

Universitat de Barcelona
Facultat de Biologia – Departament d'Ecologia

**Crustacean zooplankton dynamics in Lake Redon:
a stoichiometric, biochemical and isotopic approach**

Dinàmica dels crustacis zooplanctònics a l'estany Redon: una aproximació
estequiomètrica, bioquímica i isotòpica

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Crustacean zooplankton dynamics in Lake Redon: a stoichiometric, biochemical and isotopic approach

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Vist i plau

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A la Teresa
i a tota la família

Contents

Agräiments	III
Resum	IX
1. General introduction	3
Part I. Seasonal and inter-annual variability	
2. The main features of seasonal variability in the external forcing and dynamics of a deep mountain lake	13
3. Inter-annual life history traits variability in <i>Daphnia</i> , <i>Cyclops</i> and <i>Diaptomus</i> in an oligotrophic lake: environmental, intra- and inter-specific relationships	31
Part II. Seasonal variability in the elemental, biochemical and isotopic composition	
4. Linking biochemical and elemental composition in freshwater and marine crustacean zooplankton	55
5. Reproduction as the main cause of non-strict homeostasis in the elemental composition of zooplankton	71
6. Seasonal inter-specific and ontogenic variation in the amino acid composition of zooplankton: implications for nutritional constraints	93
7. Intra-specific variations on stable isotope composition indicate ontogenic diet changes and nitrogen limited growth in alpine lake zooplankton	109
8. Conclusions	127
References	137

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Resum

Introducció

Limnologia dels estanys d'alta muntanya

Els estanys d'alta muntanya, a causa de la seva localització remota i dificultats d'accés, han estat tradicionalment poc estudiats, especialment si els comparem amb altres estanys de zones temperades situats a menor altitud. Al contrari del que ha passat amb els estanys de poca altitud, la distància que separa els estanys d'alta muntanya de les zones urbanes ha evitat que hagin patit impactes antropogènics com els provocats històricament pels aportes de nutrients que van eutrofitzar durant la primera meitat del segle vint un nombre important d'estanys situats a menor altitud (Margalef 1983). Cap a finals del segle dinou, principis del vint, es van començar a realitzar estudis sobre diferents aspectes dels estanys alpins Europeus als Alps i als Pirineus, i també a l'Amèrica del Nord. Als Pirineus, a aquests estudis pioners (Gaurier 1933 i referències incloses; Margalef 1948; Margalef 1952) els van succeir estudis més detallats (Capblancq 1972; Rey i Capblancq 1975; Margalef et al. 1975; Vilaseca 1978; Miracle 1978a; Miracle 1978b; Campàs i Vilaseca 1979) que en els darrers anys s'han centrat en estudiar els estanys situats al vessant sud de la serralada pirinenca (p.e., Catalan 1988; Catalan 1989; Catalan et al. 1992; Gacia et al. 1994; Camarero i Catalan 1996; Felip et al. 1999).

El fet que alguns contaminants puguin ser transportats amb les masses d'aire a grans distàncies, i especialment el descobriment dels problemes que ocasionava la pluja àcida d'origen antropogènic a estanys de diferents països del nord d'Europa (Bresser i Salomons 1990) i del Nord d'Amèrica (Scholfield 1976) va despertar el interès polític en estudiar els estanys d'alta muntanya. El 1991 es van iniciar de manera coordinada una sèrie d'estudis des de diferents zones d'alta muntanya d'Europa per tal de valorar-ne el seu estat d'acidificació. Aquesta xarxa ha permès dur a terme molts estudis a estanys d'alta muntanya d'una manera coordinada (p.e., Wathne 1992; Catalan et al. 1994; The MOLAR Water Chemistry Group 1999; Catalan et al. 2002).

Dins dels estanys d'alta muntanya, l'estany Redon (Val d'Aran) ha estat el més estudiat de la serralada pirinenca. Ha estat la base de moltes tesis doctorals i tesines de llicenciatura, centrades en diferents aspectes limnològics d'interès. Des dels canvis estacionals de la química de l'aigua i la producció primària (Catalan 1987), els efectes de l'acidificació (Camarero 1994), la composició planctònica en general, i en particular la composició fitoplanctònica (Felip 1997), les característiques químiques i biològiques de la coberta de gel (Camarero 1994 i Felip 1997), l'ecologia del plàncton, especialment els rotífers (Bartumeus 1999), la composició dels grups algals utilitzant tècniques cromatogràfiques (Buchaca 2005), i també estudis paleolimnològics (Buchaca 1998; Pla 1999; Buchaca 2005). Un dels aspectes que quedaven per estudiar d'una manera específica era el grup de crustacis planctònics. Així doncs, aquest treball se centra en l'estudi de l'ecologia de les tres espècies de crustacis que viuen al plàncton de l'estany Redon.

Els crustacis del zooplàncton dels estanys dels Pirineus

La colonització del plàncton per crustacis bentònics s'ha anat produint al llarg dels darrers 650 milions d'anys. Els dos grups de crustacis d'aigua dolça més representatius són els copèpodes i els cladòcers, es creu que ambdós grups van tenir un origen bentònic que data de finals del Cretaci, malgrat alguns branquiòpodes, el gran grup que engloba els cladòcers, són molt més antics, de finals del Cambrià (Rigby i Milsom 2000). Els copèpodes tenen un origen marí, i es pensa que van migrar a les aigües continentals en diferents episodis (Huys i Boxshall 1991). Al plàncton de les aigües continentals es troben dos ordres de copèpodes, els calanoides (l'ordre més abundant al mar) i els ciclopoïdes (amb molt pocs representants marins). Als cladòcers, l'ordre dels anomòpodes i en especial els dàfnids, són els principals representants del plàncton d'aigua dolça (Freyer 1991).

Els estanys pirinencs es van originar fa aproximadament uns 15.000 anys, en retirar-se els gels de la darrera glaciació. Per tant, evolutivament parlant, els organismes que han colonitzat les seves aigües han estat vivint en aquests ambients durant relativament pocs anys. Els crustacis planctònics dels estanys pirinencs van ser descrits fa un parell de dècades (Miracle 1978a; Miracle 1978b), i un estudi més recent n'ha confirmat els resultats anteriors (Taula 1.1). A la Península Ibèrica es creu que hi ha hagut dues vies principals de colonització (Miracle 1982). Una del nord-est (principalment espècies bò-reoalpines que van entrar a través de l'est dels Pirineus) i l'altra a través del nord-oest (amb espècies atlàntiques o bé lusitànies que van entrar per Galícia o pels Picos d'Europa). Als Pirineus s'hi poden trobar espècies que provenen d'ambdues vies de dispersió (Taula 1.1).

Les dues espècies de copèpodes que es poden trobar al Redon són les espècies més abundants dins de cadascun dels seus grups respectius, en canvi *Daphnia pulicaria* és menys comú. De la seva distribució geogràfica i especialment de les co-ocurrències amb les altres espècies als Pirineus se'n poden ressaltar algunes característiques rellevants. *Diatomus cyaneus* és l'espècie dominant de copèpode calanoide (és la de major mida, Maly 1976) quan es trobava al mateix estany amb calanoides d'altres gèneres (*Eudiaptomus cyaneus* and *Mixodiatomus laciniatus*). En canvi, no s'ha trobat coexistent amb l'altra espècie del mateix gènere (*D. castaneti*). De manera semblant, no s'han trobat dues espècies de *Daphnia* al mateix estany. *Cyclops abyssorum* és el copèpode ciclopoide amb una distribució més àmplia als Pirineus i no s'ha trobat mai coexistent amb un altre ciclopoide.

Pel que fa a la reproducció, gairebé totes les espècies de crustacis d'aigua dolça produeixen més d'una posta durant la seva estació reproductora i completen el seu cicle de vida en menys d'un any (iteroparous anual, Begon et al. 1990). Els copèpodes presenten reproducció sexual obligada, mentre que els cladòcers són principalment partenogenètics i de manera ocasional també poden generar mascles per a la producció d'efipsis, el seu tipus específic d'ous perdurables. La forma de diàpauza és una estratègia reproductora d'origen filogenètic en crustacis (Hairston i Caceres 1996). Tant els copèpodes calanoides com els cladòcers poden produir ous de resistència que els permeten sobreviure períodes adversos al sediment. En canvi els ciclopoïdes no poden produir ous de resistència, però poden sobreviure enterrats al sediment en estadi de copepodit, generalment l'estadi V. Aquestes estratègies de reproducció han evolucionat per superar condicions ambientals desfavorables (Santer 1998). No totes les espècies dins de cada

grup utilitzen aquesta capacitat amb la mateixa freqüència o de la mateixa manera. Dins d'una mateixa població, l'estratègia òptima que es pot preveure, estaria governada per l'equilibri mediat per les condicions ambientals en què viuen els organismes i les seves característiques mateixes (Hairston i Bohonak 1998). La comparació de diferents espècies que viuen en el mateix ambient és una bona oportunitat per estudiar-ne les adaptacions ecològiques i la resposta fenotípica.

Les dues característiques més prominents dels estanys d'alta muntanya són la seva forta estacionalitat i les seves condicions generalment oligotròfiques. Es troben coberts de glaç al voltant de sis mesos a l'any, per la qual cosa els episodis productius estan restringits a èpoques concretes molt predictibles. És d'esperar que els organismes que habiten aquests ambients hagin desenvolupat estratègies específiques que els permetin sobreviure en aquestes circumstàncies. Els crustacis, i especialment els del gènere *Daphnia*, han estat dels organismes més utilitzats com a model, la major part de les seves característiques han estat descrites tant en experiments de laboratori com en altres tipus d'estanys diferents dels d'alta muntanya. Tot i que les espècies que poblen els estanys fa molts anys que es coneixen, les seves característiques ecològiques no han estat ben descrites. Estudis recents del zooplàncton d'estanys d'alta muntanya s'han centrat ja en aquesta direcció (p.e., Gliwicz et al. 2001; Villar-Argaiz et al. 2002; Winder et al. 2003).

Ecologia tròfica: des d'una aproximació energètica a l'ecologia estequiomètrica... i bioquímica

Els estanys han estat tradicionalment reconeguts com a paradigma del concepte d'ecosistema a causa dels seus límits clarament definits (Catalan i Fee 1994). Els estudis en estanys han revelat molts dels principis ecològics fonamentals. Una de les teories ecològiques més sòlides, el concepte trofodinàmic d'ecosistema (Lindeman 1942), es va basar en estudis duts a terme en estanys. L'aproximació tròfica de Lindeman es basava principalment en la transferència d'energia en les relacions tròfiques, i ha estat criticada per no tenir en compte les necessitats d'altres elements essencials a part del carboni (Reiners 1986). En aquest sentit, l'ecologia estequiomètrica (Sterner i Elser, 2002) supera aquesta dificultat en simplificar els organismes a la seva composició elemental (carboni, nitrogen i fòsfor principalment; Elser et al. 1996). S'ha descrit com l'estudi del balanç de l'energia i els múltiples elements químics de que estan fets els éssers vius (Reiners 1986; Elser et al. 1996), i esdevé un pas més enllà en el plantejament de l'estudi de l'ecologia tròfica basat tradicionalment en termes energètics.

L'ecologia estequiomètrica ha resultat molt útil per identificar restriccions en organismes d'ambients diversos com ara els de xarxes tròfiques d'aigua dolça, marina i terrestre (Elser i Hassett 1994, Elser et al. 2000). En el zooplàncton d'aigua dolça, s'ha trobat que els cladòcers com ara la *Daphnia* tenen un contingut de fòsfor més elevat que els copèpodes (Andersen i Hessen 1991; Hessen i Lyche 1991). S'ha trobat que la demanda nutricional d'un organisme és directament proporcional a la seva composició elemental, i que per tant els cladòcers tenen una demanda de fòsfor superior que els copèpodes (Sterner i Hessen 1994). Per tant, els diferents grups de zooplàncton reciclen nutrients en diferents proporcions, seguint la seva composició elemental respectiva (Elser i Urabe 1999).

Una dificultat que apareix quan es comparen diferents formes de vida, és la utilització de mesures comparables de les peculiaritats de les diverses espècies. En aquest sentit, l'aproximació estequiomètrica és molt útil. Precisament aquesta excessiva simplificació ha estat criticada per alguns autors que han demanat la incorporació d'elements de major complexitat (Brett 1993; Tang i Dam 1999). Els organismes són sistemes complexos amb uns òrgans o compartiments clarament definits, que alhora estan constituïts per uns pocs grups de biomolècules com ara proteïnes, lípids, glúcids, àcids nucleics i nucleòtids (Strayer 1988). Tots aquests compostos tenen papers funcionals relativament diferents en els organismes i tenen proporcions relatives diferents dels elements. Per exemple, les proteïnes es troben principalment formant part del teixit muscular i dels enzims, i contenen la major part del nitrogen de l'organisme; els lípids són la principal font de reserva energètica del cos dels metazous, i estan principalment formats per carboni hidrogen i oxigen. Per tant, la possibilitat de combinar l'aproximació elemental i la bioquímica hauria de permetre poder realitzar millors interpretacions de les històries de vida dels organismes i dels seus canvis potencials en la composició elemental (Anderson et al. 2004; Vrede et al. 2004).

Resultats recents han mostrat que en determinades circumstàncies alguns compostos específics com alguns àcids grassos i aminoàcids poden ser els compostos limitants per al creixement dels animals, més que no pas la disponibilitat total de nitrogen o fòsfor (Müller-Navarra et al. 2000; Conceição et al. 2003; Müller-Navarra et al. 2004). Aquest és el resultat de la seva naturalesa essencial per als metazous. Alguns àcids grassos, principalment alguns àcids grassos poliinsaturats com ara l'àcid eicosapentanoic (20:5W3; EPA) i l'àcid docosahexanoic (22:6W3, DHA) no poden ser sintetitzats per aquests organismes i per tant els cal obtenir-los a partir de fonts vegetals per poder incorporar-los a l'organisme (Brett i Müller-Navarra 1997). Basats en les proporcions relatives d'aquests àcids grassos essencials, els grups algals s'han classificat en funció del seu valor nutritiu pel zooplàncton herbívor (Ahlgren et al. 1992; Müller-Navarra 1995). Les diatomees, crisofícies i els criptòfits són els grups algals amb un contingut més alt d'EPA i DHA, mentre que els cloròfits i els cianobacteris són els de menor qualitat. Per tant, la presència d'alguns grups algals determina la possible limitació per àcids grassos. Com que en estanys oligotròfics com el Redon hi dominen els grups algals rics en àcids grassos, la limitació per aquests sembla ser que hi hauria de ser menys important que en ambients hipertròfics (Becker i Boersma 2003). De manera semblant, alguns aminoàcids han d'ésser obtinguts necessàriament a partir de la dieta, i una dieta deficiente en un aminoàcid essencial tindrà conseqüències negatives en el creixement de qualsevol organisme (D'mello 1994). La importància relativa dels aminoàcids en la nutrició dels organismes aquàtics és menys coneguda i se centra en alguns estudis d'aqüicultura (p.e., Conceição et al. 2003), i uns pocs estudis de camp en zooplàncton (Guisande et al. 1999; Guisande et al. 2000; Guisande et al. 2002; Guisande et al. 2003). Aquests darrers estudis, han mostrat que la composició aminoacídica d'aquestes espècies pot donar una informació important pel que fa als seus requeriments. Sembla que la component filogenètica és la més important a l'hora d'explicar-ne la seva composició.

Isòtops estables i la descripció de xarxes tròfiques

Els diagrames de xarxes tròfiques han estat sovint criticats per ser construccions subjectives, fortament esbiaixades per la nostra incapacitat d'observar en les interaccions tròfiques les variacions rellevants tant taxonòmiques, com espacials i temporals (Paine 1988). Per exemple, nombroses interaccions tròfiques no es poden observar visualment, i per tant els hàbits alimentaris dels organismes s'han basat tradicionalment en observacions directes i indirectes, i a través d'anàlisis morfològiques i dels continguts estomacals (Monakov 2003). La quantificació del que s'ha ingerit i especialment del que és assimilat per cada organisme és molt difícil de definir. Malgrat el seu alt interès en ecologia, s'ha vist que els mètodes emprats tradicionalment no eren adequats (Schindler 1971).

Una alternativa prometedora a aquests estudis tradicionals ha estat la descoberta que la proporció d'isòtops estables d'alguns elements clau (principalment carboni, nitrogen, però també s'utilitzen habitualment sofre, hidrogen i oxigen) varien d'una manera previsible en l'ambient, i especialment en les reaccions metabòliques. Els isòtops estables tenen el potencial de capturar simultàniament les complexes interaccions entre els organismes i de resseguir el flux d'energia a través de les comunitats ecològiques (Peterson i Fry 1987).

El quocient dels isòtops estables de nitrogen ($^{15}\text{N}/^{14}\text{N}$ relatiu al nitrogen atmosfèric o $\delta^{15}\text{N}$) ha estat utilitzat per estimar la posició tròfica ja que la $\delta^{15}\text{N}$ d'un consumidor és típicament més alta que la de la seva dieta, i la magnitud de la diferència és relativament consistent entre els organismes (DeNiro i Epstein 1981; Robinson 2001; Post 2002; McCutchan et al. 2003; Vanderklift i Ponsard 2003). Per referir-se a aquesta diferència generalment s'utilitza el terme d'enriquiment que es representa amb el símbol Δ , en què :

$$\Delta = \delta^{15}\text{N}_{\text{consumidor}} - \delta^{15}\text{N}_{\text{dieta}}$$

i oscil·la entre 1 i 5‰ (el valor mitjà se situa al voltant del 3,4‰). Aquest enriquiment es produeix com a resultat de les diferents afinitats d'ambdós isòtops de nitrogen a les reaccions metabòliques, essent el ^{14}N afavorit davant el isòtop més pesat en les vies metabòliques dels aminoàcids (Gannes et al. 1998; Fantle et al. 1999). S'han descrit diferències en l'enriquiment isotòpic depenent de les vies d'assimilació i excreció dels compostos nitrogenats en els organismes (Vanderklift i Ponsard 2003). Els dos factors principals són, per una banda, la ruta d'excreció de nitrogen utilitzada per cada organisme (ammoniotèlica, ureotèlica i uricotèlica) generalment constant dins un mateix grup, i per l'altra, el contingut de nitrogen de la dieta, tant en termes de disponibilitat de nitrogen total (Adams i Sterner 2000; Vanderklift i Ponsard 2003) com d'alguns aminoàcids específics (Fantle et al. 1999; McClelland i Montoya 2002).

A diferència de $\delta^{15}\text{N}$, el quocient dels isòtops de carboni ($^{13}\text{C}/^{12}\text{C}$ en relació a Pee-Dee belmite o $\delta^{13}\text{C}$) canvia molt poc a mesura que el carboni es mou a través de les xarxes tròfiques, i per tant és un bon indicador de la procedència del menjar (DeNiro i Epstein 1981; Post 2000; McCutchan et al. 2003). En els estanys s'ha vist que és de molta utilitat per distingir entre les zones litorals i els ambients pelàgics (France 1995), a causa de la diferència en el senyal de carboni entre ambdós hàbitats. Per exemple a l'estany Redon (Catalan et al. 2004), el sèston i els sediments profunds tenen un senyal

més empobrit en $\delta^{13}\text{C}$ que les zones del litoral, probablement aquest és un reflex de la presència predominant de producció primària a l'hipolímnion, sota la termoclina estacional, a causa de l'extrema transparència de la columna d'aigua (Capítol 2). La temperatura de l'hipolímnion és significativament menor que al litoral (ca. 5-10°C), la respiració dins el mateix estany seria el procés que més contribuiria a la disponibilitat de CO_2 , factors tots dos que afavoreixen un senyal isotòpic més lleuger (Rau 1980). Per altra banda, les algues bentòniques tendeixen a estar enriquides en ^{13}C , per causa d'un efecte de capa límit, que implica una major limitació de la difusió de CO_2 a les cèl·lules i per tant afavoreix la utilització de bicarbonat com a substrat de carboni (Hecky i Hesslein 1995). Aquesta diferència entre el litoral i el plàncton ha permès descriure en els peixos la contribució relativa de menjar que prové d'ambdós hàbitats (Vander Zanden i Vadeboncoeur 2002; Catalan et al. 2004). També s'han descrit moltes xarxes tròfiques en diferents estanys utilitzant la composició d'isòtops estables dels seus organismes (Kling et al. 1992; Gu et al. 1994; Yoshioka et al. 1994; Yoshii et al. 1999; Grey 2001).

Objectius

L'objectiu d'aquesta tesi és descriure la variabilitat en les històries de vida de les tres espècies de crustacis que formen el zooplàncton de l'estany Redon. S'ha fet especial èmfasi en la utilització de tècniques que donessin una informació de la condició fisiològica i de l'assimilació de nutrients de les espècies directament al camp. Malgrat que els experiments, tant al camp com al laboratori, poden oferir una comprensió sobre quins són els factors que estructuraven les comunitats ecològiques, també tenen unes limitacions importants, especialment relacionades amb la reducció de la complexitat natural a una o unes poques hipòtesis concretes (Gurevitch et al. 2000). L'aproximació que hem adoptat en aquesta tesi ha anat dirigida a utilitzar mètodes que permetin capturar els factors principals que afecten les espècies en sistemes naturals i desenvolupar o testar la utilitat d'aquestes aproximacions en donar-nos informació sobre les característiques ecològiques de les espècies.

Per assolir aquest objectiu hem realitzat dos estudis principals, que coincideixen amb les dues parts de la tesi:

1. Variabilitat estacional i interanual

S'ha fet un seguiment continuat durant un període de cinc anys a l'estany Redon amb l'objectiu de descriure'n la variabilitat estacional i interanual de les seves principals característiques limnològiques i la dinàmica associada de les tres espècies de zooplàncton. L'estudi es va dividir en dues parts, corresponents als capítols 2 i 3:

El capítol 2 descriu els canvis estacionals dels principals paràmetres físics, químics i biològics de l'estany Redon al llarg de dos cicles anuals complets. Es va posar especial èmfasi en comparar la dinàmica interna amb les forces externes que condicionaven aquesta dinàmica. Aquest capítol tenia la pretensió addicional d'aportar una descripció limnològica general, que havia de servir de base a la resta de capítols.

En el capítol 3 ens vam concentrar en la variabilitat interanual de la dinàmica i les històries de vida de les tres espècies de crustacis del zooplàncton de l'estany. Per a a-

quest treball es van utilitzar dades de cinc anys complets. Els objectius específics del capítol van ser investigar (1) si la qualitat del menjar era més important que la disponibilitat d'aquest en explicar l'abundància i els components de les històries de vida de les tres espècies; (2) si hi havia una interacció interespecífica denso-dependent en alguna de les tres espècies, i (3) avaluar si la depredació dels copèpodes era perceptible en la variabilitat interanual de la *Daphnia*.

2. Variabilitat estacional en la composició elemental, bioquímica i isotòpica del zooplàncton

Per poder descriure les possibles relacions tròfiques de les tres espècies, les seves característiques elementals i bioquímiques, i la relativa inversió energètica en la reproducció, es van mostrejar les tres espècies al llarg d'un cicle estacional complet. Els resultats d'aquest estudi es presenten en tres capítols separats (dels capítols 5 al 7), i són complementats amb el capítol 4, en què s'aborda l'establiment d'una relació entre la composició elemental del zooplàncton i els seus components bioquímics majoritaris. Els objectius específics dels quatre capítols van ser:

Capítol 4. En aquest capítol ens vam proposar determinar la variabilitat en la composició estequiomètrica dels compostos bioquímics principals (ceres, triacilglicerols, fosfolípids, proteïnes i aminoàcids lliures) en el zooplàncton marí i d'aigua dolça. En un segon estadi, es va calcular la composició estequiomètrica mitjana dels diferents compostos bioquímics, i la seva utilitat es va avaluar per estimar la composició elemental del zooplàncton a partir de la seva composició bioquímica. Els resultats d'aquest capítol es van utilitzar en el capítol següent per tal d'establir una relació entre la composició bioquímica i elemental de les tres espècies.

Capítol 5. En aquest capítol es va abordar l'esforç reproductor, un dels principals components de les històries de vida de les espècies. Es va comparar la composició elemental i bioquímica de les tres espècies estudiades, i es va examinar la importància relativa del desenvolupament i la reproducció en la composició estequiomètrica de cada espècie.

Capítol 6. La composició aminoacídica del zooplàncton es coneix ben poc, sobretot en comparació amb altres compostos com els àcids grassos. Per tant es va estudiar la variabilitat estacional en la composició aminoacídica de les tres espècies de crustacis, amb la finalitat de separar la importància relativa de la variació intraespecífica de la interespecífica. Addicionalment, com que les tres espècies coexisteixen en l'espai i el temps, es van explorar les possibles deficiències alimentàries en aquests compostos amb l'objectiu de poder predir quins aminoàcids podrien ser limitants per al seu creixement.

Capítol 7. Els objectius que es van plantejar en aquest capítol van ser (1) descriure la variació estacional en la composició isotòpica de les espècies i les seves relacions tròfiques; (2) estimar la importància relativa dels lípids i la quitina en la variabilitat isotòpica; (3) determinar si hi havia diferències significatives en l'ontogènia i entre sexes; i finalment (4) examinar si la variabilitat del fraccionament isotòpic dels copèpodes, especialment pel que fa al nitrogen, tenia alguna relació amb la quantitat o qualitat proteica de la seva dieta.

Resultats

Característiques principals de la variabilitat estacional i la dinàmica d'un estany profund

L'estany Redon (Val d'Aran), un estany dimíctic d'alta muntanya, va ser estudiat durant dos cicles estacionals complets des del juliol del 1996 fins al juliol del 1998. Es van descriure les variacions en els principals paràmetres físics, químics i biològics, amb especial èmfasi en l'influència externa (el clima i la deposició atmosfèrica) sobre la dinàmica interna de l'estany. La temperatura anual mitjana durant aquest període va ser de 3.6°C. La duració de la coberta de gel va ser de 4.5 mesos l'hivern de 1996/97 i 5.8 mesos el 1997/1998. L'aigua de l'estany era molt diluïda (conductivitat anual mitjana de 12 $\mu\text{S cm}^{-1}$), tot i que la concentració dels ions de la pluja eren menors que els de l'aigua de l'estany. Aquestes diferències estaven causades per l'aportació de calci originat a la mateixa conca. L'amoni va ser el ió més abundant a la pluja amb una concentració mitjana de 17 μM , en canvi a l'estany no va passar mai de 3 μM . La concentració de nitrogen inorgànic dissolt sempre excedia la del fòsfor reactiu soluble en dos o tres ordres de magnitud, fet que indicava que el fòsfor era el ió limitant per al creixement del fitoplàncton. Es van identificar quatre episodis de producció principals, dos coincidint amb els períodes de barreja, el de primavera i el de tardor, a la part superior de l'hipolimnion durant l'estratificació de l'estiu, i sota el gel, just al principi del període hivernal. Les concentracions de clorofil·la *a* més elevades (1.2 – 2.2 $\mu\text{g l}^{-1}$) es van mesurar durant la barreja de tardor. La clorofil·la *b* era comparativament important a la part superior de l'hipolimnion, i la clorofil·la *c* als períodes de barreja. Les taxes de consum d'oxigen al llarg del període hivernal es van estimar entre 339 $\text{mg O}_2 \text{ m}^{-2} \text{ d}^{-1}$ el 1996/97 i 281 $\text{mg O}_2 \text{ m}^{-2} \text{ d}^{-1}$ el 1997/98.

Variabilitat interanual en les històries de vida de *Daphnia*, *Cyclops* i *Diaptomus* en un estany oligotròfic: relacions ambientals, intra- i interespecífiques

Els estanys d'alta muntanya són típicament oligotròfics i tenen conseqüentment una baixa diversitat de grups d'espècies de crustacis zooplànctònics. Quan la depredació per part dels peixos és baixa, la qualitat del menjar, la denso-dependència intraspecífica i la depredació invertebrada són els factors que s'espera que afectin d'una manera més rellevant els grups d'espècies del zooplàncton d'aquests estanys. Es van mostrejar els crustacis del zooplàncton de l'estany Redon durant un període de cinc anys consecutius per determinar la importància relativa de la qualitat del menjar, la interacció intraspecífica i la depredació per part d'invertebrats en la variabilitat anual de les espècies. Els crustacis que poblen el plàncton d'aquest estany són el cladòcer herbívor *Daphnia pulex*, i dos copèpodes carnívors/omnívors, el calanoide *Diaptomus cyaneus* i el ciclopoide *Cyclops abyssorum*. El final de la coberta de gel va variar en quasi dos mesos al llarg del període mostrejat, i va ser el paràmetre limnològic més rellevant a l'hora de

caracteritzar la temporalitat dels cicles de vida de les tres espècies. Ambdós copèpodes van produir nauplis en fondre's la coberta de gel, i el moment de maduració de la *Daphnia* estava fortament correlacionat amb el dia de fusió de la coberta de gel. La densitat interanual mitjana del *Cyclops* i la *Daphnia* estava correlacionada negativament amb la seva mida màxima, i en el cas de la *Daphnia* amb la mida de maduració, suggerint que hi havia una interacció denso-dependent. Per altra banda, la mida màxima del *Diaptomus* no tenia cap relació amb l'abundància, però sí que estava relacionada amb la disponibilitat de fòsfor particulat. La quantitat de menjar no estava relacionada amb cap paràmetre poblacional o de les històries de vida de cap de les tres espècies. La comparació de la composició estequiomètrica de les tres espècies i la del sèston va posar de relleu que la *Daphnia* tenia una proporció de fòsfor més elevada que el sèston, i que els dos copèpodes tenien una major proporció de carboni i nitrogen que la *Daphnia* i el sèston. La producció anual de *Daphnia* estava fortament correlacionada amb el fòsfor particulat i la fecunditat. Per tant es va concloure que la *Daphnia* estava limitada per la disponibilitat de fòsfor. El fet que l'abundància de *Daphnia* estigués correlacionada amb la biomassa de *Cyclops* posava de manifest que no es podia descartar que de manera addicional es produís depredació del copèpode sobre la *Daphnia*.

Relació entre la composició elemental i bioquímica en crustacis del zooplàncton d'aigües dolces i marines

Els compostos bioquímics majoritaris del zooplàncton (proteïnes, lípids, quitina, àcids nucleics i nucleòtids) tenen una funció diferent. Per tant, la incorporació de la informació que podrien aportar els compostos bioquímics en les anàlisis estequiomètriques permetria ampliar el coneixement actual de les històries de vida de les espècies i les seves interaccions. La composició estequiomètrica dels principals compostos bioquímics de 43 espècies de zooplàncton d'aigua dolça i marina va resultar ser molt constant. No es van trobar diferències significatives entre les espècies marines i les d'aigua dolça, ni entre els diferents grups taxonòmics analitzats (amfípodes, copèpodes, cladòcers, eufàusids i mísids) per a la majoria dels principals compostos bioquímics (triacilglicerol, ceres, fosfolípids i proteïnes). Per tant, la variació intra- i interespecífica en la composició elemental del zooplàncton és el resultat de variacions en les diferents proporcions dels principals compostos bioquímics. La composició elemental de *Calanus finmarchicus* es va estimar a partir de les proporcions dels principals compostos bioquímics. La comparació entre la composició elemental mesurada i l'estimada va mostrar que la major part del carboni, nitrogen i fòsfor era estimada adequadament. Només un 26,3% del fòsfor no es va poder explicar, probablement a causa de que era una fracció soluble en àcids ja fossin nucleòtids o fosfats. La importància relativa dels principals compostos bioquímics en explicar la composició elemental del zooplàncton es va estimar combinant els rangs de variació de cada compost bioquímic obtinguts de la bibliografia amb la seva composició estequiomètrica. Gairebé tot el contingut de carboni quedava justificat per les proteïnes i els lípids (junts explicaven entre el 82 i el 98% del contingut total de carboni). Les proteïnes explicaven entre el 46 i el 98% del nitrogen total. El fòsfor va ser l'element amb la major variabilitat pel que fa al compost bioquí-

mic que contribuïa en major proporció al total d'aquest element. En la major part dels casos els àcids nucleics eren el compost que representava una proporció major del fòsfor, seguits dels fosfolípids i en un menor grau, els nucleòtids. Malgrat això, cadascun dels tres compostos, segons les seves proporcions relatives, podia arribar a ser el que més contribuïa al total de fòsfor en un organisme determinat.

La reproducció com a principal causa d'homeòstasi no estricta en la composició elemental del zooplàncton

La composició de carboni, hidrogen, nitrogen i fòsfor en les tres espècies de crustacis planctònics de l'estany Redon es va mesurar durant un cicle estacional complet, amb l'objectiu de determinar la contribució relativa del creixement i la reproducció a la variabilitat de la seva composició elemental. Les espècies estudiades incloïen el cladòcer *Daphnia pulicaria*, el copèpode ciclopoide *Cyclops abyssorum* i el copèpode calanoide *Diaptomus cyaneus*. En les tres espècies, la reproducció era la principal causa de variabilitat elemental, en canvi, hi havia una molt baixa influència del creixement. Les femelles de les tres espècies van perdre entre un 32% i un 48% del seu contingut de carboni i hidrogen inicial per la reproducció, que es corresponia a una pèrdua similar del seu contingut en lípids i en glúcids. El contingut en nitrogen no va canviar en cap de les tres espècies, ni el seu contingut en proteïnes i quitina. Tant la *Daphnia* com el *Diaptomus* van perdre un 35% i un 56% respectivament del seu contingut inicial de fòsfor durant la reproducció. En canvi, el contingut de fòsfor del *Cyclops* no va canviar. Les tres espècies van emmagatzemar compostos energètics en condicions desfavorables (*Cyclops* i *Daphnia*) o durant el creixement (*Diaptomus*) per gastar-los en la producció de la descendència. Però només la *Daphnia* i el *Diaptomus* van mobilitzar fòsfor emmagatzemat. Els mascles dels *Cyclops* i la *Daphnia* van viure durant un període molt més curt que les femelles, tenint a més a més una composició elemental molt més constant. Els mascles de *Diaptomus* van viure durant el mateix període que les femelles i la seva composició elemental va sofrir els mateixos canvis. Les diferències entre la variabilitat elemental dels mascles de les tres espècies poden ser interpretades pels majors requeriments de còpula que tenen els copèpodes calanoides. Es creu que la variabilitat elemental de les tres espècies és comú en la major part d'espècies que tenen segregat el període d'alimentació del de reproducció i totes elles tenen una composició elemental no homeostàtica al llarg del seu cicle de vida.

Variació estacional de la composició intraespecífica i ontogènica de la composició aminoacídica del zooplàncton: implicacions per restriccions nutritives

La composició aminoacídica com a indicadora de la qualitat de l'aliment en el zooplàncton ha estat poc estudiada, especialment si es compara amb els nutrients i els àcids grassos. En estudis anteriors s'han trobat diferències significatives en la composició aminoacídica de diferents espècies de crustacis. Es desconeix quina és la variabilitat intraespecífica deguda a factors com l'ontogènia, l'estacionalitat i els canvis entre gene-

racions. Aquesta informació és important per poder incorporar adequadament la composició aminoacídica en el marc de l'ecologia estequiomètrica. En aquest capítol s'estudia quina és la variació de la composició aminoacídica en una espècie de cadascun dels grans grups taxonòmics del zooplàncton d'aigua dolça, estudiats en el mateix hàbitat de l'estany. Les diferències interespecífiques en la composició aminoacídica van ser tres vegades més grans que la variació intraespecífica (75,3% i 24,7%, respectivament). Els dos copèpodes tenien una composició més semblant entre ells indistintament de la variació estacional i ontogènica. Dins de cada espècie, *Daphnia pulex* tenia una molt baixa variabilitat en la composició aminoacídica associada amb les diferències entre les generacions (10,9%), essent la major part de variància associada amb canvis estacionals (45,5%). *Cyclops abyssorum* tenia una variació estacional similar (49,5%), i un 19,3% de la variació era atribuïble a diferències entre estadis. *Diatomus cyaneus* va ser l'espècie amb el canvi estacional més marcat (84% de la variància intraespecífica). No hi havia diferències en la composició entre mascles i femelles en cap dels dos copèpodes, malgrat presentar un dimorfisme sexual marcat. Els desequilibris aminoacídics entre les possibles relacions tròfiques van ser estimades utilitzant dos mètodes diferents. Els resultats confirmen l'hipòtesi d'un alt grau d'homeòstasi en la composició aminoacídica dels crustacis del zooplàncton. Per tant, la composició aminoacídica pot ser utilitzada en estudis estequiomètrics conjuntament amb anàlisis elementals i d'àcids grassos.

La variació intraespecífica de la composició isotòpica indica canvis en la dieta i el creixement limitat per nitrogen en el zooplàncton d'un estany d'alta muntanya

Els canvis estacionals en la composició d'isòtops estables de carboni i nitrogen ($\delta^{13}\text{C}$ i $\delta^{15}\text{N}$) es van mesurar, durant el 1999, en els crustacis del zooplàncton d'un estany d'alta muntanya. Les tres espècies tenien un senyal isotòpic diferent, els dos copèpodes estaven un nivell tròfic per sobre el cladòcer. Malgrat això, les variacions intraespecífiques mesurades van ser significatives. Canvis en la composició isotòpica associats a canvis en la composició bioquímica estaven principalment relacionats amb canvis en les proporcions de lípids, en canvi, no es van detectar canvis associats a la concentració de quitina. Les variacions lipídiques explicaven fins a un 30% de la variació de $\delta^{13}\text{C}$ en els adults dels copèpodes, tot i que aquests canvis no es traduïen en diferències estacionals en el fraccionament isotòpic entre els copèpodes i la *Daphnia*. La major part de la variació estacional anava associada amb canvis ontogènics. El senyal de $\delta^{13}\text{C}$ i $\delta^{15}\text{N}$ de la *Daphnia* va canviar: de ser més enriquida va passar a més empobrida, reflectint un canvi en la dieta, probablement per passar de consumir bacteris o material al·lòcton a sèston. Els copepodits de *Cyclops* també presentaven un canvi isotòpic associat a un canvi de dieta amb l'ontogènia, en aquest cas era de passar de consumir algues a *Daphnia*. El canvi en *Cyclops* no era igual per a ambdós isòtops. L'ajustament d'un model de discriminació isotòpica basat en el creixement explicava un 99% de la variància del carboni i només un 60% de la del nitrogen. La resta de variància del nitrogen es va veure que estava relacionada amb la creixent distància aminoacídica entre els cope-

podits i el seu recurs, la *Daphnia*. El fraccionament isotòpic de nitrogen entre el *Cyclops* i la *Daphnia* estava directament relacionat amb la distància en la seva composició aminoacídica, que s'interpreta com una evidència d'una limitació de nitrogen en el consumidor.

Conclusions

Les característiques limnològiques més rellevants de l'estany Redon són la presència de quatre episodis principals de producció, associats amb els dos períodes de barreja, a la capa superior de l'hipolímnion durant l'estratificació de l'estiu, i sota el gel al principi del període hivernal. D'aquests quatre episodis els més productius eren sempre els associats amb la barreja. La durada de la coberta va ser la característica limnològica que més va marcar la temporalitat en el cicles de vida de les tres espècies de crustacis (*Daphnia pulicaria*, *Cyclops abyssorum* i *Diaptomus cyaneus*). El seguiment d'aquestes espècies durant un període continuat de cinc anys va permetre descriure'n les seves característiques ecològiques principals, incloent-hi determinats aspectes de les seves històries de vida. Tant la *Daphnia* com el *Cyclops* presentaven una correlació negativa entre la seva mida màxima i la densitat interanual, indicant que hi havia una interacció intraespecífica denso-dependent. La comparació de la composició estequiomètrica de les tres espècies amb la del sèston va mostrar que la *Daphnia* tenia una major proporció de fòsfor que el sèston i els copèpodes tenien més carboni i nitrogen que el sèston i la *Daphnia*. Es conclou que la *Daphnia* pot estar limitada pel contingut de fòsfor i els dos copèpodes pel de nitrogen.

Per poder establir una relació directa entre la composició estequiomètrica del zooplàncton i la seva composició bioquímica, es va fer una cerca bibliogràfica de la composició estequiomètrica dels principals compostos bioquímics de diferents espècies representatives dels principals grups taxonòmics del zooplàncton marí i d'aigua dolça. Es va concloure que els principals compostos bioquímics (triacilglicerol, ceres, fosfolípids i proteïnes) tenien una composició estequiomètrica molt constant, i per tant es conclouïa que la variació estequiomètrica observada en les diferents espècies de zooplàncton era deguda a variacions en les proporcions relatives dels compostos bioquímics majoritaris.

En les tres espècies de l'estany Redon, la principal font de variació en la composició elemental era l'esforç reproductor. Aquesta característica es creu que és pròpia d'espècies que tenen desacoblada l'obtenció d'aliment del moment en què es reproduïxen. En les tres espècies va suposar una reducció del contingut de carboni i d'hidrogen d'entre el 32% i el 48%. En canvi, només la *Daphnia* i el *Diaptomus* van reduir el seu contingut de fòsfor en una proporció similar al del carboni i l'hidrogen. El *Cyclops* va mantenir el contingut de fòsfor constant. El nitrogen va ser l'únic element que no va canviar en cap de les tres espècies. Aquests canvis en la composició elemental corresponien a canvis en la composició bioquímica, especialment de lípids i glúcids. El contingut de proteïna i de quitina es va mantenir constant. D'una manera similar, el contingut aminoacídic va variar molt poc en les tres espècies. El 75% de la variació en la composició aminoacídica corresponia a diferències interespecífiques.

L'anàlisi de la composició isotòpica va posar de relleu les característiques tròfiques de les tres espècies. El *Cyclops* apareixia com la espècie més carnívora, el *Diaptomus* apareixia una mica per sota del *Cyclops*, i la *Daphnia* era l'única espècie clarament herbívora.

General introduction

1. General introduction

Limnology of alpine lakes

Alpine lakes have been one of the less studied lake types, especially compared with other temperate lakes such as those located in the lowlands, probably due to their relatively remote location and difficult access. In contrast with lowland lakes, their distance from urban areas has prevented them from suffering direct human impacts such as high nutrient loads, which affected most European lowland lakes during the first half of the twentieth century (Margalef 1983). Pioneer limnologists started studies in European alpine lakes in the Alps and in the Pyrenees, and in North America in order to explore all possible lake types. In the Pyrenees, pioneer studies (Gaurier 1933 and references therein; Margalef 1948; Margalef 1952) were followed by relatively few but detailed studies at both sides of the mountain range (Capblancq 1972; Rey & Capblancq 1975; Margalef et al. 1975; Vilaseca 1978; Miracle 1978a; Miracle 1978b; Campas & Vilaseca 1979), which continued up today in the southern part (e.g. Catalan 1988; Catalan 1989; Catalan et al. 1992; Gacia et al. 1994; Camarero & Catalan 1996; Felip et al. 1999).

The discovery that pollutants could be transported by the air masses, and especially after the discovery of the human-induced transboundary acidification problems of some north European (Bresser & Salomons 1990) and north American lakes (Scholfield 1976), raised the political concern towards alpine lakes. In order to study the possible acidification status in the different European mountain areas a research network was initiated in 1991, which has enabled to carry out numerous research studies in alpine lakes around Europe in a coordinated manner (e.g. Wathne 1992; Catalan et al. 1994; The MOLAR Water Chemistry Group 1999; Catalan et al. 2002).

Lake Redon is the most studied alpine lake in the Pyrenees. It has been the study site of various theses and dissertations focused on the seasonal changes of water chemistry and primary production (Catalan 1987), the effects of acidification (Camarero 1994), the general plankton composition, with special emphasis on phytoplankton (Felip 1997), the chemistry and biology of the ice cover (Camarero 1994; Felip 1997), the general ecology of plankton focusing on Rotifers (Bartumeus 1999), the algal group pigment composition (Buchaca 2005) and paleolimnological studies of algal remains (Buchaca 1998; Pla 1999; Buchaca 2005). However, the crustacean zooplankton community of the lake has not been studied specifically. The present study focuses on the study of the ecology of the three planktonic crustacean species present in Lake Redon.

Crustacean zooplankton of Pyrenean lakes

Plankton colonisation from the benthos by crustaceans has been ongoing at least since the last 650 million years. Copepods and cladocerans, the two most common crustacean groups in freshwater environments, both have been described to have had a benthic ori-

gin, dated back to the end of the Cretaceous, although some brachiopods have been dated back to the Cambrian times (Rigby & Milsom 2000). Copepods migrated to inland waters from the sea in different events (Huys & Boxshall 1991), being represented by two orders with planktonic representatives, Cyclopoida and Calanoida. Amongst the cladocerans, the order anomopoda and particularly the daphniids are the main representatives of freshwater plankton (Fryer 1991).

Table 1.1. Crustacean planktonic species found in a survey of 98 lakes distributed along the whole range of the Pyrenees. The species present in Lake Redon are highlighted in black. Biogeographical distribution are obtained from Alonso (1996) and Miracle (1982).

Taxonomic group	Species	Occurrence (%)	Biogeographical distribution
Calanoida	<i>Diaptomus cyaneus</i>	17.2	Western Mediterranean
	<i>Mixodiaptomus laciniatus</i>	17.2	Boreo-Alpine
	<i>Eudiaptomus vulgaris</i>	7.5	Central European
	<i>Diaptomus castaneti</i>	7.5	Western European
Cyclopoida	<i>Cyclops abyssorum</i>	87.1	Palaearctic
	<i>Acanthocyclops vernalis</i>	3.2	Cosmopolitan
Cladocera	<i>Daphnia longispina</i>	65.6	Palaearctic
	<i>Chydorus sphaericus</i>	21.5	Cosmopolitan
	<i>Daphnia pulex</i>	12.9	Cosmopolitan
	<i>Alona sp.</i>	8.6	Cosmopolitan
	<i>Scapholeberis mucronata</i>	1.1	Cosmopolitan
	<i>Holopedium gibberum</i>	1.1	Boreo-Alpine
	<i>Acroperus harpae</i>	1.1	Cosmopolitan
	<i>Ceriodaphnia cf. quadrangula</i>	1.1	Holarctic and Neotropical
	<i>Daphnia obtusa</i>	1.1	Cosmopolitan
	<i>Daphnia rosea</i>	1.1	Palaearctic
	<i>Eurycerus lamellatus</i>	1.1	Palaearctic
<i>Simocephalus vetulus</i>	1.1	Cosmopolitan	

Pyrenean lakes originated approximately 15.000 years ago after the last glaciation, and therefore, in evolutionary terms, all the species that subsequently colonised the new habitats have inhabited these environments for a relatively short time. The species present and their distribution was first described a couple of decades ago (Miracle 1978a; Miracle 1978b). A recent survey has confirmed previous distributions (Table 1.1). Two main colonisation pathways have been identified in the Iberian Peninsula (Miracle 1982). One in the north-east (mainly Boreo-Alpine species entering through the eastern part of the Pyrenees) and another in the north-west (Atlantic or Lusitan species entering through Galicia and Picos de Europa). Within the Pyrenees species incoming from both pathways may be found (Table 1.1).

The two copepod species found in Lake Redon are the most abundant species in the Pyrenees, while *Daphnia pulex* is less common (Table 1.1). From their geographical

distribution and especially from their occurrences with other species some relevant features of the species may be highlighted. *Diaptomus cyaneus* was the dominant calanoid copepod when found, in the same lake, with other calanoids from the other genus (*Eudiaptomus cyaneus* and *Mixodiaptomus laciniatus*). However we have never found it together with the other species of the genus present in the Pyrenees (*D. castaneti*). Similarly, we have not found two species of *Daphnia* together in any lake in the Pyrenees. *Cyclops abyssorum* is the only cyclopoid copepod in most lakes.

In terms of their reproduction features, almost all freshwater crustacean species produce more than one clutch of eggs during the reproductive season and mostly complete their life-cycle within one year (iteroparous annual Begon et al. 1990). Copepods have an obligate sexual reproduction, while cladocerans are usually parthenogenetic, and occasionally may also generate males for the production of ephippia, their specific resting eggs. The mode of diapause is a reproduction strategy, which have been described to be of phylogenetic origin in crustaceans (Hairston & Caceres 1996). Calanoid copepods and cladocerans are able to produce resistance eggs, which enables them to survive adverse periods in the sediment. Cyclopoid copepods are not able to produce resistance eggs but are able to survive buried in the sediments as CV copepodites. These reproduction strategies have evolved to survive threatening environmental conditions (Santer 1998). However, not all species within each group utilise this capability with the same frequency or mode. Within a population the predicted optimal strategy will be driven by trade-offs that are mediated by the environment in which the organisms live and their specific traits (Hairston & Bohonak 1998). Comparison of different species living in the same environment will be useful for studying their ecological adaptations and their phenotypic response.

The two most prominent characteristics of alpine lakes are their strong seasonality and the general oligotrophic conditions. They are ice-covered approximately during half of the year, and production episodes are therefore highly predictable. Organisms inhabiting these habitats are likely to develop specific strategies to survive under these circumstances. While crustaceans and especially *Daphnia* have been one of the most used model organisms, most of their attributed traits have been described either in lab cultures or in other lake types. Although the zooplankton species inhabiting alpine lakes have been described in the past, their ecological characteristics are still not adequately characterised. Recent studies of alpine zooplankton communities are already focused towards this direction (e.g. Gliwicz et al. 2001; Villar-Argaiz et al. 2002; Winder et al. 2003).

Trophic ecology: from energetics to ecological stoichiometry... and biochemistry

Lakes have been traditionally recognised as a paradigm of the ecosystem concept due to their clearly defined external limits (Catalan & Fee 1994). Lake studies have elucidated many fundamental ecological principles. One of the most solid ecological theories, the trophic-dynamic theory of ecosystems (Lindeman 1942) was based on lake studies. However, Lindeman's trophic approach of community organisation was primarily based on energy transfer between the different trophic relationships, which has been criticised for not taking into account the need for other essential elements rather than

carbon (Reiners 1986). On this sense, ecological stoichiometry (Sterner & Elser 2002) overcomes such difficulty by simplifying organisms to their main body constituents (carbon, nitrogen and phosphorus; Elser et al. 1996). It has been described as the study of the balance of energy and multiple chemical elements in living systems (Reiners 1986; Elser et al. 1996), being a step forward from the traditional energy-based focus of trophic ecology.

Ecological stoichiometry has been very useful for identifying constraints in organisms from different environments such as aquatic, marine and terrestrial food webs (Elser & Hassett 1994, Elser et al. 2000). In freshwater zooplankton, cladocerans such as *Daphnia* have a higher phosphorus content than copepods (Andersen & Hessen 1991; Hessen & Lyche 1991), and this different organism's elemental composition has been shown to be directly related with their respective nutrient demands (Sterner & Hessen 1994). As a result, the different zooplankton groups recycle nutrients in different proportions, following their respective elemental composition (Elser & Urabe 1999).

One difficulty that appears when comparing different life histories, is the use of comparable measures of different species traits. The stoichiometric approach is therefore very useful in this sense. However, this simplification has been criticized by being over-simplistic (Brett 1993; Tang & Dam 1999). Organisms are complex systems with some clearly defined compartments or organs, which in turn are build up of few organic biomolecules such as, proteins, lipids, carbohydrates, nucleic acids and nucleotides (Strayer 1988). All of them have relatively different functional roles within organisms, and have relatively different proportions of the different elements. For example, proteins are mainly forming muscle tissues and enzymes, and contain most of the nitrogen content of the organism, while lipids are the most important energy reserve of the body of metazoans, and are mainly composed of carbon, hydrogen and oxygen. Therefore, the possibility of combining both, the elemental and the biochemical composition approaches, should lead to a better interpretation of organism's life histories and their potential changes in their elemental composition (Anderson et al. 2004; Vrede et al. 2004).

Recent findings have shown that under some circumstances some specific organic compounds such as some fatty and amino acids can be the limiting compounds for animal growth, rather than the bulk nitrogen or phosphorus availability (Müller-Navarra et al. 2000; Conceição et al. 2003; Müller-Navarra et al. 2004). This is a result of their essential nature for metazoans. Some fatty acids, mainly some polyunsaturated fatty acids such as eicosapentanoic acid (20:5W3; EPA) and docosahexanoic acid (22:6W3, DHA) cannot be synthesised by these organisms and since they are essential for growth, they rely on vegetation sources for their incorporation into their bodies (Brett & Müller-Navarra 1997). Based on the relative proportions of these essential fatty acids, algal groups have been classified as good or bad-quality food for aquatic herbivores (Ahlgren et al. 1992; Müller-Navarra 1995). Diatoms, chrysophytes and cryptophytes present high EPA and DHA content, whereas chlorophytes and cyanophytes have very little. The presence of some algal groups therefore determines the relative fatty acid limitation. Since oligotrophic lakes such as Redon are dominated by these fatty acid rich algal groups, the fatty acid limitation seems less likely than in hypertrophic conditions (Becker & Boersma 2003). Similarly, some amino acids are necessarily obtained from the diet of most consumers, and a diet deficient in one essential amino acid will have negative consequences in the growth of any organism (D'mello 1994). The relative im-

portance of amino acids in the nutrition of aquatic organisms, has been restricted to some aquaculture studies (Conceição et al. 2003) and very few field studies on zooplankton have been carried out (Guisande et al. 1999; Guisande et al. 2000; Guisande et al. 2002; Guisande et al. 2003). These studies showed that the amino acid composition of these species can provide information on their amino acid requirements. It seems that their phylogenetic relationships are the most relevant for explaining their amino acid composition.

Stable isotopes and the description of food webs

Food-web diagrams have been criticised as being subjective constructs, strongly biased by our inability to observe the relevant taxonomic, spatial, and temporal variations in trophic interactions (Paine 1988). For example, many trophic interactions cannot be visually observed, and therefore the feeding habits of organisms have been traditionally based on numerous direct and indirect observations, and through the analysis of morphological parameters and gut contents (Monakov 2003). However, quantification of what is actually been ingested and especially assimilated by each organism is still very difficult to achieve and despite its great ecological interest, current methods have been shown to be not very reliable (Schindler 1971).

A promising alternative to traditional feeding studies has been the discovery that the proportion of stable isotopes of some key elements (mainly carbon, nitrogen, but also sulphur, hydrogen and oxygen are being used routinely) varies in some predictable manner in the environment, and especially in metabolic reactions. As a result, stable isotopes have the potential to simultaneously capture complex interactions and to track energy flow through ecological communities (Peterson & Fry 1987).

The ratio of stable isotopes of nitrogen ($^{15}\text{N}/^{14}\text{N}$ relative to atmospheric nitrogen or $\delta^{15}\text{N}$) has been used to estimate trophic position because the $\delta^{15}\text{N}$ of a consumer is typically higher than its diet, and the magnitude of the difference is relatively consistent among organisms (DeNiro & Epstein 1981; Robinson 2001; Post 2002; McCutchan et al. 2003; Vanderklift & Ponsard 2003). This difference is generally referred to as enrichment and denoted by the symbol Δ , where:

$$\Delta = \delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{diet}}$$

and ranges between 1 and 5 ‰ (average 3.4 ‰). The trophic enrichment is a result of the different affinity of both nitrogen isotopes to metabolic reactions, being ^{14}N favoured in front of ^{15}N in the amino acid metabolic pathways (Gannes et al. 1998; Fantle et al. 1999). Differences in the isotopic enrichment have been described depending on the assimilation and excretion pathways of the nitrogenous compounds in organisms (Vanderklift & Ponsard 2003). The two main factors are the nitrogen excretion pathway used by each organism (ammoniotelic, ureotelic and uricotelic), which is generally constant within groups, and the nitrogen content of the diet, both in terms of total nitrogen availability (Adams & Sterner 2000; Vanderklift & Ponsard 2003) or some specific amino acids (Fantle et al. 1999; McClelland & Montoya 2002).

Contrasting with $\delta^{15}\text{N}$, the ratio of carbon isotopes ($^{13}\text{C}/^{12}\text{C}$ relative to PeeDee belmite or $\delta^{13}\text{C}$) changes little as carbon moves through food webs and therefore it is a

good indicator of the food sources (DeNiro & Epstein 1981; Post 2002; McCutchan et al. 2003). In lakes it has been useful for distinguishing between the littoral and the pelagic habitats (France 1995), since they have contrasting isotopic signatures. For example in Lake Redon (Catalan et al. 2004), seston and deep sediments were more depleted in $\delta^{13}\text{C}$ than in littoral algae, probably reflecting the predominant occurrence of primary production in the hypolimnion, below the seasonal thermocline, due to the extreme transparency of the water column (Chapter 2). Growing temperature in the hypolimnion was significantly lower than in the littoral (ca. 5-10 °C), and available CO_2 had a larger contribution from within lake respiration (Rau 1980). On the other hand, benthic algae tended to be enriched in ^{13}C , due to a boundary layer effect, involving a limitation of CO_2 diffusion to the cells that favours the use of bicarbonate as carbon substrate (Hecky & Hesslein 1995). This has allowed to describe the relative contribution of the pelagic and benthic habitats to the upper trophic stages such as fish (Vander Zanden & Vadeboncoeur 2002; Catalan et al. 2004). But also many lake trophic chains have been described based of stable isotope composition of their organisms (Kling et al. 1992; Gu et al. 1994; Yoshioka et al. 1994; Yoshii et al. 1999; Grey et al. 2001).

Objectives and structure of the thesis

The aim of this thesis was to describe the life history variability of the assemblage of crustacean zooplankton in Lake Redon, with special emphasis in their life-cycles, ecological characteristics and their trophic relationships. Special attention was placed in the use of techniques, which could provide information on the physiological condition and nutrient assimilation of the species in the field. Although field and laboratory experiments can offer profound insights into the importance of factors structuring ecological communities, they also have important limitations, especially related with their reduction of the natural complexity to one or few specific hypotheses (Gurevitch et al. 2000). Our approach to concentrate on methods to describe species variations and their interactions in the field were intended to capture the main factors affecting species in nature, and to develop or to test the usefulness of approaches, which could provide a better insight in the ecological characteristics of the species and their real trophic interactions.

To achieve these objectives we carried out two main studies, which coincide with the two main parts of the theses:

1. Seasonal and inter-annual variability

We carried out a five-year period continuous monitoring of Lake Redon in order to describe the main seasonal and inter-annual variability of the main limnological characteristics and the dynamics of the three zooplankton species. The study was divided into two parts, corresponding to chapter 2 and 3.

Chapter 2 focused in the seasonal changes occurring in the main physical, chemical and biological parameters of Lake Redon during the first two complete annual periods, with special emphasis on comparing internal lake dynamics with external forcing, and provided a general limnological description of Lake Redon.

Chapter 3 concentrated in the inter-annual life history variability of the three crustacean species of Lake Redon. We sampled the lake assemblage during a five year period. The specific objectives of the study were to test (1) whether food quality was more significant than food quantity in explaining the abundance and life history components of the three species; (2) if there was an intra-specific density-dependent interaction in any of the three species, and (3) to evaluate if copepod predation was noticeable in the *Daphnia* inter-annual variability.

2. Seasonal variability in the elemental, biochemical and isotopic composition of zooplankton

In order to describe the possible trophic relationships of the three species, their elemental and biochemical characteristics, and their reproduction energy allocation we sampled the three zooplankton species during a complete seasonal cycle. The results of this study are presented in three separate chapters (Chapter 5, 6 and 7), and are complemented with Chapter 4, which intends to establish a link between zooplankton elemental composition and their main biochemical compounds. The specific objectives of the four chapters are:

Chapter 4. The purpose of this study was to determine the variability in the stoichiometric composition of the main biochemical compounds (wax esters, triacylglycerols, phospholipids, proteins and free amino acids) of freshwater and marine zooplankton. At a second stage, an average stoichiometric composition for the main biochemical compounds was calculated, and its usefulness was evaluated for estimating the elemental composition from the measured biochemical composition. The results of this chapter are used in the following chapter in order to establish a relationship between biochemical and elemental composition changes.

Chapter 5. This chapter concentrated on one of the main life history component of a species, which is their reproductive energy allocation. In this study we compared the planktonic crustacean community of an alpine lake in terms of their elemental and biochemical composition, and we examined the relative importance of ontogeny and reproduction on the stoichiometric composition within each species.

Chapter 6. Since the amino acid composition variability of zooplankton is almost unknown in comparison to other organic compounds such as fatty acids, we studied the amino acid composition variation of the three crustacean zooplankton species, in order to separate the relative importance of inter-specific from the intra-specific variation. Furthermore, since the three species were known to coexist in space and time, we explored their amino acid imbalances in order to predict which amino acids would be limiting in each of the potential trophic interactions.

Chapter 7. The aims of this study were (1) to describe the seasonal isotopic composition of these species and their trophic relationships; (2) to estimate the relative importance of lipids and chitin on the isotopic variability of the three species; (3) to determine whether there were significant ontogenic and gender differences in their isotopic composition; and (4) to examine if the variability of the isotopic fractionation of copepods, especially those of nitrogen, changes depending on the protein quantity or quality of its putative food.

Part I

Seasonal and inter-annual variability

2. The main features of seasonal variability in the external forcing and dynamics of a deep mountain lake

Abstract

Lake Redon, a dimictic oligotrophic mountain lake, was monitored for two complete years from July 1996 to July 1998. The main seasonal variations in the physical, chemical and biological parameters are described, with special emphasis on the comparison of external forcing (weather and atmospheric deposition) with internal lake dynamics. Annual mean air temperature was estimated to be 3.6°C. The duration of ice cover on the lake was 4.5 months in 1996/97 and 5.8 months in 1997/98. The lake water was very ion-poor (mean annual conductivity 12 $\mu\text{S cm}^{-1}$); however, ion concentrations in the lake were higher than in the precipitation, the differences being due mainly to Ca^{2+} originating in the catchment. NH_4^+ was the main ion in the precipitation, with an average concentration of 17 μM , whereas in the lake it was always below 3 μM . The concentration of dissolved inorganic nitrogen always exceeded that of soluble reactive phosphorus by two or three orders of magnitude, thus the latter is expected to be the limiting nutrient for phytoplankton growth. Four main production episodes were identified, occurring during spring and autumn overturn, in the upper hypolimnion during summer stratification, and under the ice at the beginning of the ice-covered period. The highest chlorophyll *a* concentrations (1.2 - 2.2 $\mu\text{g l}^{-1}$) were attained during spring overturn; concentrations of chlorophyll *c* were high during both spring and autumn overturn, while chlorophyll *b* was comparatively more relevant in the upper hypolimnion during the stratification period. Winter respiration rates for the lake were estimated to be 339 $\text{mg O}_2 \text{m}^{-2} \text{d}^{-1}$ for 1996/97 and 281 $\text{mg O}_2 \text{m}^{-2} \text{d}^{-1}$ for 1997/98.

Introduction

Alpine lakes, located above the tree-line, are distributed throughout many regions of the world. Their remoteness and their exposure to extreme climatic conditions are the two most distinctive characteristics of such lake systems. Because of their geographical location - either at high latitudes or high altitudes - local anthropogenic influence tends to be low. They are, therefore, ideal sensors for monitoring transboundary air pollution and global change (Schindler et al. 1990). In alpine lakes, at least three different habitats can be distinguished: pelagic, benthic, and the most peculiar, the slush habitat, located within the snow and ice covering the lake. Felip et al. (1995; 1999b) have described the succession of organisms found in this habitat, showing how in some cases these organisms can grow at higher rates within the slush than in the lake itself, despite temperatures close to zero.

Although the main seasonal patterns have been described for lakes in the Alps (Pechlaner et al. 1970; Tilzer & Schwarz 1976; Mosello et al. 1992) in the Pyrenees (Capblancq & Laville 1983; Catalan 1988) and in other mountain ranges (Pienitz et al.

1997a; Pienitz et al. 1997b), the understanding of their seasonal variability is still partially limited by the shortness of the sampled time series, or, in the case of some events, by low sampling frequencies. In particular, there is a lack of studies relating variations in water column features to simultaneous variations in external forcing, i.e., in weather and atmospheric deposition. In this study, we describe the seasonal changes occurring in the main physical, chemical and biological parameters of Lake Redon (central Pyrenees) during two complete annual periods, with special emphasis on comparing internal lake dynamics with external forcing

Materials and Methods

Lake Redon (Fig. 2.1) is a glacial cirque lake located at 2240 m a.s.l. in the central Pyrenees (42° 38' N, 0° 46' E). It is relatively large compared with its catchment area (Table 2.1) and has an average water renewal time of 4 yr (Catalan 1988). There are two main inlet streams which dry up at the end of the summer, and one surface outlet (Fig. 2.1). The catchment is of granodiorite bedrock; 76% of its area is covered by a poorly-developed soil layer with an average thickness of 0.35 m. The main vegetation communities are *Carici-Festucetum eskiae* (36%) and *Ranunculo-Festucetum eskiae* (23%), *Festuca eskia* being the dominant species.

Table 2.1. Morphometric variables characterizing Lake Redon.

Lake area (A)	24 ha
Maximum length (NW-SE)	655 m
Maximum width	565 m
Maximum depth	73 m
Mean depth	32 m
Volume (V)	$7.75 \times 10^6 \text{m}^3$
Mean slope	46%
Catchment Area (Ac)	155 ha
Ac/A	6.5
Ac/V	0.2m^{-1}
Water renewal time	3-4.2 yr

The local meteorological conditions prevailing at Lake Redon from 1996-98 were characterized on the basis of measurements made by an automatic weather station (AWS) deployed about 30 m from the southern lake shore (Fig. 2.1). The base of the mast was 30 m above the lake surface and the sensors were located a further 6-10 m above ground level, i.e. between 2276 m a.s.l. and 2280 m a.s.l. The meteorological variables measured were air temperature, incident and reflected (short wave) solar radiation, net radiation, air pressure, relative humidity, precipitation, wind speed and wind direction. Precipitation measurements were conducted using a Geonor automatic gauge, in which a mixture of methanol and ethylene glycol was used to melt snow, allowing measurements to be made at both positive and negative air temperatures. The

data storage interval was 30 min, and with the exception of air temperature, all values stored were 30 min mean values.

Temperature and dissolved oxygen in the water column were measured monthly at 1-m depth intervals using a WTW TA-197 oxygen meter. In addition, a thermistor chain delivered continuous information on lake water temperatures from January - April 1997 and from August 1997 - February 1998. During the ice-covered period, descriptions of the ice and snow cover were made at the same time as the water column sampling using the criteria proposed by UNESCO/IAHS/WMO (1970).

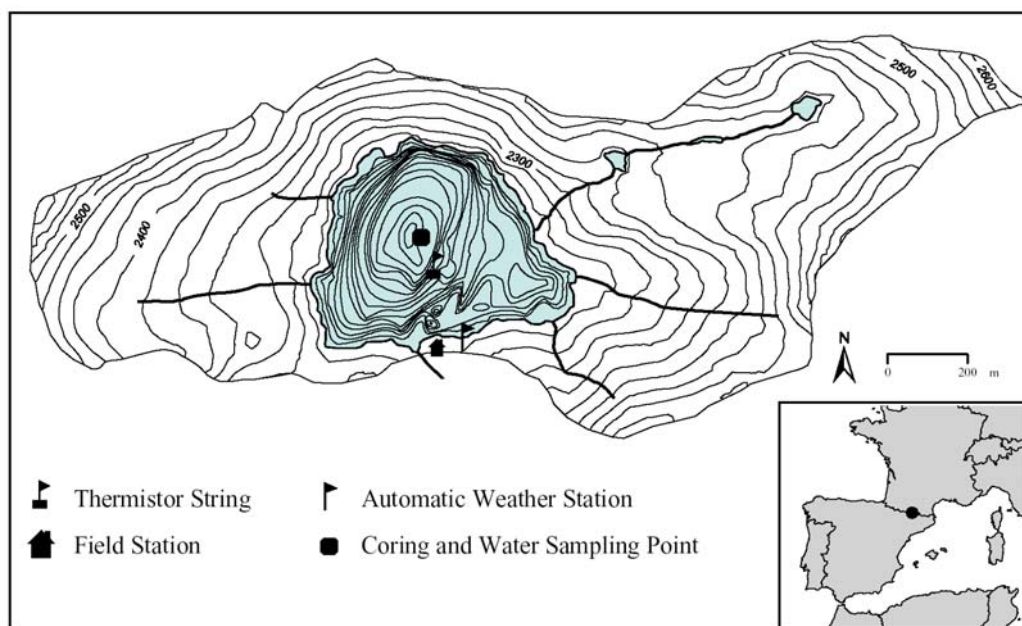


Fig. 2.1. Bathymetric map of Lake Redon (isobathic interval = 5 m). Maximum depth is 73 m at the raft.

Using a Ruttner bottle, water column samples were taken once a month at 3 m intervals from July 1996 - July 1997 and at 9 m intervals from July 1997 - July 1998. The samples were divided into sub-samples immediately for analysis in a hut at the lake shore. Glass-fibre filters were used for pigment and particulate matter analyses; samples were frozen immediately after filtration. Bulk precipitation (rainfall and snowfall) samples were also taken daily from the end of August 1997 to June 1998 at a nearby field station, which is situated at less than 1 km in distance but at a lower altitude (1600 m). This data will be referred as Lake Redon precipitation, since no altitude differences have been found in a previous study (Camarero & Catalan 1996). The procedure followed for the chemical analysis of precipitation was the same as that employed in the case of the water column samples. Finally, samples from the two main inlet streams, located at the eastern basin (Fig. 2.1) were taken once a month during the snow-free period of 1997.

Water column samples were analysed for pH, alkalinity, conductivity, Na^+ , K^+ , Ca^{2+} , Mg^{2+} , NH_4^+ , NO_2^- , NO_3^- , Cl^- , SO_4^{2-} , dissolved organic carbon (DOC), dissolved inor-

ganic carbon (DIC), soluble non-reactive phosphorous (SNRP), soluble reactive phosphorous (SRP), dissolved organic nitrogen (DON), dissolved silicon (DSi), particulate carbon (PC), particulate nitrogen (PN) and particulate phosphorus (PP). Precipitation samples were analysed for pH, alkalinity, conductivity, Na^+ , K^+ , Ca^{2+} , Mg^{2+} , NH_4^+ , NO_3^- , Cl^- , SO_4^{2-} , total nitrogen (TN) and total phosphorous (TP). Conductivity was measured using a PTI-10 conductivity meter, and pH using an Orion Research model 720 pH meter with an electrode for low ionic-strength water. Alkalinity was determined by automated potentiometric Gran titration (Edmond 1970). Na^+ , K^+ , Ca^{2+} , Mg^{2+} , NO_3^- , Cl^- and SO_4^{2-} were analysed with a model CIA-4000 Waters Capillary Ion analyser. DIC and DOC were analysed using a Shimadzu TOC-5000 analyser. Sestonic PC and PN were measured by collecting the particles on pre-combusted Whatman GF/F filters (the samples were prefiltered through a nylon mesh to remove particles larger than 200 μm) and analysing them with a Carlo-Erba C-N-H-S analyser. NH_4^+ was determined by the phenolphthalein method (Solórzano 1969). NO_2^- was determined by the sulphonylamide and n-naphthyl-ethylenediamide method (Grasshoff 1983). TN was determined by persulphate digestion (Grasshoff 1983) followed by UV spectrophotometry. PP, PT and TDP were oxidized to phosphate by acid-persulphate digestion (Koroleff, cited by Grasshoff 1983), and SRP was concentrated using chromatographic cartridges. Phosphate was analysed by the malachite green method (Camarero 1994a). For major ions, an analytical quality control was carried out by ion balance and by comparing measured and estimated conductivities (Golterman et al. 1978).

Chlorophyll (Chl) was extracted using 5 ml of 90% acetone with sonication. Chl a, b and c concentrations were calculated using the equations of Jeffrey and Humphrey (1975). The absorbance ratios A480:A665 and A430:A410 were used as a carotenoid index (Strickland & Parsons 1968) and a phaeopigment index (Moss 1967), respectively.

Results

Meteorological data

Daily mean air temperatures (Fig. 2.2a) varied between -10.5°C and $+16.2^\circ\text{C}$ during the period of observation; they were negative for at least some of the time from October to May. In winter, long periods of generally negative air temperature were often punctuated by periods of a few days in which the air temperature rose above 0°C . However, it is possible for daily mean air temperatures to remain consistently below 0°C for at least 3 weeks in midwinter (e.g., 12 January – 6 February 1998). Based on the monthly mean data, and estimating the missing monthly means in October 1997 and November 1997 by linear interpolation, the annual mean air temperature at Lake Redon was estimated to be 3.6°C .

Daily mean values of incident solar radiation (Fig. 2.2b) varied from less than $10 \text{ W}\cdot\text{m}^{-2}$ on some winter days to over $380 \text{ W}\cdot\text{m}^{-2}$ on cloudless summer days. Based on the approach described by Brock (1981), and using the atmospheric transmission coeffi-

cients of Hottel (1976), the daily mean clear-sky solar radiation at the latitude and altitude of Lake Redon could be calculated for each day of the year. A simple modification to this approach also allowed the influence of the local topography on the clear sky incident solar radiation to be calculated. Because of its southern exposure, the influence of local topographic effects on the clear-sky radiation at Lake Redon is slight, as is shown by the smallness of the discrepancy between curves B and C in figure 2.2b ($< 17 \text{ W}\cdot\text{m}^{-2}$). The percentage reduction in incident clear-sky solar radiation due to local topographic effects is strongly seasonally dependent, varying between a minimum of 4.1% in summer and a maximum of 13.4% in winter, with an annual mean of 7.6%. The radiation measurements from September to April agree well with the corresponding computed clear-sky radiation, implying generally low degrees of cloudiness. In fact, the maximum measured radiation during this period often exceeded the computed clear-sky values, sometimes by as much as 40%. During the same period, the ratio of reflected solar radiation (over land) to incident solar radiation was high (Fig. 2.2c), implying a snow-covered landscape with high albedo. The reason for the measured solar radiation exceeding the theoretical clear-sky radiation is therefore presumably the downward scattering and re-reflection of some of the radiation reflected upwards from the snow-pack. In summer, reflection, absorption and scattering by cloud cover have a considerable influence on the solar radiation incident on the lake. During May - August 1997, for instance, the monthly mean measured solar radiation varied between 59% and 73% of the theoretical clear-sky radiation; this is substantially lower than the range of 91% - 105% found for February - April 1997.

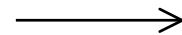
The daily mean net atmospheric (long-wave) radiation (Fig. 2.2d) was always positive, ranging from only slightly above zero to $137 \text{ W}\cdot\text{m}^{-2}$. There was no strong seasonal variation about the annual mean of $59 \text{ W}\cdot\text{m}^{-2}$. During the open-water period, the net long-wave atmospheric radiation accounted for an average of 25% of the total incident radiation and can therefore be considered to be relevant for the heat balance of the lake.

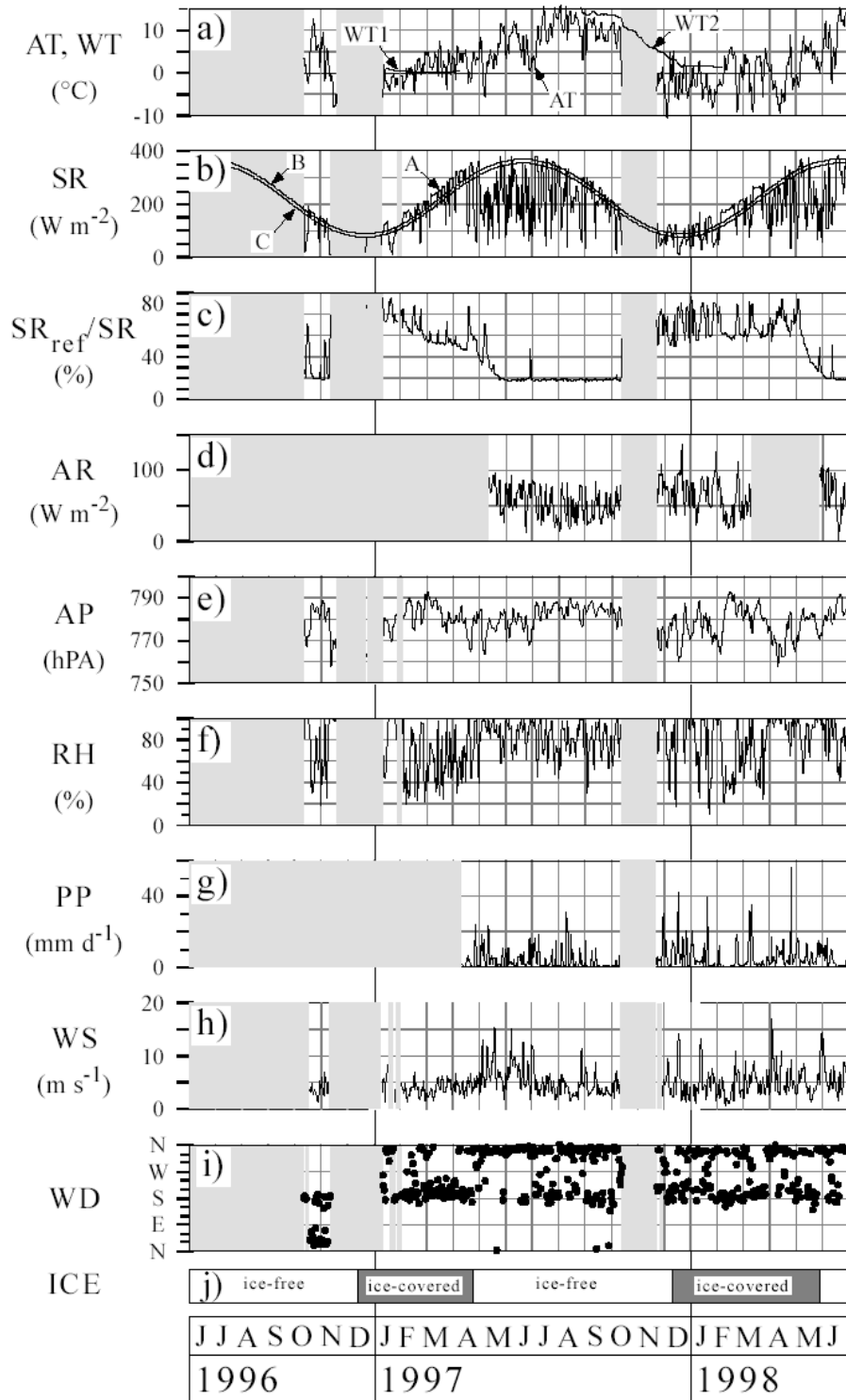
Although the air pressure at the lake (Fig. 2.2e) exhibited considerable day-to-day fluctuations, its seasonal variability was low. In summer 1997, for instance, the mean air pressure was 781.8 hPa, falling only slightly to 778.0 hPa in the following winter. The annual mean air pressure at the lake (\pm one standard deviation) was estimated to be 779.5 ± 7.0 hPa. Applying the empirical formula of Bührer and Ambühl (1975), this implies an O_2 saturation concentration of $10.1 \pm 0.1 \text{ mg O}_2\cdot\text{l}^{-1}$ at 4°C (i.e., during overturn).

Relative humidity (Fig. 2.2f) exhibited a slight seasonal variability around the annual mean of 78%. Mean seasonal values were 70% and 84% in spring 1997 and 1998, respectively, 81% in summer 1997, and 72% in winter 1997-98 (autumn means could not be computed due to lack of data).

Winds (Fig. 2.2h) were strong at Lake Redon and calm periods were rare. Daily mean wind speeds ranged from $0.8 - 17.0 \text{ m}\cdot\text{s}^{-1}$, exceeding $5 \text{ m}\cdot\text{s}^{-1}$ during 40% of the time, and $10 \text{ m}\cdot\text{s}^{-1}$ during 5% of the time. Based on all data, the annual mean wind speed was estimated to be $5.0 \text{ m}\cdot\text{s}^{-1}$. Prevailing wind directions (Fig. 2.2i), assessed in terms of vector-averaged daily means, were north (45%) and south (43%). Winds from the west (11%) were less common, and winds from the east ($<1\%$) extremely rare.

Fig. 2.2. (Following page) Meteorological measurements and observations of ice cover made at the Redon AWS from October 1996 – June 1998: a) air temperature (AT) and water temperature at 1 m depth (WT1: January - April 1997) and 2 m depth (WT2: August 1997 - February 1998); b) incident short-wave solar radiation (SR); c) the ratio of reflected to incident short-wave solar radiation (SR_{ref}/SR); d) net long-wave atmospheric radiation (AR); e) air pressure (AP); f) relative humidity (RH), g) precipitation (PP); h) wind speed (WS); i) vector-averaged wind direction (WD); and j) ice cover (ICE). In b), in addition to the measured data (A), the theoretical clear sky solar radiation calculated according to Brock (1981) using the atmospheric transmission coefficients of Hottel (1976) is shown, unmodified (B) and modified by the local skyline at the AWS (C). The grey panels indicate periods of missing data.





Physical structure

Lake Redon is dimictic; the period of study therefore included five mixing periods (Fig. 2.3). Spring overturn occurred in late June 1996, at the beginning of May 1997 and at the beginning of July 1998. Both autumn overturn periods occurred during November; however, in 1996 the lake mixed at the beginning of the month, while in 1997 it mixed two weeks later. There would therefore seem to be more variability in the timing of spring overturn than in the timing of autumn overturn, probably because of the variability in winter ice-cover characteristics that influence spring mixing.

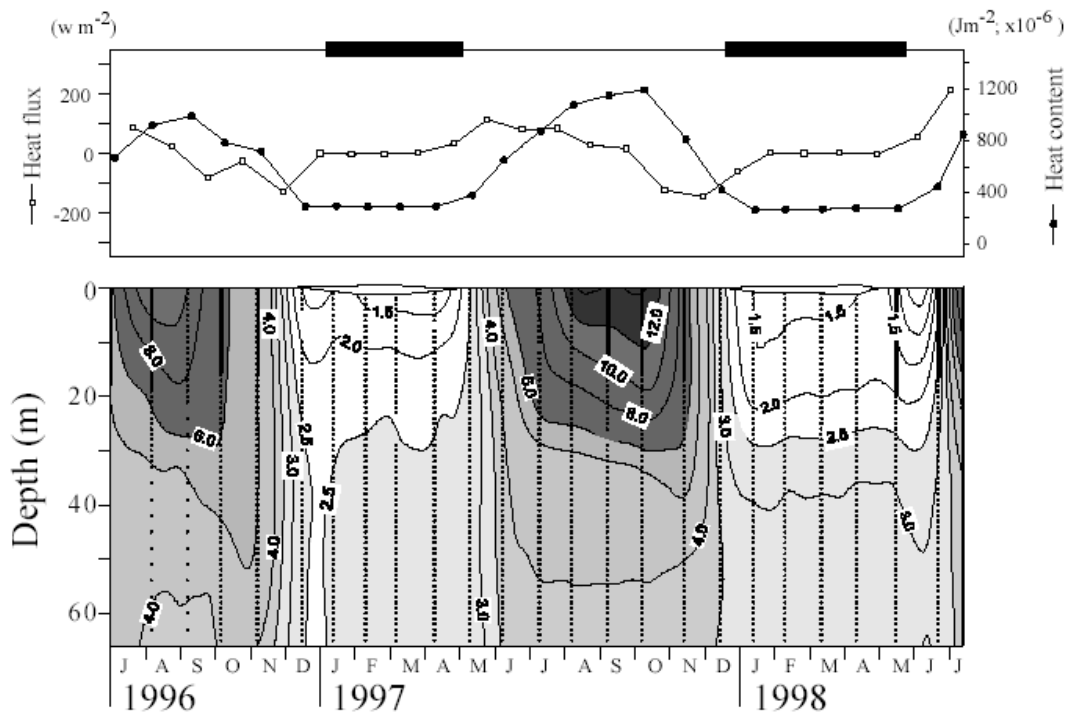


Fig. 2.3. Upper panel: Heat content and heat flux at Lake Redon during the study period (July 1996 to July 1998). The horizontal lines at the top of the panel represent periods of ice cover. Lower panel: Isotherm plot illustrating seasonal temperature changes ($^{\circ}\text{C}$). Secchi depths are shown as vertical bars. The dotted lines indicate the measured profiles on which the isotherm interpolation is based.

During the first open-water period (June to November 1996), the epilimnion had a mean temperature of 6.6°C , while during the second open-water period (May to November 1997) it was substantially higher (8.1°C), reflecting the high air temperatures prevailing in 1997 (Fig. 2.2a). Epilimnetic temperatures were generally about 5.2°C higher than the corresponding air temperatures, which is not unusual in summer and autumn in both lowland lakes (Livingstone & Lotter 1998) and alpine lakes (Livingstone et al. 1999). In contrast to the epilimnion, the temperature of the hypolimnion was es-

essentially the same during both open-water periods (4.6°C in 1996 and 4.5°C in 1997), as was the mean lake temperature during the ice-covered periods (2.2°C in 1996/97 and 2.0°C in 1997/98). Thus inter-annual variability in climate forcing would appear to be reflected only in open-water epilimnion temperatures. The annual heat budget of the lake was calculated to be 71,600 J m⁻² yr⁻¹ in 1996-97 and 90,288 J m⁻² yr⁻¹ in 1997-98. Both these values substantially exceed the annual heat budget of 63,582 J m⁻² yr⁻¹ calculated by Catalan (1988) for 1984-85.

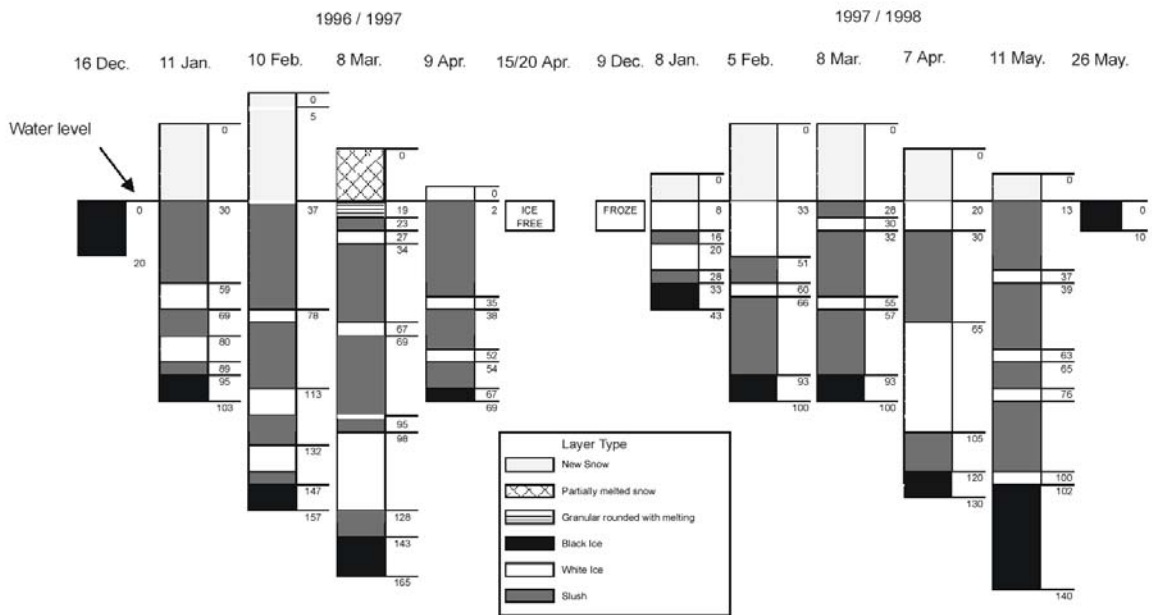


Fig. 2.4. Ice cover profiles for the winters of 1996/97 and 1997/98 in Lake Redon. The depths of the different layers are given in cm, starting from the snow surface.

Lake Redon was ice-covered from early December 1996 to late April 1997, and from late December 1997 to early June 1998. Because local topographic effects on incident solar radiation at the lake are slight, the onset and end of the period of ice cover are likely to be determined to a large extent by synoptic-scale climate integrated over several weeks (cf. Palecki & Barry 1986), and especially by air temperature (e.g. Ruosteenoja 1986). At Redon, the timing of freeze-up and break-up is thus likely to reflect the air temperatures prevailing at the lake around November and April, respectively. The fact that the lake remained frozen substantially longer in 1998 than in 1997 can thus be associated with the considerable difference in the air temperatures prevailing during spring in these two years (Fig. 2.2a). The mean spring air temperature (March - May) was 2.6°C lower in 1998 than in 1997, and the mean April air temperature was 4.7°C lower. The end of the snow cover in April 1997 is earlier than that observed in previous years (Catalan 1989; Catalan 1992; Camarero 1994b).

The structure of the ice cover (up to four layers of white ice alternating with slush) was similar during both winters (Fig. 2.4), as was the maximum ice thickness (165 cm in 1996/97 and 140 cm in 1997/98). In the first winter, ice thickness reached its maxi-

mum during February/March 1997, subsequently melting fairly rapidly (Fig. 2.4) as a result of the high air temperatures and the lack of snowfall prevailing from January to May. In the second winter, maximum ice thickness occurred later, i.e. during April/May 1998.

Light was able to penetrate quite deeply into the lake: Secchi depths ranged from about 15 - 16 m during the stratification periods to about 18.5 - 20.5 m after the snow-melt (Fig. 2.3).

Deposition chemistry and lake water chemistry

Precipitation samples were collected only from June 1997 to June 1998. Concentrations and precipitation rates are summarised in Table 2.2. A total of 1600 mm fell during this period, corresponding to a monthly mean of 122 mm. Mean conductivity was $9 \mu\text{S cm}^{-1}$. The most abundant cations were NH_4^+ , Ca^{2+} and Na^+ , while NO_3^- , SO_4^{2-} and Cl^- were the most abundant anions. The alkalinity of the precipitation was very low ($6 \mu\text{eq l}^{-1}$ on average) since most of the rain events were acidic (median pH = 5.2).

Table 2.2. Volume weighed mean chemical composition of the precipitation at Lake Redon during the second year of the study period (July 1997 to July 1998), calculated on the basis of daily samples.

	Mean	Max.	Min.
Precipitation amount ($\text{l m}^{-2} \text{ month}^{-1}$)	122	300	24
H^+ (μM)	9	23	1
Alkalinity ($\mu\text{eq l}^{-1}$)	6	38	-17
Conductivity ($\mu\text{S cm}^{-1}$)	9.0	13.1	3.9
Na^+ (μM)	16	48	4
K^+ (μM)	3	11	0
Ca^{2+} (μM)	13	47	4
Mg^{2+} (μM)	2	5	0
Cl^- (μM)	12	37	5
SO_4^{2-} (μM)	12	20	4
NO_3^- (μM)	13	30	3
NH_4^+ (μM)	17	39	4
TN (μM)	35	50	19
TP (μM)	0.176	0.308	0.024

Lake water chemistry was dilute (Table 2.3), with a mean conductivity of $12 \mu\text{S cm}^{-1}$, and an average pH of 6.3. Ca^{2+} was the most abundant cation, and HCO_3^- and SO_4^{2-} the dominant anions. Ionic concentrations were higher in the lake water than in the precipitation, mainly because of higher concentrations of Ca^{2+} and HCO_3^- . Concentrations of NO_3^- and SO_4^{2-} in the lake water and in the precipitation were similar, while concentrations of Cl^- and Na^+ were slightly lower in the lake than in the precipitation, suggesting a dilution effect because of differential elution during the thawing of the snowpack

(Camarero 1994b). There were no significant seasonal variations in the concentrations of major ions in the water column during the sampling period.

Table 2.3. Mean chemical composition of the Lake Redon water column during the study period (sampled at monthly intervals from July 1996 to July 1998).

	Mean	Max.	Min.
pH	6.3	6.6	6.0
Conductivity ($\mu\text{S cm}^{-1}$)	11.5	13.7	9.4
Alkalinity ($\mu\text{eq l}^{-1}$)	46	54	40
Na^+ (μM)	10	14	7
Mg^{2+} (μM)	4	4	3
K^+ (μM)	1	3	0
Ca^{2+} (μM)	36	42	32
SO_4^{2-} (μM)	13	15	12
Cl^- (μM)	7	14	4
NO_3^- (μM)	12	13	10
NO_2^- (μM)	0.13	0.22	0.06
NH_4^+ (μM)	1.3	2.9	0.1
DON (μM)	5	7	2
PN (μM)	1	1.6	0.6
SRP (μM)	0.009	0.021	0.003
SNRP (μM)	0.046	0.134	0.003
PP (μM)	0.061	0.193	0.000
DIC (μM)	83	100	67
DOC (μM)	125	208	50
PC (μM)	9	17	3
DSi (μM)	10	13	7
Chl <i>a</i> ($\mu\text{g l}^{-1}$)	0.70	2.24	0.07
Chl <i>b</i> ($\mu\text{g l}^{-1}$)	0.03	0.10	0.00
Chl <i>c</i> ($\mu\text{g l}^{-1}$)	0.09	0.25	0.01
O_2 (mg l^{-1})	9.1	9.9	8.2

The carbon budget in Lake Redon during 1996/97 has been studied by Camarero et al. (1999), and is illustrated in figure 2.5. DOC was the most abundant carbon fraction, followed by DIC and PC (Table 2.3). During summer 1996, variations in all three carbon fractions were similar, although DIC was highest in July, while PC and DOC were highest in August. Mean DOC and PC concentrations were highly correlated during the first year ($r^2 = 0.70$, $p < 0.001$), but much less correlated during the second year ($r^2 = 0.34$, $p < 0.05$), when the linear regression had a similar linear trend, although the data-points were much more scattered. In October of both years there was a peak in carbon concentrations; the highest concentrations of both DIC and DOC during the whole sampling period occurred in October 1997.

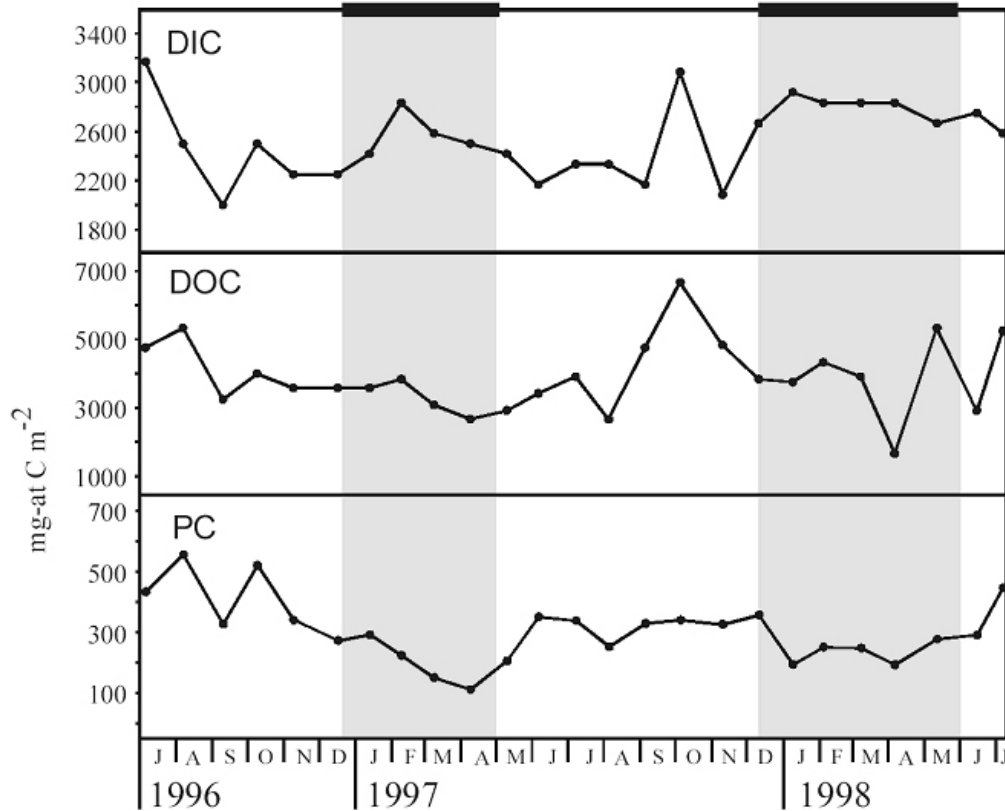


Fig. 2.5. Areal mean concentrations of DIC, DOC, PC and macrozooplankton biomass in Lake Redon during the study period (July 1996 to July 1998). The thick black lines indicate periods of ice cover.

The main nitrogen component was dissolved inorganic nitrogen (DIN, the sum of NH_4^+ , NO_3^- and NO_2^-). DON concentrations were approximately half those of DIN concentrations, and concentrations of PN much lower than DON concentrations (Table 2.3). NO_3^- was the most abundant ion of the DIN pool, as observed in most Pyrenean lakes (Catalan et al. 1994). However, concentrations of NH_4^+ , the most abundant ion in the precipitation, were very low in the lake. PN had a similar pattern to that of PC; viz. high concentrations during the open-water period that decreased during the subsequent ice-covered period. The main difference in the behaviour of the PC and PN concentrations occurred in August and October 1996, when very high concentrations of PC, but not of PN, were measured at the surface. Both sampling dates were preceded by heavy rainfall, which could have resulted in high allochthonous inputs of PC and DOC. Variations in PN and DOC were similar ($r^2 = 0.61$, $p < 0.001$), particularly during the second half of 1997 and 1998, when they were very highly correlated ($r^2 = 0.80$, $p < 0.001$). The main mismatch between DOC and PN variations occurred during the 1997 spring

overturn, when PN showed an increase very similar to that of Chl a, while DOC concentrations remained low.

Phosphorus was present mainly as PP and SNRP, the concentrations of which were similar. SRP concentrations were comparatively low (Table 2.3) and showed no significant changes throughout the sampling period.

During both winters, DSi concentrations in the deeper layers of the lake lay between 15 and 17 μM , compared to 10 μM in the upper layers, indicating a release of DSi from the sediments.

Biological activity

Four different peaks can be distinguished in the chlorophyll measurements (Fig. 2.6). Two of these are associated with the mixing periods (spring and autumn overturn), while the other two are associated with the stratification periods. A chlorophyll maximum was observed in the upper layers of the hypolimnion in summer, and another chlorophyll maximum was observed under the ice, where some algal communities are able to benefit from the stable conditions generated by the black ice (Fig. 2.7; Catalan & Camarero 1991). Among the four production episodes observed, the highest Chl concentrations occurred during spring overturn. In both years, Chl concentrations during autumn overturn were lower than during spring overturn. In 1996 there was a Chl maximum under the ice following the autumn overturn maximum. However, in 1997 no Chl maximum was detected, probably because of the rapid accumulation of 30 cm of snow on the top of the black ice after the lake froze, which prevented the penetration of light necessary for algal growth.

Mean lake Chl concentrations during spring overturn were low in 1996 (1.18 $\mu\text{g l}^{-1}$) and 1997 (1.39 $\mu\text{g l}^{-1}$), but in 1998 they were much higher (2.24 $\mu\text{g l}^{-1}$), closer to values found in other years (Catalan 1991).

As shown in figure 2.7, Chl b and c peaks did not overlap in space and time, with the exception of the two autumn overturn periods. Chl c was typical of the two mixing periods, while Chl b appeared mainly in the upper layers of the hypolimnion.

The carotenoid index, which is the ratio of the concentration of carotenoid pigments to the concentration of Chl, increased during both winters from March to April, with minimum values occurring during the episodes of maximum Chl. The carotenoid index therefore reacted more sensitively to variations in Chl concentrations than to variations in carotenoid concentrations, indicating that carotenoid concentrations were quite constant in comparison to Chl concentrations during the sampling period. The phaeopigment index, which indicates the ratio of the concentration of Chl a to that of phaeophytin a and phaeophorbids, was high during the open-water periods, decreasing both below the ice-cover and below 60 m throughout the year.

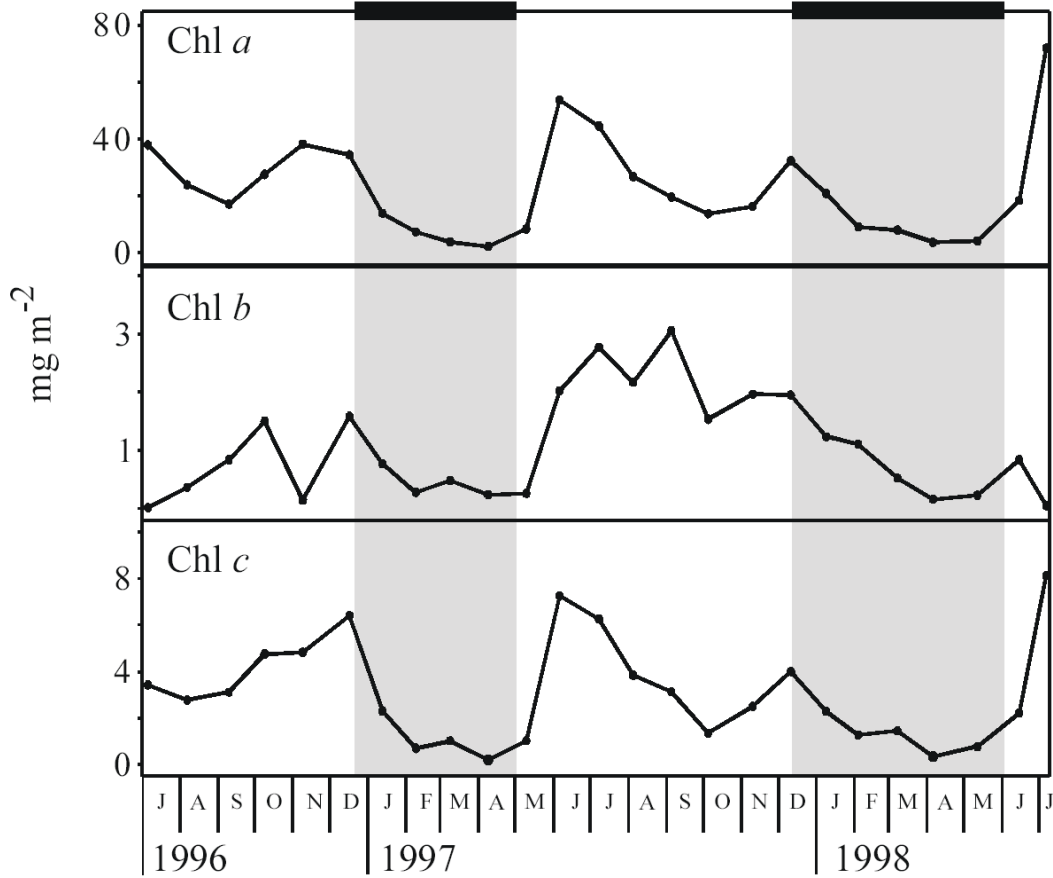
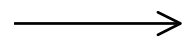
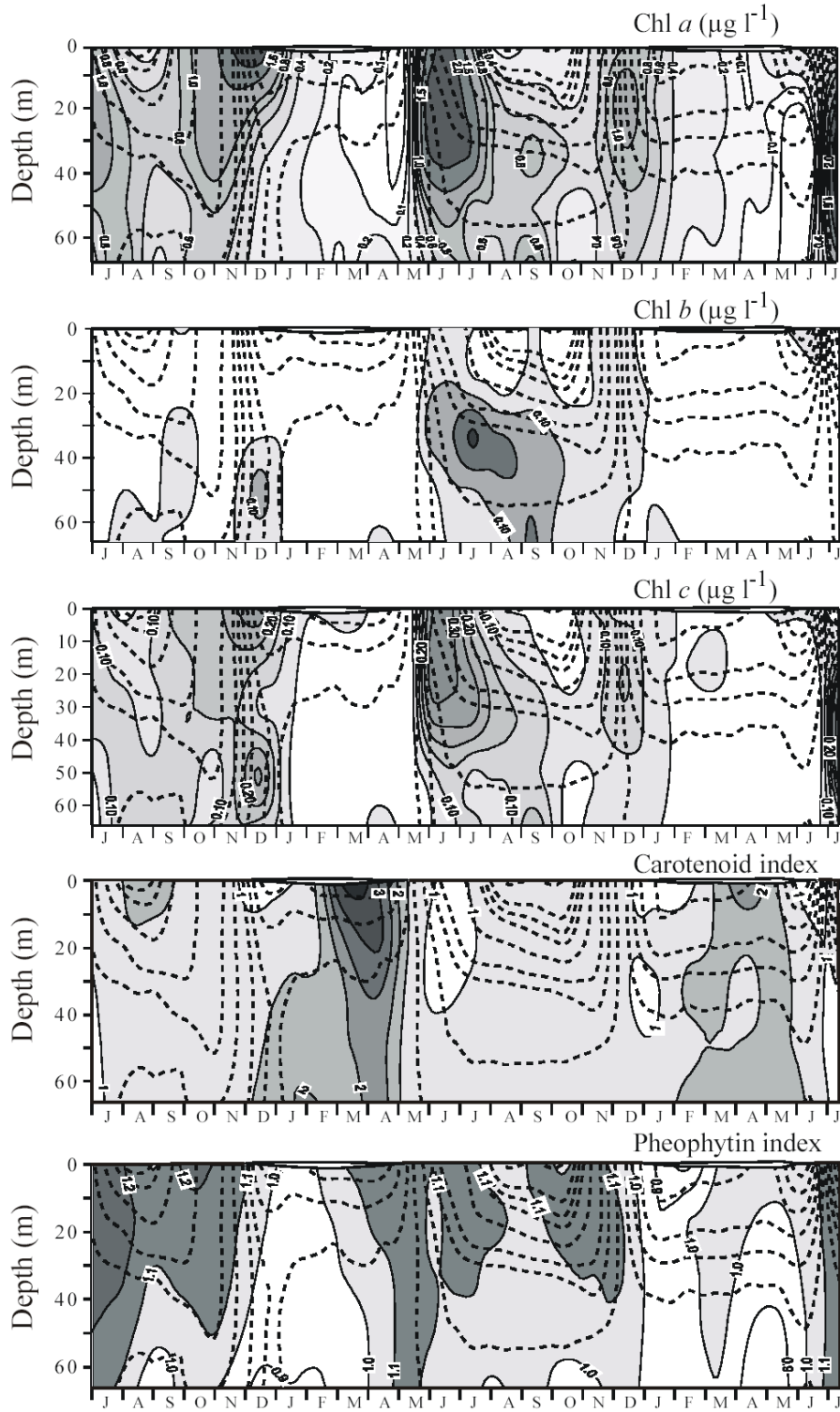


Fig. 2.6. Areal mean concentrations of Chl a, Chl b and Chl c in Lake Redon during the study period (July 1996 to July 1998). The thick black lines indicate periods of ice cover.

Fig. 2.7. (Following page) Isoline plots of Chl a, Chl b, Chl c, the carotenoid index and the phaeopigment index for Lake Redon during the study period (July 1996 to July 1998). Isotherms (dashed lines) are also shown in order to facilitate comparison with seasonal patterns of stratification and mixing. The duration and thickness of ice-cover are indicated by white polygons.





Oxygen concentrations (Fig. 2.8) decreased during winter, especially in the deeper layers of the lake. Anoxia was reached below 66 m in April 1998. In 1997, two oxygen peaks (with saturation values of 100% - 120%) occurred during spring overturn and during summer, indicating high primary production.

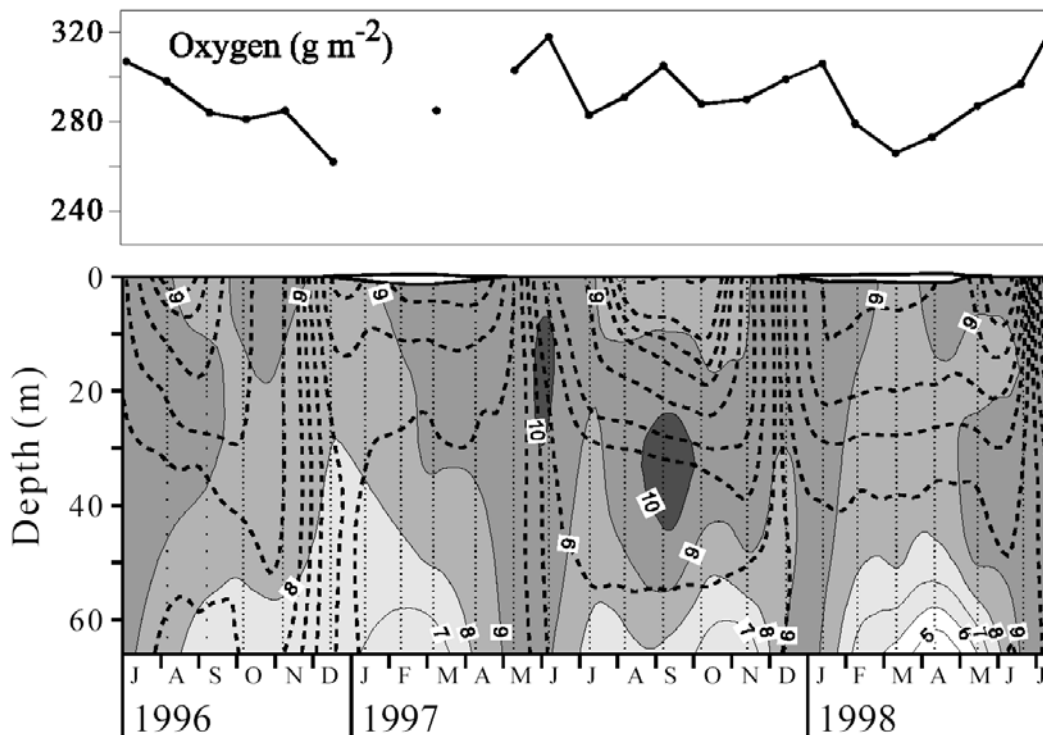


Fig. 2.8. Upper panel: Seasonal variations in the oxygen content Lake Redon (g m^{-2}) during the study period (July 1996 to July 1998). Lower panel: Isoline plot of oxygen concentration (mg l^{-1}) in Lake Redon, 1996-98 (solid lines). Isotherms (dashed lines) are also shown in order to facilitate comparison with seasonal patterns and mixing. The dotted lines indicate the measured profiles on which the isoline interpolation is based. The duration and thickness of ice-cover are indicated by white polygons.

During winter, when the lake can be considered to be isolated from the atmosphere by the ice cover and photosynthesis is practically nil because of the lack of light, total lake respiration rates can be calculated by areal integration of the measured oxygen profiles (Welch et al. 1976). Areal respiration rates were higher during the first ice-covered period ($339 \text{ mg O}_2 \text{ m}^{-2} \text{ d}^{-1}$) than the second ($281 \text{ mg O}_2 \text{ m}^{-2} \text{ d}^{-1}$). However, they were of a similar magnitude to those calculated for Lake Redon in other years ($232 \text{ mg O}_2 \text{ m}^{-2} \text{ d}^{-1}$) (Catalan 1992) and similar lakes from the Canadian Shield ($131\text{-}306 \text{ mg O}_2 \text{ m}^{-2} \text{ d}^{-1}$) (Welch & Bergmann 1985).

Discussion

The comparison of the lake chemistry with the chemistry of the precipitation falling on the lake can be very informative for understanding the origin of the chemical composition of the lake water (Margalef 1983). The chemistry of the precipitation during the second half of the present study period showed significant differences compared to previously measured values (Camarero & Catalan 1993; Camarero & Catalan 1996): NO_3^- , SO_4^{2-} , alkalinity and Ca^{2+} were lower during this study. Despite these decreases, Ca^{2+} and alkalinity were much higher in the lake than in the precipitation, indicating that, although the basin is mainly composed of granite bedrock, there was a very significant source of Ca^{2+} in the catchment. A mean Ca^{2+} concentration of $72 \mu\text{M}$ and a mean alkalinity of $130 \mu\text{eq l}^{-1}$ in the two main inlets during the snow-free period confirmed that Ca^{2+} and alkalinity must have been generated in some parts of the lake catchment.

NH_4^+ , which was the dominant cation in the precipitation, was present at much lower concentrations in the lake water. Since NH_4^+ concentrations in the inlet streams were below the limit of detection during the 1997 open-water period, this discrepancy could be explained by its retention in the soil (Schimel & Parton 1986). Thus, the only source of NH_4^+ to the lake during the snow-free period was its direct input to the lake surface in the precipitation. During the snow-covered period, when the precipitation accumulated in the snowpack, most ions were eluted during snowmelt (Camarero 1994b). In early May during the two ice-covered periods studied, NH_4^+ concentrations were very high at the surface (0-5 m: 3.1 and $5.3 \mu\text{M}$) compared with the rest of the water column (5-66 m: 1.3 and $1.9 \mu\text{M}$).

Although NH_4^+ concentrations in the lake water were much lower than in the rain, they were probably sufficient for the requirements of the algae, since the $\text{NH}_4^+:\text{SRP}$ ratio was usually significantly greater than 16, the Redfield ratio. NH_4^+ concentrations increased with increasing Chl concentration ($r^2 = 0.37$, $p < 0.001$), as did DON ($r^2 = 0.50$, $p < 0.001$), suggesting that the NH_4^+ was mainly a product of cellular exudation.

Lake Redon had very low TP concentrations during the whole study period (mean concentration: $3.6 \mu\text{g l}^{-1}$). Since $\text{TP} < 5 \mu\text{g l}^{-1}$, according to the classification scheme of Vollenweider and Kerekes (1982) Lake Redon is ultra-oligotrophic. In addition, the magnitude of the mean DIN:SRP ratio (1333:1 by atoms) indicates that Lake Redon is severely limited by P in comparison to N.

Although the chlorophyll maximum does not exactly match the biomass maximum (Felip & Catalan 2000), chlorophyll measurements are still acceptable indicators of the main patterns of primary production in the lake. Also, chlorophyll *b* and *c* concentrations may be used as a first approach to characterize the algal composition. In Lake Redon, chlorococcal chlorophytes were the most abundant group of algae containing Chl *b*, while cryptophytes, chrysophytes and dinoflagellates contributed to Chl *c*. There was a good correspondence between the concentrations of Chl *b* and *c* on the one hand (Fig. 2.7) and the algal group composition of both ice-free periods studied on the other (Felip et al. 1999a). Chrysophytes and dinoflagellates were the dominant algae during spring and autumn overturn periods; cryptophytes were associated with the deep layers, while the chlorococcal chlorophytes appeared during the stratification periods and during the 1997 autumn overturn.

Since the phaeopigment index is the ratio of Chl *a* to both phaeophytin *a* and phaeophorbids, a low index may be related to an increase in phaeophytin *a* (mainly a degradation product of Chl *a*, Moss 1967), or to an increase in phaeophorbids (grazing products of zooplankton, Moss 1967) or both. In both years, the zooplankton maximum coincided with the minimum in the phaeopigment index, indicating that phaeophorbid was probably contributing most to the decrease in the index. On the other hand, the low values of the index in the deeper layers of the lake were most probably related to the degradation of Chl *a* down the water column.

Acknowledgements

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3. Inter-annual life history traits variability in *Daphnia*, *Cyclops* and *Diaptomus* in an oligotrophic lake: environmental, intra- and inter-specific relationships

Abstract

Alpine lakes are typically oligotrophic and present low diversity of crustacean zooplankton assemblages. When fish predation is low, food quality, intra-specific density-dependence and invertebrate predation are expected to be the main factors affecting zooplankton assemblages in these lakes. We sampled the crustacean zooplankton assemblage of an alpine lake consisting of a cladoceran herbivore, *Daphnia pulicaria*, and two omnivorous/carnivorous copepods, the calanoid *Diaptomus cyaneus* and the cyclopoid *Cyclops abyssorum* during a five consecutive year period in order to evaluate the relative importance of food quality, intra-specific interaction and invertebrate predation on the annual population variability of these species. The end of the ice-covered period varied in almost two months from year to year, and was the key limnological feature characterising the timing of the life-cycles in the three species. Both copepods produced nauplii at the time the ice melted, while the timing of *Daphnia* maturation size was highly correlated with the ice-cover melting day. The average inter-annual abundance of *Cyclops* and *Daphnia* was negatively correlated with their respective maximum size and in the case of *Daphnia* with maturation size, suggesting that there was an intra-specific density-dependent interaction. In contrast, *Diaptomus* maximum body size had no relationship with abundance but was related with the average particulate phosphorus (PP) availability. Food quantity was not related with any of the population and life history traits of the three species. Comparison of the stoichiometric composition of the three species and those of seston revealed that *Daphnia* had a higher phosphorus proportion than seston, and the two copepods a higher carbon and nitrogen than *Daphnia* and seston. *Daphnia* annual production was highly correlated with sestonic phosphorus. It is suggested that *Daphnia* was limited by phosphorus availability. However, *Daphnia* abundance was negatively correlated with *Cyclops* biomass, suggesting that predation of the latter on the former might also be a relevant factor.

Introduction

Population dynamics and community structure of zooplankton have been intensively studied from a number of perspectives including field measurements, field and laboratory experiments, and theoretical models. Life history traits have been shown to be essential in describing the changes in the populations of individual species and their assemblage within communities (Gurney et al. 1990; Begon et al. 1990; McCauley et al. 1999). Fluctuations in life history traits of zooplankton have been observed for envi-

ronmental variables such as temperature and photoperiod (Hobaek & Larsson 1990; Hairston & Kearns 1995), food availability (Tessier & Goulden 1982; Lynch 1989), intra-specific interactions (Burns 1995; Rose et al. 2002), interspecific competition (Goulden & Hornig 1980; Boersma 1995) and predation (Brooks & Dodson 1965; Lynch 1979).

Among the factors affecting life history traits, food availability has received much attention, since it is a key factor for consumer's growth. Following the trophic-dynamic theory of ecosystems (Lindeman 1942) initial focus was placed on studying the effects of food quantity (Tessier & Goulden 1982; Lynch 1989; Gliwicz & Guisande 1992; Boersma 1995). More recent studies have emphasised the relevance of food quality, as well. In many environments carbon is present in excess in comparison with the other two most essential nutrients of animal tissues, nitrogen and phosphorus. The ratio of carbon to phosphorus has been found to be a good predictor of zooplankton growth in the pelagic habitat of lakes (Sterner et al. 1998; DeMott & Gulati 1999; Elser et al. 2000; Urabe & Sterner 2001).

Most of the studies on food quality effects on zooplankton have been carried out in relatively nutrient rich conditions (DeMott & Gulati 1999; Urabe & Sterner 2001; Makino et al. 2002). Studies on oligotrophic conditions are very rare, only restricted to few laboratory experiments (Boersma & Kreutzer 2002). Organisms inhabiting oligotrophic waters may have unexpected adaptations to these low food conditions that alter predictions based on short term laboratory studies or studies at high food concentrations (Sterner & Schulz 1998). There is a need of detailed field studies describing species life history adaptations within oligotrophic waters in order to evaluate whether food quality is a relevant parameter in such low food concentrations in the field.

Alpine lakes are typically ultra-oligotrophic or oligotrophic (Pechlaner 1971). Their crustacean zooplankton community is usually limited to a single grazer species such as large bodied *Daphnia* in those lakes where fish predation is low, and one or two copepods, usually a calanoid and a cyclopoid copepod (Tonolli & Tonolli 1951; Miracle 1978). Many of these copepods have been described to have an omnivorous to carnivorous diet (Gliwicz & Umana 1994; Gliwicz 1994). In these lakes, the seasonal variability is very strong, and sufficient productivity to enable juvenile survival is restricted to the ice-free period. These zooplankton species are adapted to live in this extreme environment by adjusting reproduction effort to these relatively high productive periods, which results in the production of a single cohort every year (Gliwicz et al. 2001). *Daphnia* is usually the main herbivore and coexists with a cyclopoid copepod, which predaes on *Daphnia* at least during certain development stages. In eutrophic environments the maximum densities of zooplankton species have been shown to be constrained by intra-specific interactions both in experiments (Burns 1995; Rose et al. 2002) and in the field (Declerck et al. 2003). In oligotrophic lakes, the relative importance of density dependence has been poorly evaluated. The study of the inter-annual population changes of the species inhabiting these lakes should reflect the result of the interaction between changes in food, density-dependent intra-specific interactions and differences in the predation pressure of the copepods on the cladocerans. Due to the low number of species and to the low nutrient content, they are, therefore, ideal model ecosystems for studying the relative importance of these processes on the population dynamics of zooplankton.

In order to describe the inter-annual life history variability of the three crustacean species of an alpine lake, Lake Redon, we sampled the lake assemblage during a five-year period. The specific objectives of the study were to test (1) whether food quality was more significant than food quantity in explaining the abundance and life history traits of the three species; 2) if there was an intra-specific density-dependent interaction in any of the three species, and (3) to evaluate if copepod predation was noticeable in the *Daphnia* inter-annual variability.

Materials and Methods

Sampling

The lake was sampled from July 1996 just after the lake was completely ice-free, until May 2001, just before the ice-cover melted again, covering therefore five consecutive seasonal cycles. Samples were collected at monthly intervals at the deepest point of the lake either drilling the ice-cover or on a platform anchored throughout the summer at the same point. Temperature in the water column was measured at 1-m depth intervals using a WTW TA-197 oxygen meter. Seston was sampled for particulate carbon (PC) and particulate nitrogen (PN) analysis for the five year period, and for particulate phosphorus (PP) the last four years. Collection was done using a Ruttner bottle at 2, 10, 20, 25 and 60 m and were stored in polypropylene bottles and transported cold and dark to the lab. Zooplankton samples were collected by vertical hauls from 65 m to the surface with a 200 μm net. Two replicate samples were collected; one for biomass evaluation and species counts which was immediately preserved in 5% sucrose formaline solution, while the other was used for the elemental analysis (from December 1998 until December 1999). Individuals for the latter were kept alive and transported cold (4°C) until they were frozen (-20°C) in the laboratory within few hours after collection.

Population and life history traits

In Lake Redon three planktonic crustacean species are found. The cladoceran *Daphnia pulex* Forbes, the calanoid copepod *Diaptomus cyaneus* Gurney and the cyclopoid copepod *Cyclops abyssorum* Sars. In addition to abundance and biomass, several life history traits were measured at the population level, including adult body length, fecundity (assessed as clutch size in *Cyclops* and total number of eggs produced for each ice-free period in *Daphnia*) and somatic growth rate (measured as the average length increase during the survival of the cohort). Abundance during the ice-covered period has not been considered due to the difficulty in adequately quantify abundance below the ice cover.

Animals were counted under an Olympus inverted microscope. Additionally, from each sample at least 100 individuals of *Daphnia* and at least 50 adults of the two copepod species were randomly measured for body length (upper edge of head to base of tail spine in *Daphnia* and from the anterior end of the cephalothorax to the posterior end of

the furca in the copepods), clutch size (number of eggs/embryos per clutch) and in *Daphnia*, the presence of ephippia. Zooplankton biomass was calculated from the length weight regressions obtained from individuals of the same lake. For *Daphnia* the regression equation was $\ln W = 3.552 L + 1.566$ ($r^2 = 0.940$, $p < 0.0001$), for *Cyclops* $\ln W = 2.3746 L + 1.255$ ($r^2 = 0.952$, $p = 0.0002$), and for *Diaptomus* it was $\ln W = 1.811 L + 1.508$ ($r^2 = 0.962$, $p = 0.003$). Where W is dry weight in μg and L the maximum length in mm. Conversion from biomass to carbon were done based on the measured carbon content of each species (Chapter 5). Species identification criteria followed Alonso (1996) for *Daphnia* and Einsle (1992) for the two copepod species.

Chemical analysis

Seston samples were analysed by collecting the particles on pre-combusted Whatman GF/F filters (the samples were prefiltered through a nylon mesh to remove particles larger than 200 μm). Zooplankton samples were unfrozen and from 10 to several hundreds of individuals of each species or stage were quickly sorted under a dissecting microscope and placed into pre-weighed tin for elemental carbon (C) and nitrogen (N) analysis or Teflon capsules for phosphorus (P). Individuals were kept cold ($< 4^\circ\text{C}$) during the sorting process, which was in all samples always finished within a few hours. Eggs from ovigerous females were carefully removed before analysis. Dry weight was determined for all samples after drying at 60°C for 24 h and weighed on a microbalance (Ohaus Analytical Plus, AP250D-0). Between 300 – 500 μg of dry weight was required for C, N and P analysis.

Seston and zooplankton C and N were measured on a EA 1108 CHNS-O Carlo Erba Instruments analyser after drying the samples and packing them into tin capsules with vanadium pentaoxide as catalyser to ensure complete combustion. Atropine was used as internal standard. Seston and zooplankton P were oxidized to phosphate by acid-persulphate digestion (Grasshoff 1983) and phosphate was analysed by the malachite green method (Camarero, 1994b).

Results

Abundance and biomass

Within the five years studied, both the density and biomass of the three species were very low, ranging from 0.2 to 2.5 individuals l^{-1} and 0.7 to 14 $\mu\text{g C l}^{-1}$ (Fig. 3.1). Average ice-free period zooplankton biomass was at least ten times lower and more variable from year to year than seston. The average ice-free period *Daphnia* abundance and biomass was higher in 1996, 1997 and 2000 than the other two years, when it was more than five times lower. Following an inverse pattern than *Daphnia*, *Cyclops* abundance and biomass was higher in 1997, 1998 and 1999. *Diaptomus* was as abundant as *Daphnia* in 1996, being much lower the other four years.

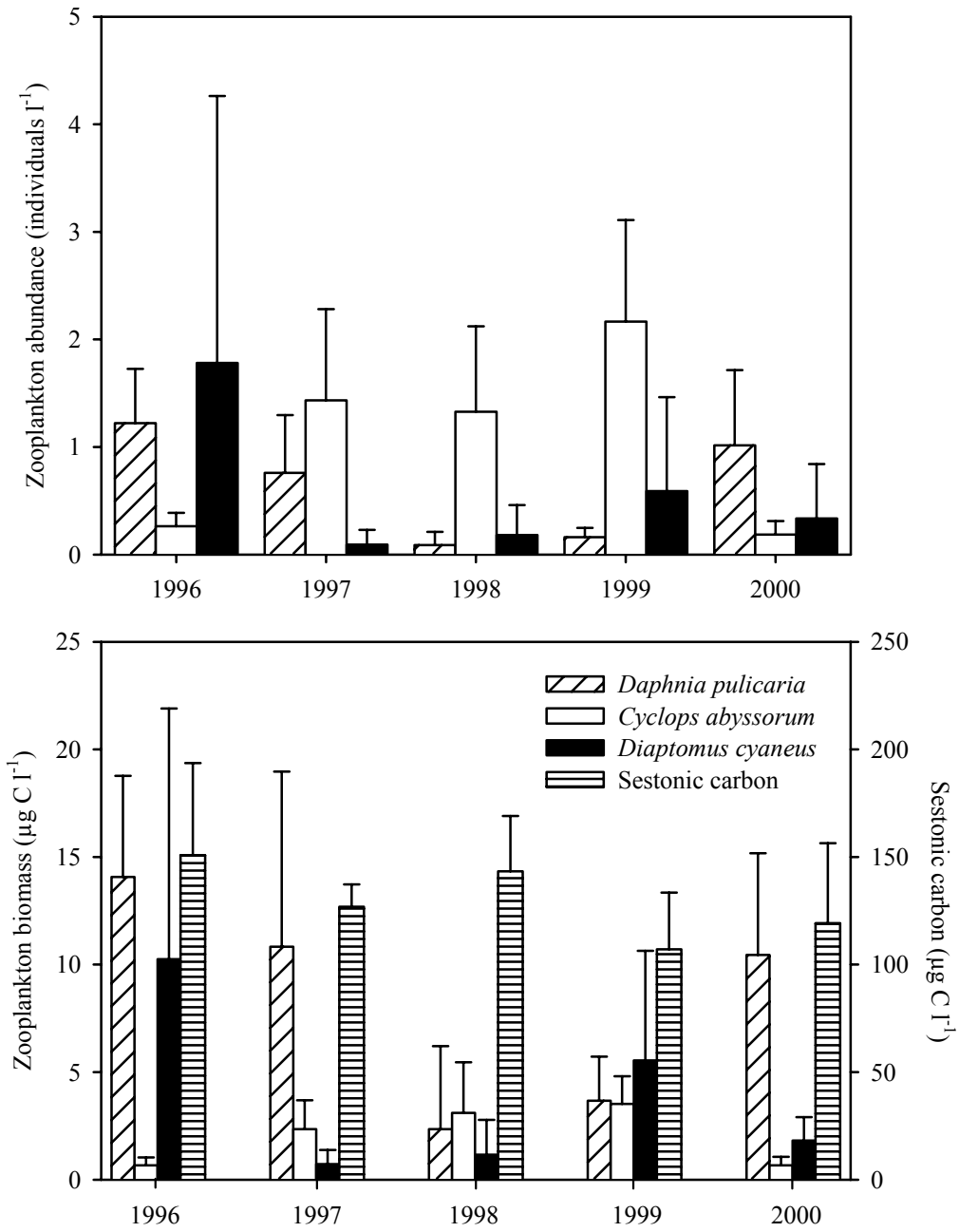
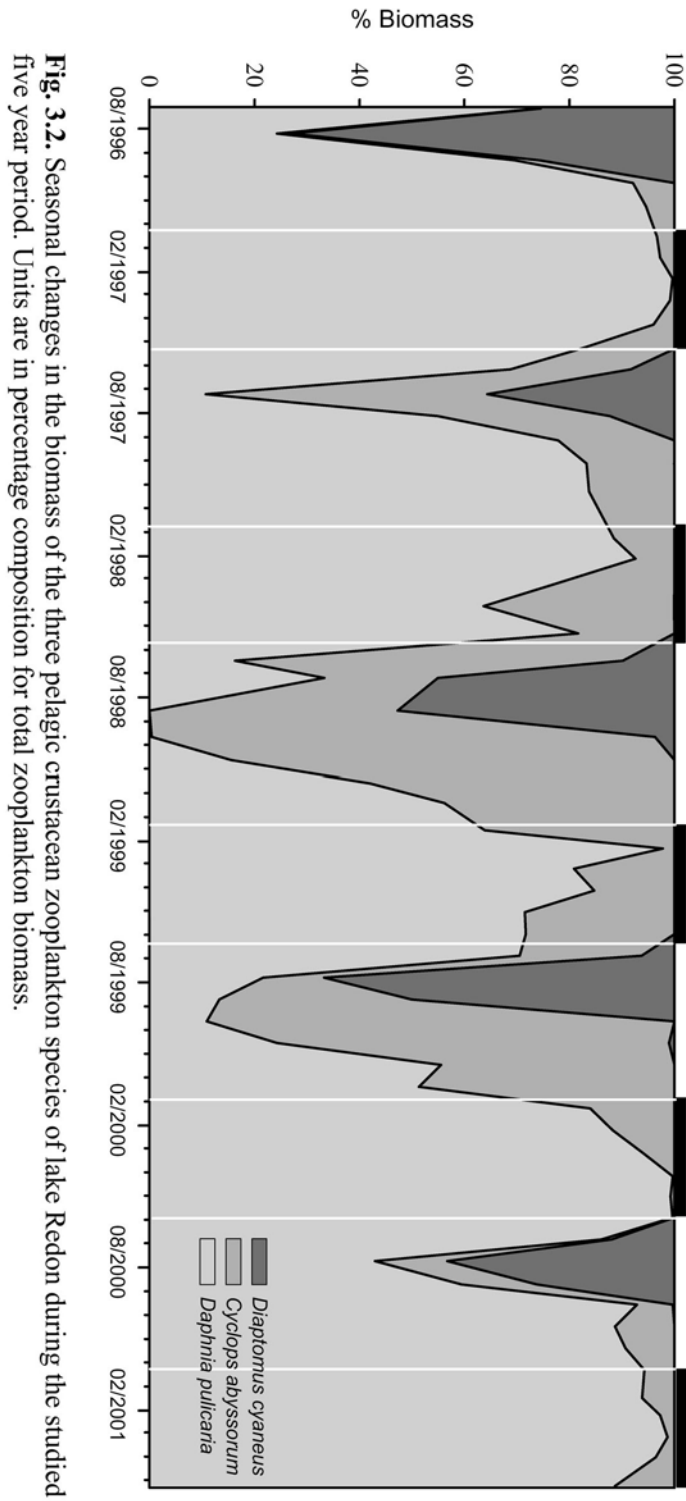


Fig. 3.1. Average and standard deviation of the ice-free period abundance (top) and biomass (bottom) of the three crustacean zooplankton species of Lake Redon during the studied five-year period.



Despite the inter-annual differences, there was a marked seasonal pattern in the three species biomass. *Daphnia* was the dominant species during most of the ice-covered period and the end of the ice-free period of every year (Fig. 3.2). *Diaptomus* was present in the plankton only from June to August, dominating the zooplankton biomass in July and August of all years with the exception of 1997, when it was *Cyclops*. In October and November of 1998 and 1999, *Cyclops* was dominating the biomass.

The two species present during the whole year, *Cyclops* and *Daphnia*, suffered a major reduction of their female abundance after the ice-cover melted compared with the abundance present in the lake before the ice-cover froze. The percentage reduction of their abundance was between 50 and 98% for *Cyclops* and for *Daphnia* in 1997, 1998 and 1999, whereas in 2000 the *Daphnia* population abundance was 35% greater than before the ice-covered period (Fig. 3.3).

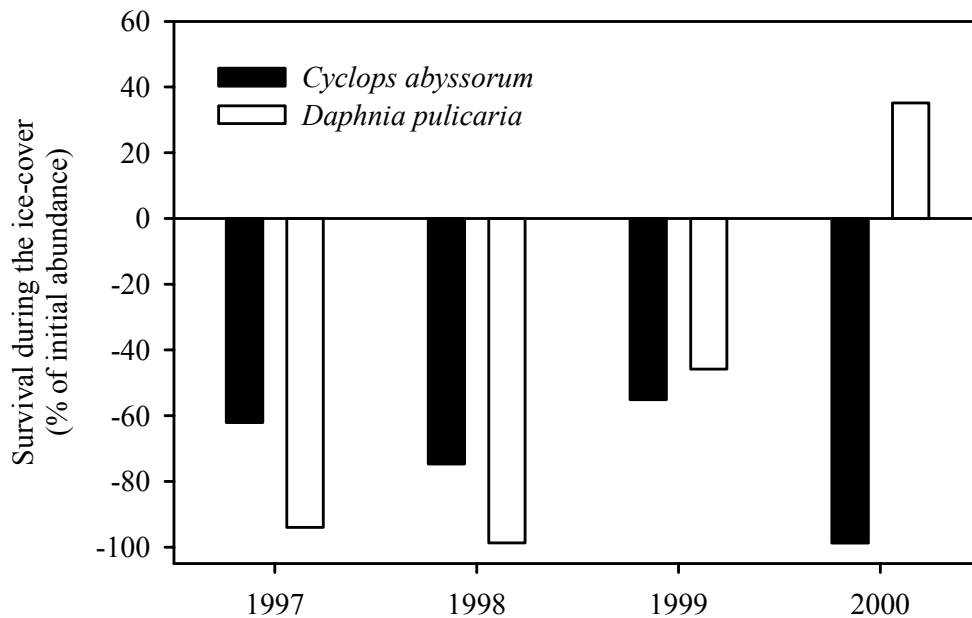


Fig. 3.3. Proportion of survival during the ice-covered period for *Daphnia pulex* and *Cyclops abyssorum* females in Lake Redon. Values are the percentage difference between the average abundance of October to December (equivalent to the new individuals produced during the ice-free period) and the abundance found after the ice cover melted. Negative values indicate a decrease in the population while positive values indicate an increase.

Life history traits

The three crustacean zooplankton species showed a clear annual seasonal cycle during the five years studied (Fig. 3.4). Amongst the three species, the calanoid copepod *Diaptomus* completed its whole life-cycle during a four month period, while the other two species were present in the lake during the whole annual cycle.

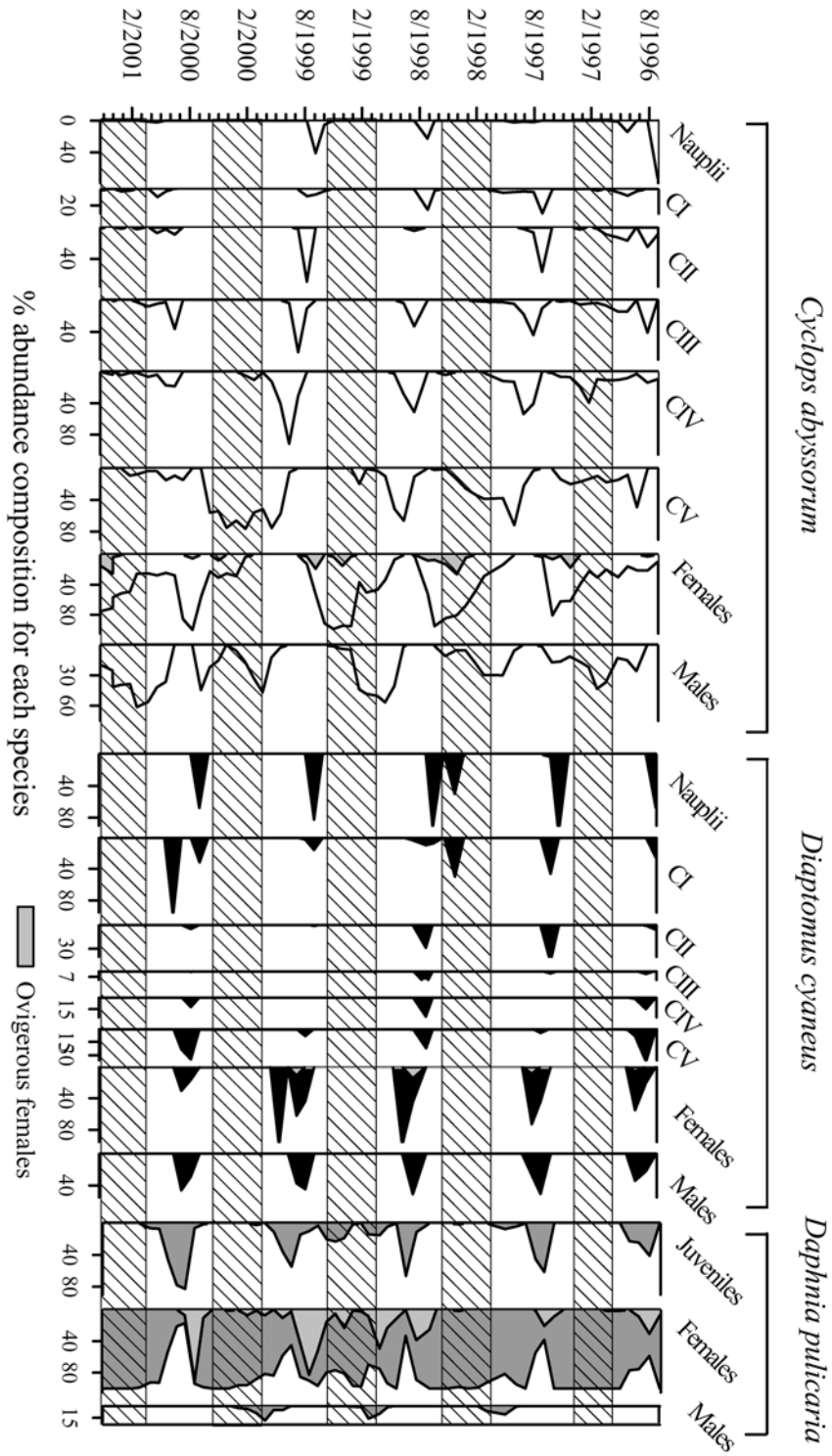


Fig. 3.4. Seasonal changes in the three pelagic crustacean zooplankton species of lake Redon during the studied five year period. Units are in percentage composition for each species.

Cyclops abyssorum

The life-cycle of this copepod followed a common general cycle during the five years studied (Fig. 3.4). Nauplii appeared at the end of the ice-cover, followed by the different copepodite stages, which moulted into adults towards the end of the ice-free period. Males always preceded females. The latter generally survived below the ice cover, and produced eggs before the cover melted. Despite this general pattern, there were some inter-annual differences in the development. During the ice-free period of 1996 and 2000, copepodites of the different stages coexisted in the lake during several months, while the other three years there was almost complete stage replacement one month after the other. A possible explanation could be related with the presence of epibionts of the green alga *Characium sp.* Colonisation of adults of *Cyclops* by this algal epibiont was observed sporadically during the sampling period, with the exception of the life-cycle of 1999/2000, when the proportion of infested females was much bigger. *Cyclops* was the only studied species infested by this algal epibiont.

Inter-annual changes in maximum body length of females (F) were more pronounced than males (M) (the coefficient of variation ranged from 5.5 to 7.9% for females and from 3.1 to 4.8% for males; Fig. 3.5a), however, there was a relatively fixed relationship between both sexes ($M = 0.76 * F$, $r^2 = 0.996$, $p < 0.0001$). In the two years where *Cyclops* density was much lower (1996 and 2000), both males and females had a longer body size: average \pm standard deviation for 1996 and 2000 was $1141 \pm 54 \mu\text{m}$ for males and $1587 \pm 94 \mu\text{m}$ for females, while the average size between 1997 and 1999 was $1060 \pm 55 \mu\text{m}$ for males and $1334 \pm 83 \mu\text{m}$ for females. There was a negative correlation between adult body size and *Cyclops* abundance ($r = -0.78$, $p = 0.062$ for females and $r = -0.95$, $p = 0.006$ for males). These size differences were closely related with different clutch sizes (Fig 3.5a): During the two years with larger females, the average clutch size \pm standard deviation was 24.4 ± 5.5 and 39.8 ± 9.3 eggs per female for 1996 and 2000 respectively, while the other three years the average clutch size was 9.6 ± 2.1 , 9.4 ± 1.8 and 8 ± 2.3 in 1997, 1998 and 1999 respectively.

Diaptomus cyaneus

Nauplii appeared immediately after the ice-cover melted (end of June) from overwintering eggs. The development time of copepodites was much quicker than in *Cyclops*, while at the end of June their population was mainly composed of nauplii and very few first stage copepodites, at the end of July most of the diaptomids were adults and copepodites of the fifth instar (Fig. 3.4). The low percentage of ovigerous females found during most of the years was probably due to the spacing in the sampling scheme, which was appropriate for the other two species but low for the diaptomid. Most of females had their ovaries ripened at the beginning of the reproductive period, or had spermatophoric sacs at the end of the reproductive period, indicating they were about to lay eggs or had already done it.

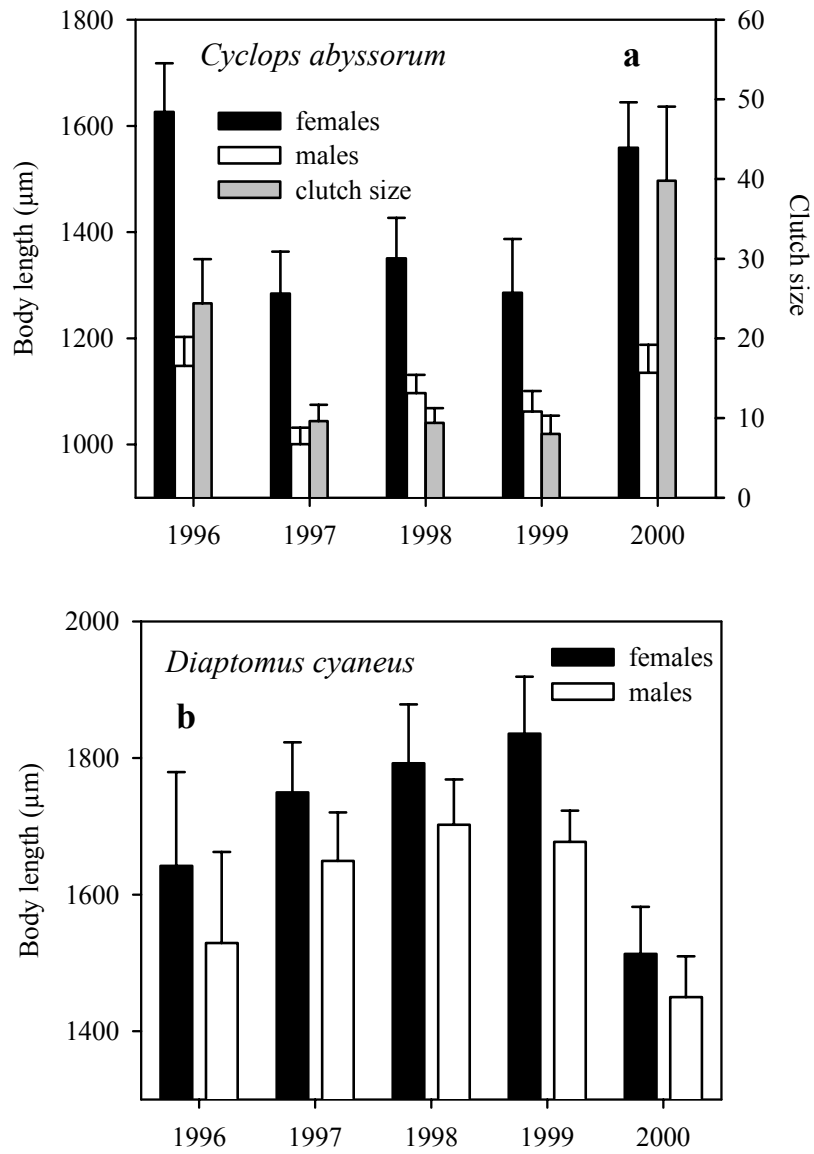


Fig. 3.5. Average body length of the adult females and males of the two planktonic copepod species present in Lake Redon. Each bar is the average for the whole period, and comprises individuals of the different sampling dates. The error bars are therefore the standard deviation of the sampled population variability. For *Cyclops abyssorum* the clutch size (number of eggs per female) is also shown.

Females maximum size ranged between $1513 \pm 69 \mu\text{m}$ in 2000 and $1836 \pm 83 \mu\text{m}$ in 1999 (average \pm standard deviation), and males between $1450 \pm 60 \mu\text{m}$ in 2000 and $1677 \pm 46 \mu\text{m}$ in 1999 (Fig. 3.5b). Both sexes experienced the same changes in size, therefore sexual size dimorphism was also constant ($M = 0.94 * F$, $r^2 = 1$, $p < 0.0001$) and less pronounced than *Cyclops*. The inter-annual differences, however, in contrast with *Cyclops*, were not density related ($p > 0.5$).

Daphnia pulicaria

Females were the dominant stage during most of the year, being juvenile production mainly restricted to the ice-free period (Fig. 3.4). Below the ice-cover the population was composed of adult females which started laying eggs when the lake was ice-free. Juveniles appeared from one to three months afterwards (Fig. 3.6), usually during July-August, and grew up during the summer. Adult females did not start reproducing until the following summer. Therefore, the reproduction strategy resulted at the end in a single annual cohort. The average population size followed a similar pattern every year, increasing steadily until reaching a breakpoint, when it slowed down to a lower rate (Fig. 3.6). This change in growth rate was considered a good estimator of the average maturation size, since most of the females were already mature at this moment. Fitting a bilinear equation to the population average sizes (Muggeo 2003), allowed to estimate several life history traits at the population level including juvenile and adult growth rate and an estimate of the population maturation size.

There were inter-annual differences in the various measured life history traits (Table 3.1). The maximum size (S_{max} , the largest population average *Daphnia* adult female size found at the end of every annual cohort) was highly correlated with maturation size (S_{mat}) ($S_{\text{max}} = 1.20 * S_{\text{mat}}$, $r^2 = 0.997$, $p < 0.0001$). Both traits were inversely related with the average ice-free period *Daphnia* abundance ($r = -0.91$, $p = 0.016$ and $r = -0.92$, $p = 0.014$ for maturation size and maximum size respectively). Adult growth rate was also linearly related with maturation size, being faster when the juveniles matured at a lower size and vice versa ($r = 0.963$, $p = 0.003$). This result is consistent with previous knowledge that *Daphnia* maximum size is a constant function of maturation size (Lynch et al. 1986), and is probably observed at the population level, due to the constant inter-annual temperature below the ice-cover. Period during which adult growth occurred. In contrast, juvenile growth rate was not related with maturation size or maximum size ($p > 0.53$).

Daphnia fecundity was highly correlated with the density of large females surviving the ice-cover ($r = 0.961$, $p = 0.009$), while it was not related to the average female size ($p = 0.145$). Clutch sizes varied from 2 to 6 eggs per female, being closely related with *Daphnia* female size ($r^2 = 0.943$, $p < 0.0001$). However, the much higher variability in *Daphnia* abundance (varying from 0.09 l^{-1} in 1998 to 1.22 l^{-1} in 1996) than maximum size (varying from 1990 to $2340 \mu\text{m}$) was the reason for the lack of correlation between fecundity and female size.

Males were found almost every year in low quantities from October to January (with the exception of 1996, when no males were found), when occasional ephippial females were found. Therefore, most of the *Daphnia* individuals during the study period originated from parthenogenetic reproduction.

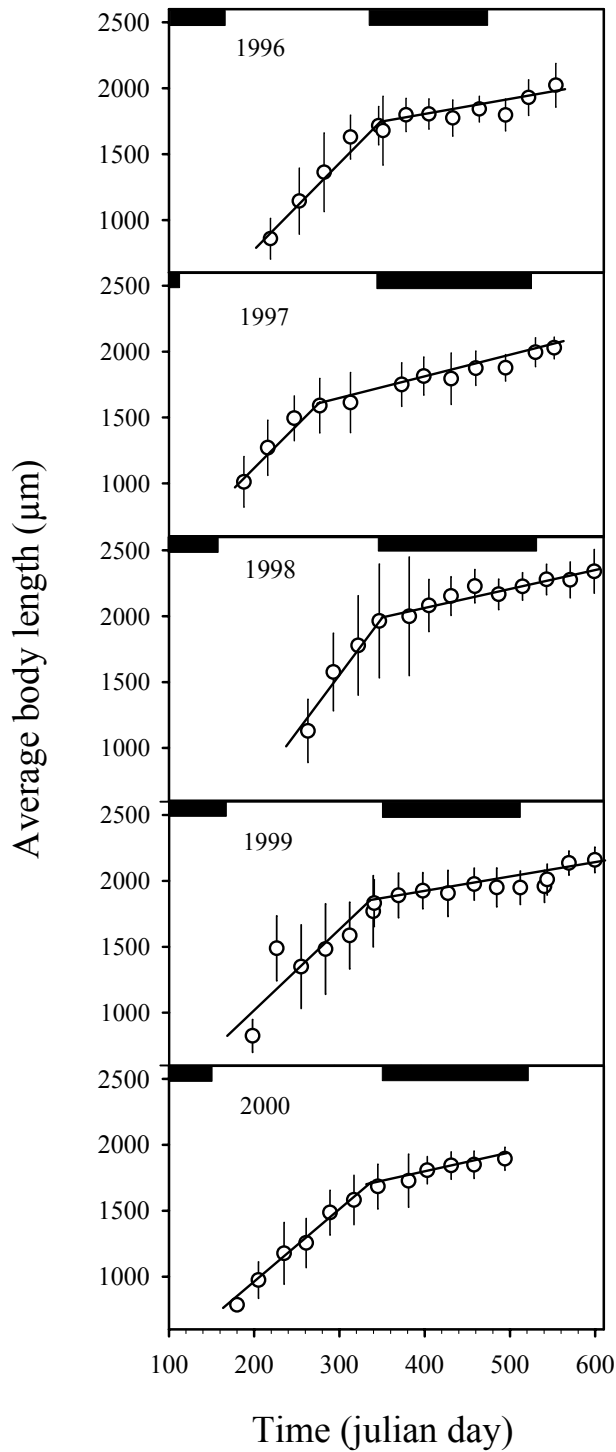


Fig. 3.6. Average body length (empty circles) and standard deviation (bars) of the *Daphnia pulicaria* population of Lake Redon. Each graph shows the population growth starting from the first juveniles appearing in the water column after the ice-cover melted for each of the five consecutive years studied. Horizontal bars indicate the ice-cover period. Straight lines indicate the *Daphnia* population specific growth estimates calculated by adjusting a bilinear equation following the method described by (Muggeo 2003) and using the library *segmented* within the R package software (Ver. 1.9.0., R Foundation, <http://www.r-project.org/>). Fitted parameters are described in Table 3.1.

Table 3.1. Results of fitting a piece-wise regression model to the *Daphnia pulex* population of Lake Redon in each of the five years studied. Growth rate are the two slopes obtained from the model \pm standard error and t-values, corresponding to the juvenile and adult growth rate (first and second slopes respectively). Breakpoint is the timing of the breakpoint \pm standard error of the estimate, and the maturation size, the size at the breakpoint. Regression coefficient (r^2), regression residual standard error (Residual SE) and degrees of freedom (df) are also indicated. Maximum size is the size of the largest average female size attained by each annual cohort, and was obtained directly from the data.

	Growth rate ($\mu\text{m d}^{-1}$)		Breakpoint Julian days	r^2	Residual SE	df	Maturation size (μm)	Maximum size (μm)
1996								
Juvenile	8.14 \pm 0.65	12.47	318 \pm 7.3	0.986	45.45	9	1668	2021.7
Mature	1.24 \pm 0.21	5.79						
1997								
Juvenile	8.16 \pm 0.70	11.70	252 \pm 5.5	0.993	29.11	8	1542	2027.5
Mature	1.57 \pm 0.11	14.36						
1998								
Juvenile	9.68 \pm 1.70	5.69	347 \pm 13.5	0.982	53.32	9	2006	2339.1
Mature	0.59 \pm 0.43	1.37						
1999								
Juvenile	5.43 \pm 0.84	6.47	354 \pm 23.9	0.910	111.9	13	1842	2158.4
Mature	1.03 \pm 0.50	2.06						
2000								
Juvenile	5.81 \pm 0.28	20.87	327 \pm 8.6	0.995	32.02	8	1669	1987.9
Mature	1.43 \pm 0.27	5.38						

Relationships with temperature and food

The possible relationship between the various measured population and life history traits with temperature, food concentration (particulate carbon and nitrogen) and food quality (phosphorus and the atomic C:P and N:P ratios) were explored with correlation analysis (Table 3.2).

Table 3.2. Correlation coefficients between the different life history parameters measured for each of the three zooplankton species of Lake Redon and the average water column water temperature, seston elemental composition and the elemental ratios. Each data point (n) corresponds to the annual mean for the period corresponding to each life history parameter, or to the average ice-free period for the species abundance and biomass. Abundance of each species (individuals l⁻¹) and biomass (µg C l⁻¹) were log transformed. Asterisks denote the significance of the correlations: where * < 0.05; ** < 0.01; *** < 0.001.

Species	Parameter	Temperature (n = 5)	PC (n = 5)	PN (n = 5)	PP (n = 4)	C:P (n = 4)	N:P (n = 4)
<i>Daphnia</i>	Juvenile growth rate	-0.30	0.70	0.80	-0.20	0.20	0.40
<i>pulicaria</i>	Adult growth rate	-0.21	0.09	0.09	0.17	-0.11	-0.06
	Maturation size	-0.61	-0.20	-0.30	-1 ***	1 ***	0.80
	Maximum size	0.11	-0.15	-0.16	-0.02	0.08	0.13
	Biomass	-0.28	-0.57	-0.21	0.90 *	-0.89	-0.86
	Abundance	-0.38	-0.07	-0.31	0.84	-0.85	-0.92 *
<i>Cyclops</i>	Female maximum length	-0.87	0.45	-0.70	0.02	-0.03	-0.75
<i>abyssorum</i>	Male maximum length	-0.89 *	0.48	-0.80 *	-0.54	0.41	-0.31
	Biomass	0.82 *	-0.30	0.63	-0.4	0.33	0.90 *
	Abundance	0.91 *	-0.33	0.71	-0.26	0.16	0.82
<i>Diaptomus</i>	Female maximum length	0.52	0.09	0.33	0.90	-0.08	-0.22
<i>cyaneus</i>	Male maximum length	0.63	0.12	0.41	0.97 **	0.14	-0.05
	Biomass	-0.84 *	0.27	-0.13	-0.14	-0.99 *	-0.81
	Abundance	-0.92 **	0.25	-0.43	-0.43	-0.81	-0.93 *

Cyclops biomass and abundance, as well as female and male maximum length were mainly correlated with the average ice-free period temperature. Being all the correlations significant with the exception of female maximum size. Biomass and abundance were positively correlated with temperature and maximum body length negatively (Table 3.2). In contrast, there was little relationship with any of the food quality or quantity parameters. N:P was the only parameter slightly correlated with abundance, biomass and maximum size.

The abundance and biomass of *Diaptomus* were negatively correlated with the average temperature, C:P and N:P ratios (corresponding to the same period as the copepod was present in the lake). In contrast, adult body length had a positive correlation with PP, and a slight correlation with the average June to August temperature.

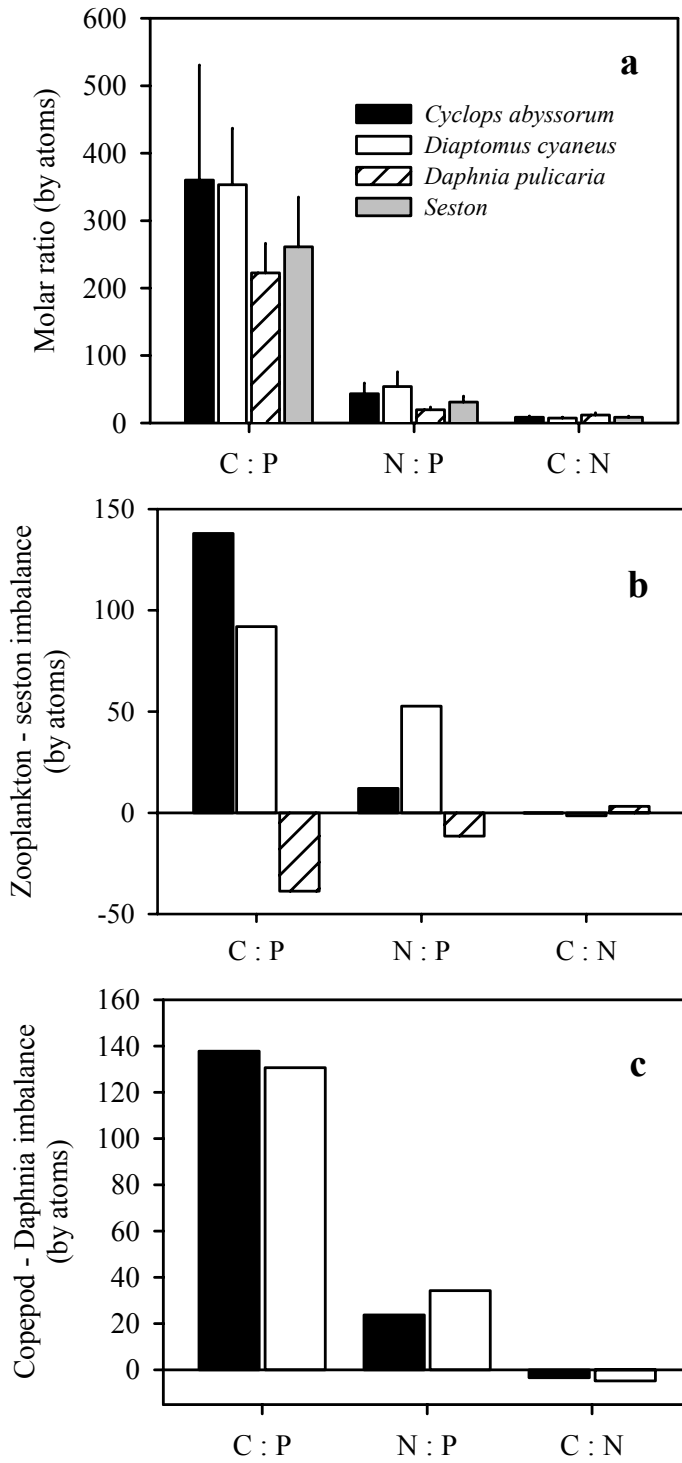


Fig. 3.7. C:P, N:P and C:N ratios (by atoms) characterising the three pelagic crustacean zooplankton species of Lake Redon and seston. (a) Average and standard deviation (error bars) of the elemental ratios. (b) elemental imbalance (Imbalance = Food Atomic ratio – Consumer Atomic Ratio) between seston and zooplankton and (c) between the cladoceran herbivore *Daphnia pulex* and the two copepods.

No dependence of any of the measured *Daphnia* parameters were found with temperature or food quantity. Whereas most parameters were highly correlated with food quality. *Daphnia* maturation size, had a negative correlation with particulate phosphorus concentration and positive with C:P and N:P ratios. Juvenile growth rate was only slightly correlated with particulate carbon and nitrogen, but the relationship was not significant. Biomass and abundance had a significant positive correlations with particulate phosphorus and negative with C:P and N:P (Table 3.2).

Since *Daphnia* abundance was found to be directly related to fecundity and correlated with PP, we investigated the relative contribution of both factors to the annual *Daphnia* production by multiple regression analysis. The average October-December *Daphnia* abundance (assumed to be a good estimate of the *Daphnia* produced during each ice-free period) inter-annual variability was explained by both the population fecundity and PP ($r^2 = 0.999$, $p = 0.033$). Further variance partitioning (Legendre & Legendre 1998) showed that population fecundity explained exclusively 20.5% of the variance, and PP 47.2%, while both variables shared 32.3% of the variance. Therefore, phosphorus availability seemed to be the driving factor of the relationship.

In order to complement the above results relating life history and population parameters with food quality, we compared the average seston stoichiometric composition with those of the three species of zooplankton (Fig. 3.7). *Daphnia* had lower C:P and N:P ratios than seston. The two copepod species had higher C:P and N:P ratios than seston and *Daphnia*. Calculation of stoichiometric imbalances between the three zooplankton species and seston revealed the differences in nutrient requirements of the two copepods and the *Daphnia*. While the two copepods were deficient in carbon and nitrogen, *Daphnia* was deficient in phosphorus.

Timing of the life history parameters

The end of the ice-covered period was the key limnological feature characterising the timing of the life-cycles of the three species. Amongst them, the two copepods had a more clear temporal synchronisation, since nauplii appeared just when the ice-cover melted, allowing copepodites to grow during the spring production maxima (*Diaptomus*) or during the whole ice-free period (*Cyclops*). Similarly to the copepods, the life-cycle of *Daphnia* was also associated with the timing of the ice-cover. Amongst the different life history parameters, the timing of maturation size (T_{ms}) was within one month of the formation of the ice-cover (which was usually around mid December) in all years excluding 1997, when the population matured three months before. This year the ice-cover melted substantially earlier than the other years (Fig. 3.6). Linear regression analysis showed that the timing of maturation size was closely related with the timing of the ice-cover melting ($T_{ice\ off}$) ($T_{ms} = 0.54 * T_{ice\ off} - 26.2$, $r^2 = 0.89$, $p = 0.016$).

Whereas there were already ovigerous females every year after the ice-cover melted, the timing of the appearance of the first juveniles was very variable (ranged from 31 days after the ice-cover melted in 2000 to 106 days in 1998) and was not related with the ice-cover melting day ($p = 0.329$). This timing together with those of the maturation size and the values themselves (the size of the first juveniles and the maturation size) are the parameters influencing the variability in the population juvenile growth rate. This trait, was the only not directly correlated with any other measured variable. A

closer look at the data could clarify which were the variables affecting juvenile growth rate variability. Initially, the density of females surviving the ice-covered period was inversely correlated with the size of the first young females found at the beginning of the ice-free period (Fig. 3.6, $r = -0.967$, $p = 0.004$). The time-lag between the ice-cover melt and the moment the first juveniles were found, was also correlated with the size of the first juveniles found ($r = 0.888$, $p = 0.022$). In other words, the higher the mother abundance, the smaller the first *Daphnia* found and the earlier they were found.

The timing of maturation size was a function of the ice-melting date. The timing of appearance of the first juveniles was related with the abundance of females surviving the ice-cover, which also together with PP predicted the abundance of females produced every summer. The female density was inversely related with the maturation size. Therefore we conclude that juvenile growth rate was dependent on three main variables, the timing of the ice-cover melt, the abundance of females surviving after the ice cover, and the concentration of particulate phosphorus (Fig. 3.8).

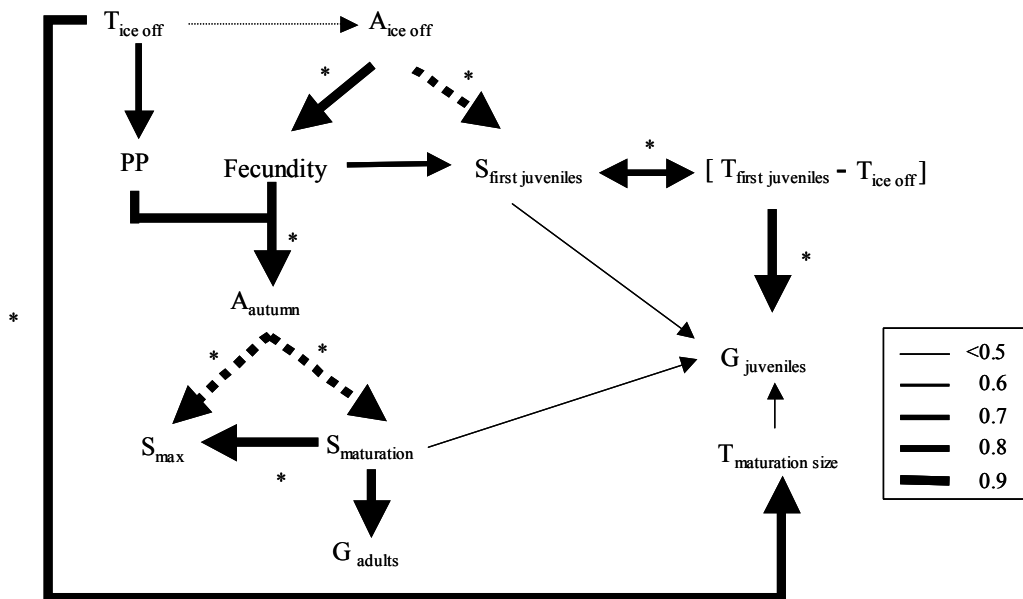


Fig. 3.8. Summary of the correlations of *Daphnia pulicaria* population and life history traits with the relevant environmental variables. T is timing of each event in Julian days; A, the abundance; S, size; G, population growth rate; and PP, seston phosphorus. Arrow thickness is proportional to the correlation according to the legend. Solid arrows correspond to positive correlations and dashed arrows to negative correlations. * $p < 0.05$.

Discussion

Food quality versus quantity

Comparison of *Daphnia* elemental composition with those of seston suggest that *Daphnia* may be primarily limited by phosphorus availability. Furthermore, *Daphnia* biomass production in Lake Redon has been found to be related to both population fecundity, which depends on the number of females surviving the ice-covered period, but especially to PP. Therefore, following (Sternler & Schulz 1998), we conclude that *Daphnia* in Lake Redon is limited by phosphorus availability. Previous stoichiometric studies have shown that under high nutrient conditions, the quality of the food is more relevant than the quantity itself, both in lab experiments (Sternler 1993; Urabe et al. 1997) and in the field (DeMott & Gulati 1999; Makino et al. 2002). In contrast, under very low food quantities, the overall energetic limitation of body growth have been traditionally thought to hide the effect of mineral limitations (Hrbacek 1985). A laboratory experiment (Boersma & Kreutzer 2002) showed that even at low food quantities, the quality of the food was relevant for zooplankton growth. Our results show that the *Daphnia* of Lake Redon is adapted to live under these oligotrophic habitats by being able to stop reproduction when the food conditions are below a certain limit. This feature has also been described in the *D. pulicaria* of Tatra mountains (Gliwicz et al. 2001), and seems therefore to be a general feature of these alpine *Daphnia* species. The ability to stop reproduction also allows the females to accumulate lipids during winter, which are subsequently used for offspring production when the ice-cover disappears. The limiting of the availability of phosphorus at this stage seems a logic consequence.

In contrast with *Daphnia*, both copepod species had a lower phosphorus and a higher carbon and nitrogen content than seston and *Daphnia*. If we assume that seston mainly represents the primary producers and *Daphnia* is a good representative of a non-selective herbivore, both seston and *Daphnia* are a good estimate of the stoichiometric composition of the potential food of the two copepods. Therefore both copepods are likely to be limited by the availability of carbon or nitrogen. Since carbon is commonly stored in copepods as triacylglycerol reserves in their body (Vanderploeg et al. 1992), but nitrogen, which is mainly present as protein, cannot be stored for a later use, the copepods of Lake Redon are likely to face a protein limitation. Nitrogen limitation has been pointed to be a general phenomenon for predators in terrestrial ecosystems (White 1993), our results suggest that this is likely the case in aquatic organisms such as copepods, too.

Possible causes for the reduction of the population after the ice-cover melting

Comparison of the abundance before and after the ice cover showed that both *Cyclops* and *Daphnia* decreased their abundance in a relevant proportion (Fig. 3.3). Several factors might contribute to the observed pattern, including those potentially reducing the population such as senescence, predation and ice-melt wash out, and those contributing

to population increase such as emergence from diapause. The first factor is associated with the length of the ice-cover and the senescence of the species. *C. abyssorum* has been described in many lakes to survive for one whole seasonal cycle (Nilssen & Elgmork 1977), and in some northern lakes other species of the genus have a lifespan of more than one year (Elgmork & Eie 1989), therefore survival due to senescence is likely not to be a problem in *Cyclops*. However, in *Daphnia*, despite the species studied in Lake Redon has been reported to have one of the longest lifespans of the genus (Dudycha 2003), its expected survival time might be close to the limit of its senescence. The age of a daphnid has been shown to depend on water temperature and the size attained by the daphnid (Geller 1987). This author derived an empirical model for several *Daphnia* species, including *D. pulicaria*, for estimating the age of *Daphnia* (understood as the post-embryonic time) based on these two parameters. Applying the model to the daphnids of Lake Redon resulted that the adult females found after the ice-cover melted had an age between 110 and 174 d, depending on the year. Comparing this values with the maximum lifespan expected for Lake Redon (302 ± 37 d; average \pm standard deviation, including the number of days elapsed between the finding of the first juveniles and the end of the ice-cover) suggest that even if the Lake Redon *Daphnia* is adapted to survive for a longer period than those described in the literature (Geller 1987; Dudycha 2003) it is possible that in some of the years with a longer ice-cover period, some individuals might die of senescence.

Both invertebrate and vertebrate predation has been described to be major factors affecting the abundance of zooplankton populations (Gliwicz & Pijanowska 1989). In small alpine lakes, the presence of trout has been shown to severely affect zooplankton densities, leading to the extinction of the cladocerans (Gliwicz & Rowan 1984). Gut contents analysis of Lake Redon brown trout revealed that this fish species had a marked shift both in quantity and quality of prey in their stomachs. After the ice-cover melted, the stomach content was full and its prey was mainly littoral invertebrates. In Autumn, following the decrease of water temperature, the stomach content was much reduced, but half of the content was zooplankton (Catalan et al. 2004). In large alpine lakes such as Redon, the effects of brown trout on zooplankton seems to be less severe than in smaller lakes (Gliwicz & Rowan 1984). However, the results from the trout gut contents indicated that trout predate on zooplankton at least during Autumn. On the other hand, predation of copepods on *Daphnia* has been documented to concentrate mainly in copepodites on eggs directly in the egg pouch (Gliwicz & Stibor 1993), and adults on juvenile cladocerans. When cladocerans reach a certain size, they are almost invulnerable to copepod predation (Gliwicz & Umana 1994). Therefore *Cyclops* predation during the mature stage of *Daphnia* is expected to be of low importance.

A third factor affecting zooplankton density is the strong wash out occurring during snowmelt. During this period a great proportion of the surface layers of the water column are renewed, and correspondingly the organisms inhabiting these layers are washed out through the outflow. The relative importance of this process has not been evaluated, however, preliminary data indicate that it can have a significant effect on zooplankton density (Ventura, personal observation).

Daphnia uses ephippial eggs as a complementary survival mechanism. Emergence from ephippia is mainly driven by environmental cues (Caceres & Tessier 2003), therefore the increase in *Daphnia* density during 2000 (Fig. 3.3) might be a result of an especially high emergence from resting ephippia.

Density-dependent intra-specific interaction

Maximum body size attained by the three species had significant inter-annual variations. In both *Daphnia* and *Cyclops*, there was an inverse relationship between abundance and maximum size, suggesting intra-specific interaction. There is a growing literature describing the negative effects of crowding on *Daphnia* growth, reproduction and body size (Burns 1995; Cleuvers et al. 1997; Burns 2000; Rose et al. 2002; Mitchell & Carvalho 2002; Lüring 2003), and rotifers (Kirk 1998). Evidences point towards an auto-regulative capacity of the populations through segregation of autotoxines. *Daphnia* densities reported by these studies were as low as 40–150 individuals l^{-1} . However, these densities are by far much higher than the densities found in Redon (from 0.09 to 1.22 individuals l^{-1} ; Fig. 3.1). At the low densities found in Redon, it seems unlikely that the effects of autotoxines regulate the *Daphnia* maximum size. However, since the annual seston concentration was more constant than *Daphnia* (the coefficient of variation was 13.7% for particulate carbon in front of 58.1%, for *Daphnia*), direct competition for food cannot be discarded. Complementary to the intra-specific interaction effects, direct predation on eggs and especially juveniles by copepods has been described in alpine lakes (Gliwicz & Umana 1994; Gliwicz & Stibor 1993). Selective predation of invertebrates to smaller juveniles has been described as a plausible mechanism for selecting larger individuals in a short time interval (Lampert 1993). In this sense, *Daphnia* started laying eggs just after the ice-cover melted, but it was not until one and three months later that daphnia juveniles were found in the water column. A different inter-annual copepod predation pressure during this period could further explain the variability in the measured size of the first juveniles found in the lake (Fig. 3.6), in addition to adult female density. Our results suggest therefore that both mechanisms, predation and density dependence food competition, can be responsible for regulating *Daphnia* population variability.

In contrast with *Daphnia*, the maximum size of *Cyclops* was negatively correlated with the average water temperature, as well as its population density. Temperature and food quantity have been shown to be negatively and positively correlated with body size respectively (Miracle 1978; Ban 1994; Mckee & Ebert 1996; Charnov & Gillooly 2003; Lee et al. 2003). However, food availability has been shown to be a more significant factor than temperature (Ban 1994). Following from the conclusions of these experimental results, in Lake Redon, a density dependent limitation of body size of *Cyclops*, which can be interpreted as an intra-specific interaction, should be more relevant than the direct effect of the temperature itself. Furthermore, the females from the two years with a higher size had a clutch size from three to five times higher than the other three years. The same range of variability in body size and clutch sizes was found in the same species in a survey of many lakes across the Pyrenees (Miracle 1978). Therefore, this variability lies within the phenotypic variability of the species, and could be interpreted as a phenotypic response to recover the abundance of previous years. For instance the average clutch size of females during 1996 was three times larger than the following three years, which is comparable to the increase of abundance from 1996 to 1997. The presence of epibionts was observed sporadically throughout the study period, but only in winter 2000 there was a general epibiont colonisation affecting most individuals. The presence of epibionts has been appointed to negatively affect copepod populations (Threlkeld et al. 1993; Xu & Burns 1991). Therefore, it is possible that the

variability in *Cyclops* abundance in Lake Redon would be influenced by the presence of these epibionts.

On the other hand, *Diaptomus* maximum size was only correlated with seston particulate phosphorus. Since *Diaptomus* had lower phosphorus content than seston and *Daphnia*, it is very likely that this is an indirect correlation. Higher phosphorus content is indicative of a higher primary production in phosphorus limited lakes, which could be propagated through the food chain to yield, for example, higher protozoan biomass. Despite the precise food consumed by these species has not been described, its size and morphological inspection of its feeding appendages suggest its not a herbivorous species (see also chapter 7). It has been shown that calanoid copepods are very efficient consumers of protozoans (Burns & Schallenberg 2001). In Lake Redon, ciliate abundance is particularly high during the period that *Diaptomus* grow, the spring overturn (Felip et al. 1999).

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Part II

Seasonal variability in the elemental, biochemical and isotopic composition

4. Linking biochemical and elemental composition in freshwater and marine crustacean zooplankton

Abstract

The major biochemical compounds of zooplankton (proteins, lipids, carbohydrates, chitin, nucleic acids and nucleotides) have a distinct functional role. Therefore incorporating biochemical compound information into stoichiometric analysis would improve current understanding of species life histories and their interactions. The stoichiometric composition of the main biochemical compounds of 43 species of freshwater and marine zooplankton revealed high constancy. No significant differences were found between marine and freshwater species or between any of the taxonomic groups considered (amphipods, copepods, cladocerans, euphausiids and mysids) for most of the main biochemical compounds (triacylglycerol, wax esters, phospholipids and proteins). Therefore, inter- and intra-specific stoichiometric variability is a result of differences in the proportions of the main biochemical compounds. The elemental composition of *Calanus finmarchicus* was estimated from the composition of major biochemical compounds. Comparison of measured and estimated elemental composition revealed that almost all of the measured carbon, nitrogen and phosphorus were accounted for, except for 26.3% of the phosphorus, related to the unmeasured acid-soluble fraction of nucleotides or phosphate. The relative importance of the main biochemical compounds to zooplankton elemental composition was derived by combining the measured ranges of the main biochemical compounds gleaned from the literature and their average stoichiometric composition. Almost all carbon content was accounted for by lipids and proteins (from 82 to 98% of the total carbon content), while proteins accounted for 46-98% of total nitrogen. Phosphorus was the element with highest variability in terms of which biochemical compound contributed the most to its total pool. In most cases nucleic acids were the main phosphorus pool, followed by phospholipids and, to a lesser extent, nucleotides.

Introduction

The main elemental constituents of freshwater and marine zooplankton have been described extensively in the literature and are highly variable, as shown by their percentage ranges on a dry weight basis: from 28-68 for carbon (C), 5-15 for nitrogen (N), 3.9-10.3 for hydrogen (H) (Båmstedt 1986; Elser et al. 2000a) and 0.3-2.1 for phosphorus (P) (Hessen & Lyche 1991; Mauchline 1998). However, the underlying mechanisms for this wide variation have been poorly investigated. Some phylogenetic constraints have been identified in some freshwater groups, such as cladocerans, which have consistently higher P and lower N content than copepods when compared within the same environment (Andersen & Hessen 1991; Hessen & Lyche 1991; Chapter 5). Elser & Hassett

(1994) found that freshwater zooplankton had lower N:P ratios than marine zooplankton. It was attributed to changes in composition across taxa (Hassett et al. 1997). As a result of their elemental composition, organisms have specific elemental requirements (Elser et al. 1996). Therefore, organisms recycle nutrients to their environment at rates which are inversely related to their own chemical composition (Sterner & Hessen 1994). For example cladocerans, due to their high P demand, release compounds with low N:P ratio (Urabe 1993). Thus, a shift in the zooplankton community from cladoceran to copepod dominance might lead to changes from P to N limitation in the water (Elser et al. 1988).

Elements do not exist as such in organisms, but are combined into biochemical compounds (Strayer 1988). Most of these biochemical compounds have a defined functional role. While some are used as energy reserves, for example triacylglycerols and wax esters (Goulden & Henry 1984; Sargent & Falk-Petersen 1988), others are used preferentially during reproductive periods, such as RNA (Acharya et al. 2004; Vrede et al. 2002), and therefore these types of compounds change in their body concentrations through the lifetime of an organism. By contrast, compounds with primarily a structural role are kept at constant proportions during the whole of an animal's life. For example, proteins are related with muscle tissues, which are directly involved in the animal's movement. Phospholipids are a the main component of cell membranes, DNA, containing the genetic information of the cell and chitin, a skeletal compound forming the support structures in invertebrates.

Several studies have focused on the description of the general biochemical composition of zooplankton (Mauchline 1998), particularly on the lipid composition in marine zooplankton. However, very few attempts have been made to directly relate the changes in elemental composition with those of the main biochemical compounds. An understanding of these relationships would improve the ecological applicability of stoichiometric arguments (Tang & Dam 1999). For example, within a species, it is known that the higher the growth rate, the higher the RNA:DNA ratio. Thus, high animal growth rates result in an increase in the phosphorus content of cells due to the relatively high phosphorus content of RNA and DNA. From these relationships, it has been possible to understand why the demand for phosphorus is higher in fast-growing species than in species of slow growth (Elser et al. 2000b; Vrede et al. 2002; Acharya et al. 2004).

The elemental composition of some of the biochemical compounds is well known because they are mostly polymers of specific molecules of known composition and structure, such as chitin, nucleic acids, nucleotides and glycogen. On the other hand, although it is often assumed that the approximate elemental composition of protein and lipid molecules is relatively constant, substantial differences have been found across species. For instance, the amino acid composition in species of three freshwater crustacean groups (cladocerans, calanoid copepods and cyclopoid copepods) was found to be species-specific (Guisande et al. 2003). Similarly, the fatty acid composition in marine zooplankton varies under different environmental conditions (Sargent & Falk-Petersen 1988). The potential effects of variability in biochemical composition of zooplankton on animal stoichiometry have not been approached in any detail yet. Therefore, the aims of this study were: i) to examine the ranges of variability in stoichiometric composition of main biochemical compounds of zooplankton (i.e. wax esters, triacylglycerols, phospholipids, proteins and free amino acids); ii) to evaluate the differences in the

stoichiometric composition of main biochemical compounds in marine and freshwater species and across main zooplankton groups (i.e. amphipods, calanoid and cyclopoid copepods, cladocerans, euphausiids and mysids); iii) to assess the potential use of an “average stoichiometric composition” for the biochemical compounds in zooplankton when calculating the elemental composition from measured biochemical composition; and IV) to define the range of stoichiometric variability in zooplankton that can be expected across the range of biochemical compositions found in zooplankton.

Materials and Methods

Stoichiometric composition of biochemical compounds

The biochemical composition of the main biochemical compounds (wax esters, triacylglycerols, phospholipids, free amino acids and proteins) was obtained from published studies. Data covered a total of 43 crustacean species belonging to the commonest taxonomic groups in both marine and freshwater habitats, such as amphipods, cladocerans, cyclopoid copepods, calanoid copepods, euphausiids and mysids (Table 4.1).

The stoichiometric composition of each biochemical compound was calculated on the basis of its molecular structure. Wax esters are a combination of fatty acids esterified with fatty alcohols, therefore wax ester mean stoichiometric composition was calculated by averaging the different proportions of the fatty acids and the fatty alcohols separately and thereafter combining them for each species. Triacylglycerols are three molecules of fatty acids combined with a molecule of glycerol, the average fatty acid stoichiometric composition was calculated for each species and then combined with that of glycerol to obtain the average composition for triacylglycerol. The calculation of a mean composition for phospholipids was more complex because phospholipids can be present as different molecules (mainly phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophosphatidylethanolamine, lysophosphatidylcholine and phosphatidic acid; Strayer 1988) of different fatty acid composition. Most papers only report the total fatty acid composition of phospholipids. Therefore, information available in the literature on phospholipid fatty acid composition and the relative proportions of different phospholipid molecules across species had to be collected separately. The composition of fatty acid and phospholipid molecules were then combined to obtain the average phospholipid composition for each zooplankton group. Thus, the range of different phospholipid molecules and that of different fatty acids could be taken into account simultaneously. The total amino acid pool was divided into protein and free amino acids. The average stoichiometric composition of the two fractions and that of the total were calculated for each species, by averaging the amino acid stoichiometric composition and multiplying by their proportions. Only studies giving complete amino acid composition data were used in order to avoid underestimating sulphur content in stoichiometric calculations. This is because sulphur-containing amino acids easily degrade and thus require special analytical methods which were not always used in the publications surveyed.

Table 4.1. Published data on freshwater (FW) and marine (MA) zooplankton species used to describe the elemental composition of the main biochemical compounds. Species belonging to the most common taxonomic groups were selected, such as amphipods (AM), cladocerans (CL), cyclopoid copepods (CO-CY), calanoid copepods (CO-CA), euphausiids (EU) and mysids (MY).

Group	Habitat	Species	Total and protein amino acids	Free amino acids	Phospholipid molecules	Phospholipid		Triacylglycerol		Wax ester	Reference
						fatty acids	fatty acids	fatty acids	fatty acids		
AM	FW	<i>Macrohectopus branicki</i>				x		x			Morris 1984
CL	FW	<i>Daphnia cucullata</i>				x		x			Farkas 1979
CL	FW	<i>Daphnia magna</i>		x		x					Farkas et al. 1984, Gardner and Miller 1981
CL	FW	<i>Daphnia pulex</i>	x	x		x		x			Farkas 1979, Dabrowski and Rusiecki 1983
CL	FW	<i>Daphnia pulicaria</i>	x								Chapter 6
CL	FW	<i>Ceriodaphnia sp.</i>	x	x							Dabrowski and Rusiecki 1983
CO-CY	FW	<i>Cyclops vicinus</i>			x			x			Farkas et al. 1984, Farkas 1979
CO-CY	FW	<i>Cyclops strenuus</i>	x	x							Dabrowski and Rusiecki 1983
CO-CY	FW	<i>Cyclops abyssorum</i>	x								Chapter 6
CO-CY	FW	<i>Acanthocyclops vernalis</i>				x		x			Farkas 1979
CO-CA	FW	<i>Epischura baikalensis</i>				x		x			Morris 1984
CO-CA	FW	<i>Eudiaptomus gracilis</i>						x			Farkas 1979
CO-CA	FW	<i>Eudiaptomus zachvatkini</i>	x	x							Dabrowski and Rusiecki 1983
CO-CA	FW	<i>Diaptomus sp.</i>		x							Jeffries and Alzara 1970
CO-CA	FW	<i>Diaptomus cyaneus</i>	x								Chapter 6
CO-CA	MA	<i>Acartia tonsa</i>		x				x			Ederington et al. 1995, Jeffries 1969
CO-CA	MA	<i>Acartia clausi</i>		x				x			Ederington et al. 1995, Jeffries 1969
CO-CA	MA	<i>Amalothrix sp.</i>						x			Lee et al. 1971a
CO-CA	MA	<i>Calanoides acutus</i>						x			Falk-Petersen et al. 1999, Albers et al. 1996, Reinhardt and Van Vleet 1986
CO-CA	MA	<i>Calanus finmarchicus</i>	x	x		x		x			Farkas et al. 1988, Sargent and Falk-Petersen 1988, Albers et al. 1996, Jeffries and Alzara 1970, Cowey and Corner 1963a

Differences in the elemental composition of each main biochemical compound were analysed using the Kruskal-wallis non-parametric test. Habitat (freshwater or marine) and taxonomic group (amphipods, cladocerans, cyclopoid and calanoid copepods, euphausiids and mysids) were factors included in this analysis.

Estimation of the elemental composition of *Calanus finmarchicus* compound proportions

To test the potential of biochemical compound specific proportions for predicting average stoichiometric compositions in a given species, I used the biochemical composition described for the marine copepod *Calanus finmarchicus* Gunnerus. Its elemental composition was estimated from the following equation:

$$W_i = \sum_{k=1}^n W_k M_{ki} \quad [1]$$

Where W_i is the mass fraction of the i elemental component (C, H, N, O, P and S), W_k is the mass fraction of the k biochemical compound (protein, triacylglycerol or wax ester, phospholipid, free amino acid, chitin, glycogen, nucleic acid or nucleotide), M is the mass fraction of the i elemental component in the k biochemical compound, and n is the number of biochemical compounds having the i elemental component. All mass fractions are referred to as ash-free dry weights.

C. finmarchicus was chosen for this study because of the wealth of data available in the literature on this species. Mayzaud & Martin (1975), for instance, measured simultaneously its detailed biochemical and stoichiometric compositions. Lipids were assumed to be composed primarily of wax esters and phospholipids, which had been separately measured by the authors, since this species has been shown to use wax esters as its main energy reserve (Sargent & Falk-Petersen 1988). The nucleic acid proportion was calculated from that of carbohydrates insoluble in tricarboxylic acid (TCA), mostly nucleic acid pentoses. The RNA:DNA ratio was assumed to be 1, which is a ratio typically found in copepod species (Badouin & Scoppa 1975; Skjoldal & Båmstedt 1976). Amongst the main biochemical compounds in zooplankton, only free amino acids and nucleotides had not been measured by the authors. Therefore, for these two compounds other values for *C. finmarchicus* were used gleaned from the literature. Thus, free amino acids were assumed to be 4% (Jeffries & Alzara 1970) and nucleotides 0.88% (Skjoldal & Bamstedt 1977).

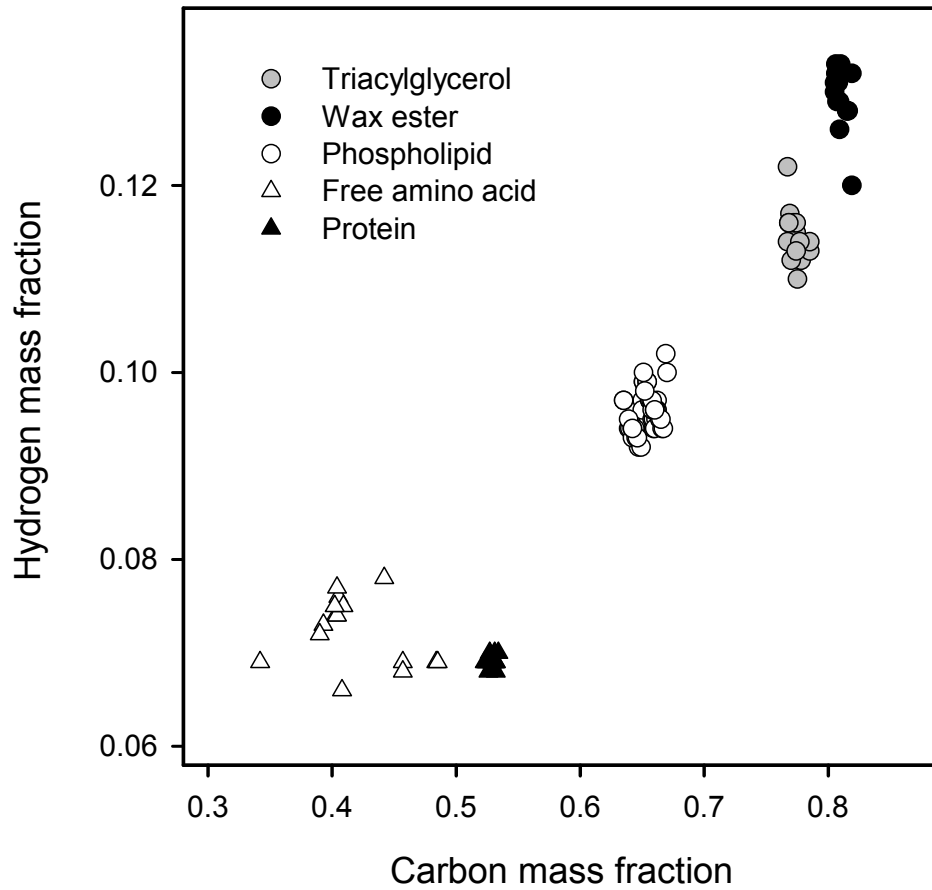


Fig. 4.1. Mass fractions on a unit basis (carbon and hydrogen) of stoichiometric compositions of main biochemical compounds of zooplankton (triacylglycerol, wax esters, phospholipids, free amino acids and proteins). Each point represents a different species and each symbol a different biochemical compound.

Table 4.2. Stoichiometric composition of the main biochemical compounds of freshwater and marine zooplankton as expressed in percentage mass composition. Means \pm standard deviations calculated for wax esters, triacylglycerols, phospholipids, proteins and free-amino acids in percentage of total mass of each compound. Other main biochemical compounds found in zooplankton are also shown (glycogen, DNA, RNA, chitin and ATP), which have known molecular compositions, with the exception of DNA and RNA for which the relationship G-C is assumed to be 40%, as demonstrated for crustacea (Smith 1964). The number of species considered for each compound (n) is indicated.

	n	C (%)	H (%)	O (%)	N (%)	P (%)	S (%)
Wax ester	14	81.0 \pm 0.5	13.0 \pm 0.3	6.0 \pm 0.4	0	0	0
Triacylglycerol	22	77.3 \pm 0.5	11.5 \pm 0.2	11.2 \pm 0.4	0	0	0
Phospholipid	25	65.3 \pm 0.9	9.6 \pm 0.2	19.6 \pm 0.3	1.7 \pm 0.1	3.9 \pm 0.3	0
Protein	15	52.8 \pm 0.5	6.9 \pm 0.1	23.2 \pm 0.6	16.0 \pm 0.3	0	0.9 \pm 0.2
Free amino acid	16	42.3 \pm 2.8	7.2 \pm 0.4	27.7 \pm 6.1	18.7 \pm 3.5	0	4.1 \pm 1.0
Total amino acid marine	6	52.8 \pm 0.4	6.9 \pm 0.1	23.2 \pm 0.4	16.1 \pm 0.2	0	1.1 \pm 0.2
Total amino acid freshwater	6	52.0 \pm 0.7	6.9 \pm 0.1	23.1 \pm 0.4	16.6 \pm 0.3	0	1.3 \pm 0.3
Free amino acid marine	10	45.5 \pm 2.9	7.0 \pm 0.4	22.6 \pm 6.4	21.6 \pm 4.0	0	3.3 \pm 1.1
Free amino acid freshwater	6	40.2 \pm 3.1	7.3 \pm 0.4	31.3 \pm 2.6	16.7 \pm 0.9	0	4.5 \pm 0.7
Glycogen		44.4	6.2	49.3	0	0	0
DNA		33.7	4.0	38.6	14.8	8.9	0
RNA		31.2	3.6	42.4	14.2	8.5	0
Chitin		45.3	6.6	41.5	6.6	0	0
ATP		23.9	2.4	41.3	13.9	18.5	0

Results and discussion

Stoichiometric composition of biochemical compounds

The analysis of zooplankton species belonging to both freshwater (calanoid and cyclopoid copepods, cladocerans and amphipods) and marine (calanoid copepods, euphausiids and mysids) habitats revealed that main biochemical compounds (wax esters, triacylglycerols, phospholipids, total amino acids, protein amino acids and free amino acids) had a very constant elemental composition (Fig. 4.1; Table 4.2). There were no significant differences in stoichiometric composition across taxonomic groups for any of the biochemical compounds analysed, nor between marine and freshwater species for the three lipid compounds or for the protein amino acids. In contrast, the elemental composition of the free amino acids differed between marine and freshwater species (Table 4.2). The freshwater species had 5.3% more C ($p = 0.002$), 4.9% more N

($p = 0.01$), 8.7% less oxygen ($p=0.013$), and 1.2% less sulphur ($p = 0.045$) than marine species because of their different proportions of amino acids. In addition, freshwater and marine species had different free amino acid concentrations (average \pm standard deviation $1.8 \pm 0.8\%$ and $6.0 \pm 2.9\%$ in weight in freshwater and marine species, respectively). Crustacean zooplankton, like other aquatic invertebrates, utilise free amino acids as main regulatory compounds for intracellular osmolarity in muscle tissue (Hochachka & Somero 2002). Furthermore, total free amino acid concentration in aquatic organisms is directly related to water salinity in their environment (Harris 1969; Jeffries & Alzara 1970). These differences among freshwater and marine species explain the different total amino acid composition found in organisms of both habitats (Table 4.2). In particular, freshwater species have 0.8% more C ($p = 0.012$) and 0.5% less N ($p = 0.018$) than marine species on average.

Because of the lack of statistical differences in elemental stoichiometry across taxonomic groups and between marine and freshwater species for all compounds except for free amino acids, an average elemental composition could be calculated for zooplankton lipids and proteins (Table 4.2).

Estimation of the elemental composition of *Calanus finmarchicus* from their biochemical compound proportions

The comparison of the directly measured elemental composition with that estimated from the proportions of main biochemical compounds showed closely agreeing values for the three key elements (C, N and P), accounting for 90.2, 89.0 and 73.8% of the total body C, N and P, respectively (Fig. 4.2A). The slight discrepancies found between direct and estimated elemental proportions could be due to not including minor biochemical compounds. In particular, this could have been the case with phosphorus, which is relatively less abundant and thus more prone to showing the effects of small unaccounted-for values. On the other hand, discrepancies between observed and estimated elemental compositions are partly explained by the limited precision of the standard biochemical methods. Zamer et al. (1989), for instance, reported underestimates of protein measurements by the Lowry method (the method used by Mayzaud and Martin 1975 for analysing proteins). Similar sources of error have been reported for standard chitin measurement methods (Cauchie 2002), and colorimetric lipid assays (Gardner et al. 1985). The estimated standard deviations for biochemical and elemental measurements were small (*circa* 1%).

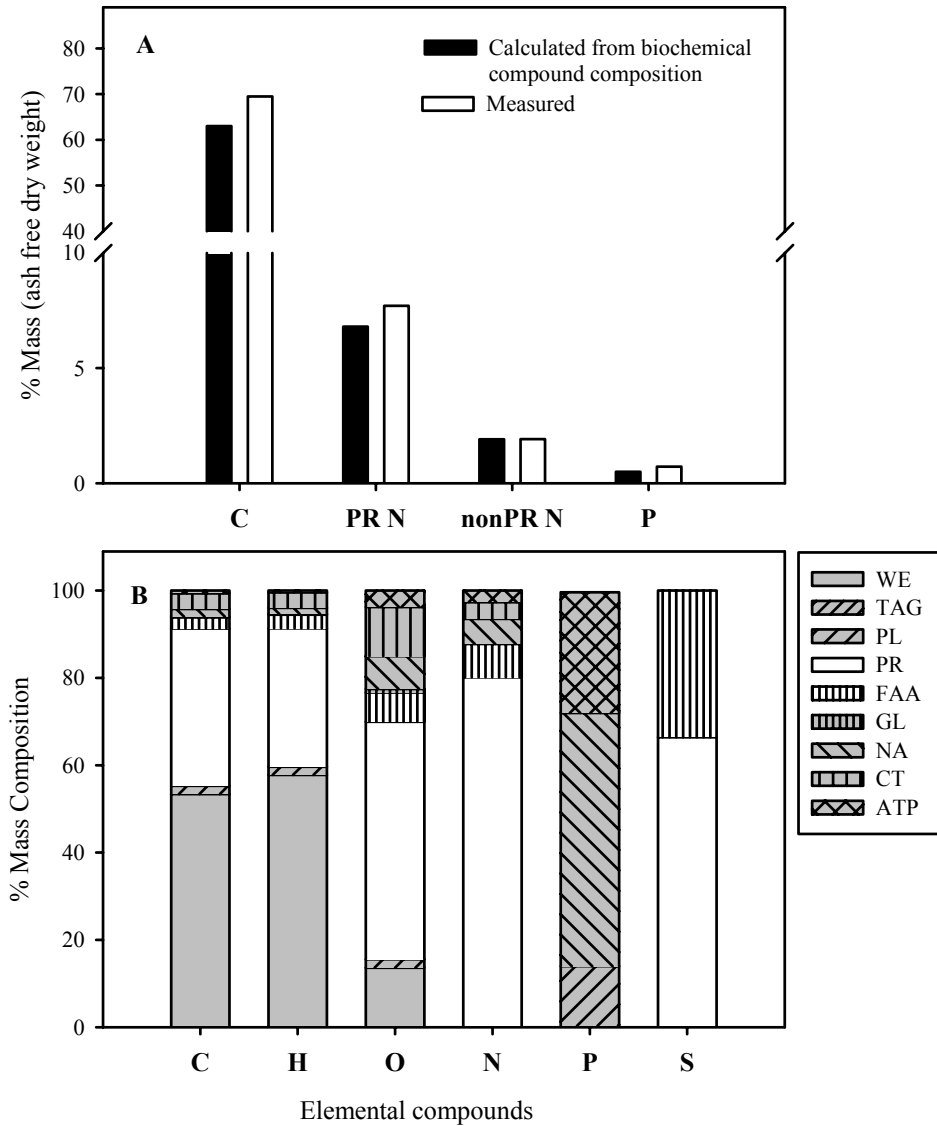


Fig. 4.2. Biochemical fractionation of the elemental compound composition in *Calanus finmarchicus*. A. Measured versus calculated elemental composition. The calculated elemental composition was obtained using main biochemical compound compositions and their stoichiometric composition (Table 4.2). See text for details. C: carbon, PR N: protein nitrogen, nonPR N: non-protein nitrogen, P: phosphorus. Measured elemental and biochemical compound data are from Mayzaud and Martin (1975). B. Major biochemical compound contribution to each element. WE, wax ester; PL, phospholipid; PR, protein; FAA, free amino acids; GL, glycogen; NA nucleic acids; CT, chitin; and ATP, adenosine-tri-phosphate.

The relative importance of each biochemical compound to the pool of each element was also calculated (Fig. 4.2B). Proteins and lipids accounted for 90% of total C and H content, whereas most of the N was accounted for by proteins and free amino acids (80 and 7.6%, respectively). Regarding the phosphorus pool, nucleic acids accounted for 42% of the total phosphorus, phospholipids accounted for 10.2%, ATP for 20.1% , while 27.7% of the phosphorus pool was unexplained. In a previous P fractionation study, Corner (1973) reported that 52.8% of the total P content of *Calanus sp.* was acid-soluble. The acid-soluble fraction contains primarily nucleotides and phosphates (Miyata & Hattori 1986). Therefore it is likely that most of the unexplained P fraction was in the form of nucleotides and phosphate. The nucleotide content was not measured by Mayzaud and Martin (1975) but the stoichiometric balance suggested that *C. finmarchicus* had either 1.7% of nucleotides or 0.88% of nucleotides and an additional 0.6% of phosphate. These results highlight the importance of the acid-soluble fraction within the P pool in copepods, which could be more important than previously suggested (Sterner & Elser 2002).

Linking biochemical with elemental composition

The composition of the main biochemical compounds in zooplankton is relatively variable. However, compositional min-max ranges (% dry weight) for most compounds are clearly bounded (Table 4.3). By combining these ranges with the average stoichiometric composition (% of total weight) of main compounds (Table 4.2), elemental compositions were generated using equation 1 above. A simple iteration procedure was used, first assigning the minimum amount of proteins to a theoretical organism, then adding the different amounts of the other biochemical compounds. Finally, lipid reserves were assigned by difference, since they are the most variable compound in zooplankton. The proportions of all compounds were altered in the simulation until all possible combinations had been covered within the measured limits of Table 4.3. Two different runs of the simulations were carried out, one with triacylglycerol as energy reserve and the second with wax esters. For facilitating the comparison with literature values, the ash content was not taken into account when generating the simulation dataset. This simulation approach was used because it gave a realistic maximum range of contribution of each biochemical compound to the elemental composition of zooplankton. It was assumed that, within the measured limits, all combinations of biochemical compounds were possible. Thus, the range of elemental compositions from the simulation may include combinations which are physiologically unrealistic. However, these data are adequate for testing whether changes in the proportions of the main biochemical compounds account for the variability in stoichiometric compositions found in the literature.

Table 4.3. Min-max range of measured compositions (% dry weight) of main biochemical compounds (protein, free amino acids, triacylglycerols, wax esters, phospholipids, glycogen, nucleotides, chitin and nucleic acids) of freshwater and marine zooplankton used for simulating the range of stoichiometric compositions (see text for details). Data from sources cited in Table 4.1 and from Båmstedt (1986) and Mauchline (1998).

% dry weight	Measured range	
	max	min
Protein	84	23
Free amino acids	9	1
Triacylglycerols	37.3	0.1
Wax esters	53.8	0.2
Phospholipids	22.4	0.7
Glycogen	5.4	0.4
Nucleotides	1.8	0.3
Chitin	10.1	2.1
Nucleic Acids	11.1	0.1

Results of the simulation showed that the maximum concentration of each element agreed very closely with those measured (Table 4.4). However, measured values had lower minimum concentrations than those found in simulations. This is due to the ash content, which in some marine species can reach 38% of total body weight (Båmstedt 1986). Ash content could increase the C content from the reported 28% to 47%, a value more similar to that calculated (48%). This is better illustrated when plotting the stoichiometric limits derived from the simulation (shaded area in Fig. 4.3) with a wide range of elemental measurements from marine and freshwater zooplankton (white circles). Virtually all measured points lie within the shaded area and most observations cluster in the lower range of the C:P and N:P axes. Some of these observations are means for a species and therefore part of the real variability is already removed. Results demonstrate that all the variation in elemental composition of freshwater and marine zooplankton can be explained by changes in the proportions of the main biochemical compounds.

Using data from simulations I also evaluated the relative contribution of the main biochemical compounds to elemental composition (C, N and P; Fig. 4.4). Almost all C content was accounted for by lipids and proteins (total lipid and total amino acid content explained between 82% and 98.2% of the total C content). Proteins were the major source of N (between 46% and 98% of the total N content). This explains why the C:N ratio is a very good predictor of both protein and lipid content (Fig. 4.5). Nucleic acids and free amino acids contributed to a similar extent to the remaining N pool. Free amino acids account for most of the non-protein N in some calanoid copepods (Cowey

& Corner 1963; Raymont et al. 1968), because of the relatively low proportion of nucleic acids in these marine calanoid copepods (Fig. 4.2B). In contrast, in freshwater cladocerans, with high proportions of nucleic acids, these may account for most of the non-protein N (Fig. 4.4). Nucleic acids were the compounds which contributed most to the total P pool on average, followed by phospholipids and, to a lesser extent, nucleotides. Despite the great importance of P for zooplankton growth, very little is known about the allocation of P and its changes in different species. In one study of phosphorus partitioning in cladocerans and copepods, Vrede et al. (1999) found that in *Daphnia galeata* the P pool not attributable to nucleic acids nor to phospholipids almost doubled when the species was grown with food of low quality and in low quantity. This change was explained by a decrease in the size of the nucleic acid P pool, due to a reduction in growth rate and reproduction under limiting food conditions. Growth, through the increase in the RNA:DNA ratio, under varying environmental conditions has been identified as an important cause for P variation in zooplankton (Saiz et al. 1998; Vrede et al. 2002; Vrede et al. 2004). However, it is not known to what extent is phosphorus variability explained by changes in the concentration of nucleic acids alone.

Table 4.4. Measured and simulated min-max ranges of stoichiometric composition (in % dry weight) from simulated combinations of biochemical compounds of the freshwater and marine zooplankton species reported in Table 4.2. DW is dry weight and n.m. stands for not measured. Measured values are from Båmstedt (1986) and Mauchline (1998) for the marine species, while for freshwater species values are from Elser et al. (2000a) and Chapter 5. See text for simulation details.

%DW	Measured range		Simulated range	
	max	min	max	min
C	69.7	28	72.9	48.5
H	10.3	3.2	10.3	6.5
O	n.m.	n.m.	28.8	11.3
N	16	5	15.3	4.1
P	2.1	0.5	2.2	0.1
S	1.8	0.3	1.1	0.3

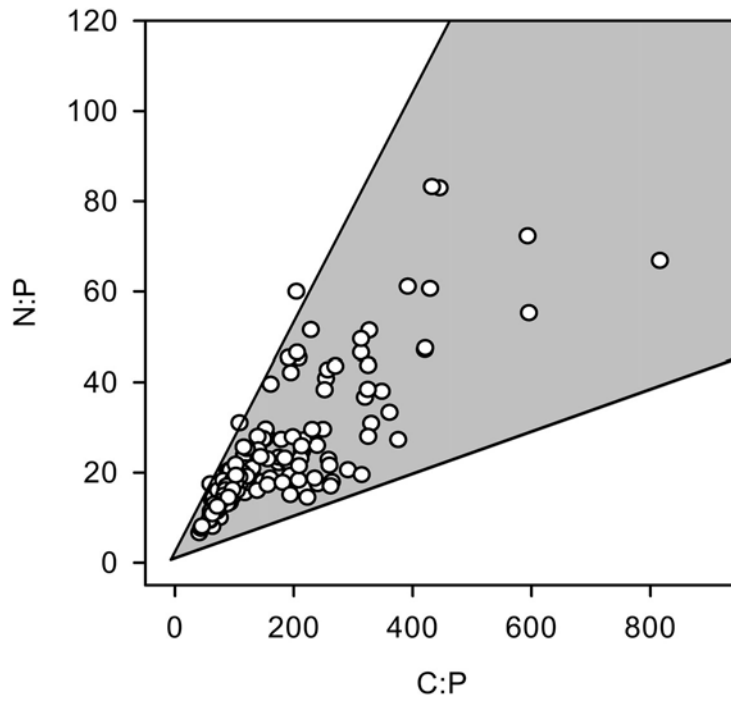


Fig. 4.3. Stoichiometric limits of zooplankton estimated from the measured biochemical composition ranges. White circles are measured stoichiometric data of different marine and freshwater zooplankton species from the same sources described in Table 4.4.

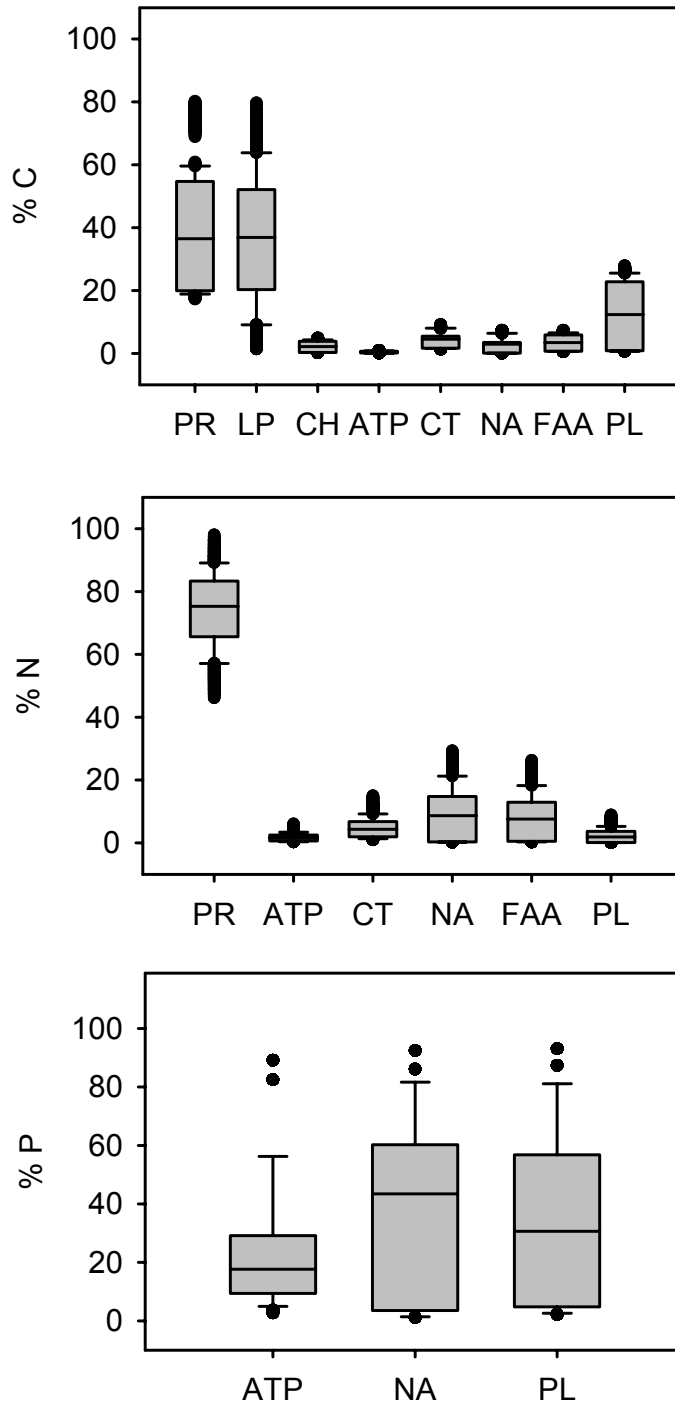


Fig. 4.4. Percentage contribution of each biochemical compound to the elemental composition of carbon, nitrogen and phosphorus. Box plots are the median, 10th, 25th, 75th and 90th percentiles. Data were obtained by generating all potential combinations of measured biochemical compound combinations. Therefore the relative contribution is proportional to the potential relevance of each biochemical compound to the composition of each element.

Several applications could be derived from the use of the results reported in this study. The elemental composition of the main biochemical compounds in zooplankton could help find links between elemental and biochemical composition. In studies measuring the biochemical composition, the simultaneous measurement of elemental compositions could be a good way for identifying the magnitude of the unmeasured fraction of body composition. In addition, in stoichiometric studies, the simultaneous measurement of biochemical compound concentrations and elemental compositions would provide complementary information regarding the functionality of the elemental compartments.

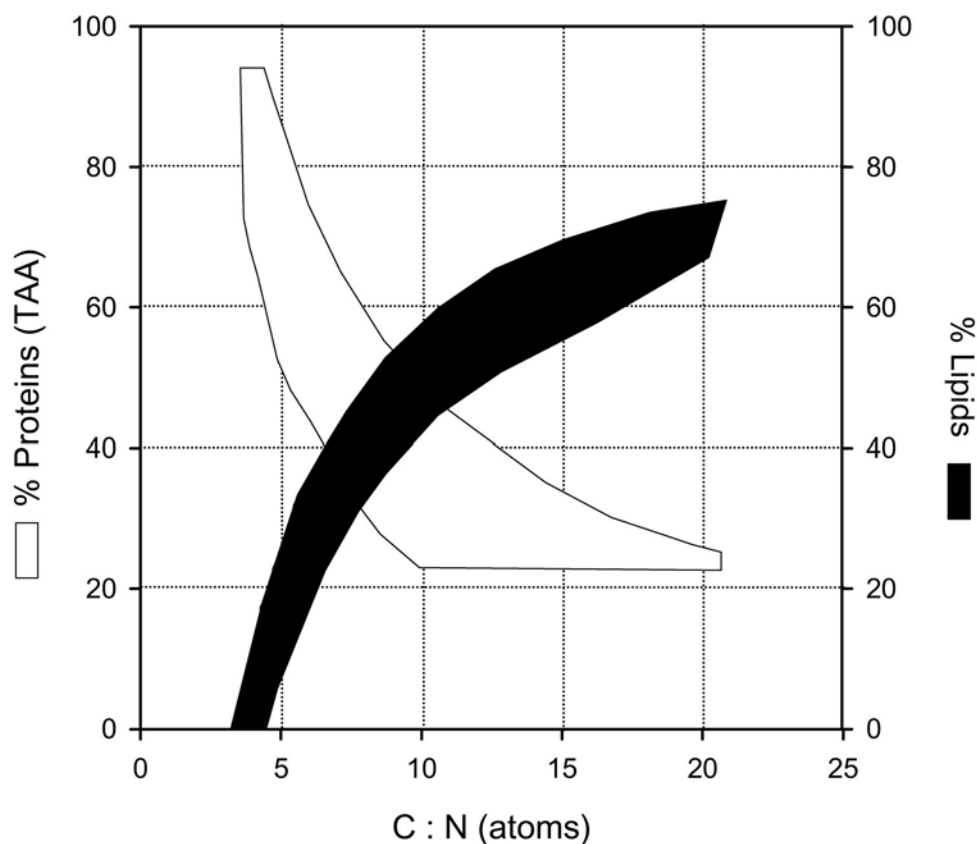


Fig. 4.5. Relationship between percentage of total lipids or the total amino acid pool (free amino acids and protein) and the C : N ratio. Percentages are calculated in relation to dry weight. Data were obtained by simulating all potential combinations of measured biochemical compound combinations.

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5. Reproduction as the main cause of non-strict homeostasis in the elemental composition of zooplankton

Abstract

The carbon, hydrogen, nitrogen and phosphorus elemental composition (C, H, N and P) of the planktonic crustacean assemblage of an alpine lake was measured for a whole seasonal cycle, in order to determine the contribution of growth and reproduction to the variability in the elemental composition of zooplankton. The species studied included a cladoceran *Daphnia pulex*, a cyclopoid copepod *Cyclops abyssorum* and a calanoid copepod *Diaptomus cyaneus*. In the three species, reproduction was the main cause of elemental variability, whereas growth had little influence. Females of the three species lost from 32 to 48% of their initial individual C and H content during reproduction, which corresponded to a similar decrease in their lipid and carbohydrate content. N content did not change in any of the three species; nor did protein and chitin content. *Daphnia* and *Diaptomus* lost 35% and 56%, respectively, of their initial P content during reproduction, while *Cyclops*' P content did not change. The three species stored energy compounds under unfavourable conditions (*Cyclops* and *Daphnia*) or during growth (*Diaptomus*) for later use in offspring production, but only *Diaptomus* and *Daphnia* mobilised stored P. Males of *Cyclops* and *Daphnia* lived a much shorter time than females and had a more constant elemental composition. *Diaptomus* males showed the same life span and elemental changes as females. Differences in males' elemental variability between the three species can be interpreted as due to greater mating requirements in calanoids than in cyclopoid copepods and cladocerans. Variability in the elemental composition of these species is thought to be general of food-independent reproduction species, which have a non-homeostatic elemental composition throughout their life-cycle.

Introduction

Trying to understand the influence of the ecological context on the evolution of traits of species and, *vice versa*, how certain species traits affect ecological dynamics are fundamental challenges of modern ecology (Partridge & Harvey 1988). One difficulty that arises when species' life histories are compared is how to select comparable species traits. In this sense, ecological stoichiometry (Sternler & Elser 2002) overcomes such difficulty by comparing the elemental main body constituents (Elser et al. 1996), usually focusing on C, N and P. This approach has been used for identifying living constraints in organisms from freshwater, marine and terrestrial environments (Elser & Hassett 1994; Elser et al. 2000e). Of animal groups, crustacean zooplankton are studied most in this sense and have shown the relevance of stoichiometry in the ecological con-

text. For instance, the homeostatic nature of the stoichiometric composition of crustacean zooplankton (e.g. N:P ratio) has a significant influence on the recycling ratios between distinct elements and, in consequence, on the entire dynamics of plankton (Urabe 1995; Sterner 1990). The assumption of elemental homeostasis in zooplankton was based on pioneering experimental (Hessen 1990) and field studies (Hessen & Lyche 1991; Andersen & Hessen 1991), which showed the rather constant elemental composition of the species. More recently, however, other studies clarified that some zooplankton species have more relevant intra-specific elemental variation (Main et al. 1997; Villar-Argaiz et al. 2002). For these species, the direct application of nutrient recycling models (Olsen et al. 1986) based on the assumption of high stoichiometric homeostasis is inaccurate. Therefore, we need to understand better why stoichiometric homeostasis is more (or less) strict in distinct species. In this sense, differences in RNA content are related to different growth rates (Main et al. 1997) and are responsible for part of the N:P variation. However, other processes such as reproduction strategies have surprisingly not yet been explored as possible causes of intra-specific stoichiometric variation. Two main reproductive strategies have been distinguished within zooplankton organisms (Gilbert & Williamson 1983). On the one hand, some species store the energy required for reproduction during their growth and use it at a certain point of their life-cycle with little complement of feeding resources during reproduction time. Therefore, there is a temporal decoupling between feeding and reproduction. The strategy is called the food-independent reproduction mode. On the other hand, there are species for which egg production is totally dependent on simultaneous food supply, a reproductive strategy that is known as the food-dependent reproduction mode. It has been suggested that food-independent reproduction is an adaptation to cases in which food availability varies greatly from season to season, such as the conditions encountered by marine calanoid copepods in the boreal and polar seas (Hirche & Kosobokova 2003; Hirche & Kattner 1993). The food-dependent reproduction mode is related to species completing their life-cycle within a time period in which food is abundant and fluctuations are scarce. This is the case of cladocerans inhabiting productive systems or completing their life-cycle within the productive period of a system (Tessier et al. 1983).

The physiological mechanism used for species with food-independent reproduction mode is related to the storage of high quantities of lipid substances, which are later used for reproduction (Vanderploeg et al. 1992a; Hagen & Schnack-Schiel 1996). Therefore, because reserve substances are formed ultimately of a few elemental compounds, we expect organisms with food-independent reproduction mode to experience relevant changes in elemental composition throughout their life-cycle. Although some studies have indicated changes in the C:N ratio during over-wintering (Tande 1982), detailed studies of elemental composition variability due to reproduction modes are lacking.

In this study we compared the elemental and biochemical changes of three crustacean species of zooplankton in an alpine lake throughout their life-cycles, by examining the influence of growth and reproduction on the stoichiometric composition of each species. The species are representative of the main freshwater crustacean groups with contrasting reproductive modes: the cladoceran *Daphnia pulex* Forbes, the cyclopoid copepod *Cyclops abyssorum* Sars, and the calanoid copepod *Diaptomus cyaneus* Gurney. *Cyclops* takes an entire year to complete its life-cycle, whereas *Diaptomus* completes it in a short period during the ice-free season (Chapter 3). *Daphnia* is also present throughout the year and has a single cohort, feature also observed in other

alpine lakes (Gliwicz et al. 2001), unlike lowland lakes in which *Daphnia* usually has several cohorts throughout the year. The three contrasting living and reproductive modes provide an excellent opportunity for studying the sources of stoichiometric variability in the field. To understand further the mechanisms behind the possible elemental changes, the seasonal variation in the major biochemical compounds (proteins, lipids, carbohydrates and chitin) was also assessed in adult individuals of the three species.

Materials and Methods

Sampling

The lake was surveyed on 14 occasions from December 1998, just after the lake became ice-covered, until December 1999, when it was ice-covered again. Samples were collected at the deepest part of the lake, either by drilling the ice-cover or from a platform anchored throughout the summer at the same point. Zooplankton samples were collected by vertical net hauls from 65 m to the surface with a 200 μm net. Two replicate samples were collected; one for individual counts, which was immediately preserved in formaldehyde, and one for elemental and biochemical analyses. Individuals from the latter sample were kept alive and transported cold (4°C) until they were frozen (-20°C) in the laboratory a few hours after collection.

Population evaluation

Individuals were counted by an Olympus inverted microscope. At least 100 individuals of *Daphnia* and 50 adults of the two copepod species were measured per sample for body length (from the upper edge of head to the base of tail spine in *Daphnia* and from the anterior end of the cephalothorax to the posterior end of the furca in the copepods). Copepods were classified at each stage. *Daphnia* females were divided into juveniles and adult females by using the criteria proposed by (Edmondson & Litt 1982), which is based on the relative length of the abdominal process.

Elemental analysis

After the zooplankton samples were thawed, from 10 to several hundred individuals of each species or stage were quickly sorted under a dissecting microscope and placed in a pre-weighed tin (for C, N, H and S analyses) or Teflon capsules (for P, protein, chitin, carbohydrate and lipid analyses). At least three combined sample replicates were analysed for each species and stage from each sampling occasion, with the exception of a few cases with not enough material available. Individuals were kept cold (< 4°C) during the sorting process, which was in all samples always within a few hours after thawing. Eggs from ovigerous females were carefully removed before analysis. Dry weight (DW) was determined for all samples after drying at 60°C for 24 h and weighed on a

microbalance (Ohaus Analytical Plus, AP250D-0). Between 300 – 500 µg of DW was required for C, N, H and P analyses, whereas for the biochemical analysis we used from 600 – 800 µg DW; and for S and ash content analyses, 3 – 5 mg DW. For all the analyses, blank capsules were used during the whole sorting and drying process. The ash content was determined after combustion at 450°C for 5 h. Elemental C, N, H and S were analysed on EA 1108 CHNS-O Carlo Erba Instruments after the samples were dried and packed into tin capsules with vanadium pentoxide as catalyser to ensure complete combustion. Atropine was used as an internal standard. Elemental P was determined by acid-persulphate digestion (Grasshoff 1983), followed by phosphate analysis using the malachite green method (Camarero 1994).

Biochemical analyses

Protein content was measured as the total amino acid composition. This method was chosen in preference over more traditional total protein methods (Zamer et al. 1989), since N-acetylglucosamine, the molecular constituent of chitin, is an amino-sugar (Strayer 1988), which after hydrolysis appears in the aminogram as glucosamine. Therefore, total amino acids and chitin could be analysed simultaneously. Samples for total amino acid analysis were vacuum-sealed and hydrolysed with HCl 6 N at 116°C for 24 h. An internal norleucine standard was included in every sample prior to hydrolysis in order to maximise reproducibility. The analysis was carried out on a Biochrom20 (Amersham-Pharmacia) ion-exchange amino acid auto analyser, following the ninhydrin method of Spackman et al. (1958). A standard solution of 20 amino acids and glucosamine was run for every 10 samples. Tryptophan was not considered in this study because it degrades under acid hydrolysis. Total carbohydrates were analysed following the phenol-sulphuric acid method, as described by Meyer & Walther (1988). Total lipids were quantified gravimetrically. Weighed dry samples were placed into a dichloromethane : methanol (2:1, v/v) solution (Folch et al. 1957) and sonicated for 30 minutes in an ultrasonic bath. Then the non-lipid constituents were collected in pre-weighed GF/F Watman filters, which were dried and re-weighed.

Results

Life-cycles

Cyclops abyssorum

At the beginning of the study in December, it was adults of the species that were found in the lake (Fig. 5.1). Initially, males and females were present in similar proportions, but males decreased suddenly in March and were not found from mid-April onwards. Females started to reproduce below the ice-cover, at the beginning of the decline of the

males in March. However, the greatest number of reproductive females was found when the ice-cover melted. On the sampling date previous to the ice-cover melting, although the proportion of females with eggs was low, almost all females had their ovaries already ripened. Nauplii first appeared after the ice-cover melted and after the over-wintering females started laying their eggs. Copepodites of the different instars peaked almost at each consecutive month: the first two stages were dominant at the end of July; the third stage in August; the fourth in September; and the fifth was dominant in October and November. Males appeared again in October but it was not until December that they, together with fifth instar female copepodites, again dominated cyclopoid abundance.

Diaptomus cyaneus

Nauplii of this copepod appeared immediately after the ice melted at the end of June. No stages were found during the ice-covered period, indicating that nauplii emerged from over-wintering eggs. Development time of copepodites was very short: while at the end of June the population was mainly composed of nauplii and a few first-stage copepodites, by the end of July most of the diaptomids were adults and copepodites of the fifth instar (Fig. 5.1). Adults were divided evenly between males and females during the two months they were present. Females had their ovaries already ripened in the sample of July, although no ovigerous females were found until the end of August, when almost all females were carrying eggs or had many spermatophores attached, indicating that they had been reproducing intensively. From September onwards, no more diaptomids were found in the surveys; thus, this species completed its life-cycle in 3 months.

Daphnia pulicaria

Males appeared from December 1998 until March 1999 below the ice cover, and again from October 1999 until the end of the study in December 1999, always in very low numbers (Fig. 5.1). Females produced offspring during most of the year, but reproducing females formed a majority of the population only in December 1998 and from June to August 1999. Population size categories (Fig. 5.2) showed that the reproductive pulse of December 1998 was less relevant than the one during the ice-free period, and that many females survived the ice-covered period and laid eggs during the following summer. After reproducing, most of them died: almost none of the old females survived after August 1999 (Fig. 5.2). During the ice-free period, females from the over-wintering cohort were easily distinguished from the 1999 newborn females, since the former had a transparent caparace and few or no lipid droplets, while the young females had melanin in the dorsal part of the caparace and lipid droplets accumulating in their body. The newborn females of summer 1999 rarely reproduced during the autumn, but waited till the following summer to lay their eggs (Fig. 5.1).

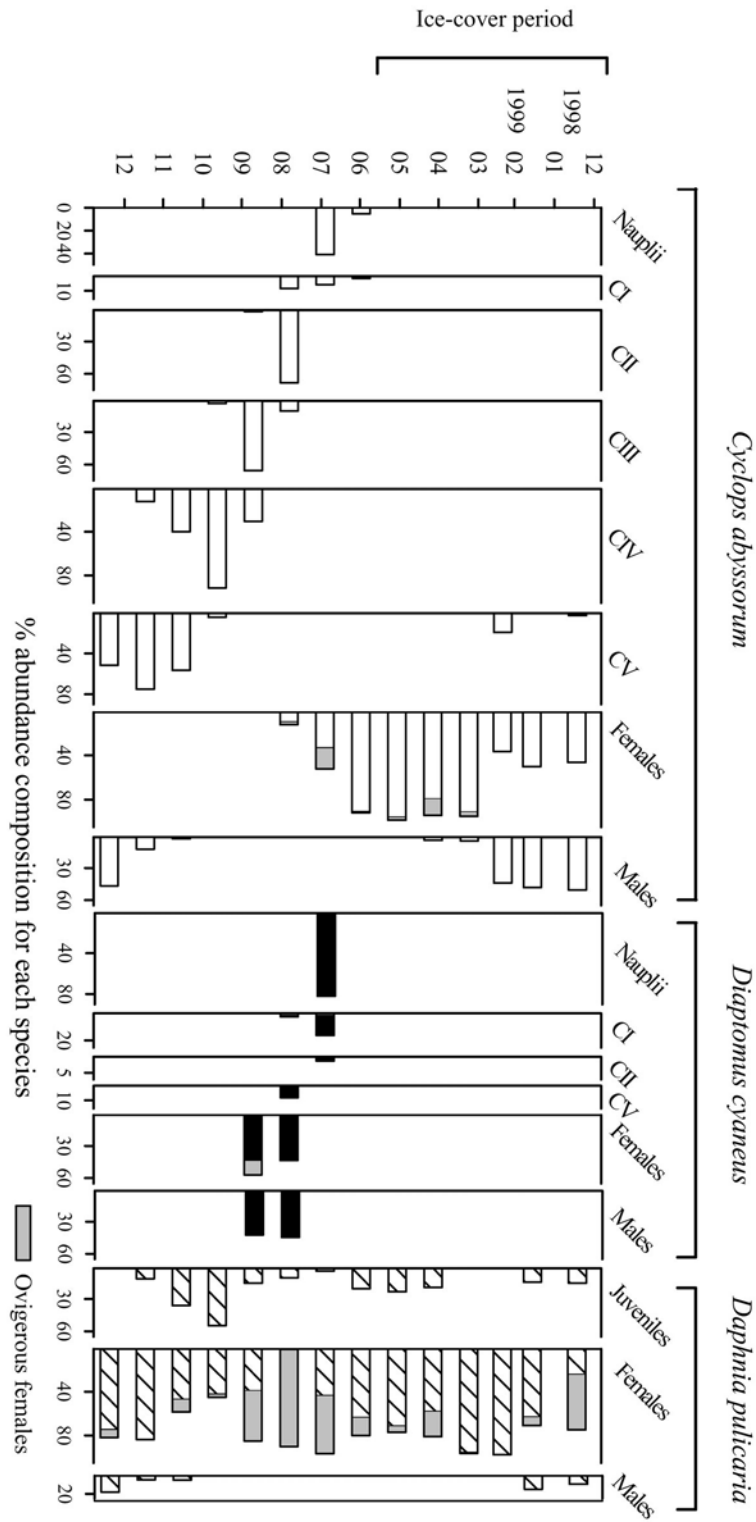


Fig. 5.1. Seasonal changes in the pelagic macro-zooplankton species in Lake Redon. Units are in percentage composition for each species

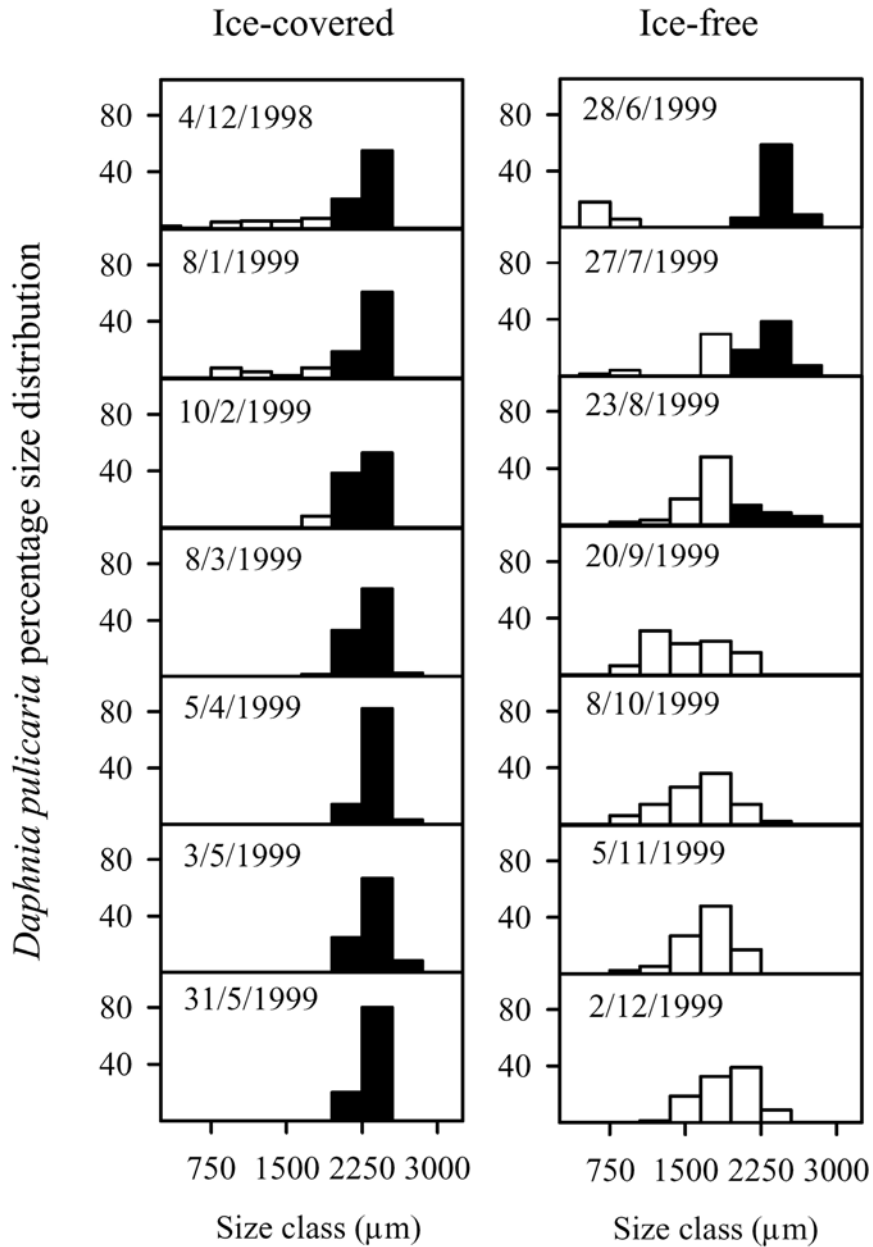


Fig. 5.2. Body size distribution in the *Daphnia pulicaria* population of Lake Redon from 14 December 1998 to 12 December 1999. Black bars correspond to over-wintering females and white bars to juveniles and females born during summer 1999.

Elemental and biochemical composition

The three species had similar C and H content, as expressed in percentage of DW (Table 5.1), with no significant differences between the three species (Table 5.2). The two copepods had significantly higher N content ($p < 0.001$) than the cladoceran, whereas within the copepods the calanoid was significantly richer than the cyclopoid ($p < 0.001$). P content was similar in the two copepods (Table 5.2), but they both had almost half the P of the cladoceran in terms of percentage of DW ($p < 0.001$). We had to use a great many individuals per S analysis because of its low concentration in animals, and, in consequence, the number of samples analysed was lower. *Diaptomus* had a higher S percentage than the other two species, although it was only significantly different from *Daphnia* (Table 5.2). In summary, the two copepods had greater similarity in their atomic ratios C:N:P:S to each other than to the cladoceran (*Cyclops* 330:31:1:0.6; *Diaptomus* 313:26:1:0.9, *Daphnia* 214:47:1:0.4).

The ash content of the three species was very low (Table 5.1). *Diaptomus* had the highest value, whereas *Cyclops* and *Daphnia* had a lower and similar content. The lipid fraction and proteins accounted for most of the biomass of the three species, whereas chitin and total carbohydrates were below 5.5%. The calanoid copepod had the highest protein and chitin content, followed by *Cyclops* and *Daphnia*, with all differences between them statistically significant (Table 5.2). However, *Diaptomus* had a significantly lower lipid content than the other two species, in which lipid substances accounted for, on average, over 50% of their DW (Table 5.1). Total carbohydrates followed an inverse pattern to proteins and chitin, with *Daphnia* having the highest content, followed by *Cyclops* and *Diaptomus*.

Intra-specific variability in the elemental composition

Variation in the elemental composition was low during ontogenic development and restricted to the last stage (i.e. adults) of the three species (Fig. 5.3). There was a significant linear relationship between individual content of each of the considered elements and the individual's DW for all the species, although the proportion of variance explained by the relationship was different for each element. For C and H the variance explained was high, between 97 and 99% (Fig. 5.3), whereas for P and N the variance explained by the regression was lower, between 63 and 89%, mainly because of greater variability in the females of *Cyclops* and *Daphnia*. Excluding females in the linear regression of *Cyclops* and *Daphnia* (Fig. 5.3, dashed lines), the variance explained increased from 2.6 to 21.4% depending on species and element, with P still the element with the highest variation. Despite the lower degrees of freedom for the case of *Diaptomus*, the results for this species are consistent with results for the other two species, with the adult stage showing the greatest variation in elemental composition.

Table 5.1. Average elemental and biochemical composition of the three species of zooplankton from Lake Redon. Data are expressed as mean values \pm standard error of the percentages; in brackets, the number of composite samples analysed. Standard errors for elemental ratios include seasonal and stage variability, and elemental and biochemical standard errors include replicate variability. Percentages are calculated in relation to DW, elemental ratios are in atoms. nc, not calculated because only 1 replicate available.

	<i>Diaptomus cyaneus</i>		<i>Cyclops abyssorum</i>		<i>Daphnia pulex</i>	
Ash (%)	3.8 \pm NC	(1)	2.4 \pm 0.1	(4)	2.5 \pm 0.2	(4)
N (%)	9.9 \pm 0.5	(16)	8.3 \pm 0.2	(42)	6.4 \pm 0.2	(55)
C (%)	56.9 \pm 1.2	(16)	58.8 \pm 1.0	(42)	58.7 \pm 0.9	(54)
H (%)	7.8 \pm 0.1	(16)	7.9 \pm 0.1	(42)	8.1 \pm 0.1	(45)
P (%)	0.47 \pm 0.04	(19)	0.46 \pm 0.03	(48)	0.71 \pm 0.02	(52)
S (%)	0.47 \pm 0.06	(4)	0.30 \pm 0.05	(8)	0.26 \pm 0.04	(14)
C:N	7.0 \pm 0.6	(7)	8.4 \pm 0.5	(18)	11.2 \pm 0.7	(19)
C:P	345.6 \pm 34.1	(7)	389.8 \pm 43.3	(18)	238.4 \pm 15.8	(18)
N:P	52.7 \pm 8.4	(7)	47.8 \pm 4.9	(18)	23.1 \pm 2.2	(19)
C:S	421.0 \pm 95.9	(4)	536.2 \pm 105.1	(3)	639.2 \pm 77.3	(8)
N:S	53.4 \pm 10	(4)	56.7 \pm 6.2	(3)	51.3 \pm 3.6	(8)
P:S	1.5 \pm 0.6	(4)	1.0 \pm 0.2	(3)	2.7 \pm 0.2	(8)
Protein (%)	58.7 \pm 4.8	(8)	43.6 \pm 1.6	(22)	27.0 \pm 1.1	(17)
Chitin (%)	5.4 \pm 0.4	(8)	3.9 \pm 0.2	(22)	2.2 \pm 0.1	(17)
Lipid (%)	35.5 \pm 5.5	(9)	51.6 \pm 2.9	(26)	53.5 \pm 2.6	(26)
Carbohydrate (%)	2.5 \pm 0.1	(12)	3.4 \pm 0.2	(36)	4.4 \pm 0.1	(23)

Table 5.2. One way ANOVAs examining inter-species differences of *Diaptomus cyaneus* (*D.c.*), *Cyclops abyssorum* (*C.a.*) and *Daphnia pulex* (*D.p.*) in terms of their elemental (C, H, N, P, S) and biochemical (lipids, protein, carbohydrate and chitin) composition. Data were previously standardised with either logarithmic or square root transformation when it was required, and a test of homogeneity of variances was carried out before ANOVA. df stands for degrees of freedom, MS for mean squares, F for the result of the F-test and *p* the probability of significance. Tukey hsd test was used to test the difference between copepod developmental phases. *Post hoc* comparison: < or > significantly higher or lower at $p < 0.05$ respectively; << or >>, significantly higher or lower at $p < 0.001$; =, not significantly different.

	Source	df	MS	F	p	Post hoc comparison
C (%)	Inter-species	2	25.9	0.7	0.511	
	Intra-species	100	38.3			
	Total	102				
H (%)	Inter-species	2	1.1	2.5	0.086	
	Intra-species	91	0.4			
	Total	93				
N (%)	Inter-species	2	0.3	34.0	0.000	D.c. >> C.a. >> D.p.
	Intra-species	101	0.0			
	Total	103				
P (%)	Inter-species	2	0.5	33.1	0.000	D.c. = C.a. << D.p.
	Intra-species	116	0.0			
	Total	118				
S (%)	Inter-species	2	0.1	3.7	0.039	D.c. = C.a. = D.p. > D.c.
	Intra-species	26	0.0			
	Total	28				
Lipid (%)	Inter-species	2	8.6	7.0	0.002	D.c. << C.a. = D.p.
	Intra-species	59	1.2			
	Total	61				
Carbohydrate (%)	Inter-species	2	0.3	21.3	0.000	D.c. < C.a. << D.p.
	Intra-species	64	0.0			
	Total	66				
Protein (%)	Inter-species	2	0.4	60.5	0.000	D.c. >> C.a. >> D.p.
	IntraSpecies	44	0.0			
	Total	46				
Chitin (%)	Inter-species	2	0.5	68.5	0.000	D.c. >> C.a. >> D.p.
	Intra-species	43	0.0			
	Total	45				

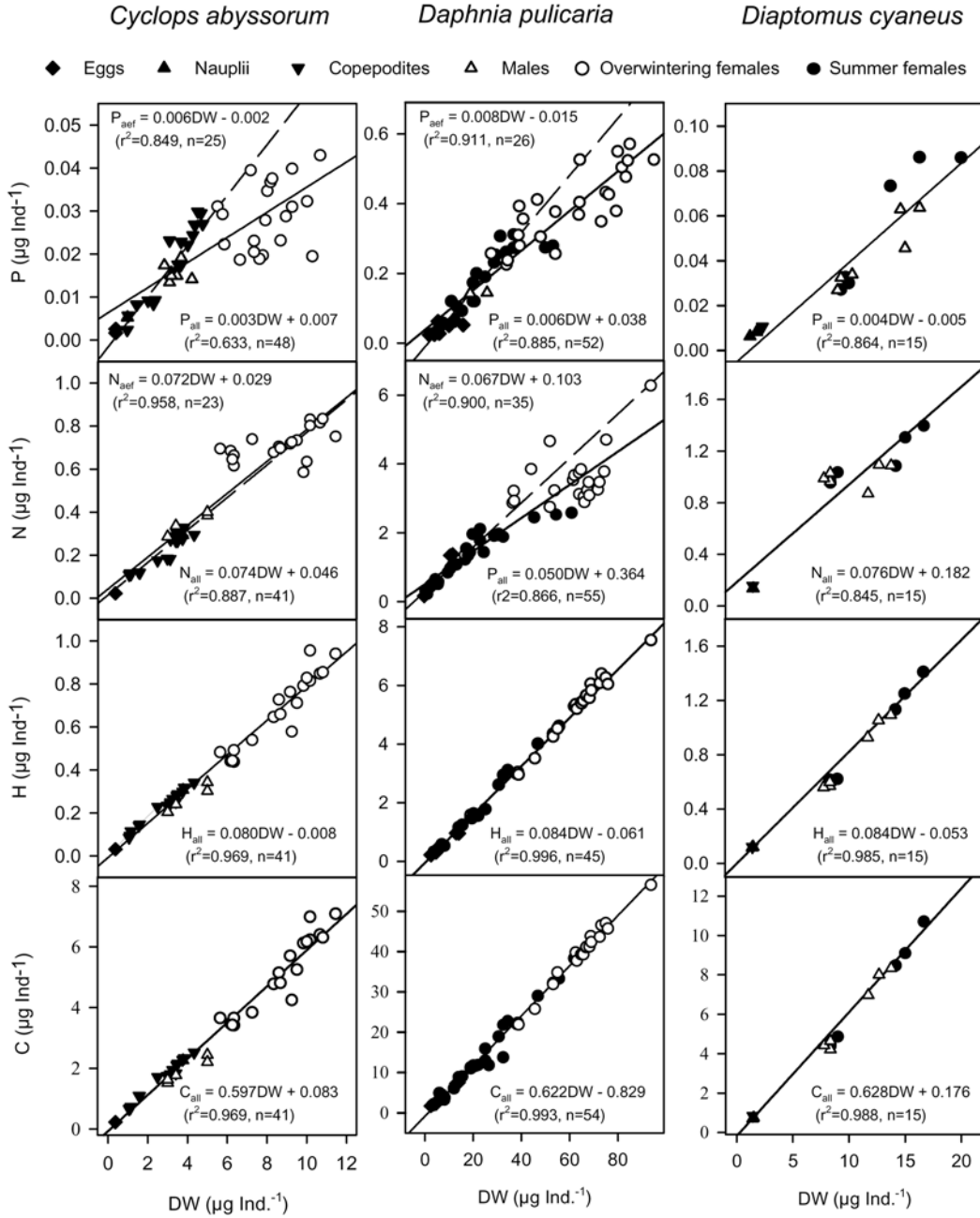


Fig. 5.3. Relationship between dry weight and elemental composition for the three crustacean zooplankton species of Lake Redon. Solid lines are regression fits including all stages (all) and dashed lines are the fits excluding females (aef).

Elemental and biochemical composition of reproducing versus non-reproducing individuals

The changes in DW of over-wintering females of both *Cyclops* and *Daphnia* followed a similar pattern: during most of the winter period the weight was constant, starting to decrease in March for *Cyclops* and April for *Daphnia*, tendency that lasted until the females disappeared (Fig. 5.4). During this time, *Cyclops* and *Daphnia* lost 34 and 52%, respectively, of their DW. The decrease in DW was mostly due to a decrease in H and C, and coincided with the reproductive period of the two species. There were significant differences between females in the reproducing period and those in the rest of the year (Fig. 5.5). The absolute content of N was rather constant during the complete seasonal cycle for the two species. However, the two species showed differences in relation to P. While *Cyclops* also had a constant content throughout its life-cycle, for *Daphnia* the P content increased until March and then steadily decreased until the end of August, losing up to 58% of the March content (Fig. 5.5). *Daphnia* had significantly lower P during the reproduction period than before reproduction. Of the females of *Daphnia* born during the summer, all the main elements changed their proportions between August and December 1999. During the last two months, however, there was a higher increase of C and H due to a relatively higher accumulation of lipids (Fig. 5.4).

There were also substantial differences in the composition of both females and males before and after reproduction in *Diaptomus* (Table 5.3). Males and females decreased their DW by 35%, which was due to a decrease in C, H and P (Fig. 5.5), but N content and total length did not change significantly from one month to the other.

Because the major elemental changes of the adults of the three species coincided with the onset of reproduction, we compared the main biochemical compounds between before and after reproduction for *Cyclops* and *Daphnia* females and *Diaptomus* adults (Fig. 5.6). As expected, differences in adult elemental composition coincided with changes in biochemical composition. The females of the three species and the males of *Diaptomus* reduced significantly their individual lipid and carbohydrate contents (U-tests, *Cyclops* $p = 0.013$, *Daphnia* $p = 0.003$ and *Diaptomus* $p = 0.014$ for lipids, and $p = 0.009$, $p = 0.003$ and $p = 0.025$, respectively, for carbohydrates). This decrease meant a reduction of 58.0, 62.7 and 74.5%, respectively, for lipids, whereas total carbohydrate loss was 55.6, 55.0 and 36.8%. Unlike these two major compounds involved in energy storage, the two main structural compounds, proteins and chitin, did not significantly change per individual in any of the three species.

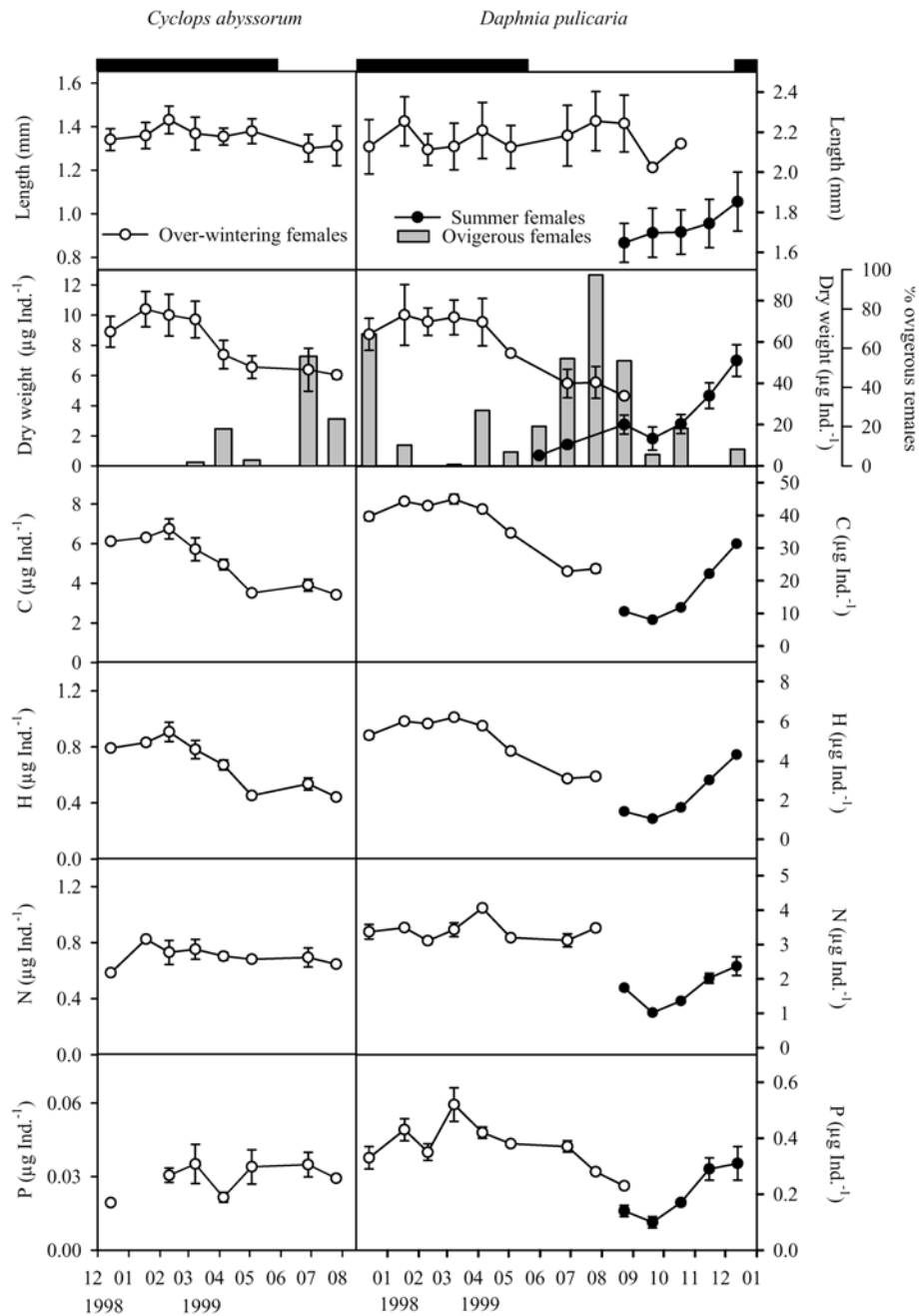


Fig. 5.4. *Cyclops abyssorum* and *Daphnia pulicaria* female seasonal changes in size, dry weight, ovigerous percentage and stoichiometric composition. The filled bar in black shows the ice-covered period. Circles are averages and error bars show the standard deviation.

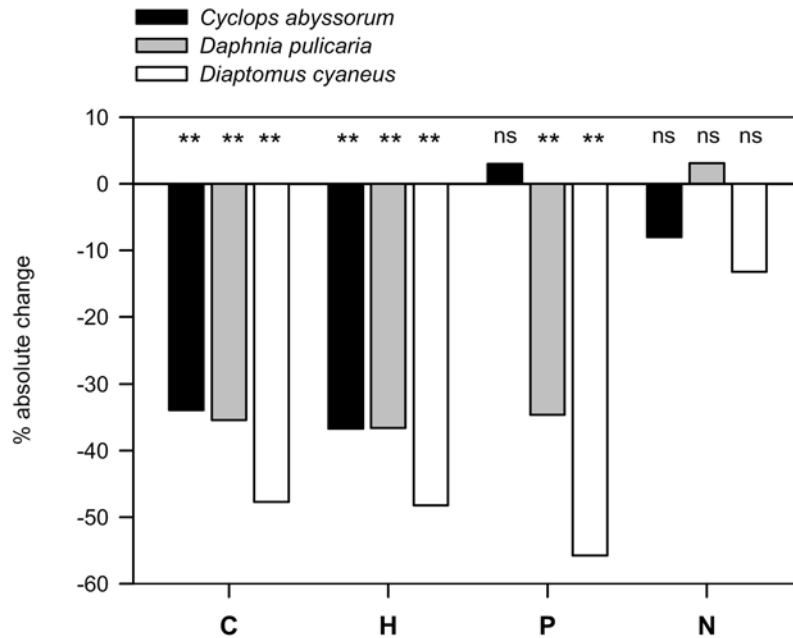


Fig. 5.5. Elemental content change before and after reproduction for the three species of crustacean zooplankton in Lake Redon. ** indicates significant differences between before and during/after reproduction at $p < 0.005$, according to a U-test (Legendre & Legendre 1998), ns, non significant.

Table 5.3. Changes in male and female *Diaptomus cyaneus* during the two months they were present in the lake. Values represent the average of three composite sample replicates \pm standard error.

	<i>Diaptomus cyaneus</i> females		<i>Diaptomus cyaneus</i> males	
	7/26/1999	8/23/1999	7/26/1999	8/23/1999
% Reproductive females	0	23		
Maximum length (mm)	1.837 ± 0.015	1.834 ± 0.015	1.689 ± 0.010	1.665 ± 0.008
Dry weight	15.7 ± 1.8	10.2 ± 1.4	13.3 ± 1.8	8.7 ± 0.8
P ($\mu\text{g Ind.}^{-1}$)	0.078 ± 0.009	0.032 ± 0.003	0.050 ± 0.009	0.028 ± 0.002
N ($\mu\text{g Ind.}^{-1}$)	1.30 ± 0.08	1.18 ± 0.02	1.07 ± 0.08	1.07 ± 0.05
C ($\mu\text{g Ind.}^{-1}$)	9.68 ± 0.36	5.45 ± 0.09	8.14 ± 0.25	4.74 ± 0.29
H ($\mu\text{g Ind.}^{-1}$)	1.30 ± 0.04	0.73 ± 0.03	1.07 ± 0.03	0.62 ± 0.02

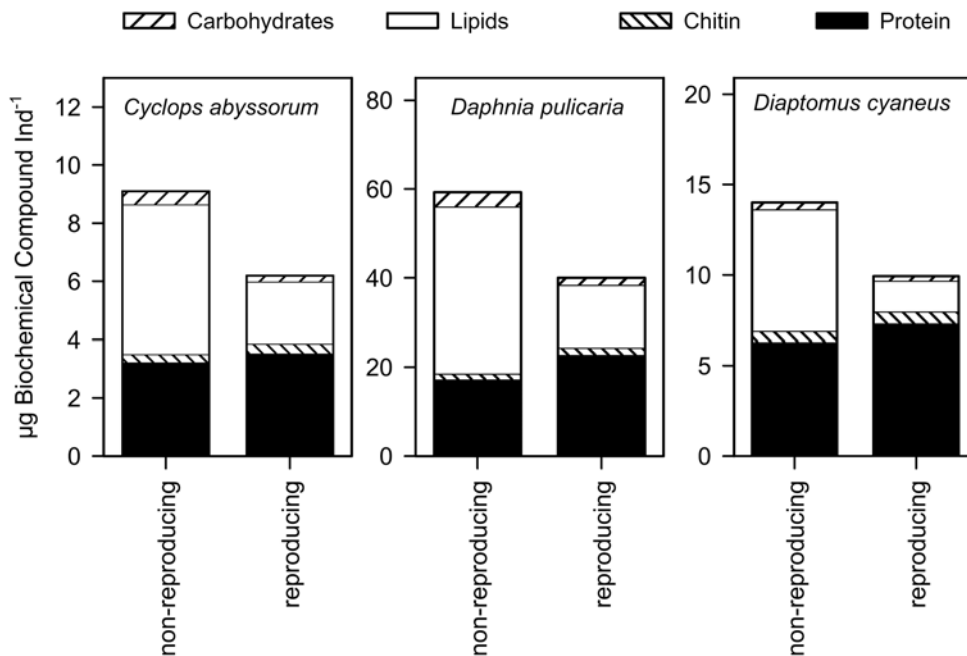


Fig. 5.6. Biochemical changes due to reproduction for adult females of the three crustacean zooplankton species of Lake Redon.

Discussion

The elemental and biochemical composition of the three crustacean species described in this study are generally consistent with the compositions found by other authors researching crustacean species (Båmstedt 1986; Hessen & Lyche 1991; Andersen & Hessen 1991; Elser et al. 2000d). C was among the highest reported values, similar to characteristic values of marine copepods living in cold-oligotrophic areas (Mayzaud & Martin 1975; Salonen et al. 1976). The high lipid content of the three species is the reason for such high C content, because lipids are C-rich compounds (e.g. 77% of triacylglycerol weight is C). H was within the range described for marine copepods (Omori 1969) and freshwater cladocerans (Badouin & Ravera 1972). The P content found in *Daphnia* was consistent with the value reported by DeMott (0.81% in weight; DeMott 1998), which confirms that, within the genus *Daphnia*, it is the species with the lowest P content. This feature may facilitate the survival of *Daphnia* in highly oligotrophic conditions (DeMott 1998), typical of large alpine lakes such as Lake Redon.

The differences in elemental composition between the three species were mainly due to their N and P content. The two copepods had, on average, half the P of the cladoceran, while they had between 2 and 4% higher N. To check the generality of these differences, we compared our results with those for 21 cladoceran species and 11 copepod species reported by (Elser et al. 2000c). We found that copepods have significantly

higher N and lower P content than cladocerans. N is $9.9\% \pm 0.4$ and $8.6\% \pm 0.2$ for copepods and cladocerans, respectively; and $0.8\% \pm 0.1$ and $1.2\% \pm 0.1$ for P (U-test, $p = 0.006$, for the two elements). Therefore, the apparently small difference in N between copepods and cladocerans (1.3%) appears to be constitutional, as is the case of P (Hessen & Lyche 1991; Elser et al. 2000b).

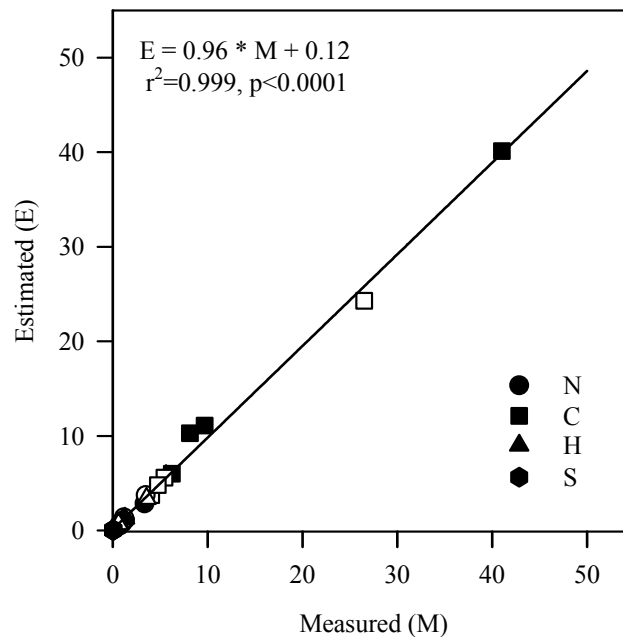


Fig. 5.7. Measured *versus* biochemically calculated elemental composition Chapter 4 for reproducing (empty symbols) and non-reproducing (filled symbols) individuals of the three species of crustacean zooplankton in Lake Redon (*Cyclops abyssorum*, *Daphnia pulex* and *Diaptomus cyaneus*).

Organisms have almost all their elements in the form of molecules, which can be grouped into a few broad types (Strayer 1988). Amongst them, the four biochemical groups measured in this study are known to be the most abundant in zooplankton (Elser et al. 1996). These can be roughly separated into structural (protein and chitin) and energy compounds (lipids and carbohydrates) (Elser et al. 1996). Proteins are mainly found in the muscular tissue (Sterner & Elser 2002); chitin in the exoskeleton (Cauchie 2002); and lipids (mainly triacylglycerol) in energy reserves (Goulden & Henry 1984), although some lipids such as phospholipids are more structural; and carbohydrates are involved in energy resources for immediate use rather than storage (Elendt 1989). These four biochemical compounds have an elemental composition that consists mainly of C, H, oxygen and N (Sterner & Elser 2002), and their average elemental composition

is very constant in zooplankton (Chapter 4). The elemental composition measured and the elemental composition calculated from the measured biochemical composition according to Chapter 4 are compared in Fig. 5.7. The good fit indicates the consistency between the biochemical and elemental composition, as should be expected. Given that the slope of the relationship is close to unity, it can reasonably be assumed that almost all the variability of C, H, N and S is explained by the variability of the four main biochemical groups. In particular, in our case, we showed that the higher N content of copepods was mainly due to a higher protein content. Copepods' active feeding gives them greater swimming capacity than cladocerans (Fryer 1991; Fryer 1957), and this higher protein content is very likely to be related to a higher proportion of muscular tissue in their bodies.

The three species studied had contrasting reproduction strategies, although they were all iteroparous annual species (Hairston & Bohonak 1998). The production of a single cohort was a common feature, while they differed in their diapausing strategy. *Daphnia* showed both diapausing and non-diapausing strategies. Coinciding with the end of the autumn overturn and the start of the ice-cover, males appeared on both occasions, with the subsequent production of ephippia. However, most females survived below the ice-cover, waiting to reproduce until the ice-free period. Very similar behaviour has been described for the same species in an alpine lake in the Tatras (Gliwicz et al. 2001), with the lack of male appearance in the Tatras the only life-cycle difference between the two populations. *Cyclops* also survived below the ice-cover as adults, probably synchronising its life-cycle with the *Daphnia*, as the former prey on the latter (Gliwicz et al. 2001). However, unlike in other northern European lakes (Nilssen & Elgmork 1977), the *Cyclops* population of Lake Redon up to now have not been observed to over-winter as copepodites in the sediments. *Diaptomus* was the only strict diapausing species, completing its life-cycle within a short period, which contrasts with other calanoid copepods, which commonly have a much longer life-cycle (Hairston & Bohonak 1998). In Lake Redon their emergence coincides with the spring production maxima (Chapter 2) and dominates the zooplankton biomass while they are present in the plankton of the lake.

Despite their three distinct life-cycles, the three species had a similar pattern of elemental changes, showing the highest variation during the last stage of their growth, whereas there was a high degree of homeostasis in the young development stages of the three species. The only previous field study examining the ontogenic changes of the elemental composition of a calanoid copepod (*Mixodiaptomus laciniatus*) (Villar-Argaiz et al. 2002) found substantial variations during ontogeny. However, due to the overlapping cohorts in the population, they also found very high seasonal variability in the elemental composition of each stage, which was responsible, at least in copepodites, for most of the ontogenic variability (Villar-Argaiz et al. 2002). In this study, the authors also concluded that another reason for the simultaneous presence of several development stages during most of the ice-free period, was the food limitation of copepodites, which were unable to moult to the following stage. Villar-Argaiz & Sterner (2002) subsequently demonstrated that copepodites of *Diaptomus clavipes* were unable to moult when fed on P-limited algae, although they were able to survive for almost one month, which provided experimental evidence of food limitation control of the timing of crustacean development. The ontogenic changes of Lake Redon species (Fig. 5.1) clearly differ from the pattern found for *M. laciniatus*. In Lake Redon, all copepodites and

Daphnia juveniles moulted successfully to the following stage, an indication that zooplankton growth was probably not limited by lack of food during the ice-free period. Our results show, therefore, that the elemental composition of zooplankton, when growth is not restricted by lack of food, is constant throughout the distinct life stages, except in adults during the reproduction period.

The consequence of allocating resources to reproduction was a marked decrease in energy-related biochemical compounds. This decrease was comparable for the three species. This behaviour has also been documented in other studies. Various authors (Smith 1990; Ohman 1987; Hirche & Kattner 1993) found reductions of lipids in marine copepods with reproduction and increased reproduction success with increasing amounts of lipids stored during previous productive periods. Vanderploeg et al. (1992b) described a decrease of lipid content in a freshwater calanoid from 33-40% for overwintering copepods to 9% during the reproduction period, which is similar to measurements in the three species of this study. In cladocerans, Tessier et al. (1983) documented a close relationship between lipid accumulation during the inter-moult cycle and egg production in an experimental study of two cladoceran species. They also found lipid content the main source of DW changes. In the *Daphnia* of Lake Redon, this inter-moult accumulation was replaced by seasonal accumulation, and then a progressive decrease in their lipid content due to offspring production. Two experimental studies of the effects of starvation on the biochemical composition of cladocerans (Elendt 1989; Lemcke & Lampert 1975) described the preferential reduction of lipids and carbohydrates after several days of starvation: reproduction was the main cause of reduction. Our results confirm that reproduction investment is the main cause of the biochemical and DW changes in crustacean zooplankton.

The elemental consequences of the biochemical changes observed were related to a decrease in C and H. Therefore, C and H are less homeostatic compounds, whose changes are closely associated with DW. Unlike these two highly variable compounds, N was the only element that remained constant when expressed in absolute value, due to its presence in the two structural components, protein and chitin. These two components are very stable, even under starvation (Elendt 1989). Differences amongst the three species existed in their P content. As shown in Fig. 5.6, *Cyclops* did not change its absolute P content during reproduction, while the other two species decreased their P content by the same proportion as their C. As *Daphnia* has almost twice as much P as *Cyclops*, if the latter preys on the former (Gliwicz 1994; Gliwicz & Stibor 1993), even if only occasionally, it has an excellent P source without any need to store it over the winter. A certain synchronisation of the life-cycles of *Cyclops* and *Daphnia* has been established (Gliwicz et al. 2001). Whatever the dependence of *Cyclops* on *Daphnia* P, our results provide field evidence that some species store P for later offspring production in addition to storage of energy compounds, and corroborate the experimental study of Urabe et al. (1997), who showed that certain *Daphnia* stored inorganic P directly.

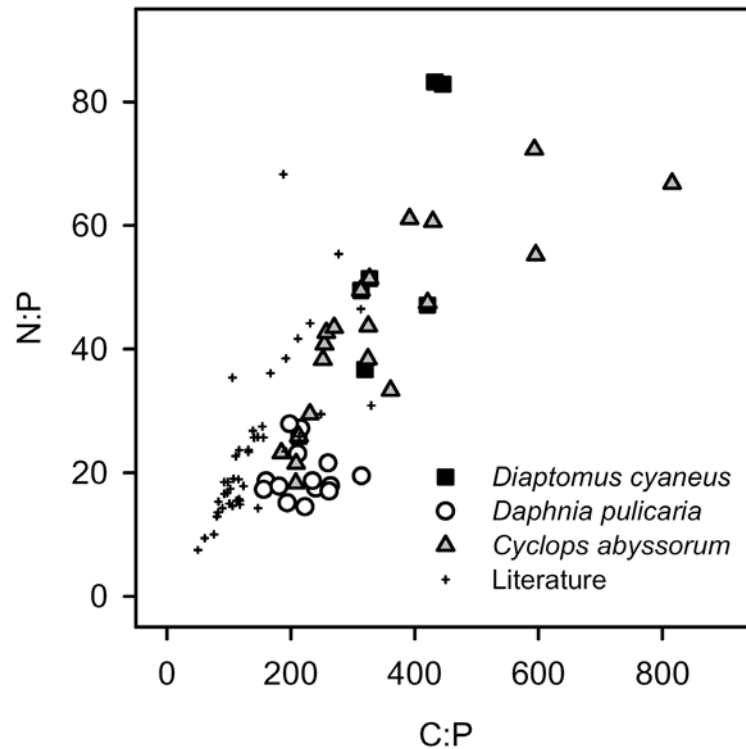


Fig. 5.8. Intra-specific variation in the elemental composition of the three species of crustacean zooplankton of Lake Redon (*Cyclops abyssorum*, *Daphnia pulex* and *Diaptomus cyaneus*) compared with inter-specific variation of 44 species, consisting of freshwater and marine copepods and freshwater cladocerans, from other studies (Omori 1969; Mayzaud & Martin 1975; Flint et al. 1991; Elser et al. 2000a; Ikeda 1988).

The ability of some sea species to store compounds for later use in reproduction (food-independent reproduction mode) has been interpreted as an adaptation to long periods of starvation, either due to occasional patchiness (Dagg 1977) or to seasonality (Hirche & Kattner 1993; Hirche & Kosobokova 2003). The two longest-lived species in this study (*Cyclops* and *Daphnia*) had a reproduction strategy that was similar to these marine species. In both habitats, females started their embryo development well in advance of the production maxima, which allowed the young stages to grow during the productive episodes. The young stages, as they are smaller and have high energy demands for growth, are usually less resistant to starvation (Gliwicz & Guisande 1992). Therefore, this strategy is an adaptation for surviving in this variable environment which nevertheless has high seasonal predictability. Unlike the two long-lived species of this study, *Diaptomus* developed its life-cycle over a short period of time. However, it also lost a considerable proportion of its body elements and biochemical components

in reproduction. Its reproduction strategy is comparable to the other two species, with the difference that it does not need to spend resources on survival during the winter. Unlike these food-independent reproduction species, other species depend entirely on immediate food supply for egg production (Hutchinson 1967). While food-independent reproduction species have a non-homeostatic elemental composition, food-dependent reproduction species are more elementally homeostatic, as they have to acquire all the resources for every clutch (Tessier et al. 1983). This is probably the case in several experimental studies in which *Daphnia magna* has been found to be very elementally homeostatic (Main et al. 1997; Hessen 1990; Vrede et al. 1999), along with other species such as *Daphnia galeata* and *Eudiaptomus gracilis* (Vrede et al. 1999), and *Daphnia obtusa* (Main et al. 1997). However, the last author, within the same experimental set-up, found another species (*Daphnia lumholzy*) with significant elemental changes during its ontogeny.

The elemental composition of males differed between the three species. The males of *Cyclops* did not change their DW significantly from December 1998 to January 1999 and from November to December 1999, and together with *Daphnia* fell within the "homeostatic" line of the pre-adult instars (Fig. 5.3). In contrast, *Diaptomus* males decreased their DW and their elemental content in the same proportions as females (Table 5.3) during reproduction, and so had a similar reproduction effort to females. In addition, the males of *Cyclops* survived for less time than females did, whereas *Diaptomus* males survived for the same period as females. The reason for such differences is probably related to a difference in mating requirements of the distinct zooplankton groups. Cyclopoid copepods need to be impregnated only once during their lifetimes, and therefore fertile subitaneous eggs can be produced for extended periods of time in the absence of males (Whitehouse & Lewis 1973). Most calanoid females, however, must mate repeatedly in order to maintain their fertility (Watras & Haney 1980). In this context, our results suggest that calanoid copepod males may have a more similar reproduction effort with females than cyclopoid males do, due to their different mating requirements.

The results reported in this study show that some species, such as those with food-independent reproduction, vary greatly in their elemental content. In Lake Redon, we found a range of C:P and N:P ratios comparable to or greater than the range among distinct species in marine and freshwater zooplankton (Fig. 5.8). Variability was not restricted to C and H, but also involved P. Therefore, assumptions that the N:P ratio would probably remain homeostatically controlled (Sterner & Hessen 1994) are not valid for all zooplankton species. Previous findings already made clear that inter-stage variability could be a relevant source of elemental variability in some species (Villar-Argaiz et al. 2002). Our results show that, in addition, reproduction strategy has to be considered for full understanding of the elemental variation of zooplankton.

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6. Seasonal inter-specific and ontogenic variation in the amino acid composition of zooplankton: implications for nutritional constraints

Abstract

Amino acid composition has been largely neglected as a food quality indicator in zooplankton, especially in comparison with nutrients and fatty acids. Significant differences in amino acid composition have been found between distinct crustacean species in previous studies. However, little is known about the intra-specific variability, concerning to ontogenic, seasonal and among generation changes. This knowledge is required for properly incorporating amino acid composition into the stoichiometric ecological theory framework. Here we present the study of three zooplankton species during a whole lake annual cycle, each species belonging to one of the main freshwater crustacean groups (*Daphnia pulicaria*, cladocera; *Cyclops abyssorum*, cyclopoid copepod and *Diaptomus cyaneus*, calanoid copepod). Inter-species differences in amino acid composition were three-fold larger than intra-specific variation (75.3 and 24.7%, respectively). The two copepods had a more similar composition to each other than the cladoceran irrespective of their seasonal and ontogenic changes. Within species, *Daphnia* had a low proportion of the amino acid variability (10.9%) related to generations, while seasonal changes accounted for 45.5%. *Cyclops* had a similar adult seasonal variability (49.5%) and 19.3% was attributable to inter-stage variations. *Diaptomus* had the highest adult seasonal change (84%). There were no differences in amino acid composition between males and females in any of the two copepod species, despite of the sexual dimorphism. Amino acid imbalances respect to potential trophic relationships were estimated using two different methods and their utility in the stoichiometric theoretical framework is discussed. The results confirm the hypothesis of high homeostasis in amino acid composition in crustacean zooplankton. And therefore, amino acid composition can be used in stoichiometric studies in conjunction with elemental and fatty acids analysis.

Introduction

In aquatic systems, food nutritional quality and its effect on consumers has received a lot of attention in recent years (Sterner & Schulz 1998; Plath & Boersma 2001). Most research effort has been focused on the effects of resource elemental composition on consumer's growth or reproduction (Sterner et al. 1993; Sterner & Hessen 1994; Urabe & Sterner 1996). Following empirical studies, a stoichiometric theory has been developed, which predicts that zooplankton growth and nutrient recycling are tightly coupled with the resource nutrient ratios (Sterner & Elser 2002). Special emphasis has been

placed in the study of phosphorus limitation, since it is the nutrient most often found to be limiting primary production in freshwaters. Application of stoichiometric arguments to other essential components for metazoan growth has revealed that in some circumstances animals may face a biochemical compound limitation (Müller-Navarra et al. 2000). Amongst these essential biochemicals (mainly some fatty acids, amino acids and vitamins) fatty acids have received most research effort (Brett & Müller-Navarra 1997). Under relatively high sestonic phosphorus concentrations (atomic C:P ratios < 300), some fatty acids, mainly highly unsaturated fatty acids (HUFA), such as eicosapentanoic acid (EPA) or docosahexanoic acid (DHP), may be the limiting compounds (Müller-Navarra et al. 2000; Boersma 2000; Wacker & von Elert 2001). It is surprising, however, that fatty acids are almost the only essential biochemical examined, and that the other essential biochemicals have been rarely explored in natural ecosystems as food quality indicators (Conklin & Provasoli 1977; Brett 1993).

The importance for animal growth of the amino acid composition of their food has been described in terrestrial organisms such as butterflies (O'Brien et al. 2003), birds (Ramsay & Houston 1998), farm animals (D'mello 1994) and humans (Reeds 2000), but also in aquatic organisms such as farmed fishes (Conceição et al. 2003; Ronnestad et al. 2003), and marine copepods (Kleppel & Burkart 1995; Guisande et al. 1999; Guisande et al. 2000). Therefore, an organism's essential amino acid (EAA) composition has been proposed to be used as a rough index of the EAA requirements (Conceição et al. 2003). Further comparison with those of its food has been suggested as a step forward in stoichiometric arguments for a better understanding of nitrogen limitation (Anderson et al. 2004). These authors recognised two main assumptions for applying stoichiometric imbalances to amino acid composition data, namely the consumer being unable to synthesise EAA in significant quantities and being homeostatic with respect to EAA composition. The former assumption has been proved in numerous studies, including crustaceans (Claybrook 1983), while the latter has only been partially demonstrated and further studies have been required (Anderson et al. 2004).

Zooplankton have received a lot of attention in stoichiometric studies, particularly *Daphnia* has been extensively used as a model organism. A better knowledge of the relative variability of the amino acid composition in zooplankton is urgently required. Previous studies of the amino acid composition of zooplankton have focused on the variability in adults compared to their food, showing that adults have a very strict amino acid composition (Cowey & Corner 1963b; Guisande et al. 1999; Guisande et al. 2000). More recently, in an extensive study of the zooplankton population of several lakes, Guisande et al. (2003) found that there was a significant inter-specific difference in the amino acid composition of females. Moreover, there was a clear phylogenetic pattern, being the species of each of the three main freshwater crustacean groups (cladocerans, calanoid copepods and cyclopoid copepods) more similar to each other than to the other groups, and the two copepods to each other than to the cladocerans. Therefore, it was shown that at least for adult females, the amino acid composition requirements were species specific and associated with their phylogenetic distances. Whether the strict amino acid homeostasis of females is extendable to their ontogeny has not been explored. Crustacean zooplankton suffer strong morphological changes during ontogeny, especially copepods. Which even change their resource utilization, from being herbivorous at the first development stages to carnivorous or omnivorous when they are adults (Fryer 1957). Therefore one could expect that following strong morphologic changes

there could be corresponding changes in the amino acid composition along the ontogeny.

We studied the amino acid composition variation of the three crustacean zooplankton species of an alpine lake throughout a whole seasonal cycle, in order to separate the relative importance of inter-specific variation from the intra-specific variation. We selected a lake from the previous extensive study (Guisande et al. 2003) which had one species of each of the main freshwater crustacean groups, a cladoceran (*Daphnia pulex* Forbes), and a cyclopoid (*Cyclops abyssorum* Sars) and a calanoid (*Diaptomus cyaneus* Gurney) copepod. Furthermore, the three species are known to have a different trophic position, being the cladoceran a generalist herbivore, the cyclopoid copepod a carnivore and the calanoid copepod an omnivore. Specifically, *C. abyssorum* has been described to feed on *D. pulex* in similar alpine lakes (Gliwicz 2002), while some other cyclopoid copepods have also been described to predate on diaptomids (Hutchinson 1967). Therefore, since the three species were known to coexist in space and time, we explored their amino acid imbalances in order to predict which amino acids would limit in each of the potential trophic interactions.

Materials and Methods

Sampling

The lake was sampled on 14 dates from December 1998 just after the lake was completely ice-covered, until December 1999, after it was ice-covered again. Samples were collected at the deepest point of the lake either drilling the ice-cover or on a platform anchored throughout the summer at the same point. Zooplankton samples were collected by vertical hauls from 65 m to the surface with a 200 µm net. Individuals were kept alive and transported cold (4°C) until they were frozen (-20°C) in the laboratory within few hours after collection.

Sample separation and chemical analysis

After thawing the samples, from 10 to several hundreds of individuals of each species depending on their individual weight were sorted under a dissecting microscope and placed into teflon capsules. Individuals were kept cold (<4°C) during the sorting process which was in all samples always carried out in a few hours after collection. Dry weight (DW) was determined for all samples after drying at 60°C for 24 h and weighed on a high precision microbalance (Ohaus Analytical Plus, AP250D-0). Between 0.6-0.9 mg of dried sample was vacuum-sealed and hydrolysed with HCl 6N at 116°C for 24h. An internal norleucine standard was included in every sample prior hydrolysis in order to maximise reproducibility. Samples were subsequently dried under vacuum at room temperature. Dried samples were re-dissolved with 0.5 to 1 ml (depending on the concentration of the sample) of buffer solution (pH 2.2) prior analysis and purified by filtration (0.65-µm Durapore filter; Millipore, Bedford, Massachusetts, USA). From two

to six replicates were analysed for each month, species and or stage, depending on the sample availability. The amino acid analysis was carried out on a Biochrom20 (Amersham-Pharmacia) ion-exchange amino acid auto analyser following the ninhidrine method of Spackman et al. (1958). A standard solution of 20 amino acids was run for every 10 samples. Tryptophan due to its degradation under acid hydrolysis has not been analysed in this study. Asparagine and glutamine due to the acid hydrolysis were analysed together with aspartic acid and glutamic acid, respectively.

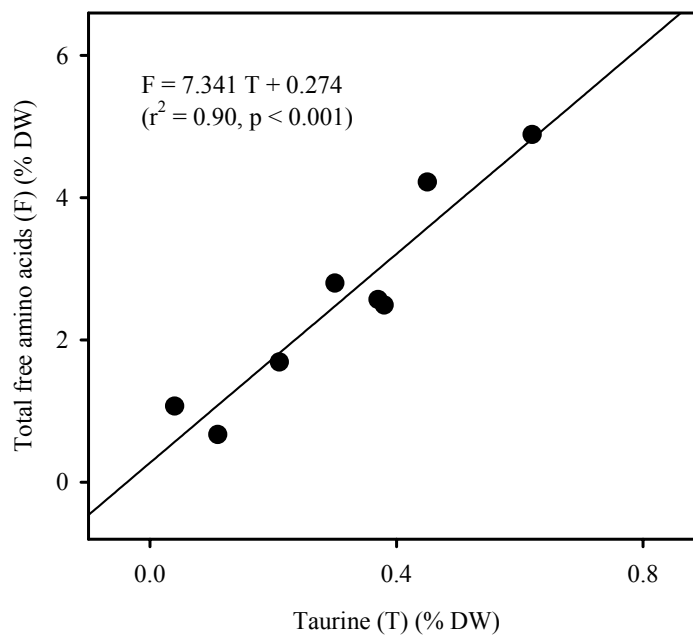


Fig. 6.1. Concentration of taurine in relation to the total amino acid pool in eight different zooplankton species found in the literature (Cowey & Corner 1963a; Suyama et al. 1965; Jeffries 1969; Jeffries & Alzara 1970; Srinivasagam et al. 1971). DW stands for dry weight.

Results

Interspecies differences

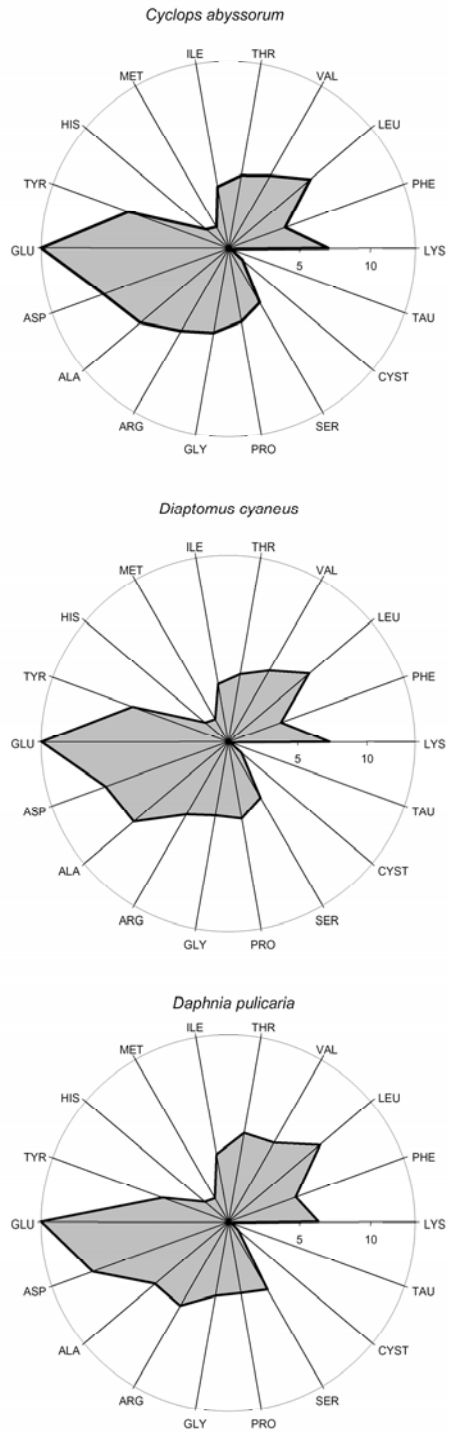
In our analysis we determined the total amino acid composition. In order to determine the relative importance of the free amino acid pool with respect to proteins, we explored if the concentration of taurine (the only amino acid not forming proteins, and one of the main free amino acids in crustaceans; Claybrook 1983) could be used as an indicator of the free amino acid pool. A bibliographic search of joint measurements of taurine and

the concentration of free amino acids were compiled, and related with a least squares linear regression (Fig. 6.1). The good fit of the linear regression ($r^2 = 0.90$, $p < 0.001$) revealed that taurine concentration could be used as a predictor of the free amino acid pool. Therefore, the relative proportion of the free amino acid fraction was estimated for each species. *C. abyssorum* had 1.9% of the total amino acid pool as free amino acids, *D. pulicaria* had 2.7% and *D. cyaneus* 1.2%. Therefore, it can be reasonably accepted that the influence of free amino acids in relation to the total amino acid pool is very small, and that our measure of total amino acids in freshwater species mainly reflects those of the protein composition.

Table 6.1. Amino acid composition of three species of zooplankton of Lake Redon. Concentrations are expressed as mg amino acid g⁻¹ dry mass. n are the number of samples analysed and sd the standard deviation.

	<i>Cyclops abyssorum</i>		<i>Diaptomus cyaneus</i>		<i>Daphnia pulicaria</i>	
	n	sd	8	sd	15	sd
glutamine	58	14	80	16	36	6
asparagine	41	1	56	10	28	5
alanine	36	8	53	13	19	3
leucine	33	8	45	9	23	4
tyrosine	33	7	45	14	14	2
lysine	31	7	43	10	18	4
arginine	30	7	36	8	19	3
glycine	27	6	32	8	14	2
valine	26	6	35	8	18	3
proline	23	6	34	9	14	2
threonine	23	5	29	7	17	3
isoleucine	19	4	25	5	13	3
serine	19	5	27	5	15	3
phenylalanine	19	5	24	4	14	2
histidine	9	2	13	3	6	1
methionine	7	2	11	2	5	1
cysteine	5	2	8	3	3	1
taurine	1	0	1	0	1	0

Fig. 6.2. Radar plot showing the amino acid composition in of the three species analysed (*Daphnia pulicaria*, *Cyclops abyssorum* and *Diaptomus cyaneus*). Concentrations are in percentage of the total amino acid composition in weight.



The three species had a relatively similar amino acid pattern (Fig. 6.2), only differing in the proportions of some of these amino acids. In all of them, the two more abundant amino acids were glutamine/glutamic acid and asparagine/aspartic acid (Table 6.1). They were followed, in the two copepods, by alanine, leucine, tyrosine, lysine and arginine, altogether accounting by 60% of the total amino acid pool. Whereas in the cladoceran, they were followed by leucine, arginine, alanine, valine and threonine accounting for 58% of the total amino acid pool.

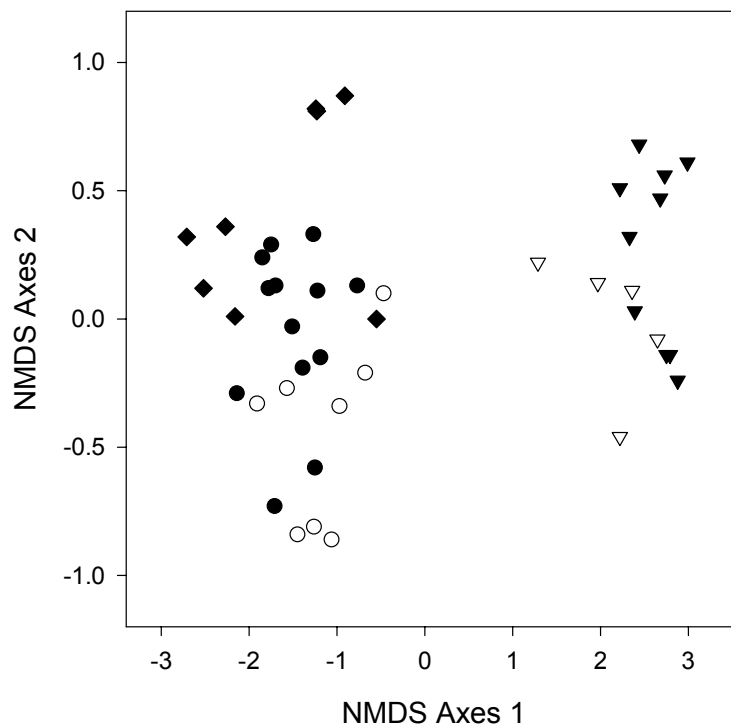


Fig. 6.3. Plot of the non-metric multidimensional scaling of the amino acid composition of the species and their stages analysed from Lake Redon. Filled diamonds stand for *Diaptomus cyaneus* Adults, filled triangles for *Daphnia pulicaria* first generation females, empty triangles for *Daphnia pulicaria* second generation females, filled circles for *Cyclops abyssorum* adults and empty circles for *Cyclops abyssorum* copepodites.

Despite the similarity in the general pattern, a bidimensional representation of an Euclidian distance matrix calculated from the amino acid percentage composition (Legendre & Legendre 1998) showed that the three species had a consistently distinctive amino acid composition (Fig. 6.3). The first axis of the non-metric multi dimensional scaling (NMDS) segregated the two copepods from the cladoceran, showing that the two copepods are more similar between each-other than from the cladoceran, while the second axis segregated the two copepods. The area covered by each species is pro-

portional to their amino acid variability. Comparison of the spread of the adults of each species revealed that each species had a relatively similar amino acid variability.

Table 6.2. Results of the four hierarchical analysis of the amino acid distance matrix using the AMOVA algorithm. 'All species' compares the inter-specific (among species) versus the intra-specific variation (Among stages and within stages). The other three AMOVAs partition the intra-specific variation of each species.

Source of variation	d.f.	Sum of squares	Variance components	% variation	P
All species					
Among species	2	175.35	5.69	75.25	0.010
Among stages within species	6	19.98	0.56	7.46	<0.001
Within Stages	37	48.38	1.31	17.29	<0.001
Total	45	243.71	7.56		
<i>Daphnia pulicaria</i>					
Among generations	1	3.17	0.15	10.95	0.226
Among time within generations	5	9.46	0.64	45.45	0.002
Within replicates	8	4.91	0.61	43.59	<0.001
Total	14	17.54	1.41		
<i>Cyclops abyssorum</i>					
Among stages	4	12.42	0.32	19.34	0.042
Among time within adults	5	11.18	0.81	49.48	<0.001
Within replicates	11	5.63	0.51	31.18	<0.001
Total	20	29.23	1.64		
<i>Diaptomus cyaneus</i> - adults					
Among time	1	8.03	1.91	83.98	<0.001
Within replicates	6	2.19	0.36	16.02	
Total	7	10.22	2.28		

Table 6.3. One way ANOVAs examining inter-species differences of *Diaptomus cyaneus* D.c., *Cyclops abyssorum*, C.a. and *Daphnia pulicaria*, D.p. in terms of their individual amino acid composition. The Tukey hsd test was used to test the difference between copepod developmental phases. Post hoc comparison: < or > significantly higher or lower at $p < 0.05$ respectively; << or >>, significantly higher or lower at $p < 0.001$; =, not significantly different.

		SS	df	MS	F	p					
PHE	Inter-groups	0.07	2	0.034	133.7	0.000	<u>D.c.</u>	>	<u>C.a.</u>	>	<u>D.p.</u>
	Intra-groups	0.01	44	0.000							
	Total	0.08	46								
ALA	Inter-groups	0.10	2	0.050	116.7	0.000	<u>D.c.</u>	>	<u>C.a.</u>	>	<u>D.p.</u>
	Intra-groups	0.02	44	0.000							
	Total	0.12	46								
TYR	Inter-groups	0.36	2	0.178	238.9	0.000	<u>D.p.</u>	<	<u>D.c.</u>	=	C.a.
	Intra-groups	0.03	44	0.001							
	Total	0.39	46								
THR	Inter-groups	0.10	2	0.049	86.6	0.000	<u>D.p.</u>	<	<u>C.a.</u>	=	D.c.
	Intra-groups	0.03	44	0.001							
	Total	0.12	46								
SER	Inter-groups	0.10	2	0.048	84.7	0.000	<u>D.p.</u>	>	<u>C.a.</u>	>	<u>D.c.</u>
	Intra-groups	0.03	44	0.001							
	Total	0.12	46								
ILE	Inter-groups	0.02	2	0.012	38.4	0.000	<u>D.p.</u>	>	<u>C.a.</u>	>	<u>D.c.</u>
	Intra-groups	0.01	44	0.000							
	Total	0.04	46								
VAL	Inter-groups	0.01	2	0.007	39.7	0.000	<u>D.p.</u>	>	<u>C.a.</u>	=	D.c.
	Intra-groups	0.01	44	0.000							
	Total	0.02	46								
GLY	Inter-groups	0.04	2	0.020	35.1	0.000	<u>C.a.</u>	>	<u>D.p.</u>	=	D.c.
	Intra-groups	0.03	44	0.001							
	Total	0.06	46								
LEU	Inter-groups	0.02	2	0.011	32.9	0.000	<u>D.p.</u>	>	<u>C.a.</u>	=	D.c.
	Intra-groups	0.02	44	0.000							
	Total	0.04	46								
CYST	Inter-groups	0.20	2	0.102	25.5	0.000	<u>D.p.</u>	<	<u>C.a.</u>	=	D.c.
	Intra-groups	0.18	44	0.004							
	Total	0.38	46								

Table 6.3. (Continued)

	SS	df	MS	F	<i>p</i>			
ASP Inter-groups	0.02	2	0.008	25.0	0.000	D.p. >	C.a. =	D.c.
Intra-groups	0.01	44	0.000					
Total	0.03	46						
ARG Inter-groups	0.02	2	0.009	17.6	0.000	<u>D.c. <</u>	<u>D.p. =</u>	C.a.
Intra-groups	0.02	44	0.000					
Total	0.04	46						
LYS Inter-groups	0.03	2	0.016	17.5	0.000	<u>D.p. <</u>	<u>D.c. =</u>	C.a.
Intra-groups	0.04	44	0.001					
Total	0.07	46						
PRO Inter-groups	0.01	2	0.006	3.2	0.052	<u>D.p. <</u>	<u>D.c. =</u>	C.a.
Intra-groups	0.08	44	0.002					
Total	0.09	46						
MET Inter-groups	0.02	2	0.011	8.1	0.001	<u>C.a. <</u>	<u>D.p. =</u>	D.c.
Intra-groups	0.06	44	0.001					
Total	0.08	46						
TAU Inter-groups	0.36	2	0.179	2.6	0.088	C.a. =	D.p. =	D.c.
Intra-groups	3.07	44	0.070					
Total	3.43	46						

The relative contribution of species, stages within species, and within stages to the overall variation of the amino acid composition was analysed with a hierarchical variance partitioning algorithm (AMOVA, Weir & Cockerham 1984; Excoffier et al. 1992) designed to partition the variation of a distance matrix into hierarchical components. This method is similar to the traditional univariate hierarchical ANOVA, but uses a series of randomisation tests to calculate the significance of the ANOVA tables (Schneider et al. 2001). AMOVA analysis revealed that most of the variation (75.3%) was attributable to species, while 7.5% was attributable to stages and the remaining 17.3% to the intra-stage variation (Table 6.2). The individual amino acids which contributed to overall species differences were detected with one-way ANOVAs (Table 6.3). The cladoceran differed significantly from the two copepods in the percentage of twelve amino acids, being especially richer in threonine, serine, phenylalanine and leucine, while it had lower content of tyrosine, alanine and lysine. Only one amino acid, arginine, was significantly different in *D. cyaneus* from the other two species, and two amino acids were significantly different (glycine was higher and methionine lower) in *C. abyssorum* than in the other two species. The two copepods differed mainly in the alanine, proline and serine content, which was higher in the cyclopoid copepod, while the calanoid had significantly more arginine, glycine, phenylalanine and isoleucine. Finally, there were only three amino acids for which the three species had no significant differences, which were histidine, glutamine and taurine.

Intra-species variation

The evaluated sources of intra-specific variation of the amino acid composition, consisted in gender, development stages and seasonality. Gender differences were only considered in the two copepod species, since *D. pulicaria* mainly reproduces asexually. No statistical differences were found between males and females of the two copepods for any of the 18 individual amino acids. Therefore, in successive analysis, males and females, were combined as adults.

Ontogenic changes were evaluated with the two species surviving during the whole seasonal cycle (*D. pulicaria* and *C. abyssorum*). Adult females of *D. pulicaria* from the previous summer survived below the ice-cover, reproduced during the first two months of the ice-free period, and disappeared from the lake afterwards, leaving the juveniles growing up during the summer (Chapter 5). We therefore compared the amino acid composition of the two cohorts. AMOVA analysis showed that there was only 10.9% of the variation attributable to the differences between the two generations. Although it was not statistically different in the randomisation test ($p = 0.226$; Table 6.2), individual amino acid tests detected significant differences for six amino acids, serine ($F_{1, 13} = 55.4, p < 0.001$), asparagine ($F_{1, 13} = 25.22, p < 0.001$), phenylalanine ($F_{1, 13} = 10.88, p = 0.006$), isoleucine ($F_{1, 13} = 9.59, p = 0.009$), glycine ($F_{1, 13} = 7.06, p = 0.02$) and lysine ($F_{1, 13} = 5.29, p = 0.039$). The remaining variation of the AMOVA analysis was attributable either to temporal changes of the two cohorts (45.5%) or to replicate variability (43.6%), both significant at the AMOVA randomisation test ($p = 0.002$ and $p < 0.001$ respectively).

The other species surviving during the whole seasonal cycle was the cyclopoid copepod *C. abyssorum*. Similarly to *D. pulicaria*, adults survived below the ice-cover, to reproduce the following summer, when the various stages moulted almost one every month. We sampled therefore two generations. The relative importance of stages, time and replicate variation on *C. abyssorum* amino acid composition was tested with AMOVA (Table 6.2). Stages analysed consisted in four copepodites stages (from CII to CV) and adults, while the temporal variation was only analysed within the adults, since they were the only stage which was present in the lake for more than one month (Chapter 5). There were significant differences between stages (19.3%), time within adults (50%) and within replicates (31.2%). The individual amino acids that differentiated copepodites and adults were lysine ($F_{1, 19} = 35.5, p < 0.001$), glycine ($F_{1, 19} = 17.99, p < 0.001$), proline ($F_{1, 19} = 9.77, p = 0.006$), valine ($F_{1, 19} = 7.96, p = 0.011$) and cysteine ($F_{1, 13} = 4.21, p = 0.054$).

The other copepod, the calanoid *D. cyaneus* dominated the plankton of July and August, spending the rest of the year as resting eggs (Chapter 5). Due to its faster development, only adults of the species were considered in this study. The summer samples of July and August coincided with the beginning and the end of the reproduction period, and therefore the samples were expected to cover the maximum range of the amino acid variability for the adults of the species. The hierarchical analysis of variance of the amino acid distances showed that most of the variation was related with the temporal changes (84%, Table 6.2), while this species had the lowest variability attributable to the replicates (16%). The amino acids that differed in the two periods were asparagine ($F_{1, 6} = 101.48, p < 0.001$), glutamine ($F_{1, 6} = 54.77, p < 0.001$), phenylalanine ($F_{1, 6} = 41.09, p = 0.001$), serine ($F_{1, 6} = 30.94, p = 0.001$) leucine ($F_{1, 6} = 27.59, p = 0.002$) and

isoleucine ($F_{1,6} = 27.56, p = 0.002$) that decreased from July to August. In contrast, tyrosine ($F_{1,6} = 56.98, p < 0.001$), cysteine ($F_{1,6} = 29.36, p = 0.002$), proline ($F_{1,6} = 23.99, p = 0.003$), glycine ($F_{1,6} = 19.05, p = 0.005$) and alanine ($F_{1,6} = 9.91, p = 0.02$) significantly increased.

Amino acid imbalances

There is a growing evidence showing that algal amino acid composition is very similar across the different algal groups (Cowey & Corner 1963b; Ahlgren et al. 1992; Ahlgren & Hyenstrand 2003; Kalachova et al. 2004). Therefore in order to compare the amino acid composition of *D. pulicaria* with those of its resources (algae), we used the average amino acid composition of distinct algal groups (Ahlgren et al. 1992). Comparison of the average composition of algae and those of *D. pulicaria* (Fig. 6.4), indicated that the overall amino acid composition was very similar (Pearson correlation, $r = 0.988, p < 0.001$; Fig. 6.4). The two copepods had a higher correlation to *D. pulicaria* than to algae ($r = 0.924$ and $r = 0.937$ for *D. cyaneus* and *C. abyssorum* and algae, $r = 0.937$ and $r = 0.947$ for *D. cyaneus* and *C. abyssorum* and *D. pulicaria*, being all correlations significant at $p < 0.001$). The two copepods had the higher correlation ($r = 0.993, p < 0.001$). These correlation analyses showed that the amino acid composition of the daphnia-algae is more similar than the daphnia-copepod.

Complementary to the overall amino acid comparison, the identification of potential amino acid imbalances should provide a better insight into these nutritional relationships. In this context, the first limiting amino acid can be defined as the EAA with the lowest, statistically significant, relative difference between its contribution to the potential diet and the consumer EAA profile (rEAA, Conceição et al. 2003):

$$rEAA_i = (\text{diet}EAA_i - \text{consumer}EAA_i) / (\text{consumer}EAA_i) * 100$$

where $\text{diet}EAA$ and $\text{consumer}EAA$ are the contributions of a given EAA to the EAA profiles of the potential diet and the consumer for a given amino acid (i). The first limiting amino acid (those with the minimum rEAA) will set the limit for amino acid utilisation above which protein synthesis cannot proceed. Therefore the absolute value of the rEAA of the first limiting amino acid has been proposed as an estimate of the amino acid loss (percent of total amino acid absorbed) for a given diet caused by its deviation to the ideal EAA profile (Conceição et al. 2003).

The relative EAA imbalances for the different EAAs of the potential trophic relationships of the three species was calculated (Fig. 6.5). Within the daphnia-algae interaction, three amino acids were significantly deficient in the diet (histidine, threonine and lysine), being histidine the EAA with the highest imbalance (rEAA = 20.6%). The two copepods if feeding on daphnia would have a deficiency in lysine and in tyrosine-phenylalanine (although phenylalanine is the only truly essential amino acid, tyrosine can only be synthesised from the former, and therefore they have been amalgamated together). *C. abyssorum* would have tyrosine-phenylalanine as the limiting amino acid (rEAA = 15.4%), and *D. cyaneus* would be firstly limited by lysine (rEAA = 13.4%). In the case that *C. abyssorum* would predate on *D. cyaneus* two amino acids would limit protein synthesis, arginine and isoleucine, being the former the first limiting amino acid (rEAA = 10.4%).

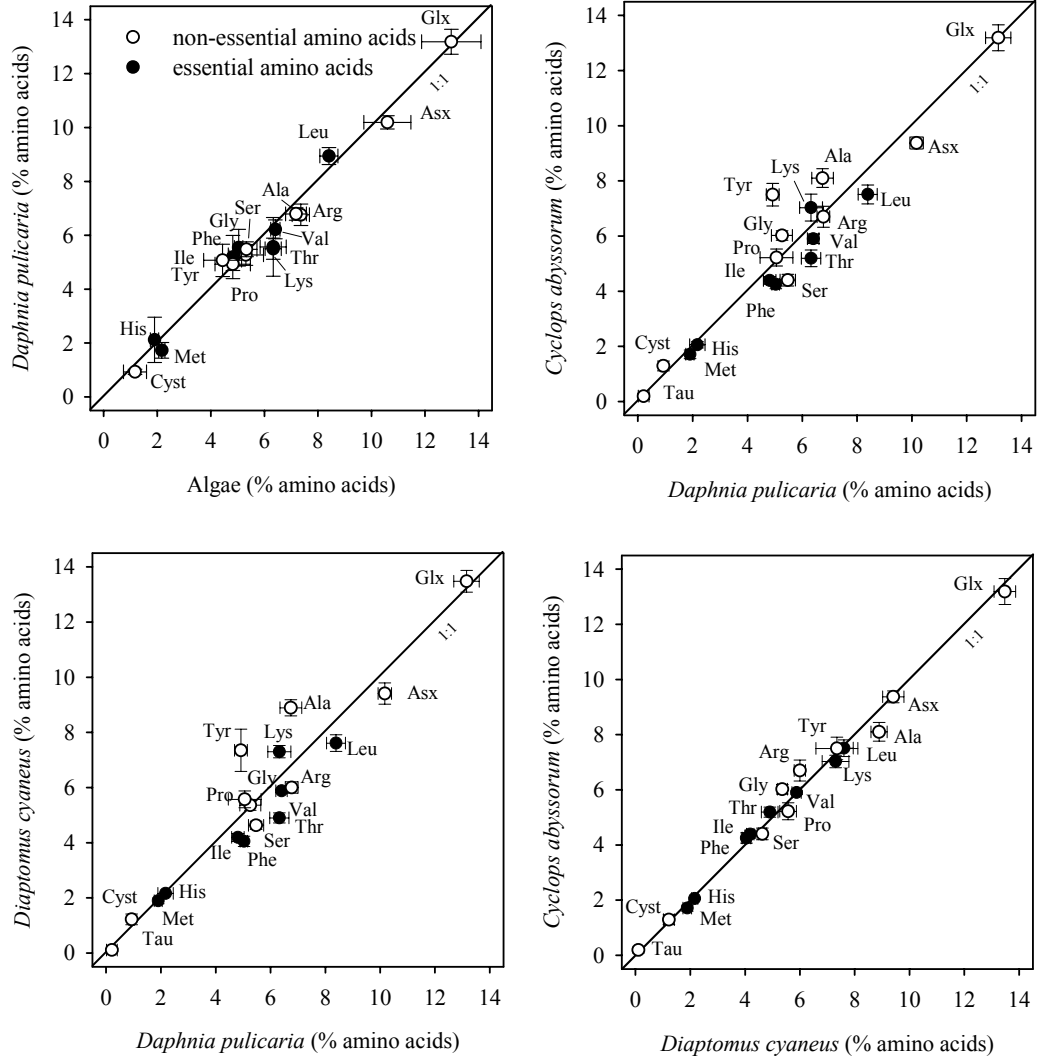


Fig. 6.4. Percentage amino acid composition of a) *Daphnia pulicaria* and algae, and b) *Cyclops abyssorum* and its potential prey, *D. pulicaria*. Each circle is a different amino acid, being the empty circles the non-essential amino acids and the filled circles the essential amino acid. Straight line is the 1:1 relationship and is shown as a reference only. Algal amino acid composition was obtained from Ahlgren et al. (1992).

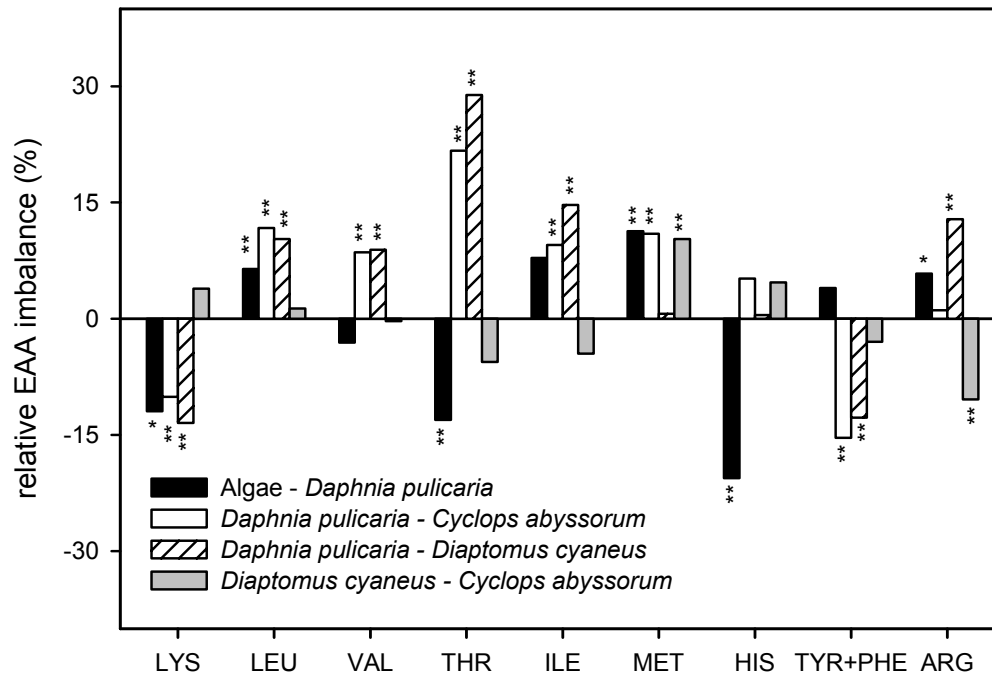


Fig. 6.5. Relative essential amino acid imbalance for the potential trophic relationships within the three pelagic crustacean species of Lake Redon. Algal amino acid composition was obtained from (Ahlgren et al. 1992). See text for index calculation procedure. Results are expressed in weight percentage of the total amino acid composition. Results from the one way ANOVA test denoted by **, $p < 0.001$; *, $p < 0.05$; no asterisk, not significant.

Discussion

Amino acid imbalances

Two different amino acid comparisons were used for characterising the possible nutritional imbalances between consumers and resources, the comparison of the overall amino acid composition (e.g. by the use of a measure of correlation) and the use of an amino acid imbalance index (the relative EAA index). Both have advantages and drawbacks, but more interestingly may be relevant at different limiting conditions. The comparison of the overall amino acid composition would have sense if all the amino acids would be essential. This is only likely to happen when there is a general nitrogen or protein limitation (Reeds 2000), which is not rare in terrestrial and marine ecosystems

(White 1993). In this case the measure of the overall amino acid similarity might be a better measure of the nutritional quality of food than total nitrogen itself. This measure has been used successfully to show that copepods had a higher reproductive success when the amino acid composition of food had a higher similarity with those of females (Guisande et al. 1999; Guisande et al. 2000). However, in freshwater ecosystems, where phosphorous is generally the most limiting nutrient, protein availability has not been typically found to limit growth, and therefore the absence of some amino acids could be more relevant. The rEAA index could be a good estimator of the degree of imbalance in the diet. The use of this index as a valid indicator of protein quality, has the underline assumption that the relative bioavailability of the individual amino acids is the same for all of them. In a study of the digestion efficiency of algae when fed by a herbivorous marine copepod (Cowie & Hedges 1996), it was found that the relative digestion efficiencies for the individual amino acids was relatively constant (ranging from 74% for threonine to 89% for lysine). The imbalances found in the species of lake Redó, are in the order of amino acid digestion efficiencies measured (average 83.8% for all amino acids; Cowie & Hedges 1996). Therefore, following this experimental evidence, the use of this index seems reasonable.

Ontogenic versus intra-specific variation

Comparison of the ontogenic amino acid composition variation with inter-specific variation showed that differences between species were much higher than intra-specific variation. This result complement therefore previous findings that adult zooplankton composition have an homeostatic amino acid composition (Guisande et al. 2003), and therefore it can be concluded that the amino acid composition of zooplankton are relatively homeostatic during their life-cycle. Other studies of adult marine zooplankton also found a strict amino acid composition (Cowe & Corner 1963b; Guisande et al. 1999), and therefore, it is very likely that our results are extensive to both freshwater and marine habitats. The higher amino acid distance between the cladoceran and the two copepods also agree with previous findings that the amino acid composition has a strong phylogenetic component (Sorimachi et al. 2000; Sorimachi et al. 2001; Guisande et al. 2003).

Despite of this relatively high degree of homeostasis, we have found that there is a certain flexibility in the amino acid composition. Distinct amino acids caused the differences between the two generations of *D. pulicaria* and *C. abyssorum*. This fact suggests that the processes behind are species specific, there is not a general and common shift in amino acid composition in the population community. However, the overall amino acid variation of the adults of each species was quite similar (as indicated by the spread of their data points in the NMDS of Fig. 6.2). This result also illustrates that the amino acid variability of each species is limited, and most interestingly of similar magnitude in all of them. The three species differed in the length of their life-cycle. *C. abyssorum* and *D. pulicaria* survived in the water column during the whole seasonal cycle, while *D. cyaneus* completed its life-cycle within three months. Therefore, the amino acid variability is not proportional to the length of their life-cycle.

The identical amino acid composition of males and females of both copepod species contrasts with their high sexual dimorphism in size, which is particularly pronounced in

cyclopoid copepods (Gilbert & Williamson 1983). Our results show that irrespectively of their differential morphological characteristics, they have the same requirements in terms of protein quality. Two main hypotheses have been suggested to explain the adaptive evolution of this dimorphism: sexual selection and ecological causation (Shine 1989). The results of the amino acid composition discard any differential nutrient requirements for protein quality for these species.

Acknowledgements

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7. Intra-specific variations on stable isotope composition indicate ontogenic diet changes and amino acid limited growth in alpine lake zooplankton

Abstract

Seasonal variations in the stable isotope composition of carbon and nitrogen ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of crustacean zooplankton in an oligotrophic alpine lake were recorded during 1999. Although the three species found had distinct isotopic signatures, being the two copepods (the cyclopoid *Cyclops abyssorum* and the calanoid *Diaptomus cyaneus*) one trophic level above the cladoceran (*Daphnia pulicaria*), there were high intra-specific isotopic variations. Isotopic changes associated with the biochemical composition were more important in the lipid fraction than in chitin, which had a negligible effect on the total isotopic composition. Lipids accounted for up to 30% of the $\delta^{13}\text{C}$ variability in the adults of the two copepods, although no effect on the seasonal isotopic fractionation between the copepods and *Daphnia* was found. Most of the seasonal changes were associated with ontogenic shifts. *Daphnia* $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ shifted from an enriched to a more depleted signal, associated with an ontogenic change in feeding, probably from bacteria or allochthonous matter to seston. *Cyclops* copepodites also presented an isotopic shift from feeding on algae to *Daphnia*. This shift however, was different for carbon and nitrogen. Fitting an isotopic growth model showed that 99% of the carbon variation could be explained by the model whereas only 60% of the nitrogen. This low fit of nitrogen to the model was related to the difference in the amino acid composition between the copepodites and its resource, *Daphnia*. In fact, the nitrogen isotopic fractionation of the two species was directly related with their amino acid differentiation, which is interpreted as an evidence of consumer nitrogen limitation.

Introduction

Stable isotopes have the potential to simultaneously capture complex interactions and to track energy flow through ecological communities (Peterson & Fry 1987). The ratio of stable isotopes of nitrogen ($\delta^{15}\text{N}$) has been used to estimate trophic position because the $\delta^{15}\text{N}$ of a consumer is typically enriched by 3-4‰ relative to its diet (McCutchan et al. 2003; DeNiro & Epstein 1981; Post 2002). In contrast, the ratio of carbon isotopes ($\delta^{13}\text{C}$) changes little as carbon moves through food webs (DeNiro & Epstein 1981; McCutchan et al. 2003; Post 2002). In lakes it has proven very useful for distinguishing between the littoral and the pelagic habitats (France 1995), which has allowed to describe the relative contribution of both habitats to the upper trophic stages (Vander Zanden & Vadeboncoeur 2002). But also many lake trophic chains have been described

based of stable isotope composition of their organisms (Kling et al. 1992; Gu et al. 1994; Yoshioka et al. 1994; Yoshii et al. 1999; Grey et al. 2001).

Limitations in the application of this analytical technique have faced mainly with the problem that most food chains have a wide omnivore structure (Sprules & Bowerman 1988) and therefore the precise food contribution to predators with more than two food sources are impossible to predict (Gannes et al. 1997). After the critical evaluation of (Gannes et al. 1997; Gannes et al. 1998) several studies have focused in some of the difficulties associated with the method. The precise fractionation from one trophic position to the other has recently been revised and slightly different fractionation factors have been shown for different trophic strategies, food biochemical compositions, and different metabolic pathways (Vander Zanden & Rasmussen 2001; Vanderklift & Ponsard 2003; McCutchan et al. 2003). Due to the high variability of the stable isotope composition of aquatic primary producers, the use of strict herbivores have been shown to be much more reliable (Kling et al. 1992; Cabana & Rasmussen 1996; Vander Zanden & Rasmussen 1999; Post 2002; Matthews & Mazumder 2003).

Perhaps one of the most neglected aspects has been the understanding of different time averaging of the organisms with different life spans, which range of several orders of magnitude (e.g. fishes live various years, zooplankton various days to months while protists hours to few days). This point was shown by (O'Reilly et al. 2002) which emphasised the importance of the temporal aspect of trophic interactions. They showed that lake Tanganyika phytoplankton and zooplankton had a different trophic position from those predicted from the upper trophic stages. They were able to relate that deviation with episodic turbulent episodes that upwelled isotopic distinctive nitrate to the upper layers and was rapidly incorporated to phytoplankton. However, the signature of this nitrate was diluted by time averaging in the upper trophic levels. This time averaging is a consequence of the fact that the isotopic signature of an organism is related to variable nitrogen sources used throughout the life of the organism.

Isotopes turnover in organisms have been studied in several occasions experimentally by switching organisms to a diet with a different isotopic composition (Fry & Arnold 1982a; Tieszen et al. 1983; Hesslein et al. 1993; Frazer et al. 1997; Herzka & Holt 2000; Grey 2000). They have shown that the isotopic "signature" change was gradual, and that turnover times were linked to metabolism, either related with maintenance or growth. The relative importance of both isotopic incorporation routes has been found very different between homeotherms and cold-water poikilotherms. In homeotherms Ponsard & Averbuch (1999) concluded that the turnover time was very quick and therefore that the isotopic composition of growing and adult animals fed on the same diet should show the same isotopic fractionation, due to a much higher importance of maintenance metabolism over growth incorporation. However, in poikilotherms living in cold environments such as fish, growth has been shown to be of much greater importance than maintenance metabolism (Hesslein et al. 1993; Herzka & Holt 2000; Grey 2000). In crustaceans Fry & Arnold (1982b) also showed that growth was the primary source of isotopic change, while in crustacean zooplankton there is only one experimental study of trophic fractionation (Grey 2000). He also showed that growth was the primary source of isotope 'signature' change. However, since crustaceans grow by episodic moulting, they found that juveniles of copepods and juveniles and adults of cladocerans (which moult with every egg clutch produced) changed their isotopic 'signature' when switched to a diet with a different isotopic composition, while adults of

copepods (which do not moult during this stage) did not change their isotopic composition during the experiment. He showed therefore that the change of the isotopic signature of zooplankton was primarily related with growth, being maintenance metabolism not 'detectable' in the isotopic composition of these crustaceans.

In previous studies of planktonic seasonal variation, it was shown that there were relevant variations in the isotopic signature of zooplankton (Zohary et al. 1994; Yoshioka et al. 1994), and (Grey et al. 2001) showed that part of this variation was due to shifts of species with different isotopic compositions, but also that the intra-species variation was significant, especially related with changes in the isotopic signature of its putative food. However, no field data exists on the ontogenic changes of the isotopic composition of zooplankton and its possible effects on the species trophic relationships.

Copepods are known for their trophic shift from the naupliar stage when they are forced herbivores to copepodites and adults when, in some species, they switch to omnivores or carnivores (Dussart & Defaure 1995). The aims of this study are therefore (1) to describe the seasonal isotopic composition of these species and their trophic relationships; (2) to estimate the relative importance of lipids and chitin on the isotopic variability of the three species; (3) to determine whether there are significant ontogenic and gender differences in their isotopic composition; (4) to examine if the variability of the isotopic fractionation of copepods, especially those of nitrogen, changes depending on the protein quantity or quality of its putative food.

Materials and Methods

Sample collection and preparation

The lake was sampled on 14 dates from December 1998 just after the lake was completely ice covered, until December 1999, after it was ice-covered again. Samples were collected at the deepest point of the lake either drilling the ice-cover or on a platform anchored throughout the summer at the same point. Zooplankton samples were collected by vertical hauls from 65 m to the surface with a 200 μm net. Sampled individuals were kept alive and transported cold (4°C) until they were frozen (-20°C) in the laboratory within few hours after collection. After thawing the samples, from 10 to several hundreds of individuals depending on the weight of each species and stage were quickly sorted under a dissecting microscope and placed into pre-weighed tin capsules for stable isotope analysis or Teflon capsules for total amino acids, chitin and lipids. At least three combined sample replicates were analysed for each species and stage for each sampling date with the exception of few cases where not enough material was available. Individuals were kept cold (<4°C) during the sorting process which was in all samples always carried out in a few hours. Dry weight (DW) was determined for all samples after drying at 60°C for 24 h and weighed on a high precision microbalance (Ohaus Analytical Plus, AP250D-0).

Chemical analysis

Dried samples for stable isotope analysis were packed into tin capsules with vanadium pentoxide as catalyser to assure complete combustion. Samples were analyzed in a Delta C Finningan MAT mass spectrometer coupled online with a Carlo Erba CHNS elemental analyzer, via a Finnigan conflo 2 interface. Specific standards were used for calibrating the isotopic signal: sucrose (IAEA CH6), polyethylene (IAEA CH7) and graphite (IAEA-USGS 24) for carbon, and ammonium sulfate (IAEA-USGS 25, IAEA-N1 and IAEA-N2) and potassium nitrate (IAEA-NO3) for nitrogen (Gonfiantini 1978). A complete batch of all standards was run at the beginning and at the end of each analytical session, and IAEA CH6 and CH7, and IAEA-N1 and IAEA-NO3 were run every twelve samples in order to control for linearity. Special care was taken in weighting the samples and the standards in order that both had similar amplitudes. Results are reported using atmospheric nitrogen and PeeDee belemnite (PDB) carbonate as references. Reproducibility was better than 0.1‰ and 0.3‰ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

The protein content was measured as the sum of individual amino acids. This analysis also allowed for the combined determination of chitin, since N-acetylglucosamine, the molecular constituent of chitin, is an amino-sugar (Strayer 1988), which after hydrolysis appears in the aminogram as glucosamine. Total amino acid samples were vacuum sealed and hydrolysed with HCl 6N at 116°C for 24h. An internal norleucine standard was included in every sample prior to the hydrolysis to increase the reproducibility. The analysis was carried out on a Biochrom20 (Amersham-Pharmacia) ion-exchange amino acid auto analyser following the ninhydrine method of (Spackman et al. 1958). A standard solution of 20 amino acids and glucosamine was run for every 10 samples. Tryptophan due to its degradation under acid hydrolysis has not been analysed in this study. Total lipids were quantified gravimetrically. Between 0.3-0.6 mg of dried zooplankton were placed into a dichlormethane:methanol (2:1, v/v) solution (Folch et al. 1957) sonicating for 30 minutes in an ultra-sound bath, for lipid extraction. The non-lipidic remains were then collected in a pre-weighed GFF Watman filters which were dried and re-weighed. Lipids were quantified by weight difference.

Results

Intra-specific and gender differences

The three pelagic crustacean species inhabiting lake Redó differed in their mean stable isotope composition of C and N (Table 7.1). These differences were significant in one way ANOVA tests for $\delta^{13}\text{C}$ ($F_{2, 77} = 8.5$, $p < 0.001$) and $\delta^{15}\text{N}$ ($F_{2, 77} = 254.2$, $p < 0.001$). In order to detect differences between species pairs we conducted a Tukey's post hoc test, which indicated that *Daphnia* and *Diaptomus* were the only species that had non significant differences in their $\delta^{13}\text{C}$ content ($p = 0.212$), while *Cyclops* had a signature significantly different from *Daphnia* ($p = 0.012$) and from *Diaptomus* ($p = 0.001$). In their $\delta^{15}\text{N}$ signature the three species had a significantly different signature ($p < 0.001$).

We analysed males and females of the two copepod species in order to evaluate if there were gender differences in their isotopic signatures. Males of *Cyclops* appeared only during January 1999, while their respective females inhabited the lake from December 1998 until July 1999. In *Diaptomus*, both sexes survived during the same period. In order to avoid potential seasonal differences in the female's diet, we compared the isotopic composition of males and females of both species when they were present together. Males and females of both species had the same $\delta^{15}\text{N}$ signature (Table 7.1), without any significant difference in one way ANOVA for *Diaptomus* ($F_{1,10} = 1.99$, $p = 0.189$) and *Cyclops* ($F_{1,10} = 0.54$, $p = 0.502$). In contrast, there were gender differences in $\delta^{13}\text{C}$ content for *Cyclops* ($F_{1,4} = 80.63$, $p < 0.001$), being females slightly more depleted (Table 7.1), whereas both sexes of *Diaptomus* had the same carbon isotopic signature ($F_{1,10} = 0.28$, $p = 0.611$).

Table 7.1. Averages and standard deviation (sd) of the stable isotopic composition of the pelagic zooplankton species of lake Redó and their survival range within the sampling period.

Species	Sex or stage	$\delta^{13}\text{C}$	sd	$\delta^{15}\text{N}$	sd	n	Data range
<i>Daphnia pulicaria</i>	1 st generation females	-31.8	0.9	-2.98	0.82	20	12/98 - 8/99
	2 nd generation females	-27.9	2.9	-2.99	0.47	12	9/99 - 12/99
<i>Diaptomus cyaneus</i>	females	-31.8	2.4	-0.77	0.51	6	8/99 - 9/99
	males	-31.2	2.0	-0.43	0.28	6	8/99 - 9/99
	nauplia	-32.9		-5.32		1	6/99
<i>Cyclops abyssorum</i>	females	-28.6	0.6	0.69	0.27	16	12/98 - 7/99
	males	-27.1	0.1	0.83	0.13	3	1/99
	copepodites	-29.5	1.6	-0.22	0.29	17	8/99 - 12/99

Seasonal variability and trophic position as inferred from stable isotope data

The plot of the $\delta^{13}\text{C}$ signatures through the whole seasonal cycle (Fig. 7.1a) showed that in both long-lived species, the adults had a much lower variability than their respective descendants. Between the two species, the herbivorous *Daphnia* was the species with the highest $\delta^{13}\text{C}$ oscillation. The adults of the calanoid copepod *Diaptomus* changed their $\delta^{13}\text{C}$ from July to August following exactly the same $\delta^{13}\text{C}$ than *Daphnia*. Nauplia of *Diaptomus* had the same carbon signature than *Daphnia* in the month they coexisted.

The seasonal changes in $\delta^{15}\text{N}$ of the different species (Fig. 7.1b) showed a much lower variability than $\delta^{13}\text{C}$. However, *Daphnia* also had a higher variability than *Cyclops*. *Diaptomus* adults had a similar isotopic composition to *Cyclops* copepodites. Nauplia of *Diaptomus* had also almost the same isotopic signature than *Daphnia*.

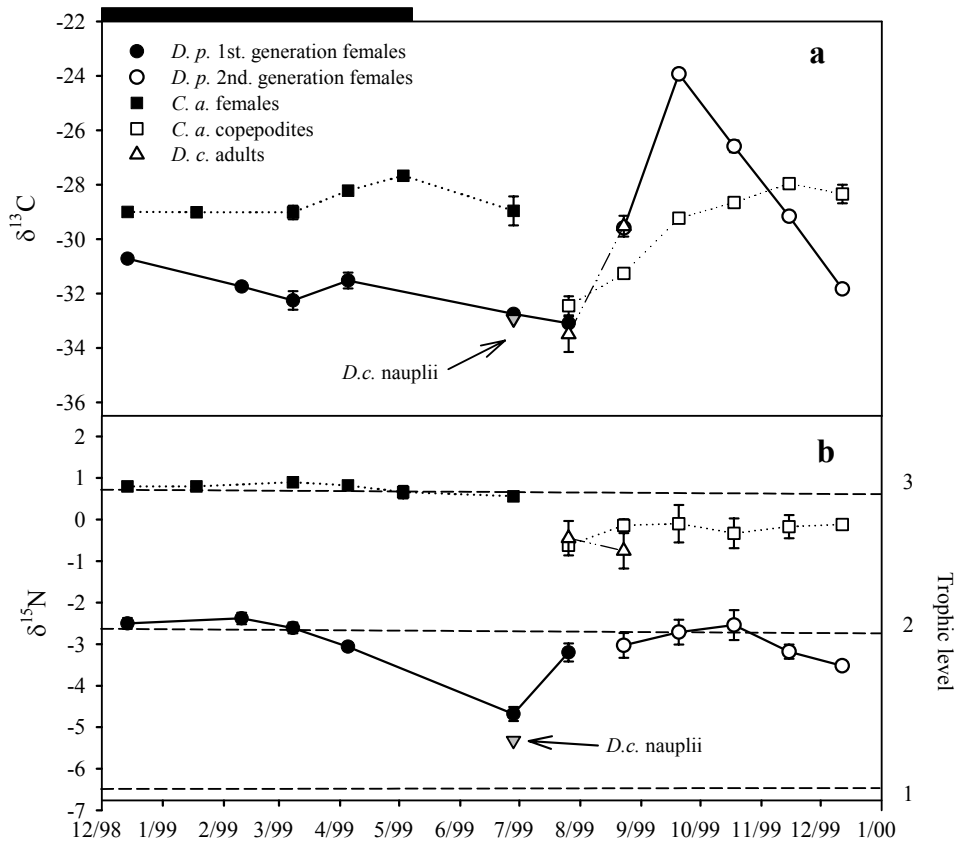


Fig. 7.1. Seasonal changes of the carbon isotopic composition (a) and nitrogen isotopic composition (b) of the pelagic crustacean species of lake Redó, *Daphnia pulex* (*D. p.*), *Cyclops abyssorum* (*C. a.*) and *Diaptomus cyaneus* (*D. c.*). The duration of the ice cover is indicated by the black line on the top of the figure. b) Theoretical trophic levels estimated under the assumption of 3.4‰ per trophic level are shown as reference by horizontal dotted lines.

In order to describe the trophic positions of the two copepod species of lake Redó we used the signature of the *Daphnia* as the best indicator of the base of the food chain, since it has been shown to be a better estimate of the base of the food chain (Cabana & Rasmussen 1996; Vander Zanden & Rasmussen 1999; Post 2002). The average fractionation factors for the two copepod species were calculated for every month when the two species coexisted, in order to avoid temporal inconsistencies. The average fractionation of nitrogen ($\Delta\delta^{15}\text{N}$, Δ denotes the change in isotope ratio between diet and consumer) for *Cyclops* was +3.5‰ (ranging from -5.3‰ to -2.2‰), while *Diaptomus* $\Delta\delta^{15}\text{N}$ was +2.7‰ (ranging from -3.0‰ to -2.4‰). Therefore, following $\delta^{15}\text{N}$ signature *Cyclops* adults were at the top of the zooplankton food chain potentially feeding on *Daphnia*, while *Diaptomus* adults were almost one trophic step above the Daphnid, but slightly more depleted than *Cyclops*. The average $\Delta\delta^{15}\text{N}$ of *Cyclops* and *Diaptomus* was

0.4‰. Calculation of $\Delta\delta^{13}\text{C}$ resulted in an enrichment of *Cyclops* of -1.4‰ with respect to *Daphnia*, although its fractionation had a very wide range (from -4.5‰ to +5.3‰). Similarly, *Diaptomus* had a $\Delta\delta^{13}\text{C}$ of -0.3‰ with respect to *Daphnia*. Following $\Delta\delta^{13}\text{C}$ results one would predict that *Diaptomus* was actually feeding on *Daphnia*, while *Cyclops* would not. These wide fractionation factors, and especially the disagreement between nitrogen and carbon isotopes suggested that a closer examination to the factors affecting the intra-specific variation was required.

Influence of the biochemical composition on stable isotope signatures

In order to determine the possible sources of these high isotope variations we analysed the concentration of lipids and chitin for each species. Lipids are known to have a more depleted signature than the whole body carbon (DeNiro & Epstein 1977), while chitin has a very depleted signature in comparison with the whole body nitrogen (Macko et al. 1990). These isotopic differences are related with their specific metabolic pathways. Calculation of the lipid and chitin free fraction was done by the following equations:

$$\delta^{13}\text{C}_{\text{LF}} = \delta^{13}\text{C}_{\text{SA}} - (M_{\text{LP}} * f_{\text{LP}}) \text{ and } \delta^{15}\text{N}_{\text{CTF}} = \delta^{15}\text{N}_{\text{SA}} - (M_{\text{CT}} * f_{\text{CT}})$$

where LF stands for lipid free fraction, SA is the isotopic composition of the sample, M_{LP} and M_{CT} are the mass fractions of lipids and chitin and, f_{LP} and f_{CT} are the isotopic discrimination of lipids and chitin respectively. In this study we used the lipid fractionation of zooplankton inhabiting a similar temperature regime ($f_{\text{LP}} = -3$); (Kling et al. 1992) and the chitin fractionation measured to be $f_{\text{CT}} = -9$ (Macko et al. 1990).

Subtraction of the lipid isotopic signature resulted in an average $\delta^{13}\text{C}$ enrichment of +1.4‰ for *Cyclops*, of +1.5‰ for *Daphnia* and +0.8‰ for *Diaptomus*. The lipid-free fraction had an overall similar seasonal variability in the isotopic content (Fig. 7.2a), and therefore the major seasonal changes were not attributed to biochemical changes. However, part of the isotopic variability of *Cyclops* females and adults of *Diaptomus* was attributable to changes in their lipid content. The adult females of *Cyclops* increased their $\delta^{13}\text{C}$ from April to July, which was attributable to a decrease in their lipid content (Fig. 7.2a). The adults of *Diaptomus* decreased their total lipid content from 45% of dry weight to only 5.5% from July to August. This decrease explained a 30% of their isotopic change (Fig. 7.2a). The effect of the lipids on the $\Delta\delta^{13}\text{C}$ was almost negligible for *Daphnia-Cyclops* (Fig. 7.2b) as well as for the fractionation between *Daphnia* and *Diaptomus* (data not shown).

Subtraction of the chitin fraction resulted in all species in a slight increase of their $\delta^{15}\text{N}$ for all the species (ranging between +0.3‰ and +0.5‰). Chitin composition was very constant for the three species, and therefore correcting for chitin changes had a negligible effect on the seasonal variability of the nitrogen isotope (Fig. 7.2c; *D.cyaneus* data not shown). Similarly, the calculated trophic fractionations were unaltered for the three species (Fig. 7.2d for *Daphnia-Cyclops*; *Daphnia-Diaptomus* data not shown).

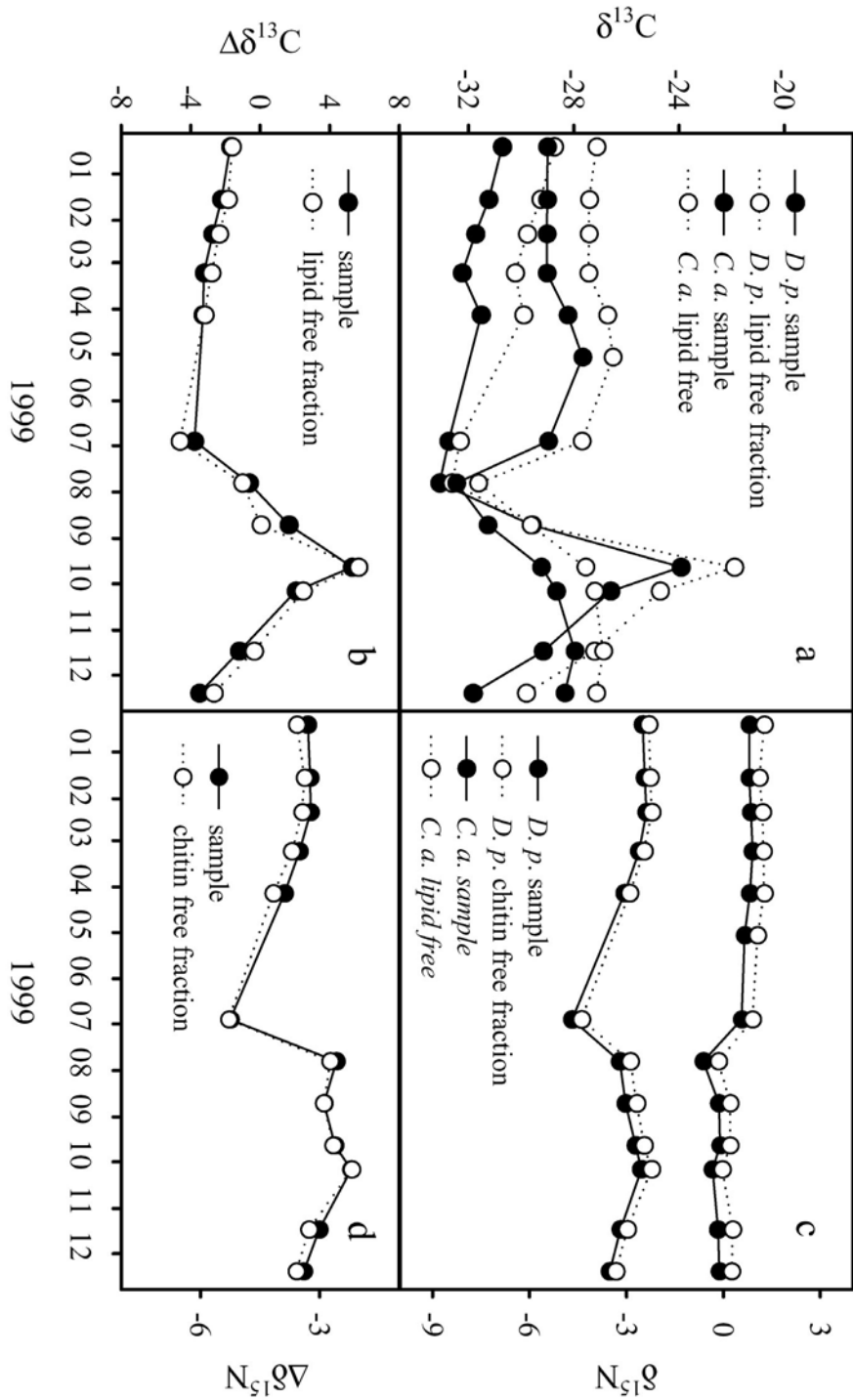


Fig. 7.2. Seasonal changes of (a) the total body and lipid free carbon isotopic composition of *Daphnia pulex* (*D. p.*) and *Cyclops abyssorum* (*C. a.*) and of (c) the total body and chitin free nitrogen isotopic composition of the same species. Their isotopic fractionation is also shown for carbon (b) and nitrogen (d).

Ontogenic isotopic shifts

Cyclops copepodites increased their isotopic composition throughout the summer, which coincided with a progressive increase in the dry weight of the different stages found in each consecutive month (Table 7.2). On the other hand, *Daphnia* juveniles did not have a clear temporal trend in their isotopic composition, being more depleted in September for carbon or in September to October for nitrogen. This pattern coincided with the average population dry weight (Table 7.2), since *Daphnia* juveniles had the highest proportion of youngest individuals in September, increasing their average weight until December. Expressing their isotopic composition in terms of their relative growth (Fig. 7.3a, b), shows that *Daphnia* second generation became more depleted in their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ with growth, indicating that isotope enrichment was related with its ontogeny (Fig. 7.3a, b). In contrast, *Cyclops* copepodites became more enriched in $\delta^{13}\text{C}$ when growing (Fig. 7.3c), but had no $\delta^{15}\text{N}$ relation with growth (Fig. 7.3d). In order to compare the rates of the isotopic shifts observed in the two species, we fitted a power dilution curve (Fry & Arnold 1982c) to each element (Fig. 7.3). For *Daphnia* $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, the depletion rate (as indicated by the exponential of the regression) was 0.14 and 0.19‰ g⁻¹ respectively, indicating that the isotopic depletion was very similar. *Cyclops* $\delta^{13}\text{C}$ had also a very good fit of the dilution curve ($r^2 = 0.99$, $p < 0.001$) and had a similar dilution rate than *Daphnia*, despite it had the opposite sign (-0.12‰ g⁻¹). In contrast, its $\delta^{15}\text{N}$ had no apparent relationship with growth ($p = 0.154$).

Table 7.2. Ontogenic changes in the population average dry weight \pm standard error of the juveniles of *Daphnia pulicaria* and *Cyclops abyssorum* born during summer 1999 in lake Redó. The average maximum length (*D. pulicaria*) or the stage dominating each month (*C. abyssorum*) are also included. When more than two stages were present their order of importance in the total sample abundance is indicated by >.

Species	Sampling date	Length (mm) or stage	Dry weight ($\mu\text{g Ind.}^{-1}$)
<i>Daphnia pulicaria</i>	23/8/1999	1.497	22.3 \pm 1.2
	20/9/1999	1.332	14.6 \pm 4.1
	18/10/1999	1.502	15.0 \pm 0.0
	15/11/1999	1.737	34.6 \pm 4.9
	12/12/1999	1.947	51.9 \pm 3.8
<i>Cyclops abyssorum</i>	26/07/1999	CII	1.2 \pm 0.1
	23/08/1999	CIII	1.7 \pm 0.1
	20/09/1999	CIII>CIV	3.0 \pm 0.3
	18/10/1999	CIV>CV	3.3 \pm 0.8
	15/11/1999	CV>CIV	3.6 \pm 0.2
	12/12/1999	CV>CIV	3.6 \pm 0.3

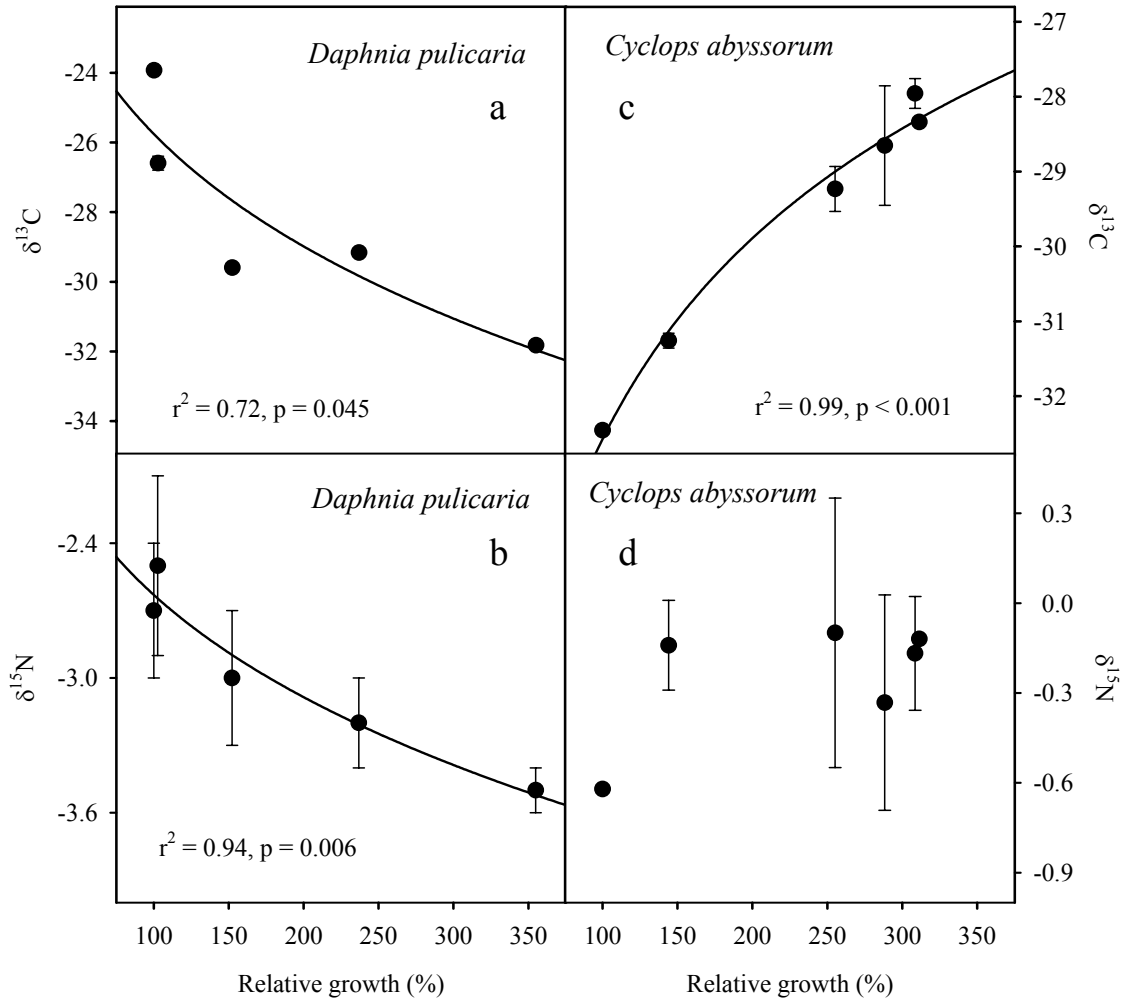


Fig. 7.3. Ontogenic changes of the *Daphnia pulicaria* carbon (a) and nitrogen isotope (b) composition. And *Cyclops abyssorum* carbon (c) and nitrogen isotope (d) composition. Relative growth was calculated as the percentage proportion to the lowest weight individuals.

In order to determine if the isotopic composition of *Cyclops* could have been a result of feeding on *Daphnia*, we applied an isotopic shift model based on growth rate of copepodites. The model simply adds ingested carbon and nitrogen from food (*Daphnia* in this case) proportionally to the average population growth rate of every time step in order to allow for isotopic food variability (Parker et al. 1989):

$$\delta_{\text{pred } t} = \delta_{\text{pred } t-1} W_{t-1}/W_t + (\delta_{\text{food}} + f_a) (1 - W_{t-1}/W_t)$$

where δ_{pred} is the carbon or nitrogen isotopic composition of the predator, W is the dry weight of the individual, δ_{food} is the average isotopic composition of food between time t and $t-1$ and f_a is the trophic fractionation (+3.4‰ for nitrogen or +0.5‰ for carbon). The model successfully predicted the $\delta^{13}\text{C}$ of *Cyclops* copepodites (Fig. 7.4a), explaining 99% ($p < 0.001$) of the measured isotopic change. However, this was not the case for $\delta^{15}\text{N}$, where predicted isotopic change only accounted for 60% ($p = 0.07$) of the measured nitrogen. This discrepancy between carbon and nitrogen suggested that for nitrogen, there was an additional fractionation mechanism in addition to those of growth. Measurement of protein content showed that *Cyclops* had a higher protein content than *Daphnia* in all months (average dry weight percentage \pm standard error was 43.6 ± 1.6 and 27.0 ± 1.1 for *Cyclops* and *Daphnia* respectively). Therefore, if *Cyclops* would be feeding on *Daphnia*, it would face a protein limitation. In this case, the residuals of the fitted model should be related with the protein content of the food, or alternatively to the protein quality of the food. Comparison of the residuals with the protein content of *Daphnia* showed that there was no relationship between both data (Fig. 7.5a), whereas the protein quality (measured as the amino acid Euclidean distance between the amino acid composition of *Cyclops* copepodites and *Daphnia*) accounted for most of the residuals variation (90%, $p = 0.004$, Fig. 7.5b). Therefore in the case of *Cyclops*, it seems that the disagreement between carbon and nitrogen ontogenic changes may be explained by a specific fractionation force acting upon nitrogen metabolism which depends on the similarity of the amino acid composition between the predator and the resource.

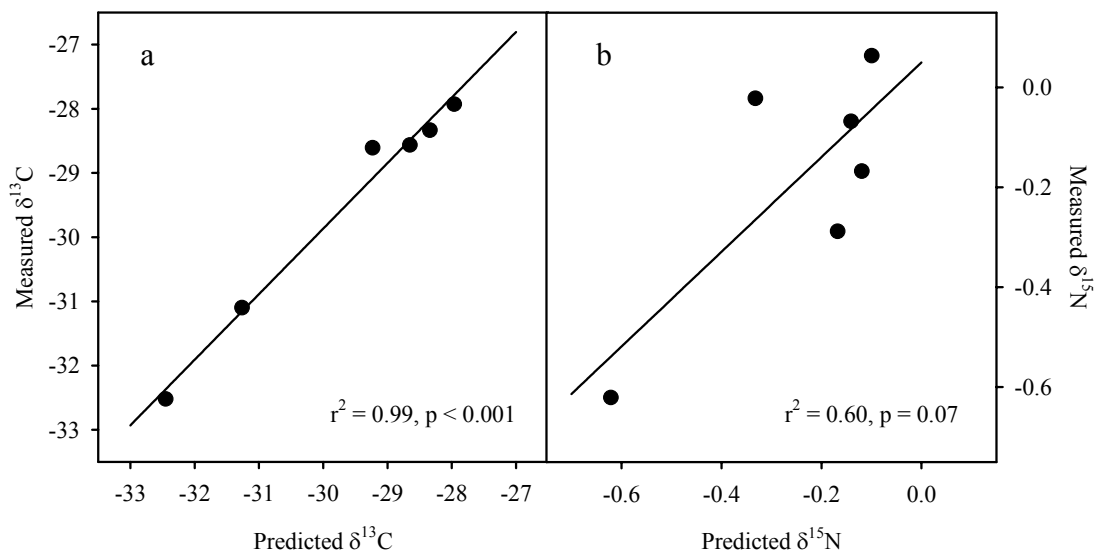


Fig. 7.4. Comparison between the measured carbon (a) and nitrogen (b) isotopic composition and modelled by an isotopic enrichment model for *Cyclops abyssorum* copepodites of lake Redó during summer 1999 feeding on *Daphnia pulex*. See text for model specifications.

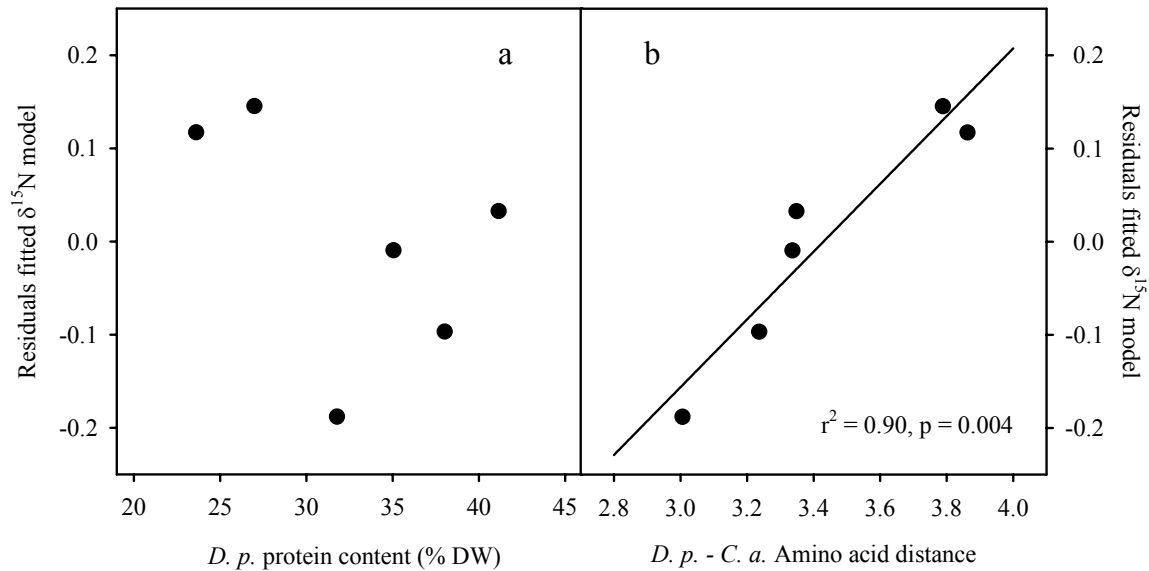


Fig. 7.5. Relationship between the residuals of the linear fit between the modeled and measured isotopic composition of *Cyclops abyssorum* (*C. a.*) copepodites shown in Fig. 7.5b and the protein content of *Daphnia pulex* (*D. p.*) (a) or the total amino acid distance between both species (b). Each point corresponds to each consecutive month from July to December 1999.

Discussion

Biochemical influence

The influence of changing the relative biochemical composition had different effects depending on the compound. The concentration of chitin was very constant in the three species and therefore the chitin corrected $\delta^{15}\text{N}$ was very similar to the non-corrected measures. In contrast, subtraction of the lipid fraction had a much more pronounced effect on the seasonal $\delta^{13}\text{C}$ of each species. In both adults of *Cyclops* and *Diaptomus*, a change of lipid content explained a significant part of the seasonal isotopic variability. A similar result was found by Kling et al. (1992), who found that lipid changes explained up to 30% of the zooplankton species variation in different arctic lakes. Similarly to *Cyclops*, *Daphnia* over-wintering females decreased their lipid content throughout winter. However, in the case of the cladoceran, correcting for lipid content had an opposite effect. In this species, depletion of $\delta^{13}\text{C}$ was not related with biochemical changes.

Despite this relatively high influence of the lipid composition on the isotopic enrichment, there was a null effect on the $\Delta\delta^{13}\text{C}$, particularly on the *Daphnia* – *Cyclops*.

This was probably related to the synchronisation of both life-cycles (Gliwicz et al. 2001). The adults of both species accumulated lipid reserves during winter, for a later use on reproduction Chapter 5. Summer juveniles of both species had a relatively low lipid content, increasing with their respective population growth. Therefore, lipid content changed similarly in both species, which was the cause of its null effect on fractionation.

Changes in adult isotopic composition

The adults of the two long-lived species presented a difference in their isotopic composition variability. *Cyclops* did not change its $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ when corrected for its lipid content, while *Daphnia* decreased its isotopic composition. A similar result was found by Grey (2000) in an enrichment experiment. He found that *Cyclops* did not equilibrate their body isotopic composition with those of the experimental food, while *Daphnia* did rapidly equilibrate its body content. He concluded that this was very likely related with differences in the molting characteristics of copepods and cladocerans. While copepods stop molting once they are adults, cladocerans continue molting even when they are adults with the production of each egg clutch (Tessier et al. 1983). *Daphnia* females of lake redo did not start their reproduction until the ice cover melted (Chapter 5). Reproductive females were more depleted in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ than non-reproductive females ($F_{1,18} = 8.5$, $p = 0.009$ and $F_{1,18} = 22.1$, $p < 0.001$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ respectively). Furthermore, non-reproductive females had a more constant isotopic composition (figures 7.1 and 7.2). Therefore it is very likely that only reproductive *Daphnia* females are truly showing the real variability occurring in its resources. In contrast, non reproductive *Daphnia* females and *Cyclops* adults isotopic composition did not show the composition of their diet but a 'summary' along their ontogeny. This conclusion is further supported by several experimental studies that demonstrated that in poikilotherms inhabiting low temperature habitats such as alpine lakes growth had a much greater importance than maintenance metabolism on their isotopic composition (Hesslein et al. 1993; Herzka & Holt 2000; Grey 2000).

The almost identical isotopic composition between males and females of both copepod species would have been interpreted in conventional isotopic arguments as they had consumed food of identical isotopic composition. However, following the above reasoning, we conclude that the identical isotopic composition of males and females is a result of a common ontogeny, feeding in similar diets. In fact, copepod males and females have an almost identical morphology during their ontogeny, and it is not until they moult from copepodites V to adults that they obtain these clear morphological differences. The elements necessary for changing to this last stage are therefore obtained during stage V, when they are morphologically similar. The answer to whether males and females feed on similar resources remains therefore open for future studies.

Daphnia ontogenic isotopic changes

The progressive depletion in the isotopic composition of the *Daphnia* generation born during summer 1999 was closely related with growth (Fig. 7.3a, b), indicating an onto-

genic shift in isotopic composition. This pattern could be explained either due to an isotopic shift in the food, or alternatively to a change in the diet throughout ontogeny. *Daphnia* juveniles have been shown to preferably feed on bacteria in those species which increase their mesh size with increasing body size (Lampert 1974; Brendelberger 1991). Furthermore, *Daphnia pulicaria* has been shown to be one of these species (Brendelberger & Geller 1985), therefore juveniles of *Daphnia* preferentially feed on bacteria while the adults on algae. Therefore, a possible explanation for this ontogenic isotopic shift would be a diet change from bacteria to algae. An alternative explanation would be a diet change from allochthonous matter to seston. The $\delta^{13}\text{C}$ of the dominant vegetation of lake Redó catchment was -24.9‰ , a value very similar to those of the smaller stages of *Daphnia* (-24.1‰), while seston of lake Redó was found with an average isotopic composition of -28.5‰ below the thermocline (where algal concentration is higher) (Catalan et al. 2004). Since seston is found to be depleted respect to plankton about 3‰ in low phosphorous lakes (del Giorgio & France 1996), algae in lake Redó could be estimated to be -31.5‰ , a value identical to those of the larger *Daphnia* in this study.

Cyclops ontogenic isotopic shift

Contrasting with *Daphnia* juveniles, the isotopic enrichment of *Cyclops* differed markedly in the two isotopes. Carbon enrichment was well predicted by the dilution model, showing that despite the higher variability of the isotopic signature in *Daphnia*, the isotopic composition of *Cyclops* copepodites could be explained if we assume that they were feeding on *Daphnia*. *Cyclops* copepodites have been previously described to predate on adults of Daphnids or even enter the egg pouch and feed on *Daphnia* eggs (Gliwicz & Umana 1994; Gliwicz & Stibor 1993). In this study we also found *Cyclops* copepodites occasionally within the egg pouch of several *Daphnia*. However, to what extent *Daphnia* represent a significant food resource for *Cyclops* copepodites it is unknown. The results derived from isotopic carbon reveal that the feeding on *Daphnia* is probably very common for copepodites.

However, the $\delta^{15}\text{N}$ was not adequately described by the dilution growth model. The residuals were correlated with the differences between the amino acid composition of *Cyclops* and *Daphnia*. This result suggested that the relatively wide isotopic fractionation between *Cyclops* and *Daphnia* could be related with the differences in the amino acid composition between the copepod and the cladoceran. Comparison of the amino acid similarity between *Cyclops* copepodites and *Daphnia* with their isotopic fractionation showed that they had a very good correlation ($r^2=0.92$, $p = 0.002$). This result is consistent with previous findings that nitrogen fractionation depends on food quality (Adams & Sterner 2000; Fantle et al. 1999) and emphasises the importance of the amino acid composition in animal nutrition.

It has been shown that copepods have almost half the phosphorus content of cladocerans (Hessen & Lyche 1991; Andersen & Hessen 1991), therefore, it seems unlikely that copepods would face a phosphorous limitation when feeding on cladocerans. The fact that in *Cyclops* both isotopes have a clearly different fractionation, while in *Daphnia*, both isotopes show a very similar ontogenic shift can be interpreted as an amino

acid limitation of *Cyclops* growth. It has been suggested that predators face primarily a nitrogen limitation, since herbivores generally have a relatively higher carbon content (White 1993). Our results therefore are in agreement with this hypothesis, and show that this limitation is basically an amino acid limitation.

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Conclusions

8. Concluding remarks

Many ecological features of the three crustacean species inhabiting the plankton of Lake Redon were species specific, as expected from their relatively large phylogenetic distance, and therefore to their very old evolutive divergence. For the same reason, the two copepods were more similar to each other than to the cladoceran. This similarity was reflected primarily on their morphological and developmental characteristics, but also on their average elemental and biochemical composition (Chapter 5, 6 and Table 8.1). Although this may seem a logic consequence, it has been poorly recognised in the literature. For example, copepods have higher nitrogen content than cladocerans (Chapter 5), because of their higher protein content. In agreement with that, I found that the copepods of Lake Redon were limited by nitrogen availability and *Daphnia* by phosphorus. To establish a relationship between the species elemental and biochemical composition and their phylogeny may be a step forward in understanding the evolutionary ecology of crustacean zooplankton. Identifying the limiting substances for each species has been suggested as a fundamental step for understanding their ecological characteristics (White 2001). The stoichiometric approach is a tool in that sense, and has traditionally focused on the main elements forming the organism's body (Sterner & Elser 2002). A drawback of this reductionist approach is to how to upscale from elements to organism characteristics, which could be better interpreted in ecological terms (Brett 1993). The major biochemical compounds have a much more clearly defined functional role in each organism, which can be more easily related with species morphological characteristics. For this reason we have paid special attention in establishing a link between elemental and biochemical composition (Chapter 4). Application of this combined approach to many species inhabiting different types of environments will be an interesting challenge for future studies.

In contrast with the species characteristics, which were related with their phylogeny, there were several life history traits, which were shared by the three species or by *Daphnia* and one of the two copepods. The three species had a food-independent reproduction mode, a survival mechanism for taking full advantage of the short productive period (Chapter 2). However, amongst the three species, *Cyclops* and *Daphnia* survived below the ice-cover waiting for the ice-cover melting, while *Diaptomus* remained in the sediment in the form of diapausing eggs. *Daphnia* was the only species using both survival strategies every year. Which might be the advantage of *Daphnia* for surviving below the ice-cover? Within the five years studied, there was a considerable variation in the ice-cover melt timing (almost two months). Emergence from diapause at the appropriate time requires therefore other stimuli than photoperiod alone. In the case of *Diaptomus* emergence cues are photoperiod and temperature (Hairston & Kearns 1995). In contrast, the emergence cues for *Daphnia* are not so clearly defined. Within cladocerans, there seems to be considerable variation among genotypes, populations and species in response to these cues (Caceres & Schwalbach 2001). For example, in Lake Washington, *Daphnia pulicaria* (the same species than in Lake Redon) seems to have an independent cue other than temperature for diapause termination (Winder & Schindler 2004). Progressive climate change over the last 20 years has advanced the timing of the spring bloom; however, *Daphnia* has not been able to respond to this change since it uses preferently diapausing eggs as over wintering strategy. In contrast, the rotifer *Ker-*

atella cochlearis was able to survive during winter in the water column and therefore advanced its reproductive peak following exactly the spring overturn. A similar response could be the reason for the survival of *Daphnia* in Lake Redon, surviving below the ice cover might be the best choice for being at the precise moment when the ice-cover melts. A further advantage might be related with their continuous growth. The females surviving below the ice-cover are close to their senescence time, and therefore are the largest females possible. *Daphnia*'s mother body size is directly related with the size of the neonates (Lampert 1993), and the size of neonates is directly related with their resistance to starvation (Gliwicz & Guisande 1992). Therefore, neonates born from over wintering females would probably be fitter than neonates born from ehippia, which in Lake Redon are produced usually during autumn. The disadvantage that Lake Redon *Daphnia* face in surviving below the ice cover is the risk of especially long ice-cover duration, when most females could die of senescence without arriving to the spring overturn. This could have been the case during the winter of 1983/1984, when the ice-cover melted at the beginning of July (Catalan 1987), and almost no *Daphnia* was found in the water column during the following ice-free period (Felip 1997).

Another characteristic that differed between *Diaptomus* and both *Cyclops* and *Daphnia* was sexual size dimorphism, which was more pronounced in the two latter species than in the former. This difference in size was even more relevant when expressed in terms of their dry weight (Table 8.1). *Diaptomus* dimorphism by weight was similar than by size, whereas in the other two species females had more than twice the weight of the males. Furthermore, both males of *Daphnia* and *Cyclops* did not change their elemental content with reproduction and survived for a very limited period, whereas males of *Diaptomus* followed the same changes and survived for the same period than females. These contrasting differences between the calanoid and, the cyclopoid and the cladoceran is not particular for these three species, but is general amongst the three taxonomic groups (Gilbert & Williamson 1983).

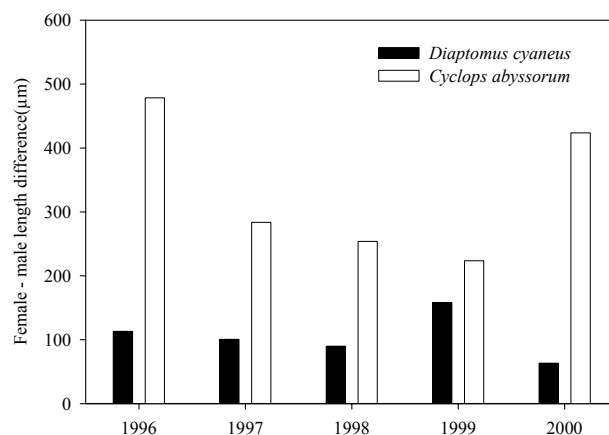


Fig. 8.1. Sexual size dimorphism in *Cyclops abyssorum* and *Diaptomus cyaneus*. Length difference between males and females.

Table 8.1. Main ecological characteristics of the three pelagic crustacean species studied from Lake Redon. Abbreviations are Naup., naupliar stage; Emb., embryo stage; Cop., copepodite stage; Juv., juvenile stage and Ad., Adult stage. C, carbon; H, hydrogen; P, phosphorus; PP, sestonic phosphorus. PR, protein; LP, lipids; CT, chitin; CH, carbohydrates.

Species	<i>Cyclops abyssorum</i>	<i>Diaptomus cyaneus</i>	<i>Daphnia pulicaria</i>
Taxonomic position	Class Maxillopoda	Class Maxillopoda	Class Brachiopoda
Order	Cyclopoida	Calanoida	Anomopoda
Development	Egg+6Naup.+5Cop.+1Ad.	Egg+6Naup.+5Cop.+1Ad.	Egg+Emb. + n Juv.+n Ad.
Growth	stop at adults	stop at adults	increase every moult cycle
Stoichiometric composition	C:N:P:S (by atoms) 3.30:31:1:0.6	313:26:1:0.9	214:47:1:0.4
Biochemical composition	PR:LP:CT:CH (% by weight) 43.6:51.6:3.9:3.4	58.7:35.5:5.4:2.5	27:53.5:2.2:4.4
Sexual size dimorphism	female/male length 1.24	1.09	1.27
	female/male dry weight 2.18	1.16	2.83
Life-history traits	Lifespan (years) 1	0.3	1
	Cohorts per year 1	1	1
Reproduction	Type sexual	sexual	asexual >> sexual
	Diapausing stage copepodites?	eggs	ephippia
	Frequency of diapause rare / unknown	always	occasional
	Reproductive period ice-free period	spring overturn	ice-free period
	Over-wintering mode adults	eggs	adults >> ephippia
	Food independence yes	yes	yes
	Stored element C, H	C, H, P	C, H, P
Food	Habit carnivorous	carnivorous/omnivorous	herbivorous
	Ontogenic changes yes	?	yes
	Limiting element N	N	P
Maximum size	Density-dependent yes	no	yes
	Temperature-dependent yes	no	no
	Food quality-dependent no	yes	yes, through abundance
Population regulation	Predators fish?	no?	fish + <i>Cyclops</i>
	Parasites <i>Charactium sp.</i>	no	no
	Resource quality amino acid composition	PP	PP

A possible explanation for these differences is in the different reproduction investment of calanoids, which require re-mating in almost every egg clutch in order to maintain their fertility. The result that males and females covary in their inter-annual size variability (Fig. 8.1) suggests that both sexes share the reproductive effort, which would prevent a divergence in sexual size dimorphism. In contrast, in *Cyclops*, the difference between female and males size has a large inter-annual variability (Fig. 8.1), which is mostly due to females. In this species, therefore, females show a much more pronounced phenotypic response to environmental or density-dependent variation. If males are only required for a very short period of time, it seems obvious that evolution has reduced males to the minimum size possible, and thus leaving more resources for females. Males are in fact of the same size and weight than copepodites V, therefore it is logic that they show a reduced response to environmental conditions.

Conclusions

The seasonal and inter-annual limnological features characteristics of Lake Redon have been described. Following these limnological features, the inter-annual population dynamics of the crustacean zooplankton have been studied during a five-year period. Several methodologies included in the ecological stoichiometry framework were applied to a detailed study of one of the five annual cycles. Analysis of elemental, biochemical and isotopic composition of the three species, complemented with description of their life-cycles, and different life history traits provided a detailed characterisation of the ecological features of the species and the factors limiting their dynamics. In the following paragraphs, an outline of the main conclusions reached is provided.

1. Seasonal and inter-annual variability of Lake Redon and its crustacean zooplankton assemblage

1. The lake water was very ion-poor (mean annual conductivity $12 \mu\text{S cm}^{-1}$); however, ion concentrations in the lake were higher than in the precipitation, the differences being due mainly to Ca^{2+} originating in the catchment.
2. NH_4^+ was the main ion in the precipitation, with an average concentration of $17 \mu\text{M}$, whereas in the lake it was always below $3 \mu\text{M}$. However, the concentration of dissolved inorganic nitrogen always exceeded that of soluble reactive phosphorus by two or three orders of magnitude, thus the latter is expected to be the limiting nutrient for phytoplankton growth.
3. Four main production episodes were identified, occurring during spring and autumn overturn, in the upper hypolimnion during summer stratification, and under the ice at the beginning of the ice-covered period. The highest chlorophyll *a* concentrations ($1.2 - 2.2 \mu\text{g l}^{-1}$) were attained during spring overturn; concentrations of chlorophyll *c* were high during both spring and autumn overturn, while chlorophyll *b* was comparatively more relevant in the upper hypolimnion during the stratification period.
4. The end of the ice-covered period varied in almost two months from year to year, and was the key limnological feature characterising the timing of the life-cycles in the three species. Both copepods produced nauplii at the time the ice melted, while the timing of *Daphnia* maturation size was highly correlated with the ice-cover melting day.
5. The average inter-annual abundance of *Cyclops* and *Daphnia* was negatively correlated with their respective maximum size and in the case of *Daphnia* with maturation size, suggesting that there was an intraspecific density-dependent interaction.

6. Food quantity was not related with any of the population and life history traits of the three species.

7. Comparison of the stoichiometric composition of the three species and those of seston revealed that *Daphnia* had a higher phosphorus proportion than seston, and the two copepods a higher carbon and nitrogen than *Daphnia* and seston.

8. *Daphnia* annual production was highly correlated with sestonic phosphorus. It is suggested that *Daphnia* was limited by phosphorus availability. However, *Daphnia* abundance was negatively correlated with *Cyclops* biomass, suggesting that predation of the latter on the former might also be a relevant factor.

2. Seasonal variability in the elemental, biochemical and isotopic composition of zooplankton

9. The stoichiometric composition of the main biochemical compounds of 43 species of freshwater and marine zooplankton revealed high constancy. No significant differences were found between marine and freshwater species or between any of the taxonomic groups considered (amphipods, copepods, cladocerans, euphausiids and mysids) for most of the main biochemical compounds (triacylglycerol, wax esters, phospholipids and proteins). Therefore, inter- and intraspecific stoichiometric variability is a result of differences in the proportions of the main biochemical compounds.

10. The elemental composition of *Calanus finmarchicus* was estimated from the composition of major biochemical compounds. Comparison of measured and estimated elemental composition revealed that almost all of the measured carbon, nitrogen and phosphorus were accounted for, except for 26.3% of the phosphorus, related to the unmeasured acid-soluble fraction of nucleotides or phosphate.

11. The relative importance of the main biochemical compounds to zooplankton elemental composition was derived by combining the measured ranges of the main biochemical compounds gleaned from the literature and their average stoichiometric composition. Almost all carbon content was accounted for by lipids and proteins (from 82 to 98% of the total carbon content), while proteins accounted for 46-98% of total nitrogen. Phosphorus was the element with highest variability in terms of which biochemical compound contributed the most to its total pool. In most cases nucleic acids were the main phosphorus pool, followed by phospholipids and, to a lesser extent, nucleotides.

12. In the three species, reproduction was the main cause of elemental variability, whereas growth had little influence. Females of the three species lost from 32 to 48% of their initial individual C and H content during reproduction, which corresponded to a similar decrease in their lipid and carbohydrate content. N content did not change in any of the three species; nor did protein and chitin content. *Daphnia* and *Diaptomus* lost 35% and 56%, respectively, of their initial P content during reproduction, while *Cyclops*' P content did not change.

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- 13.** Males of *Cyclops* and *Daphnia* lived a much shorter time than females and had a more constant elemental composition. *Diaptomus* males showed the same life span and elemental changes as females. Differences in males' elemental variability between the three species can be interpreted as due to greater mating requirements in calanoids than in cyclopoid copepods and cladocerans.
- 14.** Variability in the elemental composition of these species is thought to be general of food-independent reproduction species, which have a non-homeostatic elemental composition throughout their life-cycle.
- 15.** Inter-species differences in amino acid composition were three-fold larger than intra-specific variation (75.3 and 24.7%, respectively). The two copepods had a more similar composition to each other than the cladoceran irrespective of their seasonal and ontogenic changes.
- 16.** Within species, *Daphnia* had a low proportion of the amino acid variability (10.9%) related to generations, while seasonal changes accounted for 45.5%. *Cyclops* had a similar adult seasonal variability (49.5%) and 19.3% was attributable to inter-stage variations. *Diaptomus* had the highest adult seasonal change (84%).
- 17.** There were not differences in amino acid composition between males and females in any of the two copepod species, despite of the sexual dimorphism.
- 18.** The results confirm the hypothesis of high homeostasis in amino acid composition in crustacean zooplankton.
- 19.** Although the three species found had distinct isotopic signatures, being the two copepods one trophic level above the cladoceran, there were high intra-specific isotopic variations.
- 20.** Isotopic changes associated with the biochemical composition were more important in the lipid fraction than in chitin, which had a negligible effect on the total isotopic composition. Lipids accounted for up to 30% of the $\delta^{13}\text{C}$ variability in the adults of the two copepods, although no effect on the seasonal isotopic fractionation between the copepods and *Daphnia* was found.
- 21.** *Daphnia* $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ shifted from an enriched to a more depleted signal, associated with an ontogenic change in feeding from bacteria or from allochthonous matter to seston.
- 22.** *Cyclops* copepodites also presented an isotopic shift from feeding on algae to *Daphnia*. This shift however, was different for carbon and nitrogen. Fitting an isotopic growth model showed that 99% of the carbon variation could be explained by the model whereas only 60% of the nitrogen. This low fit of nitrogen to the model was related to the difference in the amino acid composition between the copepodites and its resource, *Daphnia*. In fact, the nitrogen isotopic fractionation of the two species was directly related with their amino acid differentiation, which is interpreted as an evidence of consumer nitrogen limitation.

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