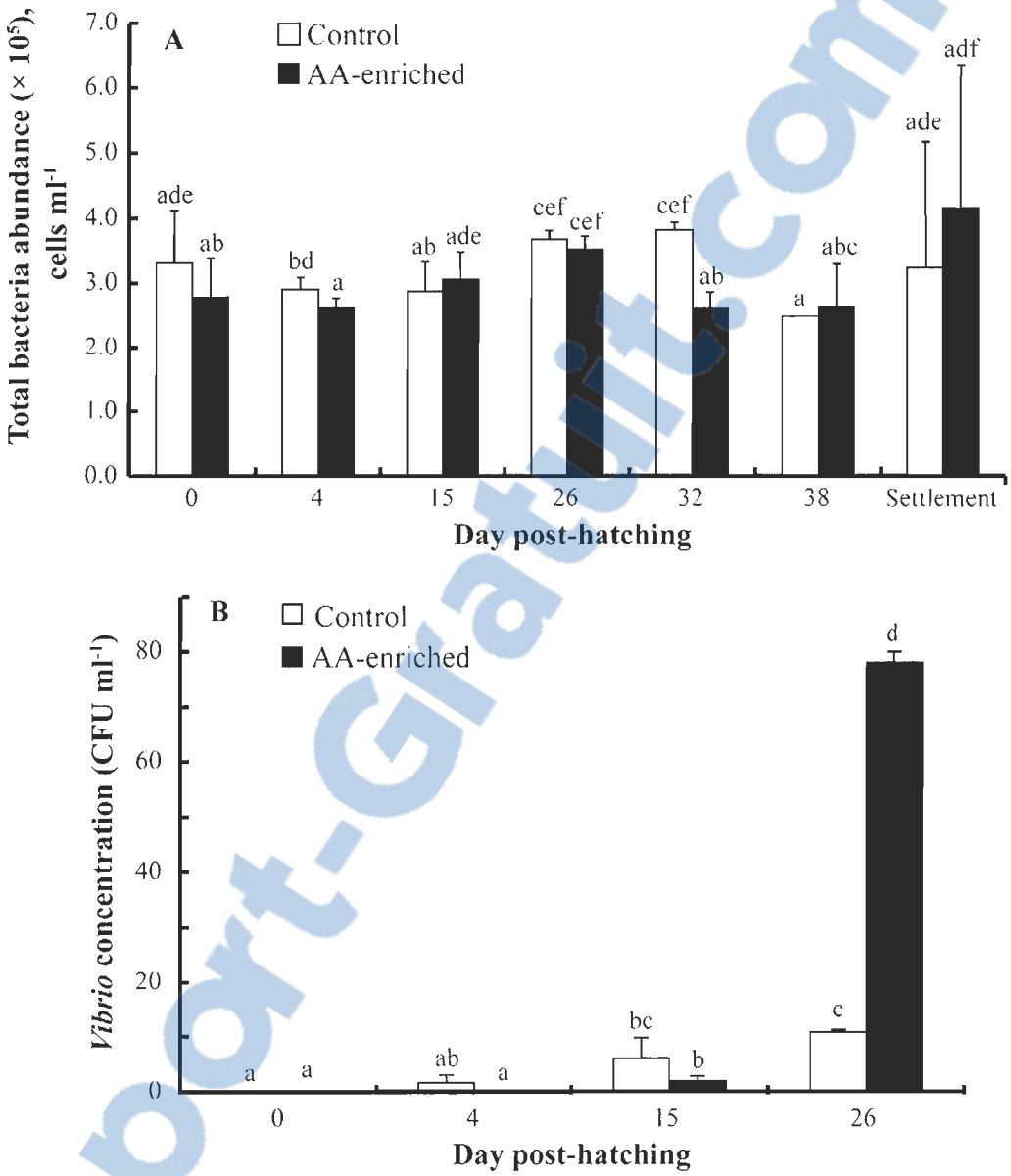

Figure III.4 A) Total bacteria abundance (cells ml^{-1} ± SD) determined using flow cytometry in control and AA-enriched larval tanks. B) *Vibrio* abundance (CFU ml^{-1} ± SD) in control and AA-enriched larval tanks. Different letters indicate significant differences among sampling times or diets.

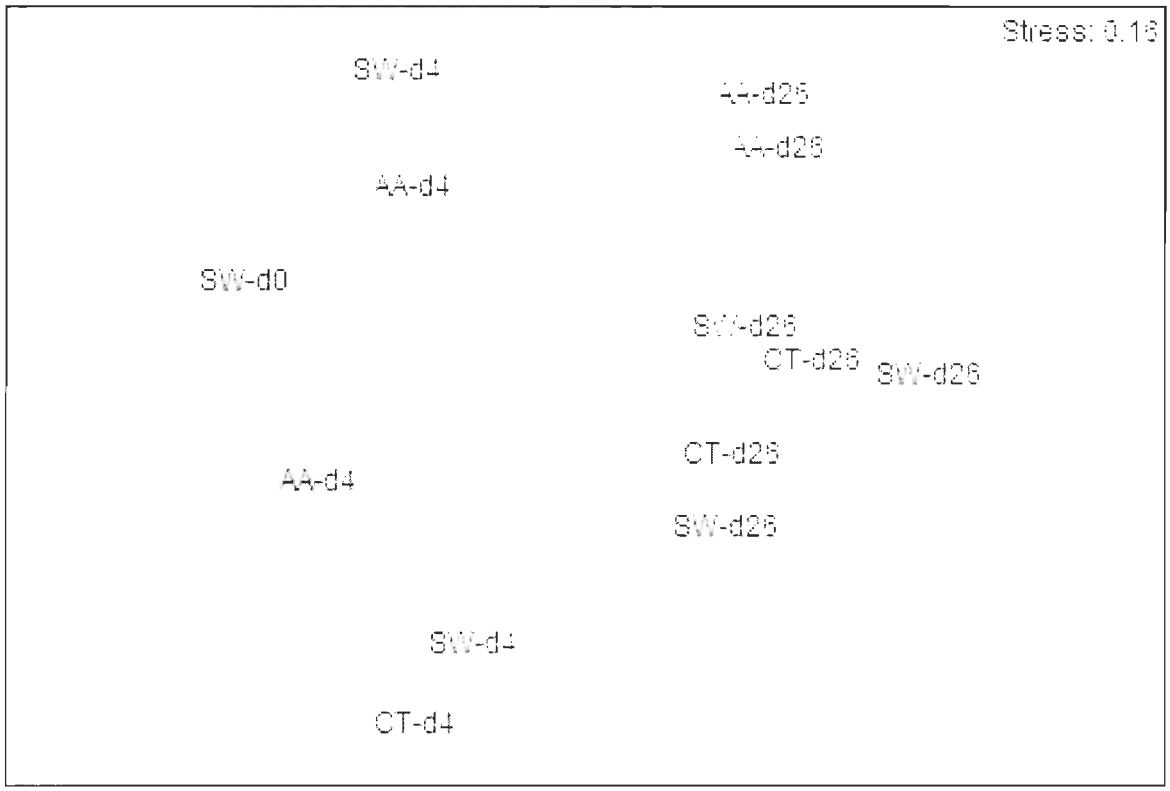

Figure III.5 NMDS diagram of the similarity matrix calculated from the binary DGGE data showing the changes in the bacterial community structure in larval rearing water. SW: source seawater (sampled prior to entering larval tanks), AA: water in AA-enriched larval tanks, CT: water in control larval tanks, d: sampling day.

Histology

Histological observations in larval intestinal lumen, gills and skin did not show any significant difference in the bacterial colonization between larval groups. Bacterial density in intestinal lumen and on gills and skin in control and larvae fed AA-enriched rotifers were respectively $2.5\times10^3 \pm 2.3\times10^3$ and $2.3\times10^3 \pm 2.5\times10^3$ bacteria mm^{-2}, $28.5 \pm 35.8\%$ and 34.5 ± 48.6 % of the gill area, and 16.3 ± 16.3 and $11.7 \pm 8.5\%$ of the fin area.

III.4 DISCUSSION

The enrichment of rotifers with the addition of commercial AA did not modify AA content in larval polar lipids, the bacterial colonization of larval tissues, or larval growth. Arachidonic acid was highly conserved in larval membranes, even though the supply was lower in the control diet. Willey *et al.* (2003) observed relatively high levels of AA within muscle, eyes and brain in summer flounder fed rotifers containing very low levels of AA. A similar AA retention was observed in other flatfish species such as turbot (*Scophthalmus maximus*) larvae (Rainuzzo *et al.*, 1994) and yellowtail flounder (*Limanda ferruginea*) larvae (Copeman & Parrish, 2002). Arachidonic acid in cell membranes reached a plateau on day 26 (average of 4.5% TFA) that was maintained until settlement. The absence of further accumulation may indicate that AA levels had reached an optimal relative level to sustain flounder larval development, as previously observed (Chapter 1). Willey *et al.* (2003) reported that levels of AA found in muscle, brain and eyes of larval summer flounder (18 days after hatch) did not significantly increase in response to increasing dietary AA levels. However, the same AA supplementation method used in one-year-old Pacific oysters (*Crassostrea gigas*) resulted in AA enrichment of gill polar lipids and was related to increases in total hemocytes (granulocytes, hyalinocytes, and agranulocytes), in the percentage of phagocytic hemocytes, and in the production of basal reactive oxygen species (ROS) by hemocytes (Delaporte *et al.*, 2006). The production of ROS is modulated by eicosanoids, which are produced from AA or EPA (Yaqoob, 2004). However, this

production was shown to be considerably different among larval marine fish species (Kadomura *et al.*, 2007).

AA accumulation in PL during larval rearing and during metamorphosis may reflect the role of eicosanoids in modulating immune and central nervous system functions (modulation of neural transmission, hypothalamic function, regulation of cerebral blood flow) (Bell *et al.*, 1994) as well as in osmoregulation and stress response regulation (Estévez *et al.*, 1997; Koven *et al.*, 2001; Tocher, 2003). In sea scallops (*Placopecten magellanicus*), AA accumulation was also observed before and during metamorphosis and could reflect stimulation of the immune function due to pathogen infection (elevated mortality observed) and apoptosis of larval organs or the role of AA in cell signaling processes associated with organ rebuilding (Pernet *et al.*, 2005). In *Hydractinia* the metamorphosis of the larva into the sessile primary polyp is induced by external bacterial stimulus (Hassel *et al.*, 1996) and involves AA and eicosanoids (Leitz *et al.*, 1994). The low accumulation of AA in PL of flounder larvae during the rotifer feeding period may indicate a low bacterial challenge in our cold water rearing system (Plante *et al.*, 2007).

In flatfish, the brain plays an important role in metamorphosis regulation and asymmetric differentiation *via* activation of the pituitary–thyroid axis (Inui *et al.*, 1995). The fish's brain is able to synthesize prostaglandins *in vitro*, with AA being the preferred substrate even when cultures are supplemented with other precursor fatty acids (Bell *et al.*, 1994). Thus, AA has been detected in relatively high amounts in neural and visual tissues

(Mourente & Tocher, 1992; Willey *et al.*, 2003). In addition, the brain controls the development of the normal pigment pattern, secreting hormones involved in the synthesis of melanin and the modulation of melanoblast dynamics on the dorsal and the blind side (Fujii, 1993).

AA accumulation at metamorphosis in flatfish may reflect osmoregulatory processes: eicosanoids including prostaglandins are involved in the stimulation of ionic transport (Castell, 1994) and environmental stress tolerance, such as salinity changes (Bell & Sargent, 2003). This was observed in Atlantic salmon undergoing adaptive transition to seawater (Tocher *et al.*, 2000). It has been suggested that the initially high tolerance to extreme salinity in early larvae is due in part to the absence of gills that could otherwise augment water loss due to the large surface area (Tytler & Bell, 1989). Another possible osmoregulatory change occurring at metamorphosis could induce the decrease of the whole-body water content during metamorphosis (Schreiber & Specker, 1999).

The less pronounced decrease of EPA and DHA in PL compared to NL may indicate a selective incorporation of both fatty acids into membranes at the expense of reserve lipids, likely in response to low dietary proportions. This has been observed in haddock (*Melanogrammus aeglefinus*) larvae (Plante *et al.*, 2007).

Water in AA-enriched rotifer tanks and larval tanks supplied with AA-enriched rotifer cultures exhibited higher *Vibrio* sp. concentrations than control rotifer and larval

tanks. The bacterial load from rotifer culture water was transferred to larval tanks, as indicated by the detection of the VFHNA bacteria in both types of rearing water on day 26. The same holds for *Vibrio* bacteria, which were more concentrated on days 15 and 26 in AA-enriched rotifer cultures than in control rotifer cultures; this was reflected in the AA-enriched larval rearing tanks. The origin of *Vibrio* sp. does not seem to be the natural seawater, where only low concentrations were detected from day 0 to day 26 of the experiment. It is well established that bacteria associated with live feed are the major influx to larval tank water and hence to the larval gastro-intestinal system (Munro *et al.*, 1994; Jensen *et al.*, 2004). Most bacteria strains associated with rotifers are located on the external surface of rotifers (Munro *et al.*, 1993): they are viable and are able to grow (Nicolas, 1989). Thus, young flounder larvae fed rotifers having high *Vibrio* sp. concentrations may have been challenged with high *Vibrio* bacteria content in their gastro-intestinal tract and external tissues. However, we observed a small increase of the total bacteria abundance with the increase in *Vibrio* sp. between days 15 and 26 in AA-enriched larval tank water. Thus, *Vibrio* sp. bacteria was relatively low in larval tank water.

The NMDS plot obtained from the PCR-DGGE of larval rearing water indicated two major groups of bacterial communities on day 26. A first group was composed of bacterial communities associated with AA-enriched larval rearing water and a second group was composed of bacterial communities associated with control larval rearing water, this latter group was similar to that found in the seawater source. The enrichment treatments probably

affected the microbiota in rotifer and larval culture waters both quantitatively and qualitatively, as mentioned by Korsnes *et al.* (2006).

This study should be regarded as a first step to understanding the problem of mortality events that are chronically observed in winter flounder larval cultures. Though no significant effect of AA enrichment on larval growth was detected, there was a significant effect on *Vibrio* sp. concentration in rotifer rearing water, which could be transferred to larval tanks. The appearance of these *Vibrio* bacteria and their possible interactions with larval development need further investigation. Furthermore earlier metamorphosis in smaller AA-enriched larvae is surprising and will need to be investigated more closely.

DISCUSSION GÉNÉRALE

La discussion générale ne reprend pas les discussions spécifiques à chacun des chapitres, mais aborde plutôt des points plus généraux.

Dans cette thèse, nous avons mis en évidence que des enrichissements présentant différents profils lipidiques affectent les communautés bactériennes dans des élevages larvaires de plie rouge, en terme qualitatif et quantitatif. En effet, l'utilisation de différents enrichissements lipidiques pour rotifères a eu pour effet de modifier les communautés bactériennes dans les bassins de proies vivantes et larvaires (Chapitre 3), ainsi que l'abondance totale des bactéries dans la lumière intestinale des larves (Chapitre 1). Les larves dont l'alimentation était composée de rotifères élevés dans l'émulsion la plus riche en DHA et n-6 DPA (traitement AlgaMac 2000, *ALG*) présentaient une concentration bactérienne totale six fois plus faible à l'intérieur de la lumière intestinale. Korsnes *et al.* (2006) ont montré que des enrichissements commerciaux pour rotifères affectent également la concentration de bactéries cultivables dans le tractus gastrointestinal des larves de morue. Ces auteurs ont observé une plus faible concentration de bactéries dans le tractus digestif des larves dont l'alimentation était composée de rotifères élevés dans une émulsion riche en DHA (AquaGrow), par rapport à celles alimentées sur des rotifères enrichis avec trois autres types d'émulsion (AlgaMac 2000, Protein Selco et Marol E).

Au terme de la phase expérimentale (alimentation sur proies vivantes uniquement), les larves de plies présentant la plus faible densité bactérienne intestinale étaient plus grandes que celles dont l'alimentation était composée de rotifères élevés dans l'émulsion commerciale la moins riche en DHA, EPA et AA (traitement *SEL*, Chapitre 1). Il est intéressant de noter qu'en fin de période d'élevage larvaire, les néo-juvéniles issus du traitement le plus riche en DHA, AA et n-6 DPA (Chapitre 1) semblaient toutefois moins vigoureux que ceux issus des autres traitements. En outre, le taux de survie à la métamorphose des larves soumises à ce même traitement (11,1%) était plus faible que celui des larves du traitement intermédiaire moins riche en DHA et AA (traitement *DPS*, chapitre 1) (16,6%). Des expériences récentes de sevrage de larves de morues sur des artémies enrichies en DHA et n-6 DPA (AlgaMac 2000) ont montré une croissance certes plus élevée chez ces larves, mais également un indice de survie parmi les plus faibles (Garcia *et al.*, 2008). Notons que ces observations sont contraires à ce qui est généralement observé en milieu naturel, où le taux de survie augmente en fonction de l'augmentation en taille des larves, comme démontré chez des larves de morues marquées génétiquement et relâchées en milieu naturel (Kristiansen *et al.*, 1997) et des juvéniles de plie rouge (Howell, 1993). D'autres facteurs liés aux conditions d'élevage interviennent probablement sur le faible taux de survie et la faible vitalité observés chez les larves de plie enrichies en DHA, AA et n-6 DPA.

Dans son ensemble, cette thèse met en évidence la présence d'une rétention de l'acide arachidonique dans les membranes cellulaires de la plie rouge, traduite par une

accumulation dans les lipides polaires, comme d'ailleurs précédemment observé chez d'autres espèces de poissons plats (turbot, Rainuzzo *et al.*, 1994 ; limande à queue jaune, Copeman & Parrish, 2002 ; cardeau d'été, Willey *et al.*, 2003). Chez les poissons plats, cet acide gras joue probablement un rôle dans la promotion de la croissance et la survie, l'augmentation de la résistance à des stress aigus, la pigmentation et la métamorphose (Bell *et al.*, 2003).

Après le sevrage, une diminution de l'ensemble des acides gras essentiels au sein des membranes larvaires a été décrite. Cette diminution est plus marquée dans les lipides neutres des larves et semble indiquer une déficience alimentaire. Ainsi, nous avons pu montrer que les artémies non-enrichies n'étaient pas satisfaisantes pour combler les besoins nutritionnels des larves de plie dès le sevrage. Au cours de la seconde expérience sur les larves (Chapitre 3), la chute de la composition en acides gras à l'intérieur des membranes pourrait être due à un problème de sevrage sur la moulée sèche.

Le deuxième chapitre de cette thèse a confirmé qu'il était possible d'utiliser des microalgues concentrées congelées pour enrichir des proies vivantes (rotifères) en acides gras essentiels. Néanmoins, il reste encore à expérimenter et à optimiser ces mélanges afin de permettre une croissance optimale des larves de plie rouge. Lors des expériences du troisième chapitre, nous avions envisagé d'utiliser des microalgues concentrées congelées. Cependant, la méthode d'enrichissement en AA initialement développée par Seguineau *et al.* (2005) avait été réalisée sur des cultures microalgales fraiches. Par conséquent, nous

avons utilisé des microalgues concentrées uniquement afin de se rapprocher des conditions d'enrichissements de Seguineau *et al.* (2005). D'autres espèces de microalgues très riches en DHA et dépourvues d'EPA telles que *Crypthecodinium cohnii* et *Schizochytrium* sp. (50% et 32% des acides gras totaux respectivement) ouvrent de nouveaux horizons pour la production algale tant sous forme congelée que concentrée qui permettrait d'obtenir des concentrations à la carte en acides gras essentiels.

L'une des préoccupations actuelles les plus importantes en aquaculture, est la substitution des huiles issues des captures de pêches par des huiles d'origine végétale. Un bon nombre d'études ont été réalisées sur le remplacement des huiles de poissons par des huiles d'origine végétale terrestre, dépourvues d'AGPI essentiels. Le bilan est mitigé et les effets semblent être problématiques surtout chez les espèces marines. Chez la truite arc-en-ciel, Murray *et al.* (2007a) ont comparé des régimes alimentaires de plus en plus riches en huile et protéines végétales au détriment d'huile et de protéines de poissons. Après 140 jours d'expérience, les chercheurs ont observé une diminution significative de DHA et d'EPA (75 et 85% des acides gras totaux, respectivement) dans la chair des truites nourries à 100% de produits végétaux par rapport à celles nourries à 100% de farine et d'huile de poissons. Tocher *et al.* (2006) ont également observé une baisse significative de la teneur en DHA et EPA dans la chair (en moyenne 27,5 et 23,5% des acides gras totaux, respectivement) et le foie d'omble chevalier (*Salvelinus alpinus*) dont le régime alimentaire était composé d'huile végétale terrestre. Or, l'approvisionnement alimentaire en acides gras n-3 est un des enjeux majeurs de l'aquaculture mondiale étant donné les

effets bénéfiques sur la santé de l'homme. Grâce à leur richesse naturelle en AGPI essentiels, les microalgues (à l'instar de certaines bactéries et protistes marins) permettraient de palier à ces problèmes d'élevages aquacoles.

Perspectives de recherche

Faible concentration des bactéries intestinales et faible taux de survie (infection)

L'étude de la microflore intestinale indigène est importante et permettrait de mieux comprendre ce qu'il advient de celle-ci lors d'une infection (Ringø *et al.*, 2003) ou d'un enrichissement en AGPI essentiels. Ringø *et al.* (2002) ont montré une diminution d'un facteur 100 des quantités de bactéries associées au tractus digestif d'ombles chevalier infectés avec *Aeromonas salmonicida* ssp. *salmonicida*. Lødemel *et al.* (2001) ont montré une diminution des bactéries associées aux microvillosités de l'omble chevalier après infection avec *A. salmonicida*, ainsi qu'une augmentation du nombre de cellules à mucus au niveau du système digestif. Suite à une infection bactérienne, des études ont montré une augmentation du nombre de cellules à mucus dans le système digestif du saumon keta (*Oncorhynchus keta* : Ransom *et al.*, 1984) et les branchies de la truite arc-en-ciel (Ferguson *et al.*, 1992), ainsi qu'au niveau de la peau de larves de flétan Atlantique exposées à de fortes concentration bactérienne (Ottesen & Olafsen, 2000). Ringø *et al.* (2002) ont alors émis l'hypothèse qu'une augmentation de production de mucus, *via* l'augmentation du nombre de cellules à mucus, entraînait la perte de la microflore

protectrice associée. Cette microflore intestinale indigène empêcherait les bactéries potentiellement pathogènes d'adhérer et de coloniser la muqueuse intestinale. L'électrophorèse sur gel en gradient dénaturant (DGGE) pratiquée à partir des homogénats de larve semble être un outil adéquat pour cette tâche (Hovda *et al.*, 2007).

Chez des individus adultes de plie rouge, des bactéries et des protozoaires sont retrouvés en étroite association avec la membrane des cellules épithéliales de l'intestin et du cæca pylorique (sites d'absorption des lipides), sans que des signes histopathologiques aient été observés (Murray *et al.*, 1996). Ces bactéries pourraient être impliquées dans le processus de digestion (Lindsay & Gooday, 1985). Nous avons vu que la microflore du système digestif tirait son origine de l'eau d'élevage et des proies vivantes (cf. introduction générale). Ainsi, il est intéressant de noter qu'à la surface des œufs de plie rouge, il existe une bactérie indigène (*Leucothrix mucor*) qui est résistante à l'action de la pleurocidine, un puissant peptide antimicrobien découvert chez la plie rouge (Cole *et al.*, 1997). Chez la larve, l'expression du gène codant pour la pleurocidine est activée dès le treizième jour après l'éclosion (Douglas *et al.*, 2001). Chez la plie rouge adulte et juvénile, ce peptide antimicrobien est sécrété par les cellules à mucus de la peau (Cole *et al.*, 1997), de l'intestin (Cole *et al.*, 2000) et des branchies (Murray *et al.*, 2003b). Or, au niveau des branchies de la plie rouge, la pleurocidine a la particularité d'être sécrétée par les granulocytes éosinophiles (Murray *et al.*, 2003b) et stockée à l'intérieur des granules cytoplasmique de ces cellules (Murray *et al.*, 2007b). Les granulocytes éosinophiles sont des cellules de défense non-spécifique largement répandues chez la plupart des poissons

téléostéens et les vertébrés supérieurs et constituent une première ligne de défense contre des organismes pathogènes (Leknes, 2007). Ils sont observés dans de nombreux tissus, dont les régions sujettes à des réactions inflammatoires et aux infections bactériennes et parasitaires (Sitjà-Bobadilla *et al.*, 2007 ; Alvarez-Pellitero *et al.*, 2008), comme le tractus gastrointestinal, la peau et les branchies. Murray *et al.* (2007b) ont suggéré que ces cellules de défenses immunitaires seraient directement impliquées dans la destruction des pathogènes. La présence de ces cellules chez la plie rouge au stade juvénile et adulte semble indiquer que celles-ci jouent un rôle dans la première ligne de défense chez cette espèce.

Faible concentration des bactéries intestinales et meilleur taux de survie

D'autre part, des travaux ont montré qu'une faible densité bactérienne à l'intérieur de la lumière intestinale était associée à une meilleure survie larvaire. Des larves de morue présentant les plus faibles concentrations de bactéries cultivables dans le tractus digestif présentent un meilleur taux de survie (2,1%), bien que relativement faible, que les larves présentant de plus fortes concentrations bactériennes (0,4% en moyenne) (Korsnes *et al.*, 2006). Chez cette espèce, les bactéries du genre *Vibrio* sont parmi les plus dominantes. De même, une corrélation inverse entre la survie larvaire et la concentration bactérienne (>96% du genre *Vibrio*) a été démontrée dans l'estomac des larves de turbot (Thomson *et al.*, 2005) et le tractus digestif des larves de cardeau hirame (*Paralichthys olivaceus*) (Kim *et al.*, 2004).

L'importance des bactéries autochtones

En fait, la survie larvaire n'est pas uniquement fonction de la concentration bactérienne intestinale, mais elle est surtout fonction du type de bactéries en présence (cf. introduction générale). Certaines bactéries peuvent être à l'origine de mortalité lorsqu'elles sont en assez grand nombre, tandis que d'autres peuvent augmenter la survie (Hjelm *et al.*, 2004). Encore une fois, l'identification des espèces bactériennes autochtones en présence dans le système digestif permettrait de mieux comprendre leurs interactions avec l'hôte.

L'acide docosapentaénoïque (n-6 DPA)

Tout récemment, Garcia *et al.* (2008) ont suggéré que l'apport en acide docosapentaénoïque (n-6 DPA, 22:5n-6) dans l'alimentation de larves de morues aurait affecté positivement leur croissance et leur survie. Cet acide gras maintiendrait la fluidité membranaire nécessaire aux cellules d'un organisme subissant des changements de température ambiante, comme c'est le cas pour la morue Atlantique. Chez l'être humain, un apport alimentaire déficient en DHA ou excédentaire en acides gras n-6 peut mener à la biosynthèse de n-6 DPA (Alessandri *et al.*, 2004 ; Guesnet *et al.*, 2005). Ce dernier peut remplacer le DHA dans les phospholipides membranaires des cellules cérébrales, mais n'empêche pas l'apparition des troubles fonctionnels du système nerveux chez l'homme. L'effet de cet acide gras sur la croissance des larves de plie rouge reste encore à déterminer.

Les outils d'analyse

L'électrophorèse sur gel en gradient dénaturant (DGGE) est un outil efficace d'analyse des changements de communautés bactériennes à l'intérieur d'un écosystème aquacole, comme l'ont démontré tout récemment Brunvold *et al.* (2007) dans une écloserie de morue. Toutefois, les auteurs préconisent l'emploi d'une sonde ARN ciblant la sous-unité beta de l'ARN-polymérase, permettant de caractériser plus précisément jusqu'à l'espèce bactérienne. La méthode actuelle (ADN 16S) permet de révéler aussi bien l'empreinte génétique d'une seule espèce bactérienne que celle d'un ensemble de communautés bactériennes. Cet outil nous a permis de mettre en évidence que l'une des deux préparations utilisées pour enrichir les rotifères a affecté la composition des communautés bactériennes à l'intérieur des bassins d'élevage de rotifère (Chapitre 3). Les communautés bactériennes présentes dans les bassins de rotifères ont été transférées vers l'eau d'élevage larvaire. Ainsi, nous démontrons une nouvelle fois à quel point il est essentiel d'étudier la flore bactérienne des proies vivantes.

Le système d'analyse d'image en microscopie photonique nous a permis de caractériser la colonisation bactérienne sur les branchies et la peau des larves, ainsi que la densité bactérienne dans la lumière intestinale. Les grands écart-types observés pour les données d'une même population expérimentale montrent à quel point la microflore fluctue entre les individus. Ce type de variabilité a également été montré au niveau de la microflore intestinale du tilapia (*Oreocrhomis mossambicus*), de la carpe (*Cyprinus carpio*) et du poisson rouge (*Carassius auratus*) (Asfie *et al.*, 2003).

L'originalité de ce travail tient en partie au fait que nous avons utilisé des outils peu familiers dans les études scientifiques en milieu aquacole. L'utilisation de la cytométrie en flux, utilisée abondamment en écologie, permet de mesurer très précisément l'abondance bactérienne totale au sein des élevages. La méthode classique d'ensemencement sur milieu de culture ne permet la culture que de 0,1 à 1% des bactéries totales. L'outil DGGE, fortement utilisé en écologie, est également très efficace dans le suivi des communautés bactériennes dans un système aquacole. Le couplage histologie et système d'analyse d'image est globalement satisfaisant pour une première approche. D'autres techniques peuvent être envisagées afin de comprendre la dynamique bactérienne chez la larve (microscopie électronique, immuno-histochimie, marquage de bactéries avec la protéine fluorescente verte) et également le devenir des acides gras essentiels apportés par les aliments (marquage isotopique stable).

CONCLUSION GÉNÉRALE

L'objectif de cette thèse était de déterminer si l'apport en acides gras polyinsaturés dans l'alimentation des larves de plie rouge affectait la communauté bactérienne tant au niveau du système d'élevage que de l'organisme. Nous avons mis l'emphase sur les acides gras essentiels nécessaires à la croissance larvaire et impliqués dans la production d'eicosanoïdes (EPA, AA) et de docosanoïdes (DHA). Des outils moléculaires (PCR-DGGE, cytomètre en flux) jusqu'alors employés en écologie ont été utilisés dans le cadre de cette étude, afin d'étudier les communautés bactériennes dans un milieu aquacole. Un système d'analyse d'images de microscopie photonique a permis de déterminer la densité bactérienne dans la lumière intestinale larvaire, ainsi que sur les branchies et la peau. La composition en acides gras des larves a été analysée séparément dans la fraction polaire et neutre afin de différencier la fraction lipidique allouée à l'énergie de celle allouée aux structures membranaires.

Les résultats de cette thèse de doctorat montrent clairement que les enrichissements lipidiques pour proies vivantes peuvent affecter la dynamique des communautés bactériennes à l'intérieur des systèmes d'élevages des proies vivantes et des larves de plies rouges, ainsi que la densité bactérienne du tractus digestif des larves. Les larves alimentées avec des rotifères enrichis avec l'émulsion la plus riche en DHA, et n-6 DPA présentaient une densité bactérienne six fois moins élevée et une croissance plus élevée. Nous avons observé une augmentation de bactéries du genre *Vibrio* à l'intérieur des bassins d'élevages

des proies vivantes enrichies en AA, qui par la suite, ont été ensemencées dans les bassins d'élevage larvaire, sans qu'il y ait eu d'effet sur la densité bactérienne totale dans la lumière intestinale ni la croissance larvaire. Des études approfondies sur la flore microbienne intestinales des larves de plie rouge sont nécessaires afin de nous renseigner sur l'évolution de cette microflore lors d'un enrichissement en acides gras essentiels ou lors de transitions trophiques (artémies, moulées). Ces bactéries intestinales peuvent intervenir dans la croissance et la survie des larves. Par ailleurs, l'expérience d'enrichissement en acides gras essentiels des rotifères à partir de microalgues concentrées et congelées est concluante et constitue un premier pas vers l'affranchissement d'utilisation de suppléments commerciaux riches en huiles de poissons sauvages. Dans le futur, il serait intéressant de travailler avec d'autres espèces de microalgues ouvrant de nouvelles perspectives en terme de composition en acides gras essentiels.

Cette étude se veut avant tout une première approche dans la compréhension de la dynamique des communautés bactériennes dans les élevages larvaires de la plie rouge, caractérisés par des épisodes de mortalité. Au terme de ce travail de doctorat, il nous apparaît clairement indispensable d'intégrer l'étude des communautés bactériennes aux expériences d'enrichissement en acides gras polyinsaturés chez les larves de poissons marins. L'étude des bactéries indigènes présentes au niveau de l'intestin semble incontournable dans la compréhension globale d'un enrichissement lipidique et est un préalable dans le contrôle des communautés bactériennes. Nous pourrions alors envisager

l'expérimentation de probiotiques dans le but d'améliorer la croissance, le taux de survie et la croissance des larves.

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