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Pour l'obtention du grade de

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Discipline : Biologie marine, Écologie, Écotoxicologie

Présentée par
Paul DAYRAS

"*Paracyclopina nana* : un petit copépode à fort intérêt en écotoxicologie et en aquaculture"

Dirigée par le Pr. **Sami SOUISSI**, Université de Lille, France

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Presented by
Paul DAYRAS

"*Paracyclopina nana*: a small copepod with a strong interest in ecotoxicology and aquaculture"

Supervised by Prof. **Sami SOUISSI**, University of Lille, France

Publicly defended on December 19, 2019 in Wimereux (France)

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DEDICATION

This thesis is dedicated to my partner Joffrey, my dad, my mom and my sister Marie, whose unwavering love, support and encouragement, despite the distance and the hard blows, held me day by day and inspired me to pursue and complete this research.

I love you.

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ABSTRACT

The cyclopoid copepod *Paracyclopina nana* plays a key role in the trophic chains of the aquatic environments of Eastern Asia. It has a small adult size (600 µm), a short life cycle, a high fecundity, and can be easily cultured under medium salinity (15 psu) and a wide range of temperatures. Its whole genome has also been recently sequenced, assembled and annotated. All these assets give it a very interesting double potential for current research: as a test organism for risk assessment associated with aquatic pollutants (bioindicator), and as a live prey in mass culture for the feeding of fish larvae in aquaculture.

In the framework of this PhD project, we aimed: (i) to test the productive and qualitative potential of *P. nana* in aquaculture in relation to the nature of the ingested microalgae diet; (ii) to establish the profile of *P. nana* as an ecotoxicological model through metal contaminant exposure tests.

The effects of seven different microalgal diets constituted by *Rhodomonas salina* (R), *Tisochrysis lutea* (T), and *Pavlova lutheri* (P) on *P. nana* productivity in culture were explored. The R+T and R diets induced the highest population growth and the greatest reproductive investment in ovigerous females. Those same diets also generated the highest total fatty acid content in copepods, and the highest total monosaccharide content has been found in copepods fed R+T+P. Overall results demonstrated that all the diets including *R. salina* lead to an increasing productivity of *P. nana*, and particularly when combined with *T. lutea* in a mixed diet.

Another study examined the effects of cadmium (Cd), copper (Cu), and nickel (Ni), on two subpopulations of *P. nana*. A first experiment conducted on a regular

P. nana culture showed a decreasing population growth but an increasing metal bioaccumulation in copepods. Cd was also more accumulated when it was alone than in the mixture with Cu and Ni, confirming the hypothesis of metal competition recently demonstrated in a calanoid copepod. A second experiment performed on a *P. nana* culture already exposed to a higher Cu concentration for several generations revealed a lesser impact on population growth and a lower metal accumulation in copepods. Increasing metal concentrations in the experimental water reflected the depuration happening in this metal-loaded population already acclimated to metal exposure.

Overall results are the first ones showing that *R. salina* is a suitable microalga for productive mass culture of *P. nana* for use as live food for marine fish larval aquaculture, and to investigate the parameters influencing the bioaccumulation capacity of *P. nana* in response to metals in contaminated aquatic ecosystems.

KEYWORDS

Aquaculture, bioaccumulation, copepod, ecotoxicology, microalgae diet, *Paracyclopina nana*

RÉSUMÉ
Résumé développé

Le copépode cyclopoïde *Paracyclopina nana* peuple les baies et estuaires des pays d'Asie Du Sud-Est (Japon, Taïwan et Corée du Sud). Constituant majeur du zooplancton, ce crustacé joue un rôle central et essentiel dans les chaînes trophiques marines. Comme la majorité des copépodes, il possède une alimentation à base de phytoplancton et constitue à son tour une source de nourriture pour de nombreux organismes planctivores marins, en particulier pour tous les stades larvaires, notamment les poissons. *P. nana* présente une petite taille au stade adulte (600 µm de longueur totale), un cycle de vie court (15 jours à 18°C), une fécondité élevée et peut être facilement cultivé sous une salinité moyenne (15 psu) et dans une large gamme de températures. Tous ces paramètres de vie particulièrement conciliants et prolifiques couplés à sa petite taille ont fait de *P. nana* un candidat de choix dans la recherche d'une espèce de copépode standard pour l'étude des milieux aquatiques. La recherche expérimentale sur les écosystèmes marins manque encore d'une espèce modèle présentant des paramètres de culture rapides, une utilisation facile en laboratoire et des réponses claires aux tests scientifiques. *P. nana* se démarque d'autant plus des autres espèces de copépodes que son génome entier a été récemment séquencé, assemblé et annoté. Depuis, l'espèce a été abordée comme un nouveau modèle génétique prometteur, notamment en se focalisant sur ses biomarqueurs d'intérêt, c'est-à-dire ses gènes spécifiques permettant d'identifier directement un processus physiologique particulier. Les changements d'expression des biomarqueurs de ce copépode face aux changements de ses paramètres environnementaux,

principalement les expositions aux contaminants, peuvent ainsi être étudiés. Tous ces atouts confèrent à *P. nana* un double potentiel très intéressant pour les recherches en cours : en tant qu'organisme d'essai pour l'évaluation des risques associés aux polluants aquatiques (bioindicateur), et en tant que proie vivante en culture de masse pour l'alimentation des larves de poissons en aquaculture.

Dans le cadre de ce projet de thèse, nous avions pour objectifs : (i) de tester le potentiel productif et qualitatif de *P. nana* en aquaculture en lien avec la nature du régime de micro-algues ingérées ; (ii) d'établir le profil de *P. nana* en tant que modèle écotoxicologique au moyen de tests d'exposition à des contaminants métalliques.

Les effets de sept régimes de micro-algues différents sur la productivité de *P. nana* en culture ont été explorés, incluant des régimes monospécifiques de *Rhodomonas salina* (R), *Tisochrysis lutea* (T) et *Pavlova lutheri* (P), deux régimes à deux espèces de micro-algues (R+T, T+P, R+P), et un régime constitué des trois espèces (R+T+P). Les expériences ont été menées à grande échelle, en utilisant une culture de copépodes de 300 L répartie dans des béchers de 10 L, en commençant uniquement avec les stades nauplii. Chaque bécher fut nourri spécifiquement avec son régime attitré pendant 15 jours. Passé cette période, des études de comptage, des mesures morphologiques, et des dosages d'acides gras et de monosaccharides ont été pratiqués sur des échantillons de copépodes prélevés dans chacun des béchers. Les résultats ont montré que le régime R+T a induit la plus forte croissance de population, en particulier pour les copépodites et tous les stades femelles (femelles pré-adultes, femelles adultes, et femelles ovigères), des stades clés dans le cycle de vie de toute espèce de copépode. Les régimes R+T et R ont généré les plus grandes tailles individuelles et les nombres d'œufs et

volumes d'œufs les plus élevés chez les femelles ovigères, se traduisant par l'investissement reproductif le plus accru. Ces mêmes régimes ont aussi induit les plus fortes concentrations en acides gras totaux chez les copépodes, et également les ratios DHA/EPA les plus faibles en raison de leur forte teneur en EPA, tandis que R suivi de T ont induit la plus forte teneur en DHA. La plus forte concentration en monosaccharides totaux a été trouvée dans les copépodes nourris avec R+T+P, les régimes plurispécifiques semblant induire un effet additif sur la richesse en monosaccharides des copépodes. Les résultats globaux ont montré que tous les régimes incluant *R. salina* entraînaient une augmentation de la productivité de *P. nana*, en particulier en association avec *T. lutea* dans un régime mixte. Parmi les trois micro-algues testées, *R. salina* et *T. lutea* semblent présenter des profils complémentaires d'acides gras et de monosaccharides, confirmant l'idée que le régime R+T semble être la meilleure combinaison de micro-algues pour une culture productive de *P. nana*. A l'inverse, *P. lutheri* n'est pas apparue comme une micro-algue efficace dans chacun des paramètres de productivité testé chez *P. nana*, aussi bien pour améliorer sa fécondité que son profil nutritionnel.

Une autre étude exposant deux sous-populations de *P. nana* au cadmium (Cd), au cuivre (Cu) et au nickel (Ni) a examiné les effets de ces contaminants sur la croissance et la structure de la population afin de comprendre les paramètres affectant la bioaccumulation des métaux par les copépodes. Une première expérience a testé l'hypothèse de concurrence entre ces métaux en mélange, par rapport aux sites spécifiques de fixation, pour la bioaccumulation par les copépodes. Une culture de masse classique de *P. nana* a été mise à profit et répartie dans des béchers de 10 L préalablement inoculés avec les métaux seuls

ou en mélange à hauteur des concentrations sublétales (1/3 de CL50) déterminées pour le copépode estuarien *E. affinis*. Une deuxième expérience a suivi le même protocole mais en utilisant une culture de *P. nana* préalablement exposée à une concentration plus élevée en Cu pendant plusieurs générations et en utilisant les concentrations sublétales de métaux appropriées pour *P. nana*. Après 96h d'exposition, des prélèvements d'eau et de biomasse ont été effectués dans chaque bécher pour des études de comptage et des analyses élémentaires afin d'évaluer la bioaccumulation. Les résultats de la première expérience ont montré une population décroissante mais une augmentation de la bioaccumulation des métaux dans les copépodes. Le Cd a également été plus accumulé lorsqu'il était seul, confirmant l'hypothèse de la compétition des métaux en mélange. Les résultats de la deuxième expérience ont révélé des effets moins prononcés et moins délétères sur la population. Alors que les concentrations en métaux augmentaient dans les béchers, elles diminuaient chez les copépodes, traduisant une activité de dépuration survenue chez cette population déjà adaptée à l'exposition aux métaux.

Les résultats obtenus au cours de cette thèse sont les premiers montrant que *R. salina* est une micro-algue appropriée à une culture de masse productive de *P. nana* pour son utilisation en tant que proie vivante pour l'aquaculture des larves de poissons marins, et investiguant les paramètres influant sur les capacités de bioaccumulation de *P. nana* en réponse aux métaux dans les écosystèmes aquatiques contaminés.

MOTS-CLÉS

Aquaculture, bioaccumulation, copépode, écotoxicologie, régime de micro-algues, *Paracyclopina nana*

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CHAPTER I

INTRODUCTION

Copepods are small planktonic crustaceans living in all aquatic environments of the planet. They are found everywhere, from freshwater to saltwater, from tropical to polar areas. There is a great copepod diversity. The number of copepod species described at the end of 1993 was estimated at 11500 (Humes, 1994). But this number is very underestimated according to the analysis of experts in taxonomy and diversity of copepods. The species identified to date are divided into 10 orders, 200 families and 1650 genera. Some of them are parasites and therefore live at the expense of hosts corresponding to different aquatic macro-organisms, mainly fish. Other copepods are benthic (harpacticoid copepods) and may play an important role in the functioning of the meiofauna living in the sediment. But most of the copepod species are free and establish their entire life cycle within zooplankton, therefore called holoplanktonic. In the ocean, copepods represent most of the biomass of zooplankton and play a central and essential role in trophic chains (Puelles et al., 2003, Thompson et al., 2013). The majority of them have a diet based on phytoplankton and are in turn a source of food for many marine planktivorous organisms, especially for all marine larval stages. Marine copepods therefore represent a fundamental trophic level of transformation from primary production to secondary production in addition to other pathways from the microbial food web.

This special trophic position gives them a double potential that is particularly interesting for current research. First, copepods represent an ideal model in marine ecotoxicology because they are the gateway to any disturbance to which the trophic chain is subject (Kwok et al., 2015). Thus, they prove to be excellent bioindicators of the quality of their environment, that is to say that copepods are organisms that can be used as an indicator of the state of health of marine

ecosystems, especially in terms of pollution. Their very short life cycle and their sexual reproduction mode are also undeniable assets for experimental research. And in a second part, the growing control of their mass culture in different volumes also reveals their innovative potential as live prey for the feeding of fish larvae in aquaculture and aquariology. Particularly, their small size and capacity to grow at high density offer real advantages to perform larval cultures of delicate species having small mouths.

As part of a recent collaboration between Prof. Sami Souissi and Prof. Jae-Seong Lee (Sungkyunkwan University, South Korea) initiated by the Franco-Korean project PCH STAR 2014-2015, the cyclopoid copepod *Paracyclopina nana* (Smirnov, 1935) has been added to the copepod strains collection of the laboratory. This emerging copepod species is euryhaline and lives at an average salinity of 15 psu. It is mainly found in the bays and estuaries of East Asian countries (Japan, Taiwan, and South Korea). It is a small copepod, not exceeding 600 µm on average (**Fig. 1**).

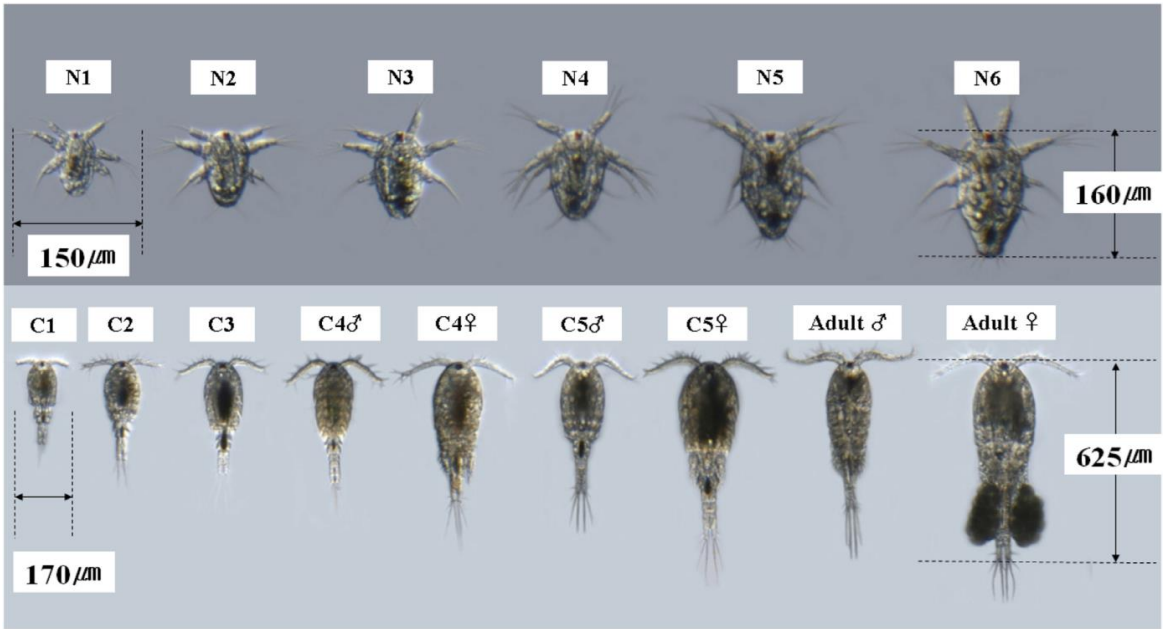

FIGURE 1. Successive life stages of *Paracyclopina nana*, from Dahms et al., 2016. Adapted from Hwang et al., 2010.

Like most copepod species, the life cycle of *Paracyclopina nana* consists of 12 successive development stages:

- 6 larval stages called nauplii (N1-N6)
- 5 juvenile stages called copepodites (C1-C5)
- 1 adult reproductive stage (male or female)

The transition from stage N6 to C1 is the metamorphosis, and the number of pairs of legs and segments increases unitarily during each successive moult. Reproduction is achieved by a mating from sexual partners. After fertilization, females of cyclopoid copepod species such as *P. nana* present two egg sacs to store the produced eggs (up to 40 in good conditions) and are from then on called ovigerous females. They will reform new ones after the hatching of N1 nauplii as soon as they are fertilized again. The generation time of *P. nana* is short: a new mature individual is obtained in about two weeks (15 days) at 18°C. Likewise, its average longevity is estimated at 2 months and the female is therefore able to reproduce several times during her life.

All these particularly fast, obvious, and prolific life parameters coupled to its small size have made *P. nana* a prime candidate in the search for a standard copepod species for the study of aquatic environments. Experimental research on marine ecosystems still lacks a model-type species with fast, non-binding culture parameters, easy laboratory use, and clear responses to scientific tests.

Problem statement

P. nana is a copepod species benefiting from several studies related to molecular responses to different stressors as well as genomic tools. All these studies were

conducted in our collaborator's lab in South Korea who was the first to take an interest in this species. Its whole mitochondrial genome has even been recently sequenced, assembled and annotated (Ki et al., 2009) (**Fig. 2**).

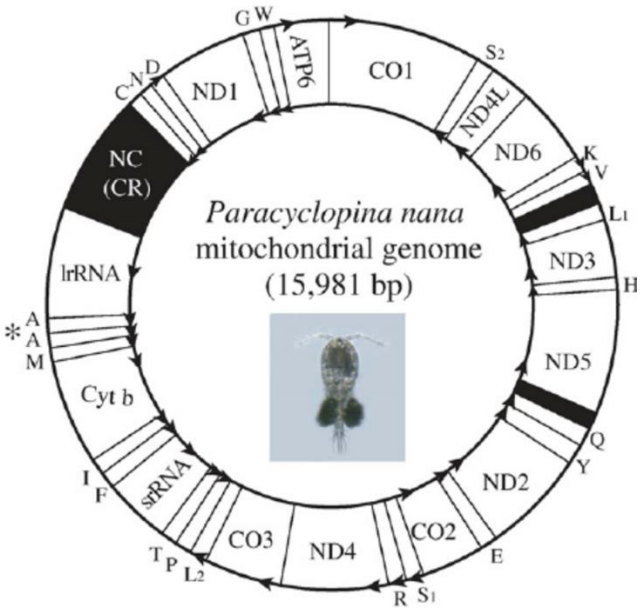

FIGURE 2. Gene organization of the mitochondrial genome of *Paracyclopina nana*, from Ki et al., 2009.

Since, the species has been approached as a new promising genetic model, especially by focusing on its biomarkers of interest, that is to say its specific genes which make it possible to directly identify a particular physiological process. Expression changes of *P. nana* biomarkers facing changes of its environment parameters, mainly expositions to contaminants, have been studied (Hwang et al., 2010; Won et al., 2014; Dahms et al., 2016; Lee et al., 2016; Jeong et al., 2017).

But this new model demonstrates a lack of purely experimental ecophysiology and ecotoxicology work to account for the physiological effects that contaminants and changes in abiotic parameters may have on its behaviour and physiology. Moreover, studies at the population level rather than at the individual level would be more appropriate to better reflect the responses of its entire populations from

an ecosystem perspective and explore the potential that a large culture of this copepod may present. *P. nana* can be easily cultured in large volumes, under medium salinity, and a wide range of temperatures, allowing it to be a good invertebrate model for experimental research. This asset makes it an appropriate copepod species to be used as a test organism for risk assessment associated with pollutants in aquatic environments (bioindicator), as well as an interesting candidate as a live prey for the feeding of fish larvae in aquaculture. A reference review published by Dahms et al. in 2016 compiled all the knowledge and results acquired to date on *P. nana*, demonstrating its undeniable potential as a new invertebrate model to complete the range of investigation in marine ecotoxicology. Its potential in aquaculture has also been enlightened. Lee et al. (2013) showed that *P. nana* is the copepod species with the highest growth rate (0.234 ± 0.0131) in mass monoculture in comparison with another cyclopoid copepod *Apocyclops royi* (0.096 ± 0.0138) and the harpacticoid copepod *Tigriopus japonicus* (0.086 ± 0.0143), thus reinforcing the interest for this copepod species. According to Lee & Park (2005), *P. nana* can also support very high culture densities: it still has a survival rate of 18.4% for a density of 300 nauplii/mL. Its optimal culture density to obtain nauplii is 7 females/mL and that to obtain adults is 200 nauplii/mL, which is considerable. However, these interesting results were obtained in small volumes and have to be confirmed in larger experimental volumes. Our research team has already made key findings by investigating the physiology and potential of more commonly used copepod species in experimental research. We conducted several experiments to investigate the parameters influencing the bioaccumulation capacity of trace metals in the calanoid copepod *Eurytemora affinis* (Zidour et al., 2019) and it was necessary to carry out similar experiments on *P. nana*, as

bioaccumulation in copepods depends on the nature of the contaminants and the particular life traits of the targeted species. Kadiene et al. (2019) also made key hypothesis about the routes of exposure to metals in calanoid copepods by introducing the idea that oral water intake may be involved in high metal body accumulations observed in copepods exposed to dissolved metals, and that feeding would then reduce the stress associated with the exposure by filling the guts, preventing it from the contact with dissolved metals. However, these studies targeted two calanoid copepods and little is known about the possible extrapolation of these funding to cyclopoid copepods. On the aquaculture field, conclusive microalgae diet optimization tests were performed on the larger cyclopoid copepod *Apocyclops royi* (Pan et al., 2018) and inspired to test the potential of the small cyclopoid *P. nana* in the same field. Results allowed to showcase the different fatty acid compositions of *A. royi* fed on different microalgal diets. It was interesting to apply similar approach to *P. nana* and even to explore its saccharide profile, as sugar composition have been poorly studied in copepods.

The aim of this thesis is to investigate the double potential of *P. nana* in ecotoxicology and aquaculture. This multidisciplinary project is therefore divided into two parts. At the beginning of this thesis, it first seemed essential to study and describe the various fitness parameters of the maintained *P. nana* strain (generation time, fecundity, survival), as well as the ecotoxicological factors influencing their modification, thanks to individual reproduction experiments and LC50 tests. These preliminary results have laid the groundwork for bioaccumulation studies, which are needed to investigate how *P. nana* bioaccumulates contaminants, and what conditions and factors alter its bioaccumulation capacity. In a second part, the potential of *P. nana* in aquaculture

was investigated through a large-scale microalgae diet test to determine which diet leads to the most productive *P. nana* culture in terms of population growth, fecundity, and fatty acid and saccharide synthesis.

The major asset of this thesis lies in the expertise of the host laboratory to maintain for several years several large-scale monospecific copepod cultures. These unique culture systems correspond to 300 L cylindrical acrylic tanks and provide a privileged framework for studying copepods in correlation with their natural living conditions and massive amounts of experimental copepods for the research work of this project. It also benefits from the achievements of the COPEFISH project (2011-2015) resulting from a partnership established with the aquarium Nausicaá (Boulogne-sur-Mer, France). The aim of this aquaculture project is to develop and optimize a large-volume copepod production pilot for larval fish rearing. This initiative can also be used to study in practice the phenomena of bioaccumulation and biomagnification of contaminants between the different links of the trophic chain constituted by micro-algae, copepods, and fish.

Research objectives

This thesis aims to complete and deepen the research conducted to date on *P. nana* according to two distinct themes.

In aquaculture, the objectives are to optimize the know-how and knowledge of copepod mass culture in order to constitute a living food source for fish larvae through experiments based on *P. nana* testing the microalgae diets inducing the maximum productivity of copepods (population growth, fecundity, fatty acid and saccharide synthesis).

In ecotoxicology, the objectives are to test and understand the impact of some metal contaminants on the life traits and adaptive strategies of copepods. And more specifically, to understand the parameters influencing the bioaccumulation capacities of *P. nana* facing metals (food source, generational factor, acclimation).

Various methods are used to carry out this project and gives it a multidisciplinary aspect, including stage-specific copepod counting and morphological measurements practiced on ovigerous females. Elementary chemical analysis are performed to estimate and understand the bioaccumulation of trace metals by copepods. Extraction and quantification of copepods fatty acids and saccharides also provide key informations on the benefits of different microalgae diets. And finally, the contributions of molecular biology enable the acquisition of innovative results thanks to ARN-extraction and q-PCR.

CHAPTER II

POTENTIAL OF *PARACYCLOPINA NANA* IN AQUACULTURE

Part 1

Effects of microalgal diet on the population growth and fecundity of the cyclopoid copepod *Paracyclopina nana*

Dayras, P., Bialais, C., Lee, J. S., & Souissi, S. (2020). Effects of microalgal diet on the population growth and fecundity of the cyclopoid copepod *Paracyclopina nana*. *Journal of the World Aquaculture Society*.

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1. INTRODUCTION

In marine ecosystems, copepods are a common component of zooplankton and play a key role as a trophic link between phytoplankton and secondary consumers (Breteler et al., 1990; Mauchline et al., 1998; Støttrup, 2000). They represent an important food source for many fish and crustacean larvae (Sun & Fleeger, 1995; Turner, 2004). Rotifers and Artemia are commonly used for larval rearing of aquaculture species. They are easy to grow in large volumes at relatively low cost (Sorgeloos, 1980; Lubzens, 1987). But they are not the natural prey of marine fish and do not always provide optimal fish larval growth because of their unsuitable size or fatty acid content and require nutritional enrichment (Watanabe et al., 1983; Léger et al., 1987; Fernández-Reiriz et al., 1993; Sorgeloos et al., 2001; Lubzens et al., 2003). Mass culture of several marine copepod species has now been attempted in order to use them as live feed for marine fish cultures (Zillioux, 1969; Støttrup et al., 1986, Ohno et al., 1990; Støttrup & Norsker, 1997; Payne & Rippingale, 2000; Van der Meeren et al., 2014; Blanda et al., 2017). It has been shown that their superior nutritional value is more suited to the growth and development of many species of commercial interest (McEvoy et al., 1998; Shields et al., 1999, Payne & Rippingale, 2000; Payne, Rippingale & Cleary, 2001; Olivotto et al., 2008). Copepods have a high content of highly unsaturated fatty acids (HUFAs) such as docosahexaenoic acid (DHA) and E- pentaenoic acid (EPA), which are required by fish larvae (Izquierdo, 1996; Sargent et al., 1997; McEvoy et al., 1998; Pan et al., 2014). Therefore, a copepod with a high efficiency for aquaculture is characterized by high fecundity and high lipid concentration. In fact, the productivity of copepods is translated in terms of female fecundity and individual

storage of fatty acids in the form of lipid droplets (Souissi et al., 2016a; Lee et al., 2017), and therefore in amounts of carbon chains ingested through their diet. It is therefore essential to provide copepod cultures with optimal microalgal diets for maximal fecundity, development, and nutritional storage (Pan et al., 2014).

The evaluation of a suitable microalgal diet for captive copepods has been investigated with some species that belong to the orders Calanoida (Camus et al., 2009; Milione & Zeng, 2007; Ohs et al., 2010; Jeyaraj & Santhanam, 2012; Pan et al., 2014; Siqwepu et al., 2017), Harpacticoida (Pinto et al., 2001; Arndt & Sommer, 2014) and Cyclopoida (Lee et al., 2006; Rasdi & Qin, 2016; Pan et al., 2018). Overall, results indicated that mixed algal diets are more productive than monoalgal diets. Moreover, feeding microalgal species from the genus *Isochrysis* result in the greatest population growth (Milione & Zeng, 2007; Jeyaraj & Santhanam, 2012; Siqwepu et al., 2017), whereas species from the genus *Rhodomonas* provide the highest fecundity (Ohs et al., 2010; Siqwepu et al., 2017). But there is still a lack of information on the optimal diets for cyclopoid copepod species.

The brackish water cyclopoid copepod *Paracyclopina nana* Smirnov 1935 has demonstrated a strong potential for aquaculture (Ki et al., 2009; Lee et al., 2006; Lee et al., 2013). Native to the bays and estuaries of Eastern Asia (Japan, Taiwan, and South Korea), it provides a small size at adult stage (600 µm on average), a high tolerance to salinity and temperature ranges, and can be cultured at high densities (Lee et al., 2012b; Lee et al., 2017). Recent studies on this species tried to define the optimal diet for its growth in aquaculture (Min et al., 2006; Lee et al., 2012a). However, most of these studies used monoculture algae and it is unknown

for *P. nana* if different microalgae affect production parameters (Pan et al., 2014). It is therefore important to define the optimal algae diet for a copepod culture.

The aim of this study was to investigate the effects of different microalgal diets on the population growth and fecundity of *Paracyclopina nana* populations in culture. This work relies on the expertise of a laboratory in copepod culture and the utilization of microalgal species known for their high nutritional value.

2. MATERIALS AND METHODS

2.1. Establishment of microalgae diets

Three species of microalgae, *Rhodomonas salina*, *Tisochrysis lutea,* and *Pavlova lutheri*, were individually grown in the laboratory using standardized methods and were obtained from the Roscoff Culture Collection of Marine Microalgae (http://roscoff-culture-collection.org/). These three species were distinguished by their belonging to different genera and families of microalgae. As a result, their physiology, their physico-chemical composition and their nutritional quality were known to be different (Brown, 2002).

In order to test the nutritional value of the selected microalgal species on *P. nana*, they were fed to copepods in carbon equivalents so only the species of algae varied. In this study, carbon was used as a proxy in order to establish equivalent rations among the three microalgae species (Pan et al., 2014). A CHN analysis was performed on samples of each of the three microalgal cultures at their exponential phase of growth using an *elemental analyzer* (FLASH 2000 Series CHNS/O Analyzer, Thermo Fisher Scientific, Waltham, MA). The aim was to know their

respective cellular concentration of carbon. Obtained values were compared with standard reference values (Berggreen et al., 1988; Verity et al., 1992; Flynn et al., 1994; Olenina et al., 2006) in **Table 1**.

TABLE 1. Average carbon amount per cell for each species of microalgae compared with standard reference values.

Microalga	Average carbon amount /cell	Range of values from literature
R. salina	53.6 pg ± 0.70 pg	23-47,4 pg[1,2]
T. lutea	15.2 pg ± 1.53 pg	6.97-13.6 pg[1,3]
P. lutheri	7.86 pg ± 0.85 pg	10.1-16.5 pg[2,4]

References : 1 : Olenina et al., 2006; 2 : Berggreen et al., 1988; 3 : Flynn et al., 1994; 4 : Verity et al., 1992

Then the different possible microalgal diets were constituted between the different combined species so that the total given volume remained the same.

With these three species of microalgae (*R. salina* (R), *T. lutea* (T), *P. lutheri* (P)), seven combinations of different diets were established:

- 3 single-species diets: R, T and P
- 3 two-species diets: R+T, T+P and R+P
- 1 three-species diet: R+T+P

Because of the variability of cellular carbon concentration and cell density in microalgal cultures, we standardized the volume of microalgal mixtures used for this study. According to the standard feeding protocol used to feed the permanent copepod cultures maintained in the laboratory, it was necessary for our study to give 9.1 mL of microalgal diet per day for each beaker. This volume of algal cells corresponded to a carbon amount that was not limiting for this species (Lee et al., 2012a). Thus, in order to make the different combined diets, volume ratios between each of the microalgal species were deduced so that the combined specific sub-volumes within each diet contained the same amount of carbon in proportion to the

same total volume of 9.1 mL and that only the species parameter needed testing.

2.2. Microalgal cultures

Microalgae were grown in batch cultures in several autoclaved 2 L flasks following the protocol of Sadovskaya et al. (2014). The standardized microalgal culture conditions in the laboratory used natural autoclaved seawater (33-35 in PSU), a constant temperature of 18°C and a 12 h light: 12 h dark photoperiod. These culture conditions were those used before the CHN analysis and maintained during the whole experiment. The microalgae were grown in a culture medium gathering 0.2 µm filtered, autoclaved, and gently aerated seawater, 2 mL of Conway solution and 200 µL of vitamins. At their exponential growth phase, the *R. salina* cultures were transferred to a new culture medium every three days and the *T. lutea* and *P. lutheri* cultures were changed and put in a new culture medium once a week.

2.3. Copepod cultures and initial sampling

Paracyclopina nana used in current experiments were initially obtained from Prof. Jae-Seong Lee from Sungkyunkwan University of South Korea. Copepods were cultured in the laboratory in 300 L cylindrical acrylic tanks filled gently with aerated water of 15 salinity in PSU, at a constant temperature of 18°C with a 12 h light: 12 h dark photoperiod, and were usually fed twice a week with *Rhodomonas salina*. Our experiment started with nauplii of *P. nana* cultured for a period of 15 days, which corresponds to the average duration of *P. nana* life cycle (Lee et al.,

2006). The whole column of 300 L of *P. nana* was filtered successively on a 120 µm mesh and a 33 µm mesh to separate adults from nauplii. Half of the amount of nauplii were kept for the experiment and concentrated in a beaker containing 1.2 L of 15 g/L water (filtered and autoclaved seawater + distilled water). This salinity was fixed using a multiparametric probe. The adult copepods and the second half of nauplii not used for the experiment were transferred to a new clean 300 L culture. Seven 10 L beakers containing 8 L of culture medium were set up, one for each of the diets tested.

The use of a single replicate of these large beakers, like in Zidour et al. (2019), is an inevitable compromise to perform all the planned analyses for this project. In order to perform an experimental protocol allowing the accurate evaluation of the effects of diet composition on copepod production and population structure (current work) and also the nutritional value of copepods (ongoing work), some preliminary tests and choices were realised. First, because *P. nana* is a very small copepod, we validated the minimum quantity (dry weight) of biomass needed from each subsample to later evaluate the nutritional value of copepods in terms of fatty acid composition as well as polysaccharides. Consequently, it was not possible to apply the protocol that the same research group used from another larger cyclopoid *Apocyclops royi* (Pan et al., 2018). Rather, another protocol was applied in the same preliminary experiment to assess simultaneously the effects of trace metals on copepod structure and bioaccumulation (Zidour et al., 2019). The choice of a single microcosm per condition is also justified by our preliminary personal observations confirming low variability between replicates of *P. nana* cultures. In fact, *P. nana* has low swimming activity compared to calanoid copepods we have in our culture collection and can be easily used to inoculate several beakers with the same initial

composition and density. Regular copepod culture monitoring counting carried out in the laboratory, using up to 8 replicates (n varied between 4 to 8), even by different operators, show very low coefficients of variation and prove the robustness of our protocol (CV=4.96; difference between two operators: 5.44%).

Each beaker was filled with 8 L of 15 ppt water and placed at a constant temperature of 18°C with a 12 h light: 12 h dark photoperiod. The 1.2 L of concentrated nauplii was divided in 12 equal parts of 100 mL. Seven of them were each diluted in one of the seven beakers of the experiment, three of them were individually filtered and concentrated with 70% ethanol for initial counting of the population, and the last two parts of 100 mL were filtered to remove any water, concentrated in cryotubes, and stored at -80°C for further analyses. Initial counting of the 100 mL triplicates was performed using a manual counter and a serpentine slide under a stereomicroscope (model Olympus SZX12, Tokyo, Japan) and shows after extrapolation that 9,344 (± 478,46) nauplii were introduced into each beaker, or 1,154 (± 59.07) per liter.

2.4. Experimental monitoring

Daily feeding with microalgal diets

Each beaker was fed daily with a total volume of 9.1 mL for each combination of microalgal diet, in relative proportions of microalgae. Microalgal cultures were always fed to copepods at their exponential growth phase. Each of the seven microalgal diets were daily mixed in a 10 mL tube with its respective sub-volume sampled with a syringe and then centrifuged. After centrifugation, the excess

water was removed from the tubes and the pellets of each diet were re-diluted in the water of their corresponding beaker for feeding.

Copepod sampling

After eight days, the first copepod sampling occurred at each condition for estimating the population density and composition in the beaker. A 500 mL sample was filtered through a 33 µm mesh and copepods were collected, concentrated and preserved in 70% ethanol for collection of population data. The exact same sampling protocol was carried out at the end of the experiment after 15 days, supplemented by sorting of 20 ovigerous females from the fixed samples of each condition intended for morphological measurements (prosome length), and fertility measurement (number of eggs per ovigerous female).

2.5. Copepod individual counting

Copepods fixed with 70% ethanol were individually counted and their sex and life stage were recorded using a stereomicroscope (model Olympus SZX12, Tokyo, Japan) and a manual counter. Nauplii, Copepodites (C1-C4), C5 Males, C5 Females, Adult males, Adult females and Ovigerous females were differentiated according to morphological details (Hwang et al., 2010).

2.6. Ovigerous female morphological measurements and clutch size

The 20 fixed ovigerous females from each diet group were individually photographed and studied under an inverted microscope (model Olympus IX71, Tokyo, Japan) with 10X magnification coupled with a ToupCam camera (model UCMOS05100KPA, Zhejiang, China) connected to a computer using ToupView software (ToupTek Photonics version 3.7). For each single ovigerous female, a photo of the whole body was taken for measurements. Then the egg sacs were manually broken and photos of all the eggs were taken with the software.

Morphological measurements were then performed on each photo using ImageJ software (version 1.48v) as described in Souissi et al. (2016a). Prosome length was manually measured and eggs were also counted for each ovigerous female from each microalgal diet.

2.7. Statistical analysis

In order to test the effect of diet on the population composition using three life stages (nauplii, copepodites and adults), a Pearson's chi-squared test was used. Moreover, the tests were applied to all combinations of two treatments within the seven diets at two end-points corresponding to 8 days and 15 days of culture. All statistical tests were conducted at the 95% confidence level. These analyses were performed using Matlab Software (Mathworks Inc., Version, 7.5).

3. RESULTS

3.1. Effects of microalgal diet on population growth of *Paracyclopina nana*

Total population growth

Fig. 3 shows the total number of *P. nana* individuals and respective number of nauplii for each tested diet after 8 days and after 15 days of feeding.

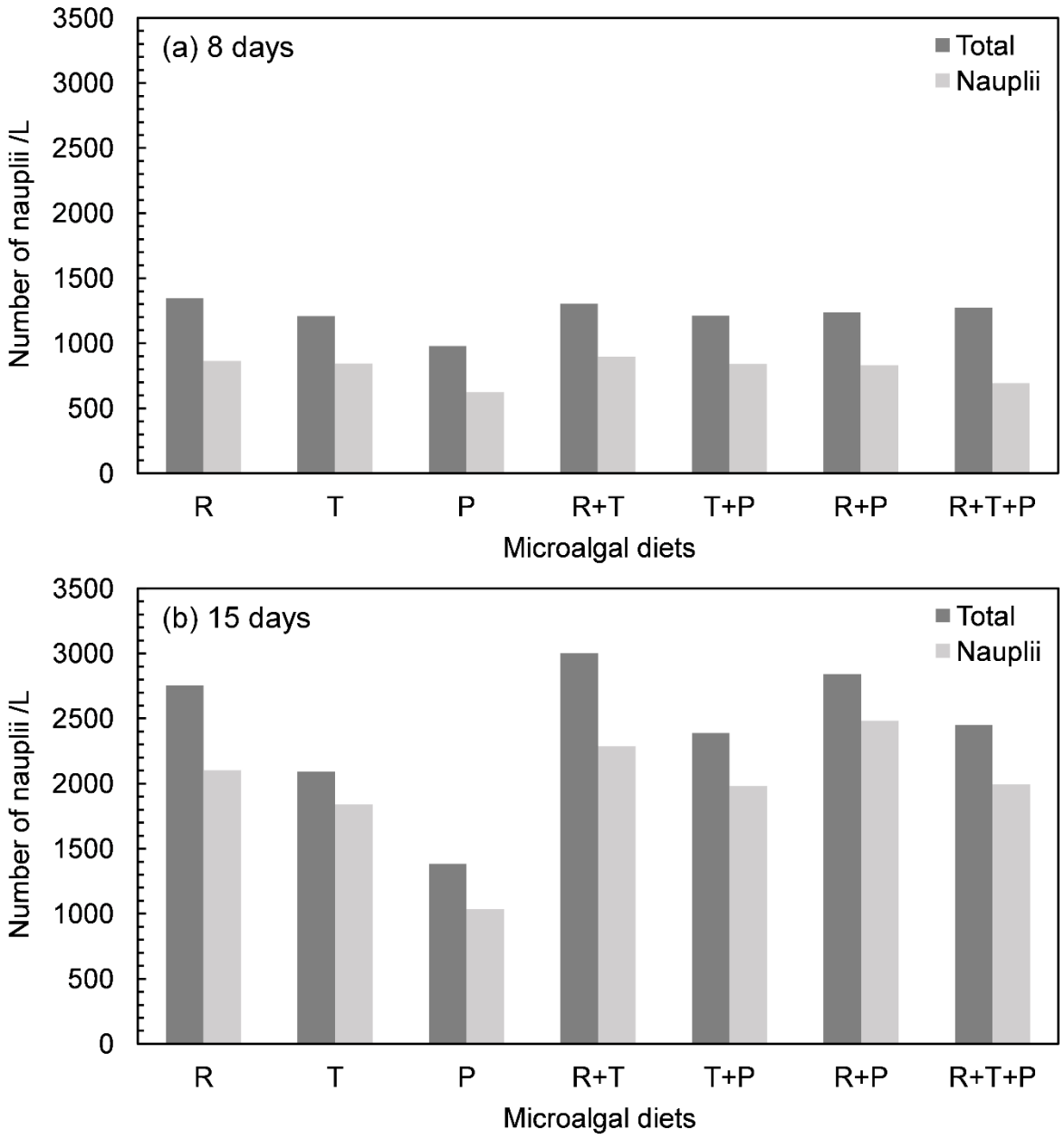

FIGURE 3. Total numbers of individuals and respective numbers of nauplii of *Paracyclopina nana* when feeding on different microalgal diets after 8 days (a) and after 15 days (b). R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

On the first day, 1,154 (± 59.07) nauplii per liter were introduced into each beaker. After 8 days, no significant differences can be seen between each condition, except that the R+T and R diets showed slightly higher densities than the other diets and that the P diet was the lowest for both the total number of individuals and the number of nauplii. After 15 days, the R+T diet resulted in the highest total number of individuals with 3,000 individuals L^{-1}, with a relative increase of 259.97% of individuals (of which 2,286 were nauplii, which corresponds to almost to the double compared to the initial conditions). It was followed by the R+P diet (+146.10% of individuals L^{-1}), also showing the highest number of nauplii (2,482 nauplii, that is to say +1,328 nauplii), the R diet (+138.65% of individuals L^{-1} and +948 nauplii) and the R+T+P diet (+112.13% of individuals L^{-1} and +838 nauplii). The T+P, T and P diets resulted in the lowest increase of the total number of *P. nana* individuals after 15 days of treatment (+106.76% of individuals L^{-1}, +81.11% of individuals L^{-1}, and +19.76% of individuals L^{-1} respectively) and the P diet even resulted in a negative naupliar recruitment (-118 nauplii).

Table 2 presents M:F sex ratios of *P. nana* populations fed with each tested diet after 8 days and after 15 days.

TABLE 2. M:F sex ratio of *Paracyclopina nana* populations after 8 days and after 15 days of feeding on different microalgal diets. R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

Microalgal diet	Sex ratio after 8 days (%)	Sex ratio after 15 days (%)
R	43.64	44.51
T	48.84	36.59
P	54.41	51.85
R+T	55.56	44.00
T+P	46.27	51.08
R+P	57.61	61.42
R+T+P	38.64	52.78

After 8 days of feeding, the lowest proportions of males were obtained by the diets R+T+P (38.64%), R (43.64%) and T+P (46.27%). After 15 days, the T (36.59%), R+T (44.00%) and R (44.51%) diets were the ones showing the lowest proportions of males.

Stage-specific composition

Fig. 4 shows the number of *P. nana* individuals for each life stage after 15 days of feeding with each diet.

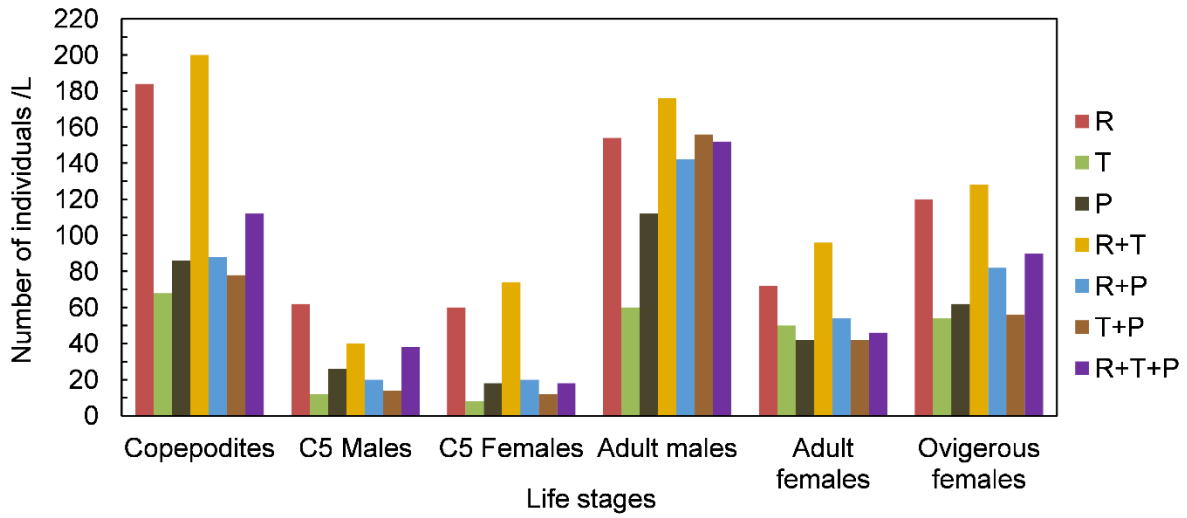

FIGURE 4. Comparison of the effects of the different monospecific diets (a), plurispecific diets (b), diets containing R (c) and diets containing T (d) on the number of *Paracyclopina nana* individuals in each life stage. R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

Among the monospecific diets, the R diet produced the highest number of individuals in each life stage, particularly for copepodites (184 individuals L^{-1}), adult males (154 individuals L^{-1}) and ovigerous females (120 individuals L^{-1}), compared to the T and P diets that almost never reach 100 individuals for each life stage (only 112 adult males individuals L^{-1} with the P diet). The R+T diet induced

a significantly higher number of individuals in each life stage in comparison with the other plurispecific diets ($p < 0.05$), particularly for copepodites (200 individuals L^{-1}), and all female stages (74 C5 females L^{-1}, 96 adult females L^{-1}, and 128 ovigerous females L^{-1}). Among the diets containing R, the R and R+T diets were the ones generating a significantly higher number of individuals in each life stage in comparison with the two other diets ($p < 0.05$), particularly for copepodites and all female stages.

3.2. Effects of microalgal diet on the size and fecundity of *Paracyclopina nana* ovigerous females

Ovigerous females prosome length and clutch size

The distribution of ovigerous females prosome length after 15 days of feeding with each tested diet is shown in **Fig. 5a**.

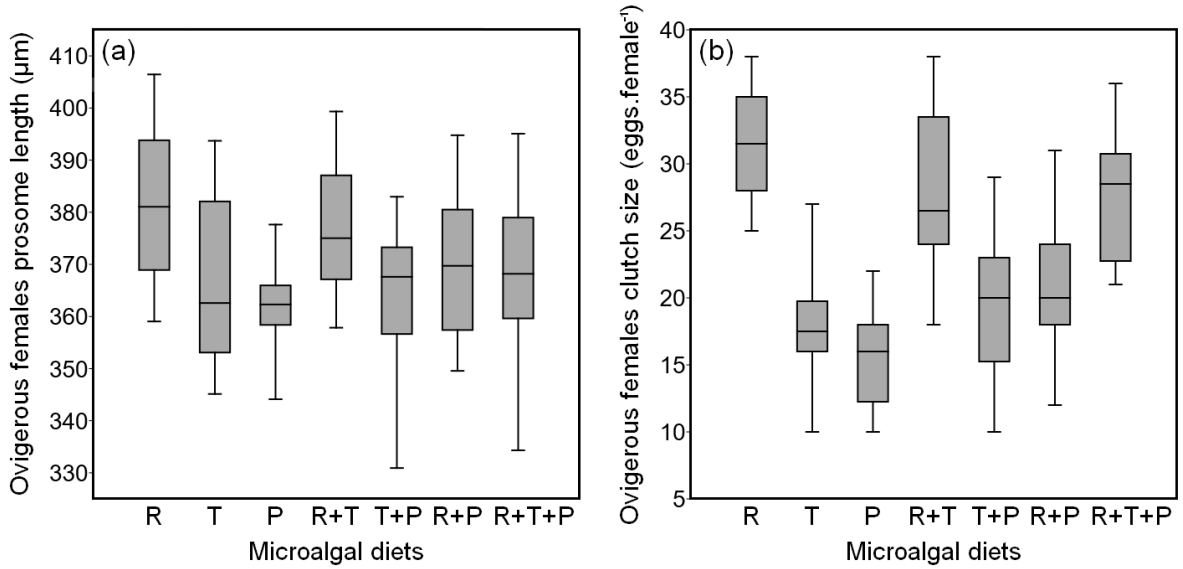

FIGURE 5. Boxplot showing the distribution of prosome length (a) and clutch size (b) of *Paracyclopina nana* ovigerous females fed on different microalgal diets. The shaded boxes represent the first and third quartile with the middle bar being the

median. The end of the whiskers extends from the hinge to the lowest and highest value within a 1.5 inter-quartile range. R: *Rhodomonas salina,* T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

The R and R+T diets induced the longest prosome lengths in ovigerous females (R: 381.72 µm ± 14.58 µm, R+T: 377.37 µm ± 11.93 µm) whereas the P and T+P diets resulted in the lowest prosome lengths (P: 361.79 µm ± 8.24 µm, T+P: 360.62 µm ± 19.71 µm).

The effect of the different diets on fecundity after 15 days of feeding (**Fig. 5b**) confirmed that the R, R+T and R+T+P diets induced the greatest egg number per ovigerous female (R: 31.7 ± 3.81, R+T: 28.0 ± 5.56, R+T+P: 27.8 ± 4.80) whereas the T and P diets resulted in the lowest egg number (T: 17.7 ± 3.40, P: 15.6 ± 3.42).

4. DISCUSSION

4.1. Effects of microalgal diet on population growth of *Paracyclopina nana*

The evaluation of copepod productivity may be linked to several factors, such as the egg production, the survival and development of the nauplii and copepodites, or the nutritional profile of the harvested copepods (Knuckey et al., 2005). Therefore, assessing the effects of microalgal diets on the population dynamics of a whole copepod culture rather than focusing on a single stage of the copepod life cycle and in restricted numbers of individuals may provide more complete information for an aquaculture perspective. Studying population growth provides an overview of the effects of diets as it shows correlated life cycle parameters. The

relative increase of the population density in a culture of living organisms is the first indicator of the general good state of health and good growing conditions of the culture (Pan et al., 2018). And in the particular case of copepod cultures, food quality is one of the key parameters influencing the population density (Pinto et al., 2001; Camus et al., 2009; Ohs et al., 2010; Jeyaraj & Santhanam, 2012; Pan et al., 2014).

Starting from nauplii (<120µm) and after 15 days of feeding, the diet that has resulted in the highest total number of individuals of *Paracyclopina nana* was R+T, the one combining *Rhodomonas salina* (R) and *Tisochrysis lutea* (T). It was followed by the R+P diet, the R diet and the R+T+P diet. Our results confirm that microalgal diets combining two species are always more productive than monospecific diets. In a previous study of Milione and Zeng (2007) conducted in 500 mL conical bottles, all the microalgal monospecific diets tested (*Nannochloropsis*, *Isochrysis*, *Tetraselmis* and *Rhodomonas*) on *Acartia sinjiensis* produced lower population growth and hatching success than the binary ones. The different nutritional qualities of microalgae may explain these results as some species may present complementary nutritional qualities increasing the nutritional bonus of the copepods that feed on them in a mixture, finding all the necessary nutrients for their growth that may be lacking in a monospecific diet. This idea is sustained by Knuckey et al. (2005) who reported that a combined diet of *Isochrysis galbana* and *Tetraselmis suecica* induced a better nauplii development of *Acartia sinjiensis* than fed either *I. galbana* or *T. suecica* alone. Microalgal diets combining two species are then thought to provide a better nutritional balance than single microalgal ones (Coutteau, 1996; Southgate, 2003).

Our results also show that all the diets including *Rhodomonas salina* (R) are the ones inducing the highest population growth of *P. nana* individuals. This could be linked to the fact that *R. salina* is the microalga containing the highest cellular carbon amount among the three species tested here, with 53.6 pg. At its exponential growth phase, our culture of *R. salina* was twice as concentrated in carbon as the literature, as was the case for *T. lutea*, while our *P. lutheri* had half the concentrated of carbon as reported the literature (**Table 1**). It's known that cellular carbon concentration can vary because of microalgae culture conditions (temperature, light, salinity) (Renaud et al., 1995; Hu, 2004; Pal et al., 2011). For example, *P. lutheri* is cultured with F/2 medium and at a temperature of 20-21°C by Verity et al. (1992), which may explain the different carbon concentration reported compared to ours. That's why only the values obtained from the CHN analysis performed on the current microalgae cultures of the laboratory were used as a reference here.

Indeed, a high nutritional carbon intake induces a high lipid and carbohydrate concentration in copepods (Souissi et al., 2016a; Lee et al., 2017) and so an increased individual quality and fertility (Støttrup & Jensen, 1990; Shin et al., 2003; Pan et al., 2018), resulting in an increase in population density. Further studies have also confirmed increased productivity of *Rhodomonas* among other microalgal species for copepod cultures, being as effective as a single diet or as part of a mixed microalgal diet (Berggreen et al., 1988; Støttrup & Jensen, 1990; Koski et al., 1998; Knuckey et al., 2005; Dahl et al., 2009). This microalga is also the one with the largest average cell size here with 12.9 x 7.7 µm (average coefficient of variation 19.4%) (Renaud et al., 1999; Schipp et al., 1999), probably making it the most productive for copepod to feed on to consume the same amount of carbon, thus

copepods have to eat less of it than another species, probably making it also the least expensive in terms of grazing energy.

On the contrary, the T+P, T and P diets have induced the lowest total number of *P. nana* individuals and number of nauplii after 15 days of feeding. This confirms that *R. salina*, which was absent from these three diets, really is the most productive microalga species among those three. These results also indicate that *P. lutheri* (P) is the microalga associated with the lowest copepods population growth. Camus & Zeng (2012) also found that a diet based on *Pavlova salina* produced the lowest survival at the naupliar stage in the harpacticoid copepod *Euterpina acutifrons* among all different tested diets. Our results show that this microalga is also the one containing the lowest cellular carbon amount with 7.86 pg. This could explain its low nutritional productivity and so the low population densities obtained when feeding with this species. *P. lutheri* is also a small microalga with a cell size of 4-6 µm (Kamiyama & Arima, 2001; Rehberg-Haas, 2014) meaning it may be the most expensive one among the three species in terms of grazing energy for *P. nana* or it may not be adapted to the ingestion of this copepod species. Buttino et al. (2009) showed that off of the ten diets tested on the copepod *Temora stylifera*, the monoalgal diet made of *Isochrysis galbana* was unable to sustain egg production and adult survival possibly because adults were unable to ingest this microalga due to its small size. Many studies have examined the food preferences of copepods and the different parameters that can induce and modify them. It has been shown that copepods are selective grazers (Azovsky et al., 2005; Meunier et al., 2016; Tackx et al., 2003) and each copepod species possess its own mode of feed detection based on different signals such as tactile and olfactory stimuli (Gonçalves et al., 2014). But the most important factor for

successful ingestion is prey size suitability. The retention efficiency of the copepod maxillae, which corresponds to the filtering mouthparts, increases with particle size (Boyd, 1976). A reference study by Frost (1972) showed that when the copepod *Calanus pacificus* is in the presence of two microalgal species of different cell size, it will tend to preferentially feed on the microalga presenting the largest cell size. Later studies based on other copepod species have reached the same conclusions (Kiørboe et al., 1982, Barthel et al., 1983, Libourel Houde & Roman, 1987, Lee et al., 2012a). *Rhodomonas salina* is the largest microalgae here and this is most likely why it is preferentially ingested by *P. nana*.

When comparing the obtained number of individuals of each life stage after 15 days, the diets R+T and R are again the most productive. They are respectively the plurispecific and the monospecific diet always giving the highest numbers of individuals for each life stage. They particularly stand out from the others for the numbers of copepodites and females from all stages, mainly C5 and ovigerous females. These results tend to demonstrate that *Rhodomonas* is more efficient than other microalgaes for the naupliar survival and development to copepodite stages and also the female fecundity of *P. nana*. Koski et al. (1998) showed that this microalga species induces the fastest rate of development and the lowest mortality in the copepod *Pseudocalanus elongatus* in comparison with the other tested diets. A subsequent study by Ohs et al. (2010) proved that the survival of nauplii to adult of *Pseudodiaptomus pelagicus* was the highest with a *Rhodomonas* diet and that *Rhodomonas* is the tested microalgae giving the highest percentage of ovigerous females for a feeding with a non-limiting cell density.

The P diet exclusively composed of *P. lutheri* is also the least productive one here when looking at each individual life stage. Beside P, the T and T+P diets

were overall the least productive ones for each *P. nana* life stage. All the diets that did not contained R were the least interesting ones for mass culture of *P. nana*.

4.2. Effects of microalgal diet on the size and fecundity of *Paracyclopina nana* ovigerous females

The size of copepods females is a major parameter determining the reproductive success of a population (Kiørboe & Sabatini, 1995; Sichlau & Kiørboe, 2011). Copepod individual size is controlled by food quality and is the key to the reproductive potential. The larger a female copepod is, the more it indicates that it has accumulated essential fatty acids, and the more it will present a high egg production potential and will also generate juveniles of good quality. A study carried out by Ban (1994) on the copepod *Eurytemora affinis* demonstrated that the prosome length of food-limited females was 75% of well-fed ones, and an equation correlating the clutch size of well-fed females with their respective prosome length was even established. Other environmental conditions such as temperature and salinity can also affect clutch size that is linearly related to the prosome length (Souissi et al., 2016b). The present study used a population of *P. nana* grown at constant temperature and salinity for several generations in a large volume (300 L). The same conditions were used in the 10 L experimental beakers. Consequently, the observed results can be principally attributed to diet composition. They show that the R and R+T diets are the ones that have induced the greatest prosome length in *P. nana* ovigerous females after 15 days of feeding, whereas the P and T+P diets produced females presenting the smallest prosomes.

And the R+T diet has also induced the greatest prosome width in ovigerous females whereas the T diet generated in the lowest size.

Female copepod fecundity is an essential factor to look at when investigating the optimal productivity of a population in culture (Parrish & Wilson, 1978; Lacoste et al., 2001). Egg production is one of the main parameters determining population growth in copepods, and several studies have shown it is largely influenced by food quality (Uye, 1981; Castro-Longoria, 2003; Ianora, 2005), and particularly the fatty acids composition (Støttrup & Jensen, 1990; Lee et al., 2006). Our results led to the same conclusion as morphological features for female's individual fecundity. The largest clutch sizes per ovigerous female was induced by the R, R+T and R+T+P diets and the greatest egg equivalent spherical diameter (ESD) has also been obtained with the R+T diet. On the contrary, the T+P, T and P diets had the lowest egg numbers and the lowest egg equivalent spherical diameters per ovigerous female, the same diets that produced the smallest ovigerous females in terms of prosome length. Final results on clutch size and prosome length are found to be similar. The R and R+T diets are the ones giving the best results for both parameters. The representation obtained by plotting clutch size against prosome length for each microalgal diet confirms this idea (**Fig. 6**).

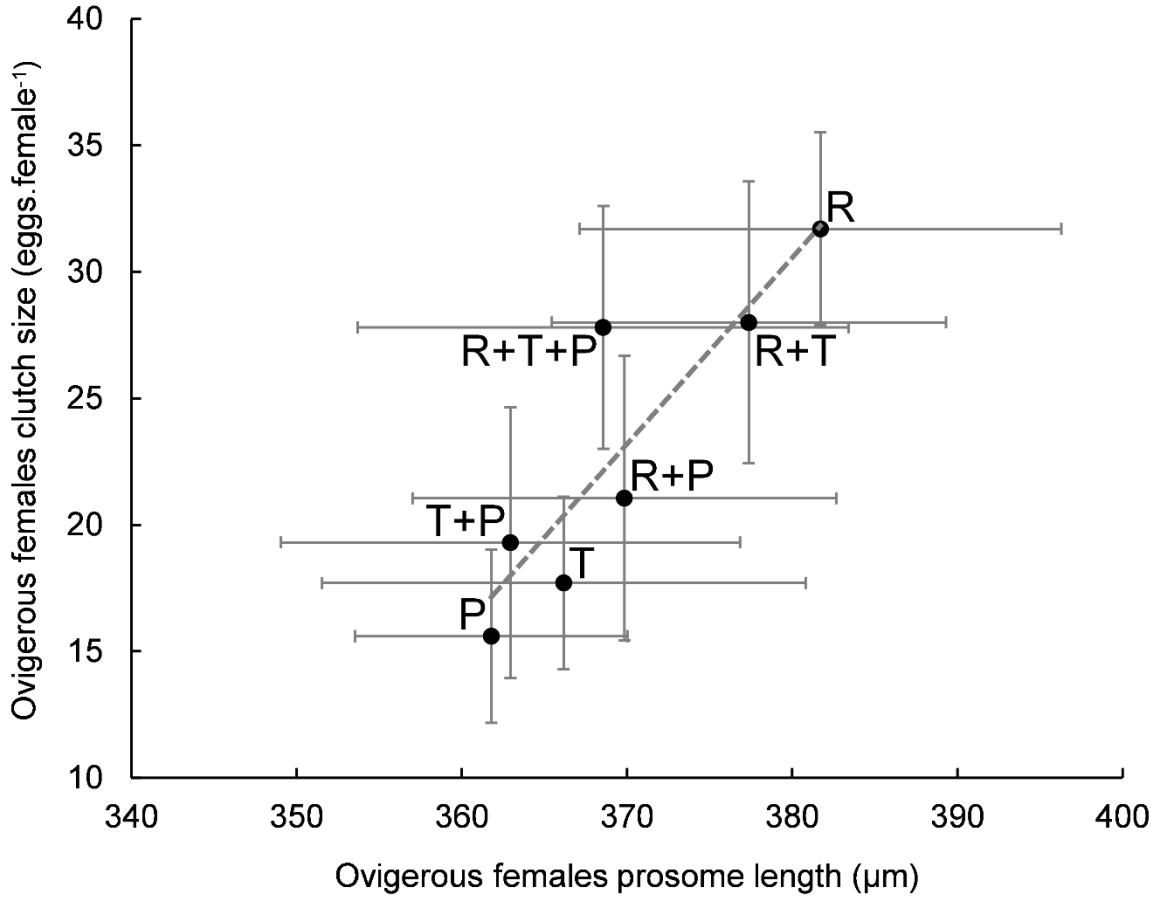

FIGURE 6. Relationship between the average prosome length and clutch size of *Paracyclopina nana* ovigerous females for each microalgal diet. Error bars show the standard deviation obtained from n=20 females. The dashed line corresponds to the linear regression (CS=0.7365xPL-249.32, R^2=0.7867, p<0.01). A Pearson's test was performed to define the significance of the coefficient of determination R^2. R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

A linear relationship does exist between ovigerous females clutch size and prosome length (y=0.7365x-249.32), with a significative correlation coefficient (R=0.8869, p<0.01).

Many studies have proved that copepods fed on two-species microalgal diets present a higher fecundity than those fed on single-species diets (Støttrup, 2000; Payne & Rippingale, 2001; Knuckey et al., 2005; Lee et al., 2006). Pan et al. (2018) found that females of *Apocyclops royi* fed on binary microalgal diets attest a higher fecundity compared with those fed on monospecific microalgal diets. In fact,

copepod reproduction requires high levels of nutritional and energetic intake. Therefore, the microalgal diet must provide an optimal nutritional balance (Milione & Zeng, 2007), which only a mixed diet can provide. In their study, Lacoste et al. (2001) proved in the copepod *Calanus helgolandicus* that the apparent female reproductive incapacity was linked to the number and combination of the microalgae species in the diet.

Our results also confirm that *R. salina* is the microalgae species providing the best fecundity parameters with *P. nana* ovigerous females. Several studies already proved the benefit of *Rhodomonas* on copepod fecundity and egg production. McKinnon et al. (2003) found that *Rhodomonas* supported relatively high egg production rates of *Acartia sinjiensis*. A further study by Siqwepu et al. (2017) showed that *Pseudodiaptomus hessei* individuals fed *R. salina* exhibited the highest fecundity in comparison to those fed *Isochrysis galbana* and the 50:50 binary diet of those two microalgae species.

The R+T, R and T diets also resulted in the lowest M:F sex ratios compared to the other diets by the end of the experiment, meaning a majority of females made up the respective populations. These results again show that these microalgae enhance the female fecundity, as more females also means more egg production and an ever increasing population.

5. CONCLUSIONS

Copepod aquaculture requires a microalgal diet of a nutritional quality that ensures increased population growth and female fecundity. Based on the results of this study, *R. salina* was shown to be the most effective microalga tested for a good

productivity of *P. nana* in culture. This is even more effective when combined with *T. lutea*. The monoalgal diet exclusively composed of *R. salina* and the binary one made of *R. salina* and *T. lutea* both induced the highest population growth and for each life stage, greatest individual size and clutch sizes in ovigerous females. To the contrary, *P. lutheri* appears to not be a very effective microalga as feed of *P. nana* for aquaculture purposes. In practice, a regular feeding equally composed of *R. salina* and *T. lutea* should be provided for efficient *P. nana* aquaculture. Our present study provides first results that demonstrate the high productivity provided by feeding *Paracyclopina nana* in culture with *Rhodomonas*.

Part 2

Effects of microalgal diet on the nutritive quality and reproductive investment of the cyclopoid copepod *Paracyclopina nana*

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1. INTRODUCTION

Copepods are the primary component of zooplankton and present a key role as the trophic link between phytoplankton and secondary consumers in marine ecosystems (Breteler et al., 1990; Mauchline et al., 1998; Støttrup, 2000). They serve as an important food source for numerous fish and crustacean larvae (Sun & Fleeger, 1995; Turner, 2004). Commercial species are bred in aquaculture using brine shrimps and rotifers for feeding, known to be easy to grow in large volumes and to present a relatively low cost (Sorgeloos, 1980; Lubzens, 1987). But these preys remain unnatural and do not always bring optimal fish larval growth because of their inadequate size or fatty acid content and even need a nutritional enrichment (Watanabe et al., 1983; Léger et al., 1987; Fernández-Reiriz et al., 1993; Sorgeloos et al., 2001; Lubzens et al., 2003). Mass culture of several marine copepod species has now been tried in order to use them as live feed for marine fish cultures (Zillioux, 1969; Støttrup et al., 1986; Ohno et al., 1990; Støttrup & Norsker, 1997; Payne & Rippingale, 2000; Van der Meeren et al., 2014; Blanda et al., 2017). It has been demonstrated that their superior nutritional value is more adapted to the growth and development of many species of commercial interest (McEvoy et al., 1998; Shields et al., 1999, Payne & Rippingale, 2000; Payne, Rippingale & Cleary, 2001; Olivotto et al., 2008). Copepods present a high content of highly unsaturated fatty acids (HUFAs) such as docosahexaenoic acid (DHA) and eicosa- pentaenoic acid (EPA), both really important nutrients for the development of fish larvae (Izquierdo, 1996; Sargent et al., 1997; McEvoy et al., 1998; Pan et al., 2014). A copepod inducing a high yield for aquaculture is therefore characterized by high lipid concentration and high fecundity. Copepod productivity

is actually translated in terms of female fecundity and individual storage of fatty acids consisting of lipid droplets (Souissi et al., 2016a; Lee et al., 2017), and thus in amounts of carbon chains ingested through the diet. Carbohydrates, and particularly monosaccharides, on the other hand, are only rarely stored in the crustacean organism, but they are not less important because they are rapidly metabolized after ingestion to be used for some vital functions and thus have a key role in biological performances (Cuzon et al., 2000; Hohnke & Scheer, 2012). It is therefore essential to supply copepod mass cultures with optimal microalgal diets inducing maximal fecundity, larval development and nutritional storage (Pan et al., 2014).

Some works have been published on optimising diets for copepod cultures but many questions remain to be explored. The assessment of an appropriate microalgal diet for captive copepods has been attempted on some species that belong to orders such as Harpacticoida (Pinto et al., 2001; Arndt & Sommer, 2014), Calanoida (Camus et al., 2009; Milione & Zeng, 2007; Ohs et al., 2010; Jeyaraj & Santhanam, 2012; Pan et al., 2014; Siqwepu et al., 2017) and Cyclopoida (Lee et al., 2006; Rasdi & Qin, 2016; Pan et al., 2018). Results globally indicated that mixed algal diets are always more effective than monoalgal diets. However, these studies did not use the same set of microalga and can not be generalized to all copepod species. Moreover, several studies already underlined the high quality of red microalgae from the genus *Rhodomonas* that improves mainly the female fecundity compared to other diets (Ohs et al., 2010; Siqwepu et al., 2017). 2017). The biochemical composition of *Rhodomonas sp.* is quite known, especially its fatty acid profile. Renaud et al. (1999) stated that its lipid content represents 18.7% of dry weight and is mostly composed of polyunsaturated fatty acids at a very high

rate of 65.8%. However, its carbohydrate profile is poorly known. Another micro-algae genus widely used in aquaculture is *Isochrysis*. The lipid content of *Isochrysis sp.* can reach 23.4% of dry weight (Renaud et al., 1999). The carbohydrate constituents of *Tisochrysis lutea*, the species from the genus *Isochrysis* chosen for our study, also represent up to 23% of dry weight and were reported to be a complex mixture of polysaccharides containing glucose, galactose, mannose, xylose, arabinose, xylose and rhamnose in various proportions, all of them being essential for copepod metabolism (Chu, Dupuy & Webb, 1982; Gnouma et al., 2017). However, a lack of studies looking into the understanding of feeding conditions of cyclopoid copepod species still persists.

The brackish water cyclopoid copepod *Paracyclopina nana* Smirnov 1935 has revealed a strong potential for the aquaculture industry (Ki et al., 2009; Lee et al., 2006; Lee et al., 2013). Native to the bays and estuaries of Eastern Asia (Japan, Taiwan, and South Korea), it has a small size at adult stage (600 µm on average), a high tolerance to salinity and temperature ranges, and easily endures being cultured at high densities (Lee et al., 2012b; Lee et al., 2017). Recent studies conducted on this species tried to establish the optimal diet for its growth in aquaculture (Min et al., 2006; Lee et al., 2012a). But these studies often use algal monoculture, while work carried out on other copepod species demonstrated that the microalgae quality can affect productivity (Pan et al., 2014). It is therefore essential to optimize copepod culture conditions by selecting the right diet and the right combination of microalgae.

The aim of this study was to examine the effects of different microalgal diets aiming to optimize the nutritive quality and reproductive investment of *Paracyclopina nana* populations in mass culture. This work relies on multi-year

skills in culture of several species of copepods and the use of microalgal species whose high potential and interesting nutritional value have been demonstrated.

2. MATERIALS AND METHODS

The materials and methods used are the same as those described in the previous paper since the results presented here are from the same experiments. This part will therefore be described more succinctly and the reader is invited to refer to the first paper for further details.

2.1. Establishment of microalgae diets

Three species of microalgae, *Rhodomonas salina*, *Tisochrysis lutea,* and *Pavlova lutheri*, were individually grown in the laboratory using standardized methods and were obtained from the Roscoff Culture Collection of Marine Microalgae (http://roscoff-culture-collection.org/). These three species were distinguished by their belonging to different genera and families of microalgae. As a result, their physiology, their physico-chemical composition and their nutritional quality were known to be different (Brown, 2002).

In order to test the nutritional value of the selected microalgal species on *P. nana*, they were fed to copepods in carbon equivalents so only the species of algae varied. In this study, carbon was used as a proxy in order to establish equivalent rations among the three microalgae species (Pan et al., 2014). A CHN analysis was performed on samples of each of the three microalgal cultures at their exponential phase of growth using an *elemental analyzer* (FLASH 2000 Series CHNS/O

Analyzer, Thermo Fisher Scientific, Waltham, MA). The aim was to know their respective cellular concentration of carbon. Obtained values were compared with standard reference values (Berggreen et al., 1988; Verity et al., 1992; Flynn et al., 1994; Olenina et al., 2006) and can be found in the first paper.

Then the different possible microalgal diets were constituted between the different combined species so that the total given volume remained the same.

With these three species of microalgae (*R. salina* (R), *T. lutea* (T), *P. lutheri* (P)), seven combinations of different diets were established:

- 3 single-species diets: R, T and P
- 3 two-species diets: R+T, T+P and R+P
- 1 three-species diet: R+T+P

Because of the variability of cellular carbon concentration and cell density in microalgal cultures, we standardized the volume of microalgal mixtures used for this study. According to the standard feeding protocol used to feed the permanent copepod cultures maintained in the laboratory, it was necessary for our study to give 9.1 mL of microalgal diet per day for each beaker. This volume of algal cells corresponded to a carbon amount that was not limiting for this species (Lee et al., 2012a). Thus, in order to make the different combined diets, volume ratios between each of the microalgal species were deduced so that the combined specific sub-volumes within each diet contained the same amount of carbon in proportion to the same total volume of 9.1 mL and that only the species parameter needed testing.

2.2. Microalgal cultures and initial sampling

Microalgae were grown in batch cultures in six different autoclaved 2 L flasks

following the protocol of Sadovskaya et al. (2014). The specific culture conditions used can be found in more details in the first paper. Three other 2 L flasks were set up with the exact same protocol, one for each species, exclusively intended for fatty acids and carbohydrates analysis. At first respective exponential phase, three samples of 1 mL were made for each microalga species and fixed with Lugol's solution for cellular counting. The entire remaining volume of culture of each species was then centrifuged and lyophilized for further fatty acids and carbohydrates analysis.

2.3. Copepod cultures and initial sampling

Paracyclopina nana used in current experiments was initially obtained from Prof. Jae-Seong Lee from Sungkyunkwan University of South Korea in 2015. Copepods were cultured in the laboratory in 300 L cylindrical acrylic tanks. The specific culture conditions and collecting protocol used can be found in more details in the first paper. Adult stages were removed and half of the amount of nauplii contained in the tank was kept for the experiment.

Seven 10 L beakers containing 8 L of culture medium were set up, one for each of the diets tested. The use of a single replicate of these large beakers has been justified in the first paper. Each beaker was filled with 8 L of 15 ppt water and placed at a constant temperature of 18°C with a 12 h light: 12 h dark photoperiod. The 1.2 L of concentrated nauplii was divided in 12 equal parts of 100 mL. Seven of them were each diluted in one of the 7 beakers of the experiment, two of them were individually filtered to remove any water, concentrated in cryotubes,

and stored at -80°C for both fatty acids and carbohydrates analysis, and the three other parts of 100 mL were used for other analysis.

2.4. Experiment monitoring

Daily feeding with microalgal diets

Each beaker was fed daily with a total volume of 9.1 mL for each combination of microalgal diet, in relative proportions of microalgae. Microalgal cultures were always fed to copepods at their exponential growth phase. More details about the procedure can be found in the first paper.

Daily and final sampling

At the end of the experiment after 15 days, two volumes of 500 mL were sampled from each condition. Each one was individually filtered to remove any water, and copepods were collected and concentrated in cryotubes, and stored at -80°C for both fatty acids and carbohydrates analysis. This sampling was supplemented by sorting of 20 ovigerous females from the fixed samples of each condition intended for morphological measurements (prosome length and width), and fertility study (number and size of eggs per ovigerous female).

2.5. Ovigerous females morphological measurements

The 20 fixed ovigerous females of each diet condition were individually photographed and studied under an inverted microscope (model Olympus IX71, Tokyo, Japan) with 10X magnification coupled with a ToupCam camera (model UCMOS05100KPA, Zhejiang, China) connected to a computer using a ToupView software from ToupTek Photonics (version 3.7). For each single ovigerous female, a photo of the whole body was taken for measurements. Then the egg sacs were manually broken and photos of all the eggs were taken with the software.

Morphological measurements were then performed on each photo using ImageJ software (version 1.48v) as described in Souissi et al. (2016a). Prosome length and width and eggs diameter were manually measured for each ovigerous female from each microalgal diet. Then prosome volume was calculated using the formula for calculating the volume of an ellipsoid: $V=\frac{4}{3}\pi ab^2$, where a and b used two times are the three semi-axis, a corresponding to half of the body length, and b corresponding to half of the body width.

2.6. Fatty acid analysis

To highlight the variations of internal fatty acid quantity induced by the different tested diets, the fatty acid methyl esters (FAMEs) of the copepods and microalgae samples stored at -80°C were prepared by a direct transesterification. The employed method is the same as that detailed by Pan et al. (2018). The used quantity of lyophilized copepods and microalgal pellets (n = 1 and n = 3, respectively) was 5 mg each. The extracted FAMEs were then conserved at −20°C until gas chromatography-mass spectrometry (GC-MS) analysis which was performed on a Trace GC ULTRA system (Thermo Scientific, Waltham, MA, USA)

equipped with a capillary column NMTR-5MS (30 m × 0.25 mm) using a temperature gradient of 170°C (3 min) → 250°C at 5°C/min and with a DSQ II MS detector. Obtained results for copepods were then converted and directly expressed in µg/mg DW. For microalgae, the counting of the number of cells in 1 mL triplicates was converted to the initial tested volume of 1700 mL for each species and then extrapolated for 5 mg. Obtained results were then also expressed in µg/mg DW.

2.7. Carbohydrate analysis

Internal carbohydrate quantity of the different copepods and microalgae samples were also studied. An analysis focused on monosaccharides was conducted following the same method as Sadovskaya et al. (2014). After lyophilization of all the samples, 5 mg of dry cells in triplicates were used for microalgae and each of the whole sample was used for copepods, with its respective weight previously noted. Samples were defatted by extractions with CHCl–MeOH, 2:1 and 1:2 (v/v), and air-dried. The residue was hydrolyzed with 4 M TFA (110°C, 3 h) in the presence of a known amount of myo-inositol, the internal standard. 50 µL of myo-inositol concentrated at 180 µg/100 µL (corresponding to 90 µg) was used for microalgae samples and 50 µL of myo-inositol concentrated at 9 µg/100 µL (corresponding to 4.5 µg) was used for copepod samples. The released monosaccharides were then dried with nitrogen before being reduced with NaBH4 for one night. The solution was then dried three times with nitrogen with addition of AcOH to destroy the reagent, and then dried twice with addition of MeOH. Monosaccharides were then acetylated (alditol acetates) with 0.4 mL Ac_2O–0.4 mL

pyridine mixture for 1 h at 100°C, and dried with toluene. They were finally cleaned up two times with successive addition of chloroforme and distilled water and concentrated by drying before being analyzed by gas chromatography-mass spectrometry analysis. GC–MS was performed using the same method as for fatty acid analysis. Obtained results were then converted and directly expressed in µg/mg DW.

4. RESULTS

3.1. Effects of microalgal diet on fatty acid composition of *P. nana*

Present results (**Table 3**) are based on 5 mg of each lyophilized sample. The total fatty acid amounts have been calculated from the initial standard C17 quantity introduced in each sample (10 µg). Detailed DHA and EPA amounts are shown. Three replica (n=3) were made for the microalgae samples and a mean value was then calculated. For copepods, the fatty acid increase ratios have been deduced from the FA quantity found in the T0 sample made at the very beginning of the experiment.

TABLE 3. Total fatty acid amounts and respective EPA and DHA amounts for each copepod and microalgae lyophilized sample.

Microalgae samples	Total FA amount, n=3 (µg)	Fatty acid ratio	µg total FA/mg DW, n=3	µg DHA/mg DW, n=3	µg EPA/mg DW, n=3	DHA/EPA ratio
R. salina	331.9 ± 36.09	C17 x331.9	66.38 ± 7.22	5.04 ± 0.90	8.06 ± 0.90	0.63
T. lutea	426.9 ± 21.80	C17 x426.9	85.38 ± 4.36	8.31 ± 0.56	0.69 ± 0.24	12.04
P. lutheri	375.5 ± 70.16	C17 x375.5	75.10 ± 14.03	7.68 ± 0.84	25.43 ± 1.27	0.30

Copepod samples	Total FA amount (µg)	Fatty acid increase ratio	µg total FA/mg DW	µg DHA/mg DW	µg EPA/mg DW	DHA/EPA ratio
T0	22.8	T0	4.56	0.404	0.106	3.81
R	251.7	T0x11.04	50.34	5.57	2.66	2.09
T	246.2	T0x10.80	49.24	5.37	2.08	2.58
P	157.6	T0x6.91	31.52	3.08	1.36	2.26
R+T	291.1	T0x12.77	58.22	3.40	3.19	1.07
T+P	182.3	T0x8.0	36.46	4.38	1.04	4.21
R+P	159.2	T0x6.98	31.84	4.25	1.28	3.32
R+T+P	168.5	T0x7.39	33.7	3.04	1.40	2.17

T. lutea (T) is the microalgae presenting the highest total fatty acid amount per mg compared to the other two species (85.38 µg ± 4.36 µg) whereas *R. salina* (R) is the one presenting the lowest total fatty acid amount per mg (66.38 µg ± 7.22 µg) and also the lowest DHA amount per mg (5. 04 µg ± 0.90 µg). It shows a DHA/EPA ratio of 0.63. *T. lutea* is also the one containing the highest DHA amount per mg (8.31 µg ± 0.56 µg) but it however presents the lowest EPA amount per mg (0.69 µg ± 0.24 µg) among the three tested microalgae species, making it the one with the highest DHA/EPA ratio (12.04). *P. lutheri* (P) contains a much higher EPA amount than the other two species (25.43 µg ± 1.27 µg) and a relatively high DHA amount (7. 68 µg ± 0.84 µg), leading it to present the lowest DHA/EPA ratio (0.30).

The R+T (291.1 µg), R (251.7 µg), and T (246.2 µg) diets led to the highest fatty acid amounts in copepods after 15 days of feeding, which corresponds to increase ratios of 12.77, 11.04 and 10.80, respectively. The diets inducing the weakest fatty acids increase in copepods are R+T+P (168.5 µg), R+P (159.2 µg), and P (157.6 µg), which corresponds to increase ratios of 7.39, 6.98 and 6.91, respectively. R+T is also the diet inducing the highest EPA amount per mg in copepods among all the seven tested diets with 3.19 µg, and so the lowest DHA/EPA ratio (1.07). The highest DHA amounts per mg have been detected in copepods fed with the R and

T diets with 5.57 µg and 5.37 µg respectively. The highest DHA/EPA ratio has been found in copepods fed with the T+P diet, due to the really low EPA amount per mg it induced (1.04 µg).

3.2. Effects of microalgal diet on monosaccharide composition of *P. nana*

Results gathered in **Table 4** are based on 5 mg of lyophilized sample for microalgae and each of the whole sample for copepods. Respective final amounts have been calculated from the initial standard myo-inositol quantity introduced in each sample (90 µg for microalgae and 4.5 µg for copepods). Detailed amounts of 7 specific monosaccharides are shown. Three replica (n=3) were made for the microalgae samples and a mean value was then calculated.

TABLE 4. Total monosaccharide amounts and respective amounts of 7 specific monosaccharides for each copepod and microalgae lyophilized sample.

Micro-algae samples	µg total MonoS/ mg DW, n=3	µg Glc/mg DW, n=3	µg Man/mg DW, n=3	µg Gal/mg DW, n=3	µg Rib/mg DW, n=3	µg Fuc/mg DW, n=3	µg Ara/mg DW, n=3	µg Xyl/mg DW, n=3
R. salina	46.60 ± 4.71	36.46 ± 3.31	1.86 ± 0.15	2.95 ± 0.34	2.17 ± 0.32	1.23 ± 0.17	0.87 ± 0.41	1.07 ± 0.24
T. lutea	28.28 ± 9.20	3.15 ± 1.32	3.35 ± 1.03	7.55 ± 2.82	2.01 ± 0.42	-	9.55 ± 2.53	2.66 ± 1.22
P. lutheri	32.67 ± 3.97	19.21 ± 0.51	2.17 ± 0.34	3.26 ± 0.51	3.96 ± 1.50	-	1.65 ± 0.48	2.42 ± 0.68
Copepod samples	**µg total MonoS/ mg DW**	**µg Glc/mg DW**	**µg Man/mg DW**	**µg Gal/mg DW**	**µg Rib/mg DW**	**µg Fuc/mg DW**	**µg Ara/mg DW**	**µg Xyl/mg DW**
T0	4.08	0.29	0.43	0.40	2.22	0.12	0.32	0.30
R	6.28	1.65	0.55	0.86	1.88	-	0.58	0.76
T								
P	3.83	0.74	0.49	0.95	0.67	-	0.46	0.53
R+T	15.09	1.85	1.56	1.39	7.70	0.43	1.11	1.05
T+P	22.89	5.19	3.46	5.18	2.61	0.49	2.75	3.22
R+P								
R+T+P	40.69	5.43	4.79	8.81	7.33	0.45	7.41	6.45

R. salina (R) is the microalgae presenting the highest total monosaccharide amount per mg compared to the other two species (46.60 µg ± 4.71 µg) whereas *T. lutea* (T) is the one presenting the lowest total monosaccharide amount per mg (28.28 µg ± 9.20 µg). *R salina* (R) also presents a much higher amount of glucose per mg than the other two species (36.46 µg ± 3.31 µg). It is also the only one of the three microalgae species in which fucose has been found (1.23 µg ± 0.17 µg). *T lutea* (T) is the one containing the highest amounts of manose (3.35 µg ± 1.03 µg), galactose (7.55 µg ± 2.82 µg), arabinose (9.55 µg ± 2.53 µg) and xylose (2.66 µg ± 1.22 µg). *P. lutheri* (P) is always the one containing lower amounts of the different monosaccharides studied except for ribose (3.96 µg ± 1.50 µg).

The R+T+P diet (40.69 µg) induced by far the highest total monosaccharide amount per mg in copepods after 15 days of feeding among the seven tested diets. The T+P (22.89 µg) and R+T (15.09 µg) diets also led to high total monosaccharide amounts in copepods. We notice that fucose has only been found in copepods with these three diets. R+T+P and T+P both led to relatively high amounts of each of the studied monosaccharides in comparison with the five other diets, with the R+T+P diet always being the highest. The R+T diet only surpasses them for the ribose amount (7.70 µg). The diet P induced the lowest total monosaccharide amount in copepods (3.83 µg), being even weaker than the initial amount in the T0 copepods sample (4.08 µg). P always presents the weakest amounts for each of the studied monosaccharides among the seven tested diets, except for galactose (0.95 µg).

Table 5 compares both fatty acids and monosaccharides final results in microalgae and copepods samples.

TABLE 5. Fatty acids and monosaccharides results comparative tables.

Microalgae samples	µg total FA/mg DW, n=3	µg total MonoS/mg DW, n=3
R. salina	66.38 ± 7.22	46.60 ± 4.71
T. lutea	85.38 ± 4.36	28.28 ± 9.20
P. lutheri	75.10 ± 14.03	32.67 ± 3.97

Copepod samples	µg total FA/mg DW	µg total MonoS/mg DW
T0	4.56	4.08
R	50.34	6.28
T	49.24	
P	31.52	3.83
R+T	58.22	15.09
T+P	36.46	22.89
R+P	31.84	
R+T+P	33.7	40.69

We notice that *R salina* presents the highest total monosaccharide amount per mg (46.60 µg ± 4.71 µg) and the lowest total fatty acid amount (66.38 µg ± 7.22 µg), whereas *T. lutea* presents the lowest total monosaccharide amount (28.28 µg ± 9.20 µg) and the highest total fatty acid amount (85.38 µg ± 4.36 µg). The same tendency is observed in copepods as the highest total monosaccharide amount is found with R+T+P (40.69 µg), which led to a relatively low total fatty acid amount (33.7 µg), whereas R+T and R induced the highest total fatty acid amounts (58.22 µg and 50.34 µg, respectively) but not the most successful total monosaccharide amounts in copepods (15.09 µg and 6.28 µg, respectively).

3.3. Effects of microalgal diet on reproductive investment of *P. nana* ovigerous females

Ovigerous females prosome volume and clutch volume

The distribution of ovigerous females prosome volume after 15 days of feeding with each tested diet is shown in **Fig. 7a**.

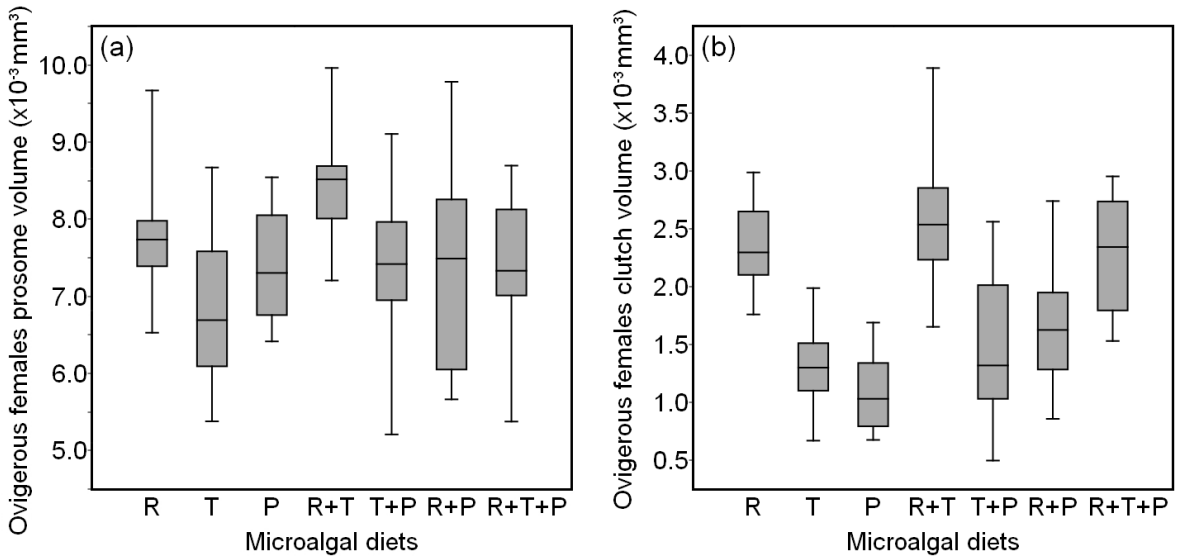

FIGURE 7. Boxplot showing the distribution of prosome volume (a) and clutch volume (b) of *Paracyclopina nana* ovigerous females fed on different microalgal diets. The shaded boxes represent the first and third quartile with the middle bar being the median. The end of the whiskers extends from the hinge to the lowest and highest value within a 1.5 inter-quartile range. R: *Rhodomonas salina,* T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

The R+T and R diets induced the greatest prosome volume in ovigerous females (R+T: 8.40 X 10^{-3} mm^3 ± 0.66 X 10^{-3} mm^3, R: 7.82 X 10^{-3} mm^3 ± 0.82 X 10^{-3} mm^3) whereas the R+P and T diets resulted in the weakest prosome lengths (R+P: 7.37 X 10^{-3} mm^3 ± 1.16 X 10^{-3} mm^3, T: 6.82 X 10^{-3} mm^3 ± 0.93 X 10^{-3} mm^3). The average clutch volume of these same ovigerous females (**Fig. 7b**) is also the greatest for the R+T, R and R+T+P diets (R+T: 2.59 X 10^{-3} mm^3 ± 0.55 X 10^{-3} mm^3, R: 2.36 X 10^{-3} mm^3 ± 0.37 X 10^{-3} mm^3, R+T+P: 2.27 X 10^{-3} mm^3 ± 0.47 X 10^{-3} mm^3) whereas the P diet resulted in the weakest clutch volume (1.09 X 10^{-3} mm^3 ± 0.31 X 10^{-3} mm^3).

The representation obtained by plotting clutch volume against prosome volume for each microalgal diet confirms this idea (**Fig. 8**).

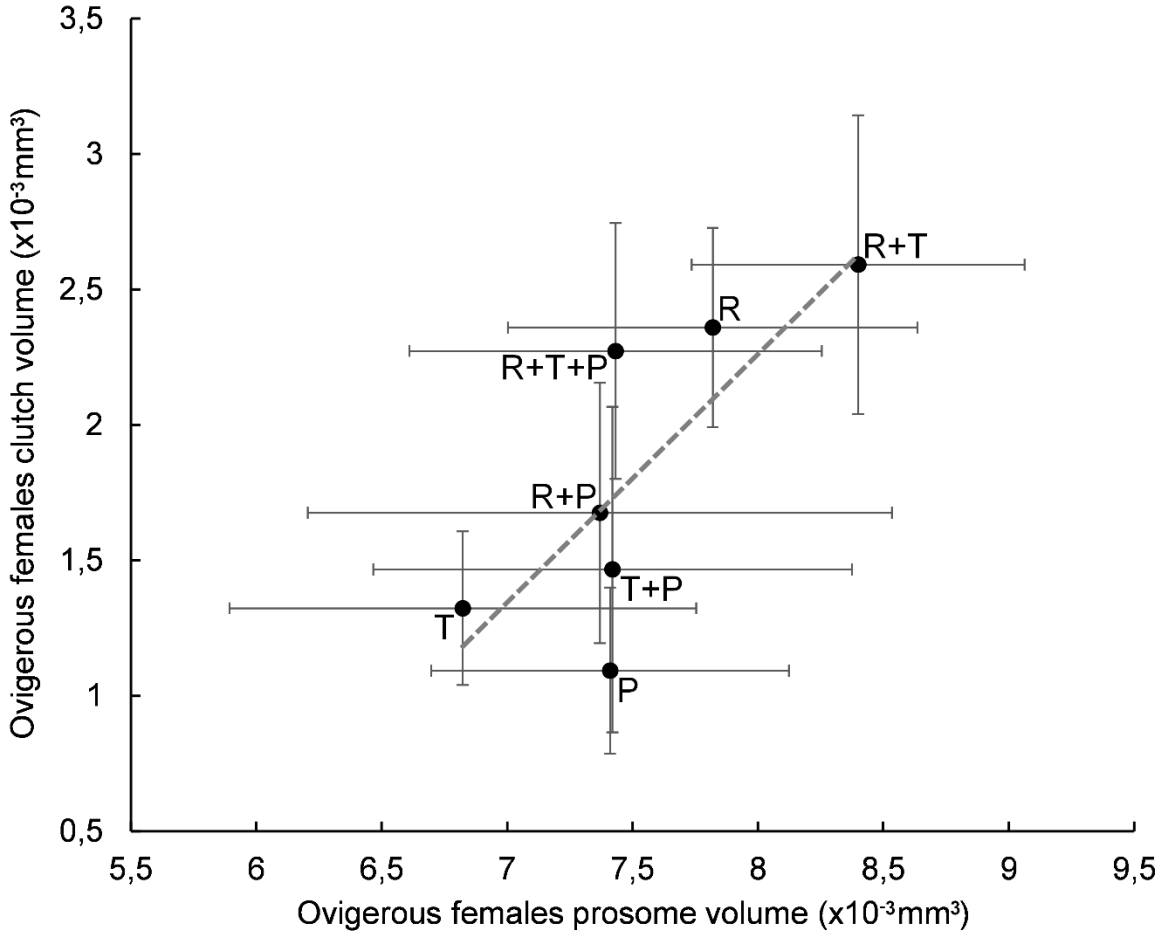

FIGURE 8. Relationship between the average prosome volume and clutch volume of *Paracyclopina nana* ovigerous females for each microalgal diet. Error bars show the standard deviation obtained from n=20 females. The dashed line corresponds to the linear regression (EV=0.9163xPV-5.069, R^2=0.5841, p<0.01). A Pearson's test was performed to define the significance of the coefficient of determination R^2. R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

The R+T diet gave the highest values for both parameters and a quite linear relationship does exist between ovigerous females clutch volume and prosome volume (y=0.9163x-5.069), with a significant correlation coefficient (r=0.5841, p<0.01).

Representative photos of the morphology of the different *Paracyclopina nana* ovigerous females fed with each diet are presented in **Fig. 9**.

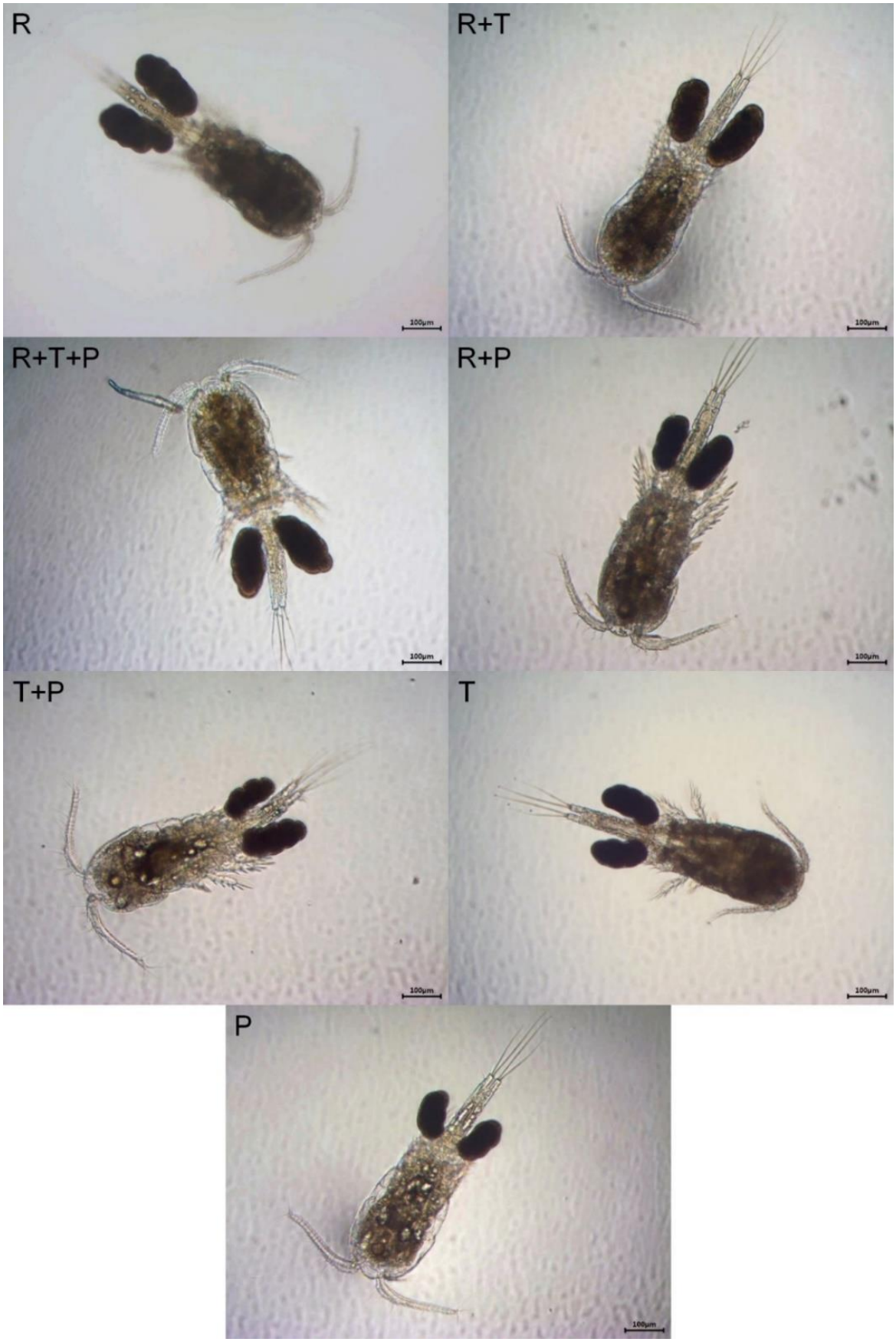

FIGURE 9. Visual comparison of the size of the egg sacs of *Paracyclopina nana* ovigerous females fed on different microalgal diets. R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

Photos showed that the R-fed and R+T-fed ovigerous females are the ones looking the biggest and presenting the biggest egg sacs compared to the others. They are followed by the ones fed with the R+T+P diet and the R+P diet. The ovigerous females looking the smallest and presenting the smallest egg sacs are the ones fed with T+P diet, the T diet and finally, the P diet.

4. DISCUSSION

4.1. Effects of microalgal diet on fatty acid composition of *P. nana*

The fatty acid profile of copepods is directly linked to their diet and its own fatty acid composition as these nutrients are stored in the copepod body as lipid droplets (Lee et al., 2017). It is therefore essential to investigate both of them in order to produce productive copepods for their use in aquaculture (Van der Meeren et al., 2008) and also understand their trophic ecology in marine ecosystems (El-Sabaawi et al., 2009).

In the present study, the highest final fatty acid amounts in copepods have been detected in those fed with the R+T, R and T diets. The diets inducing the weakest fatty acids increase in copepods are R+T+P, R+P and P, comforting the idea put forward by the previous results that *P. lutheri* is not the best microalgae to feed *P. nana* with. However, analysis show that *P. lutheri* is the microalgae containing the highest fatty acid amount and the one containing the lowest is *R. salina*. Copepods fatty acid profile must then lie in the nature and quality of the fatty acids contained in their diet and not in their relative quantity. This idea is confirmed when we study the respective DHA/EPA ratios. The highest DHA/EPA

ratio in individual microalgae is shown by *T. lutea* due to a very low EPA amount found in this microalga, but the T diet led to one of the highest DHA and EPA amounts in *P. nana* individuals. This phenomenon therefore implies a conversion capacity of DHA via EPA in the copepods. Desvilettes, Bourdier and Breton (1997) have already reported that *Paracyclopina nana* would be able to convert the high amounts of α-linolenic acid (ALA) from *Tetraselmis suecica* to EPA and DHA when fed on it. Thus, cyclopoid copepods seem to have the ability to incorporate dietary short-chain fatty acids from microalgae into their own EPA and DHA.

Conversely, the lowest DHA/EPA ratio is presented by *P. lutheri* because of its EPA amount being very high, the highest among the three tested species, but the copepods fed on the P diet did not reflected its EPA richness, revealing one of the lowest DHA and EPA amounts among all the tested diets. Although *P. lutheri* contained a lot of potential precursor fatty acids for DHA synthesis, they may not be efficiently absorbed by the copepods. It may be related to the small cell size of *P. lutheri* (4-6 μm) (Kamiyama & Arima, 2001; Rehberg-Haas, 2014), probably making it the most expensive one among the three species in terms of grazing energy for *P. nana* or it may not be adapted to the ingestion of this copepod species.

In our work, *R. salina* looks like an intermediate microalga with its average DHA and EPA amounts but its diet combination with *T. lutea* (R+T) led to the lowest DHA/EPA ratio in copepods, with a record EPA amount, and the diet its forming with *P. lutheri* (R+P) led to the highest DHA/EPA ratio in copepods, with a high DHA amount. The diet its composed alone (R) comes to be one of the most prolific for copepods fatty acid profile, with a record DHA amount and one of the highest for EPA.

Many studies pointed at the crucial roles of dietary DHA and EPA in fish

development (Izquierdo et al., 2000; Rainuzzo, Reitan, & Olsen, 1997; Sargent et al., 1999). These particular fatty acids contribute to maintain the function of visual and neural cells and the membrane structure. Therefore, a diet with DHA and EPA deficiency may lead to delayed growth and increased mortality in fish larvae (Izquierdo, 1996). DHA requirement is even of greater importance than that of EPA for marine fish as it is present in high concentration in fish larval tissue and has a high physiological efficiency for development. It was stated that the relative proportion of DHA to EPA in larval diets is essential, with an optimal ratio of DHA to EPA established at 2:1 (Sargent, McEvoy, & Bell, 1997). In the present study, the ratio of DHA to EPA in *P. nana* was always higher than 2:1 among the different tested microalgal diets, except for R+T being rather at 1:1. Particularly high DHA-to-EPA ratios has been observed in copepods fed with the T+P and R+P diets, probably because the coupling of *P. lutheri*, which has by far the highest amount of EPA, with either one of the two other microalgae with an additional amount of DHA, induced a record conversion rate of EPA to DHA and therefore particularly low amounts of EPA in these two diets.

Comparisons between microalgae and copepods fatty acid results enlightened the occurrence of a conversion capacity in *P. nana*. These complementary results confirm again that R+T and R are two interesting diets for the culture and enrichment of *P. nana,* inducing record internal amounts of DHA and EPA and globally increasing its fatty acid content. In another perspective, it appears that the diets T+P and R+P are the preferable ones here to feed *P. nana* with for use as fish larvae prey in aquaculture as these diets induced the highest DHA/EPA ratios in copepods.

4.2. Effects of microalgal diet on monosaccharide composition of *P. nana*

Carbohydrates in aquatic animals have been mostly studied on fish species and mainly to investigate fish abilities to use dietary carbohydrates, but few studies have been performed on crustaceans and even fewer on copepods. Most of the research focused on the carbohydrate metabolism of shrimp and crab species because of the nutritive interest of their high quality protein for humans (Wang, Li & Chen, 2016). The amount of knowledge in crustaceans is still quite limited. Carbohydrates in copepods are poorly known and previous investigations provided rather divergent results because the carbohydrate content in copepods turns out to vary a lot depending on the study conditions and various intrinsic factors (Raymont & Conover, 1961). These nutrients appears to be rarely stored in the crustacean organism as they are rapidly metabolized and directly used for some vital functions (Cuzon et al., 2000).

Present results show very different manosaccharide profiles between the three tested microalgae. *R. salina* presents by far the highest total monosaccharide content per mg, almost one third more than the two others. However, overall results show that its respective individual amounts of monosaccharides are always the lowest, except for glucose which is really high in *R. salina* and explains by itself its high total monosaccharide content. *R. salina* therefore seems to be a glucose-specialized microalgae. Conversely, results show that *T. lutea* is the one presenting the lowest total monosaccharide content, with a really low glucose amount per mg, more than ten times less than in *R. salina*, but always show higher, and most of the time the highest individual amounts for other monosaccharides. *T. lutea* profile therefore seems to be characterized by a variety of monosaccharides, without any

predominant one. *P. lutheri* presents an intermediate monosaccharide profile, with a majority of glucose content, but not as much as *R. salina* and not as much far from the other monosaccharides.

When looking at monosaccharides results in copepods, the R+T+P diet, which is the only one gathering the three tested microalgae species, is by far the most efficient one, giving copepods containing both the highest total monosaccharide content but also often the highest amounts for each of the individual studied monosaccharide. These results tend to demonstrate the existence of an additive effect of microalgae species on the monosaccharides richness. The more the diet is composed of various sources of monosaccharides, the more it will be concentrated in monosaccharides. This idea is confirmed by the rest of the present results as the following diets inducing the richest copepods in monosaccharides are T+P and R+T, both binary microalgal diets. Diets giving the lowest total monosaccharides content in copepods are the single-species diets, often resulting in individual monosaccharides amounts less than 1 µg/mg. The diet P corresponding to feeding with only *P. lutheri* is once again the least efficient one, resulting in very low monosaccharides amounts in copepods, corresponding to the very low concentrations already found in the cells of this microalgae.

Comparing both fatty acids and monosaccharides profiles in microalgae, it appears that *R. salina*, which was the most concentrated one in monosaccharides, was the lowest concentrated one in fatty acids. Inversely, *T. lutea* presented the lowest monosaccharides content but the highest one in fatty acids. The same phenomenon has been highlighted in copepods after feeding with the different established diets. R+T+P was the diet inducing the highest monosaccharide concentration in copepods but a low fatty acid content, whereas the R+T and the R

diet have given the most concentrated copepods in fatty acids but not the best ones for monosaccharide content. Our results therefore suggest that, for fish larvae rearing purposes, it is preferable to propose a varied diet to copepods, and thus combining several species of microalgae, and that the R+T diet is here the best compromise for a productive culture of *P. nana*.

4.3. Effects of microalgal diet on reproductive investment of *P. nana* ovigerous female

Our study showed that the three diets R+T, R and R+T+P have given the best results for both the clutch volume and the ovigerous female prosome volume in *P. nana*. Our previous results showed that these three diets are also the ones inducing the highest egg numbers per ovigerous female. As proved by our photos added to this study (Fig. 3), the diets R+T, R and R+T+P have thus induced both large females with bulky prosomes and many eggs forming a big clutch. When looking back at the fatty acid results, it's these three precise diets that have given the lowest DHA/EPA ratios in copepods. A low DHA/EPA ratio, and so a high EPA content, would therefore be linked to an increased fecundity in *P. nana* copepod females, both in number of eggs and morphological quality of eggs and females. Our results have precisely shown the highest EPA amounts in the copepods fed with the R+T and R diets, and also the highest total fatty acid amounts. The ovigerous females rich in fatty acids and particularly in EPA thus appeared to be the biggest and the most fertile.

Conversely, the P diet made of *P. lutheri* was the one showing the lowest clutch volumes and also the lowest egg numbers in our previous results in

ovigerous females. Our photos showed that the P-fed females are the ones looking the smallest and carrying the smallest egg sacs. All the diets in which *P. lutheri* was involved were the lowest ones in terms of EPA content in copepods, confirming again its poor performances in the culture of *P. nana*.

Ovigerous females prosome volume and clutch volume seem to be not only influenced in the same way by the EPA richness but also correlated to one another. Our results demonstrated a linear relationship and a significant correlation coefficient between prosome volume and clutch volume in *P. nana* ovigerous females. Thus, EPA seems to be a predominant fatty acid in the fecundity of *P. nana*. Arendt et al. (2005) found a positive relationship between the EPA amount ingested by the copepod *Temora longicornis* and its egg production rate. It appears that the higher the EPA content of the copepod is, the higher its egg production increase, and our present results with *P. nana* confirm this idea. Some studies have already shown that copepods fecundity may be influenced by the DHA and EPA content in their diets (Kleppel et al., 2005; Støttrup & Jensen, 1990). As found by Pan et al. (2018) with *Apocyclops royi*, our results indicate that dietary DHA levels do not appear to be leading in *P. nana* fecundity and may rather have an indirect role. Just like Lee et al. (2006) already reported on this species, *P. nana* may be able to synthesize DHA by itself from short-chain fatty acids. This ability would allow this copepod species to maintain its particularly high level of reproduction.

5. CONCLUSIONS

According to the overall results of this study, *R. salina* is the microalga that gives

the best results on all the aspects leading to a high productivity of *P. nana* in culture, and it leads to optimal parameters when combined with *T. lutea*. The mixed diet composed of *R. salina* and *T. lutea* both induced the highest total fatty acid content in copepods, a good monosaccharide profile, and the greatest prosome volumes and clutch volumes in ovigerous females. Conversely, *P. lutheri* does not appear as an effective microalga to enhance the potential of *P. nana* in aquaculture. These are the first published results proving that a feeding with *Rhodomonas* induces a high productivity of *Paracyclopina nana* in culture.

CHAPTER III

POTENTIAL OF *PARACYCLOPINA NANA* IN ECOTOXICOLOGY

Effects of different routes of exposure to metals on bioaccumulation and population growth of the cyclopoid copepod *Paracyclopina nana*

Dayras, P., Bialais, C., Ouddane, B., Lee, J. S., & Souissi, S. (2020). Effects of different routes of exposure to metals on bioaccumulation and population growth of the cyclopoid copepod *Paracyclopina nana*. *Chemosphere*, *248*, 125926.
https://doi.org/10.1016/j.chemosphere.2020.125926

1. INTRODUCTION

Most aquatic ecosystems are increasingly contaminated with a mixture of trace metals, such as cadmium (Cd), copper (Cu), and nickel (Ni) which became common contaminants in freshwater and oceanic environments (Rollin & Quiot, 2006). Their high concentrations in natural waters are originating from several anthropogenic activities including agriculture and aquaculture, industry, or urbanization (Thévenot et al., 2009; Förstner & Wittmann, 2012). Some metals such as Cu and Ni appear to be functionally essential to the metabolism of certain organisms at low concentrations (Langston & Bebianno, 1998) but can become toxic when they exceed a certain threshold concentration (Rainbow, Phillips & Depledge, 1990; Ansari et al., 2004). Cadmium was shown to be toxic only without known physiological functions in any organism (Larsson et al., 1976; Yang et al., 1996; Godt et al., 2006). It is recognized as one of the most toxic non-essential metals for aquatic organisms (Taylor, 1983), which leads to changes of the endocrine system, development, reproduction, and behaviour (Sullivan et al., 1983; Toudal & Riisgård, 1987). Besides showing these direct toxic effects, metals show combined effects and have a high potential of bioaccumulation within organisms and within ecosystems (Rainbow, 1990; Pavlaki et al., 2017). Metals commonly bioaccumulate within organisms, and can reach harmful levels, causing physiological changes and cascading problems in trophic chains (Dobbs, Cherry & Cairns, 1996; Kouba, Buřič & Kozák, 2010).

In aquatic ecosystems, copepods represent an important trophic link between lower trophic levels such as phytoplankton producers and secondary consumers like fish or invertebrate larvae (Breteler et al., 1990; Mauchline et al., 1998;

Støttrup, 2000). Therefore, they can be an important factor in the biomagnification of toxic contaminants in aquatic food webs (Fisher et al., 2000; Stewart & Fisher, 2003). Due to their central position in marine food webs, copepods are often used as bioindicators and are used as invertebrate models in aquatic ecotoxicology studies (Raisuddin et al., 2007; Kwok et al., 2015; Dahms et al., 2016). In addition, they are easy to cultivate because of their short life cycle and high fecundity (Raisuddin et al., 2007; Kulkarni et al., 2013). Of them, the brackish water cyclopoid copepod *Paracyclopina nana* Smirnov, 1935 has demonstrated a high potential both for the aquaculture industry and for ecotoxicological research (Lee et al., 2006; Lee et al., 2013; Dahms et al., 2016). Native to the bays and estuaries of Eastern Asia (Japan, Taiwan, and South Korea), it presents a small size at adult stage (600 µm of total length on average), a high tolerance to temperature and salinity changes, and supports to be cultured at high densities (Lee et al., 2012; Lee et al., 2017). Its whole genome has even been sequenced and annotated (Ki et al., 2009; Han et al., 2015; Lee et al., 2015; Jeong et al., 2017) (http://rotifer.skku.edu:8080/Pn). The first exposure tests of *P. nana* to environmental contaminants and other stressors showed interesting results on its molecular and biochemical responses. For example, when they are exposed to various metals (Cd, Cu, and As), the expression of vitellogenin transcripts was induced in a stronger and increasing manner throughout its life cycle (Hwang et al., 2010). Also, in *P. nana* ovigerous females, increasing antioxidant enzyme and DNA repair activities coupled to an increasing mortality were shown in a dose-dependent manner in response to gamma radiation (Won and Lee, 2014). However, these studies were mostly carried out at a small scale with low individual numbers and often using a single contaminant for each treatment. Given the current

ecotoxicological challenges with combined effects of many contaminants at the same time, it is demanding to use large individual numbers in larger mesocosms and several contaminants which is closer to the real world situation. An understanding of the effects of contaminant cocktails, such as metals, can provide a better understanding about ecological risks in natural situations. To date, the combined effects of metals are poorly known and only some studies showed their additive or synergetic effects (Bræk et al., 1976). Similarly, the bioavailability of metals is an important parameter to consider and only a few studies show that this parameter is closely correlated to the chemical speciation of metals (Batley, Apte & Stauber, 2004). Recently, the bioaccumulation rates of Cd, Cu, and Ni were studied in the estuarine calanoid copepod *E. affinis* (Zidour et al., 2019). The authors found that the accumulation rates of each metal was higher in treatments alone than the combined effect in a three-metal mixture. A lower bioaccumulation with the mixture could be explained by a competitive complexation occurred between the three metals, resulting in a saturation phenomenon of metal-binding sites. It would be interesting if this phenomenon applies also to another copepod species. Moreover, quantitative studies about the bioaccumulation of metals in cyclopoid copepods are still lacking. Furthermore, for tiny cyclopoids like *P. nana*, any experimental protocol for bioaccumulation study should consider a high number of individuals to reach the threshold value of a few mg of dry weight required for metal detection.

We used 1/3 50% lethal concentration (LC50) of each metal based on the values to *E. affinis*. Then, the LC50 concentrations of *P. nana* for Cd, Cu, and Ni were accurately measured by using the same protocol described in Tlili et al. (2016). This step allowed to perform a second bioaccumulation experiment using

1/3 LC50 of *P. nana*. Furthermore, we slightly modified the long-term mass culture conditions between both experiments by using a higher concentration of Cu in the second culture, as Cu seems to be the most labile metal in copepod bioaccumulation. It is important to compare both kinetics of bioaccumulation when initial conditions, in terms of initial loading of Cu in copepods, are totally different. Thus, we first replicated the experiment performed with *E. affinis* in the same conditions but by changing the copepod species (Experiment 1). Then we performed another experiment focused on the mixture using 1/3 LC50 of Cd, Cu, and Ni specific for *P. nana* but started the experiment with individuals already saturated in terms of Cu bioaccumulation (Experiment 2).

In this study, we used the animal model copepod *P. nana* to quantify the bioaccumulation of Cd, Cu, and Ni *via* waterborne uptake and the effect of these trace metals on population growth and structure. We particularly, challenged the competition hypothesis between these metals when combined.

2. MATERIALS AND METHODS

2.1. Copepod cultures and initial sampling

The copepod *P. nana* used in this experiment was initially obtained in 2015 from Prof. Jae-Seong Lee's lab from Sungkyunkwan University, South Korea and has been added to the copepod strain collection of Lille University in the Marine Station of Wimereux, France. Copepods are grown in the laboratory in large-scale culture systems of 300 L cylindrical acrylic tanks. The experimental conditions were the same as the regular culture conditions and also the same as in Zidour et

al. (2019): T = 18°C, salinity = 15 psu, 12h light: 12h dark photoperiod, and fed with the microalga *Rhodomonas salina* as in Arias et al. (2017). The protocol used for these two experiments is also the same as the one used in this study and as described below, with only few minor differences between the two of them.

For each experiment, a whole column of 300 L of *P. nana* was filtered on a 33 µm mesh to keep the whole population. Half of the amount of copepods was kept for the experiment and concentrated in a large beaker containing 15 psu seawater (filtered and autoclaved seawater + distilled water). This salinity was kept constant using a multiparametric probe. The remaining copepods was transferred to a new clean 300 L culture.

Several experimental 10 L beakers were set-up. We tested the effect of adding food or not on the copepod bioaccumulation capacity in Experiment 2. The respective experimental set- up and beaker labels used for each experiment were detailed in **Fig. 10**.

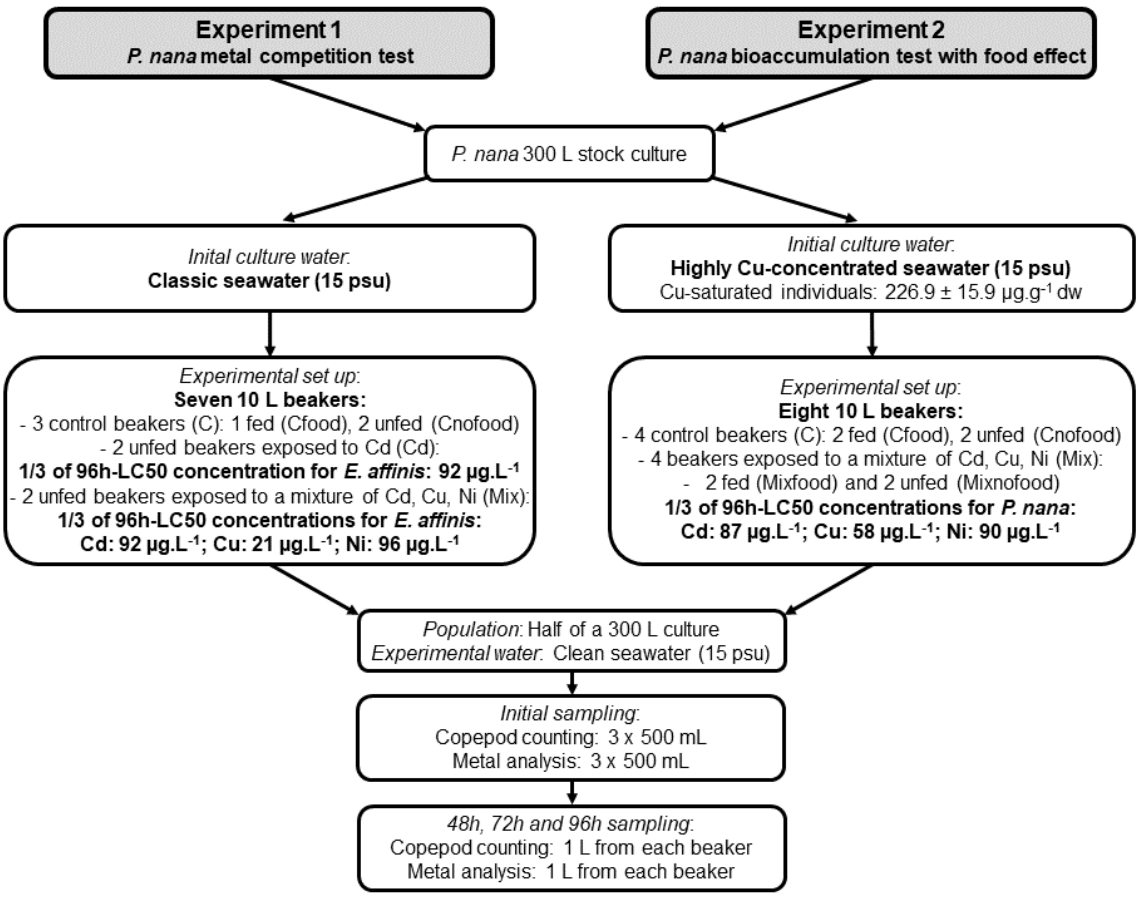

FIGURE 10. Flow chart of the experimental design and beaker labels used for each experiment.

Each beaker was filled with 8 L of 15 psu seawater. For each experiment and after homogenization, 1 L of the volume of concentrated copepods was added in each beaker of the experiment, and 3 L were used for initial sampling. Three volumes of 500 mL (n = 3) were individually filtered on a 33 µm mesh and concentrated with 70% ethanol for initial counting of the population structure, and three volumes of 500 mL (n = 3) were filtered through cellulose nitrate filters of 0.45 µm porosity (Whatman International Ltd., Maidstone, England). Filters containing copepods were dried in a desiccator for 72h and weighted for later metal analysis. Each time, 50 mL (n = 3) of the filtrate corresponding to culture water was collected and conditioned immediately in 50 µL pure nitric acid (HNO_3, Merck

Suprapur 65%) for further analysis of residual metal concentrations. For each experiment, the fed-treatments were initially fed with the microalga *R. salina* according to the protocol provided by Souissi et al. (2010).

2.2. Experimental monitoring

Daily sampling was the same as the initial sampling and was performed at 48h, 72h and 96h. The only difference was that a volume of 1 L was sampled from each beaker and filtered on a cellulose nitrate filter for metal analysis. Obtained filters were also weighed and dried daily in a desiccator throughout the whole experiment and the same volume of 50 µL of filtrate was collected and fixed. A volume of 1 L was also sampled daily from each beaker and fixed with 70% ethanol for population structure analysis. Also, the fed beakers were given *R. salina* daily following the same method as mentioned above.

2.3. Copepods individual counting

Copepods fixed with 70% ethanol were individually counted according to their sex and life stage using a stereomicroscope (model Olympus SZX12, Tokyo, Japan) and a desk manual counter with several units. Nauplii, copepodites (C1-C4), C5 males, C5 females, adult males, adult females and ovigerous females were differentiated according to morphological details (Hwang et al., 2010).

2.4. Metal analysis

Metal analysis was performed in the LASIR Laboratory, University of Lille (France) as described by Ouddane et al. (1999) with some modifications. Once filters containing copepod samples were dry, they were mineralized with 3 mL ultrapure nitric acid (HNO_3, Merck Suprapur, 65%) in a Block Digestion System (Environmental Express HotBlock® SC100, Charleston, SC, USA) at 105°C for two hours. Obtained solutions were then diluted with ultrapure water to a final volume of 10 mL. Respective elemental concentrations of the three targeted metals in the copepod and water samples were measured in triplicate against multi-element standards using an inductively coupled plasma atomic emission spectrometer (Agilent Technologies ICP-AES 5110, dual view, Santa Clara, CA, USA). Data obtained were expressed as the mean ± standard deviation (SD).

2.5. Statistical analysis

In order to compare the concentration of each metal (Cd, Cu and Ni) in copepods and in water, two similar databases were built for each experiment. In the first column, supposed to be our dependent variable, we considered the metal concentration. Then we encoded in four different columns all sources of variability starting by replicate number, treatments (food, no food), metal (Cd, Cu, Ni) and exposure duration (48, 72, 96h). Then for each metal we first performed a multiway (n-way) analysis of variance (ANOVA) to test the effects of multiple factors on the mean of metal concentration either in copepods or in water. Then in a second step and because no significant differences between the treatments having two

replicates were detected, we performed a two-way analysis of variance by considering only two factors treatment (food, no food) and exposure duration (48, 72, 96h). We also considered the interaction between these two factors in a two-way ANOVA model.

The same procedure was applied to the population structure of *P. nana* by using a similar database with the number of individuals per L as dependent variable and 4 factors composed of replicate, treatment, exposure duration, and life stage. The latter factor considered the following seven life stages: nauplii, copepodites (C1-C4), C5 males, C5 females, adult males, adult females, and ovigerous females. An additional variable composed by the sum of pre-adult (C5) and adult stages was also considered. The multi-way ANOVA and two-way ANOVA were applied in the same way as described for metals but by considering each developmental stage separately as well as a combination of them (8 variables in total).

For all two-way ANOVA analyses, a post-hoc test was performed by using the interactive graphical interface of MULTCOMPARE function of Matlab.

These analyses were performed using Matlab Software (Mathworks Inc., Version, 7.5).

5. RESULTS

3.1. Effects of metals on population growth of *Paracyclopina nana*

Total population growth

The total number of *P. nana* individuals obtained for each condition of each experiment is shown in **Table 6**.

TABLE 6. Detailed labels of the different conditions and their respective total population at 96h for each experiment.

Treatment description	Microcosm number	Label	Total population in Experiment 1	Total population in Experiment 2
Control with food	#1	Cfood1	8268	1960
Control with food	#2	Cfood2		1698
Control without food	#1	Cnofood1	7476	1532
Control without food	#2	Cnofood2	7616	1374
Cadmium without food	#1	Cd1	5916	
Cadmium without food	#2	Cd2	5956	
Mixture (Cd+Cu+Ni) with food	#1	Mixfood1		2094
Mixture (Cd+Cu+Ni) with food	#2	Mixfood2		2152
Mixture (Cd+Cu+Ni) without food	#1	Mix1 / Mixnofood1	7216	1340
Mixture (Cd+Cu+Ni) without food	#2	Mix2 / Mixnofood2	6440	1252

For Experiment 1, the three control conditions showed more individuals than in the conditions exposed to the contaminants, Cd and Mix. Nevertheless, the Cnofood conditions corresponding to the unfed controls showed less individuals after 96h than the fed control (Cfood) which led to the highest population density with 8268 individuals/L. At exposed conditions, the Cd-exposed beakers (Cd1 and Cd2) final populations appeared still below those of all other conditions, with 5916

individuals/L and 5956 individuals/L, respectively. Conversely, Mix1 and Mix2 beakers presented higher final populations than those of beakers exposed only to Cd. At the end of Experiment 2 at 96h, all control beakers (fed and unfed) showed a higher population density than the unfed exposed beakers (Mixnofood), while Cfood was always higher than Cnofood. However, the Mixfood1 and Mixfood2 beakers presented the highest populations at 96h, even higher than all the control ones, with 2094 individuals/L and 2152 individuals/L, respectively. Comparing the two experiments, overall results demonstrated that the one based on *E. affinis* LC50 concentrations was presenting always more individuals at each condition than the one using the proper *P. nana* LC50 concentrations.

Stage-specific composition

For the first experiment, all 3-way ANOVA (replicates, treatments and exposition duration as factors) performed on the 8 life stages (or aggregated stages) showed significant levels ($p<0.05$) for only two factors (treatment and duration). Consequently, we performed 2-way ANOVA using only those factors and by adding their interaction.

Fig. 11 presents the stage-specific composition of Experiment 1 over time.

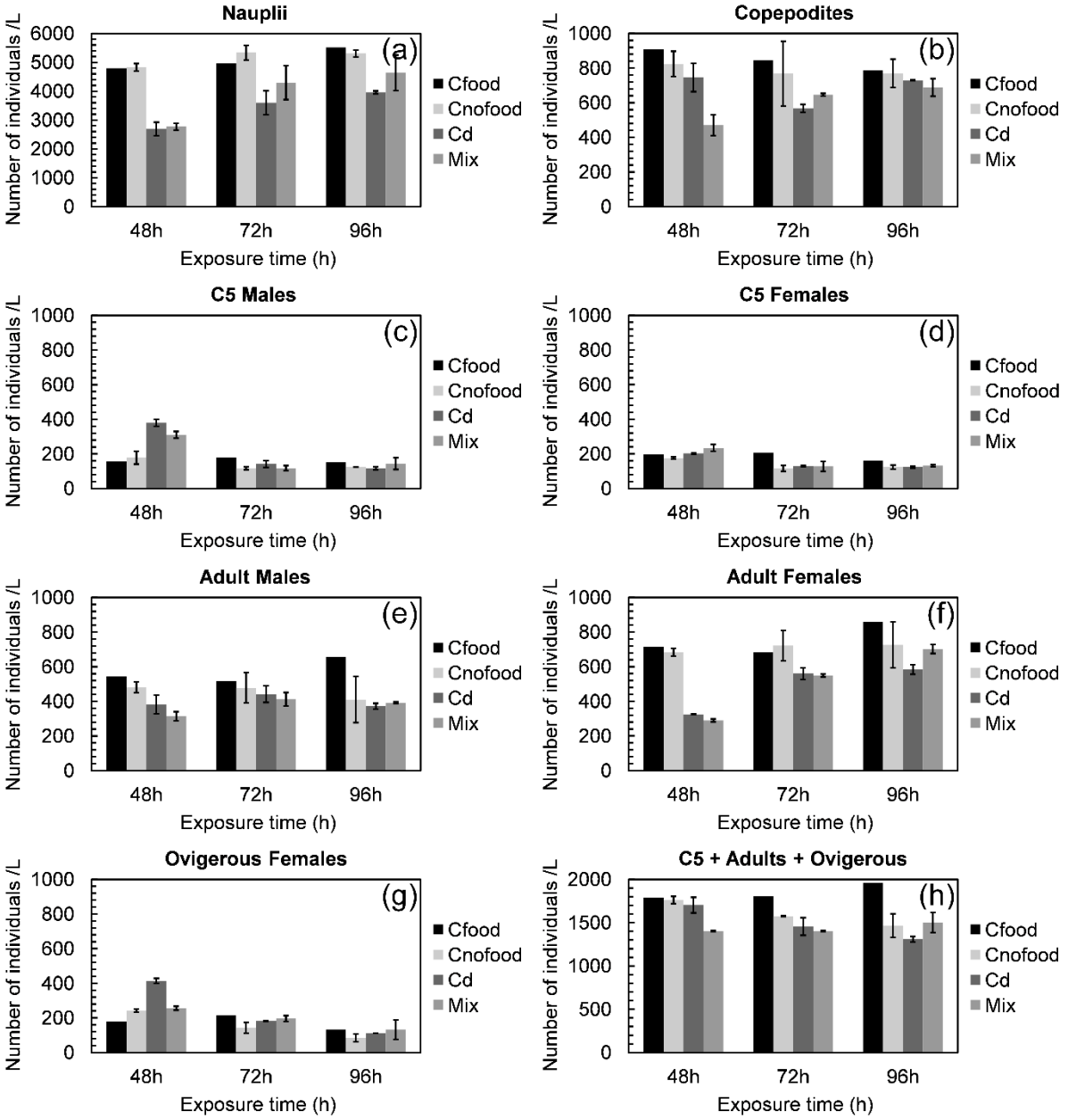

FIGURE 11. Stage-specific compositions of the *Paracyclopina nana* populations for each condition during Experiment 1: Nauplii (a), Copepodites (b), C5 Males (c), C5 Females (d), Adults Males (e), Adult Females (f), Ovigerous Females (g), C5+Adults+Ovigerous (h).

The density of nauplii (**Fig. 11a**) was relatively high in every condition of the experiment. Both factors were significant but not their interaction ($p = 0.188$). In fact, nauplii showed an increasing density over time, and particularly in contaminants-exposed beakers, they reached 3956 ± 51 individuals.L^{-1} for the Cd beakers and 4638 ± 614 individuals.L^{-1} for the combined treatments. Copepodite

(**Fig. 11b**) populations gradually aligned over time between the different conditions, slightly decreasing in the control but increasing in the treatments, reaching 730 ± 3 individuals.L^{-1} for the Cd and 688 ± 51 individuals.L^{-1} for the Mix treatments. However, only the treatment factor was statistically significant ($p<0.05$). In the pre-adult stages (C5), the observed patterns in females (**Fig. 11c**) and males (**Fig. 11d**) were similar to the highest densities observed in the treatments of Cd and Mix at the beginning of the experiment, and then their numbers decreased over time, reaching the lowest density after 96h of exposure. Both tested factors and their interaction were statistically significant ($p<0.05$). The density of adult males (**Fig. 11e**) increased during the experiment in the fed control (Cfood), but their number varied slightly in other conditions over time. Only a slight decrease in their number was observed both in the unfed control (Cnofood) and in Cd and Mix conditions (372 ± 17 individuals.L^{-1} and 392 ± 6 individuals.L^{-1}, respectively). Only the treatment factor was statistically significant. Contrary to adult females (ovigerous and non-ovigerous), the interaction of both factors were statistically significant. Adult females (**Fig. 11f**) showed an increasing number during the experiment in the fed control (Cfood) and a rather stable population in the unfed control (Cnofood). Their density was increasing over time in both Cd and Mix-exposed conditions (584 ± 28 individuals.L^{-1} and 702 ± 25 individuals.L^{-1}, respectively). The number of ovigerous females (**Fig. 11g**) globally decreased during the experiment in the control beakers (Cfood and Cnofood) but it decreased even more in the Cd and Mix-exposed beakers (112 individuals.L^{-1} and 132 ± 57 individuals.L^{-1}, respectively, after 96h).

The stage-specific composition of Experiment 2 over time is shown in **Fig. 12**.

When 3-way ANOVA was applied to all stages, only two factors (treatment and duration) showed significant levels. Consequently, we performed 2-way ANOVA using only those factors and their interaction.

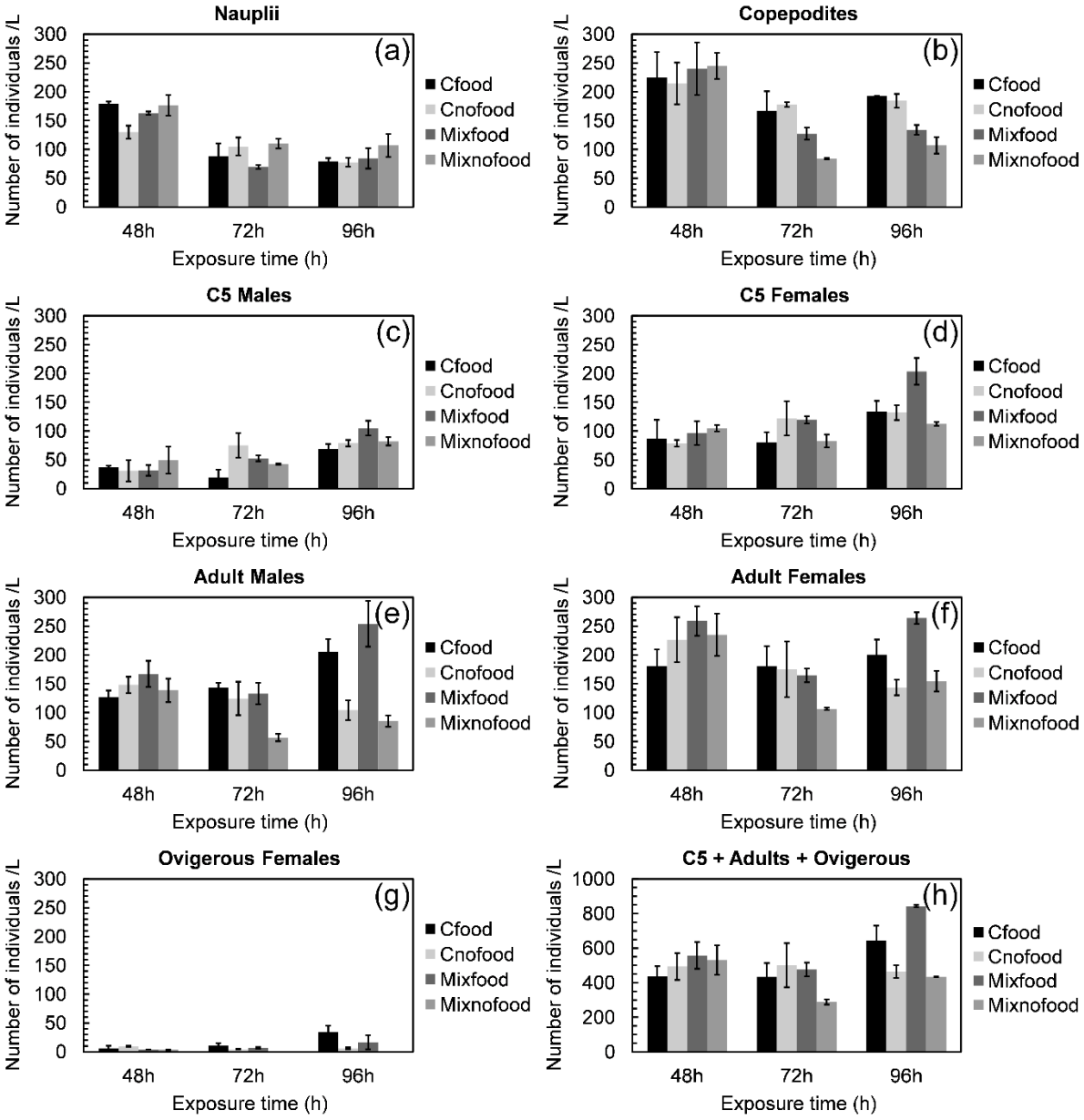

FIGURE 12. Stage-specific compositions of the *Paracyclopina nana* populations for each condition during Experiment 2: Nauplii (a), Copepodites (b), C5 Males (c), C5 Females (d), Adults Males (e), Adult Females (f), Ovigerous Females (g), C5+Adults+Ovigerous (h).

The density of nauplii (**Fig. 12a**) was decreasing until the end of the experiment for all conditions. Only a slight increase was noted in the Mixfood beakers at 96h (Mixfood: 84 ± 18 individuals.L^{-1}; Mixnofood: 107 ± 20 individuals.L^{-1}). Both factors were significant ($p<0.05$) but not their interaction. Copepodites observed a fall in all the beakers at 72h, particularly for Mix conditions but their number slightly increased at each condition at 96h with the Mix ones always lower than the control ones (Mixfood: 134 ± 8 individuals.L^{-1}; Mixnofood: 107 ± 14 individuals.L^{-1}) (**Fig. 12b**). However, statistically only the treatment factor was significant ($p<0.05$). The density of C5 males (**Fig. 12c**) was increasing in all the beakers during the experiment with Mixfood and Mixnofood being the highest after 96h with 105 ± 13 individuals.L^{-1} and 82 ± 7 individuals.L^{-1}, respectively. Both factors and their interaction were significant. C5 females observed a global increase also in each condition, with a very high final number in Mixfood beakers (203 ± 23 individuals.L^{-1}) (**Fig. 12d**), while Mixnofood beakers were lower than the control ones (112 ± 4 individuals.L^{-1}). The two-way ANOVA provided similar results for C5 females. The density of adult males increased after 96h in both the fed conditions Cfood and Mixfood (205 ± 22 individuals.L^{-1} and 254 ± 40 individuals.L^{-1}, respectively) (**Fig. 12e**), whereas it decreased in the unfed treatments Cnofood and Mixnofood (104 ± 17 individuals.L^{-1} and 85 ± 10 individuals.L^{-1}, respectively). Only the treatment factor was significant for males whereas both factors and their interaction were significant for females. The numbers of adult females increased in the fed treatments after 96h (Cfood: 200 ± 26 individuals.L^{-1}; Mixfood: 264 ± 10 individuals.L^{-1}) but decreased in Cnofood (143 ± 13 individuals.L^{-1}) and Mixnofood (154 ± 18 individuals.L^{-1}) (**Fig. 12f**). Initial densities of ovigerous females were very low but increased substantially after 96h

in the fed conditions Cfood and Mixfood (34 ± 11 individuals.L^{-1} and 16 ± 12 individuals.L^{-1}, respectively) (**Fig. 12g**). Both factors and their interaction were significant ($p<0.05$).

3.2. Bioaccumulation of metals in *Paracyclopina nana*

Initial concentrations in copepods and water

The concentrations of the three metals were measured both in copepods and water from the initial copepod stock cultures used for each experiment (**Table 7**).

TABLE 7. Concentrations of cadmium (Cd), copper (Cu) and nickel (Ni) both in copepods and water from initial stock cultures for each experiment.

Experiment 1	Concentration in copepods (µg.g^{-1} dw)	Concentration in water (µg.L^{-1})
Cd	0.02 ± 0.04	0.26 ± 0.5
Cu	20.7 ± 1.6	44.3 ± 5.9
Ni	3.7 ± 0.2	36.5 ± 0.9
Experiment 2	**Concentration in copepods (µg.g^{-1} dw)**	**Concentration in water (µg.L^{-1})**
Cd	0.08 ± 0.02	0.45 ± 0.05
Cu	226.9 ± 15.9	2.4 ± 0.1
Ni	4.4 ± 0.6	1.1 ± 0.4

Concentrations in copepods for the pre-exposed population always showed higher values for each metal than the copepods used for Experiment 1, particularly for Cu which was reaching 226.9 ± 15.9 µg.g^{-1} dw, but was 20.7 ± 1.6 µg.g^{-1} dw for Experiment 1. Conversely, concentrations in the water of each initial culture were generally higher for Experiment 1 than for Experiment 2, except for Cd, which reached 0.45 ± 0.05 µg.L^{-1} in the culture intended for Experiment 2 but was 0.26 ±

0.5 µg.L^{-1} for Experiment 1. Initial Cu and Ni concentrations in water for Experiment 1 were really high, with 44.3 ± 5.9 µg.L^{-1} and 36.5 ± 0.9 µg.L^{-1}, respectively, whereas it was at 2.4 ± 0.1 µg.L^{-1} and 1.1 ± 0.4 µg.L^{-1}, respectively, in the water of the pre-exposed culture.

Concentration in copepods

Final concentrations of the three tested metals in the copepod samples are presented in **Fig. 13**.

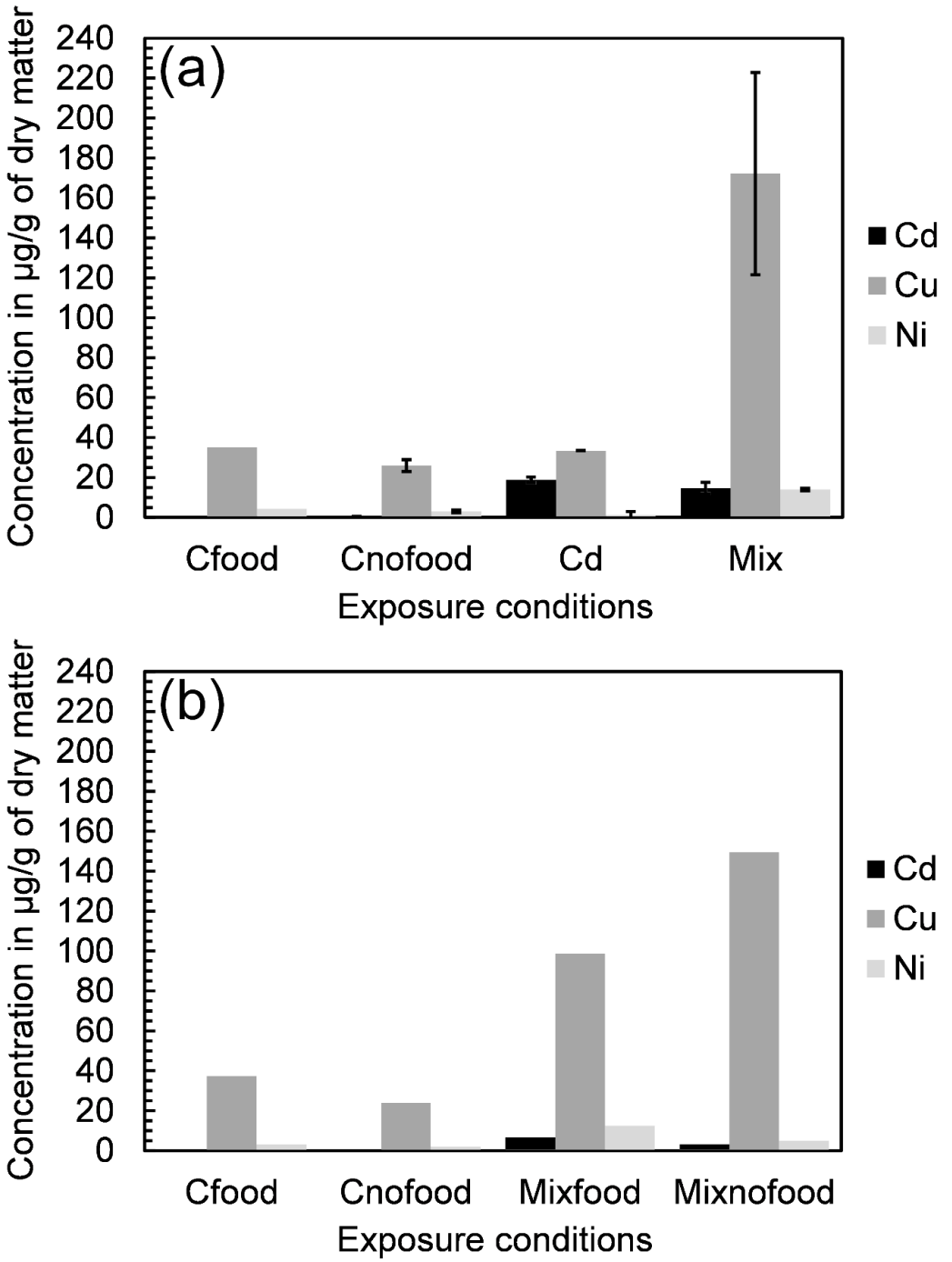

FIGURE 13. Final concentrations of cadmium (Cd), copper (Cu) and nickel (Ni) in copepods of each treatment of Experiment 1 (a) and Experiment 2 (b) after 96h of exposure.

Looking at the results of Experiment 1 (**Fig. 13a**), cadmium was present in very low concentrations in controls (Cfood: 0.16 $\mu g.g^{-1}$ dw; Cnofood: 0.21 $\mu g.g^{-1}$ dw) and in high concentrations in the Cd and Mix conditions (18.8 ± 1.5 $\mu g.g^{-1}$ dw and 14.8 ± 2.8 $\mu g.g^{-1}$ dw, respectively), but still higher for Cd alone. The two-way

ANOVA performed on Cd loadings showed that only the treatment factor was significant ($p<0.05$). Ni was only noticeably present at separate exposure; Mix (14 ± 0.6 $\mu g.g^{-1}$ dw). Statistical analyses showed that only treatment and the interaction 'treatment x duration' were significant ($p<0.05$). Cu appeared as the most detected metal in copepods, showing high concentrations in the individuals from the Mix beakers, the only ones exposed to it (172.2 ± 50.6 $\mu g.g^{-1}$ dw). However, Cu was also present in high and quite similar concentrations in both controls and Cd condition (33.5 ± 0.03 $\mu g.g^{-1}$ dw for the Cd treatment). Both factors were significant but not their interaction. Additionally, both Cu and Ni appeared to be higher in the fed control copepods than in the unfed ones. In Experiment 2, Cd was present at very low concentrations in the control (Cfood: 0.04 $\mu g.g^{-1}$ dw; Cnofood: 0.06 $\mu g.g^{-1}$ dw) but more concentrated in copepods from the Mix conditions (**Fig. 13b**). Thus, its accumulation was higher when copepods were fed (Mixfood: 6.7 $\mu g.g^{-1}$ dw; Mixnofood: 3.3 $\mu g.g^{-1}$ dw). The same tendency appeared for Ni, which is present at higher concentrations under Mix conditions as well, and is very high in the Mixfood one (Mixfood: 12.5 $\mu g.g^{-1}$ dw; Mixnofood: 4.9 $\mu g.g^{-1}$ dw). Cu was also the most detected metal in copepods, showing high concentrations in the exposed Mix conditions, with 98.7 $\mu g.g^{-1}$ dw for Mixfood and 149.5 $\mu g.g^{-1}$ dw for Mixnofood. Like Cd and Ni, Cu was higher in the fed controls than in the unfed ones, but was higher in the copepods from Mixnofood than in the ones from Mixfood. Only for all metals the treatment factor was satatistically significant ($p<0.05$).

The concentrations of the three tested metals during the whole experiments in the copepods from the Mixfood conditions are shown in **Fig. 14**.

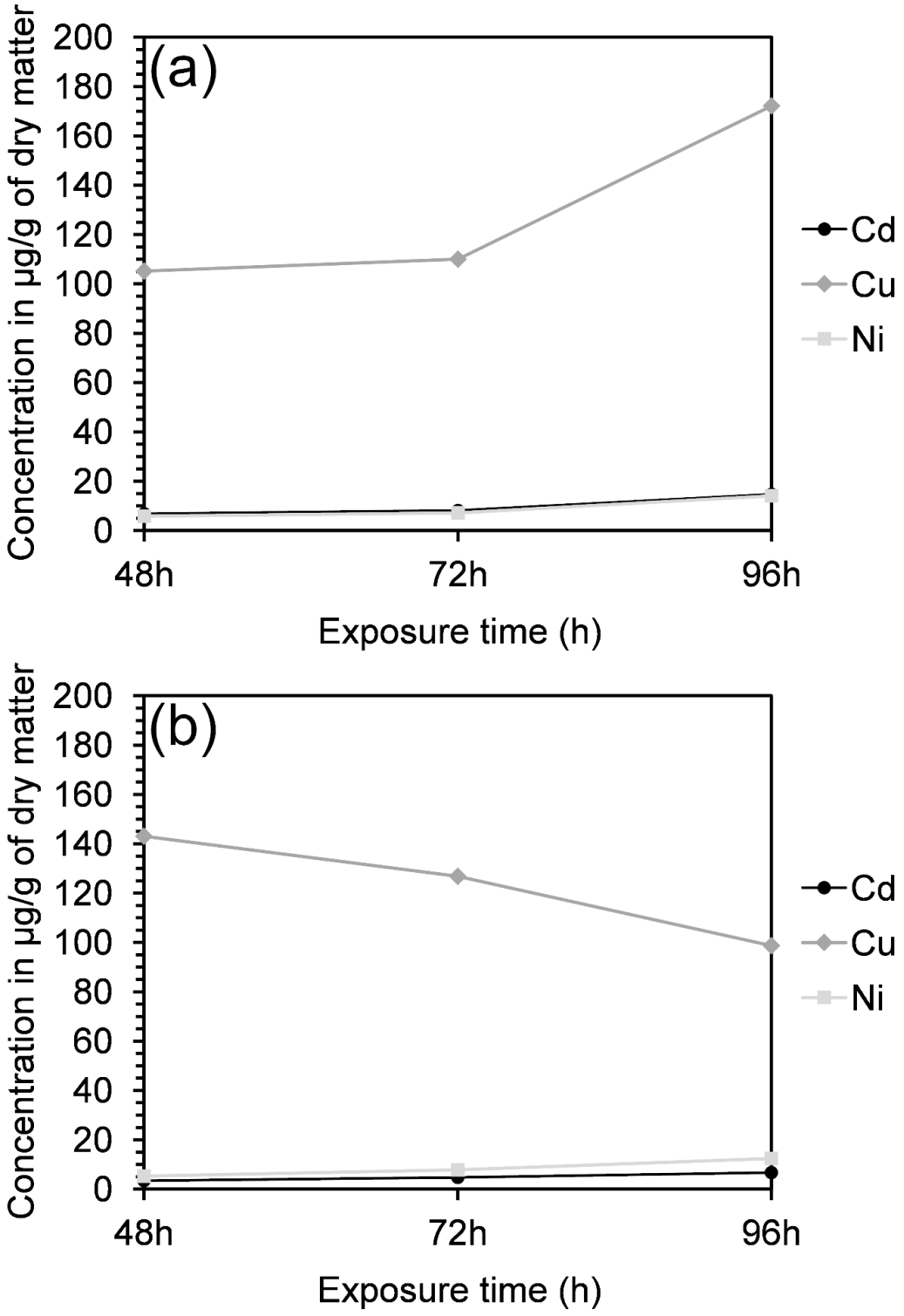

FIGURE 14. Concentrations of cadmium (Cd), copper (Cu) and nickel (Ni) in copepods of the Mixfood condition during Experiment 1 (a) and Experiment 2 (b).

The relative bioaccumulation values for Experiment 1 (**Fig. 14a**) showed that Cu concentrations in copepods always increased over time. It was by far the most

accumulated metal in copepods, whereas it was the one introduced at the lowest concentration. Cd and Ni, for their part, showed similarly increasing bioaccumulation rates, in a much lower range than Cu. Looking at the results for Experiment 2 (**Fig. 14b**), Cu appeared once again as the most concentrated metal in copepods, but its concentration in the sample was decreasing over time. The accumulation of Cd and Ni was quite identical as for the other experiment and still low compared to Cu. Both of their concentrations increased in copepods during the experiment.

Concentration of metals in water

The final respective concentrations of the three tested metals in the water samples are shown in **Fig. 15**.

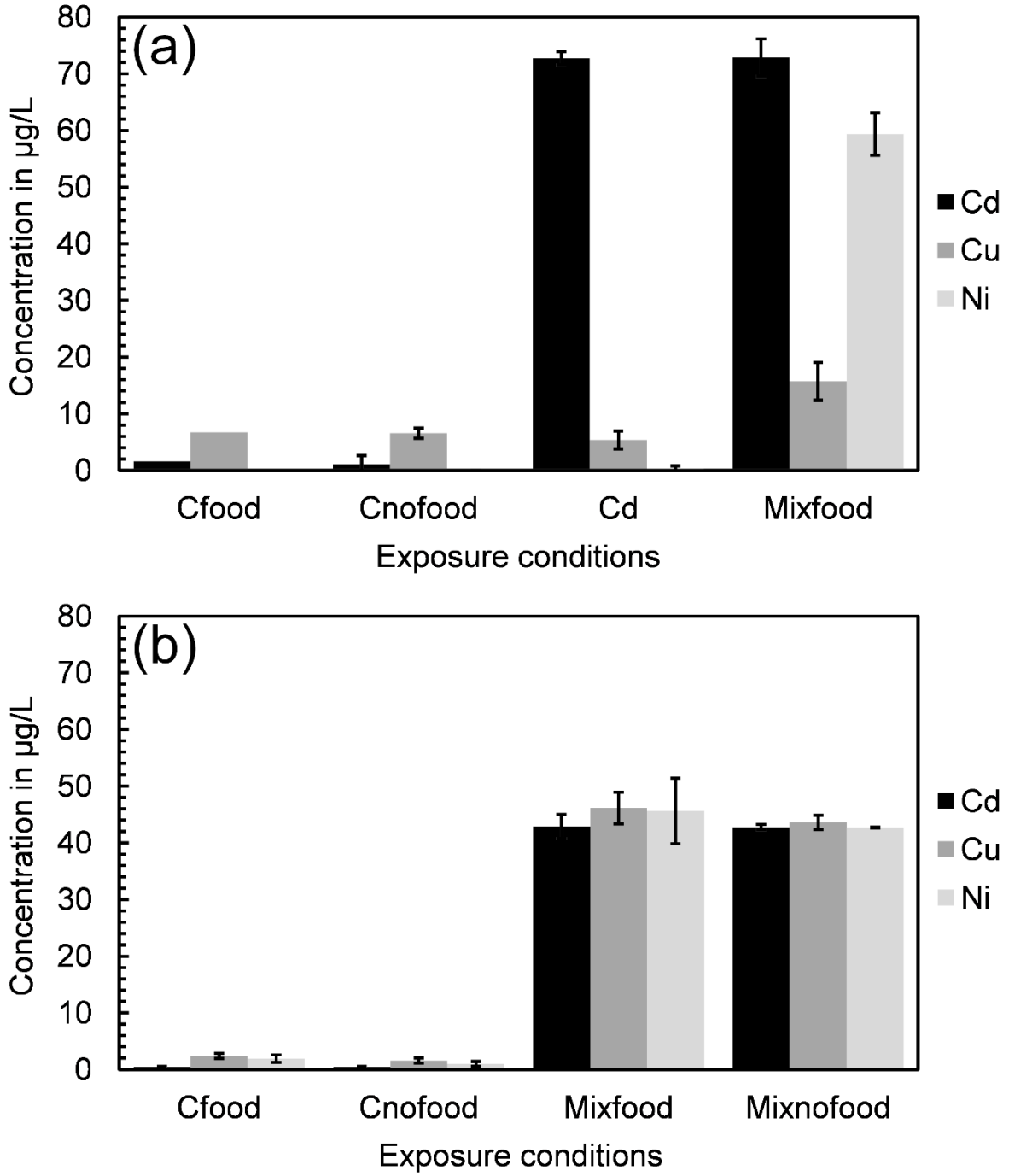

FIGURE 15. Final concentrations of cadmium (Cd), copper (Cu) and nickel (Ni) in the water of each condition of Experiment 1 (a) and Experiment 2 (b) after 96h of exposure.

Results for Experiment 1 (**Fig. 15a**) showed that Cd concentrations in the water of the beakers at the end of the experiment was low in all the controls but were very high in all the exposed ones, with 72.7 ± 1.2 $\mu g.L^{-1}$ for Cd and 72.9 ± 3.3 $\mu g.L^{-1}$ for Mix. The same trend was found with Ni, which was very low in all

controls and the Cd-exposed beakers (Cd) but very high in the water of the exposed Mix beakers (59.4 ± 3.7 $\mu g.L^{-1}$). Cu was the highest in the Mixfood beakers (15.7 ± 3.3 $\mu g.L^{-1}$) but generally low compared to the Cd and Ni concentrations. These trends were obviously due to the initial addition of high concentrations of metals in the exposed beakers. The two-way ANOVA performed on each metal showed similar results for Cd and Ni, with treatment, duration and their interaction significant ($p<0.05$). But for Cu only treatment factor was significant. **Fig. 15b** shows the results for Experiment 2. Very low concentrations of the three tested metals were observed in the water of all the control beakers, fed and unfed, but high concentrations appeared in both Mixfood and Mixnofood beakers at the end of the experiment, with similar values for the three metals, all of them between 42 and 46 $\mu g.L^{-1}$. Only the treatment factor was statistically significant in all metals.

The respective concentrations in the water of the Mixfood beakers during the experiments were compared in **Fig. 16**.

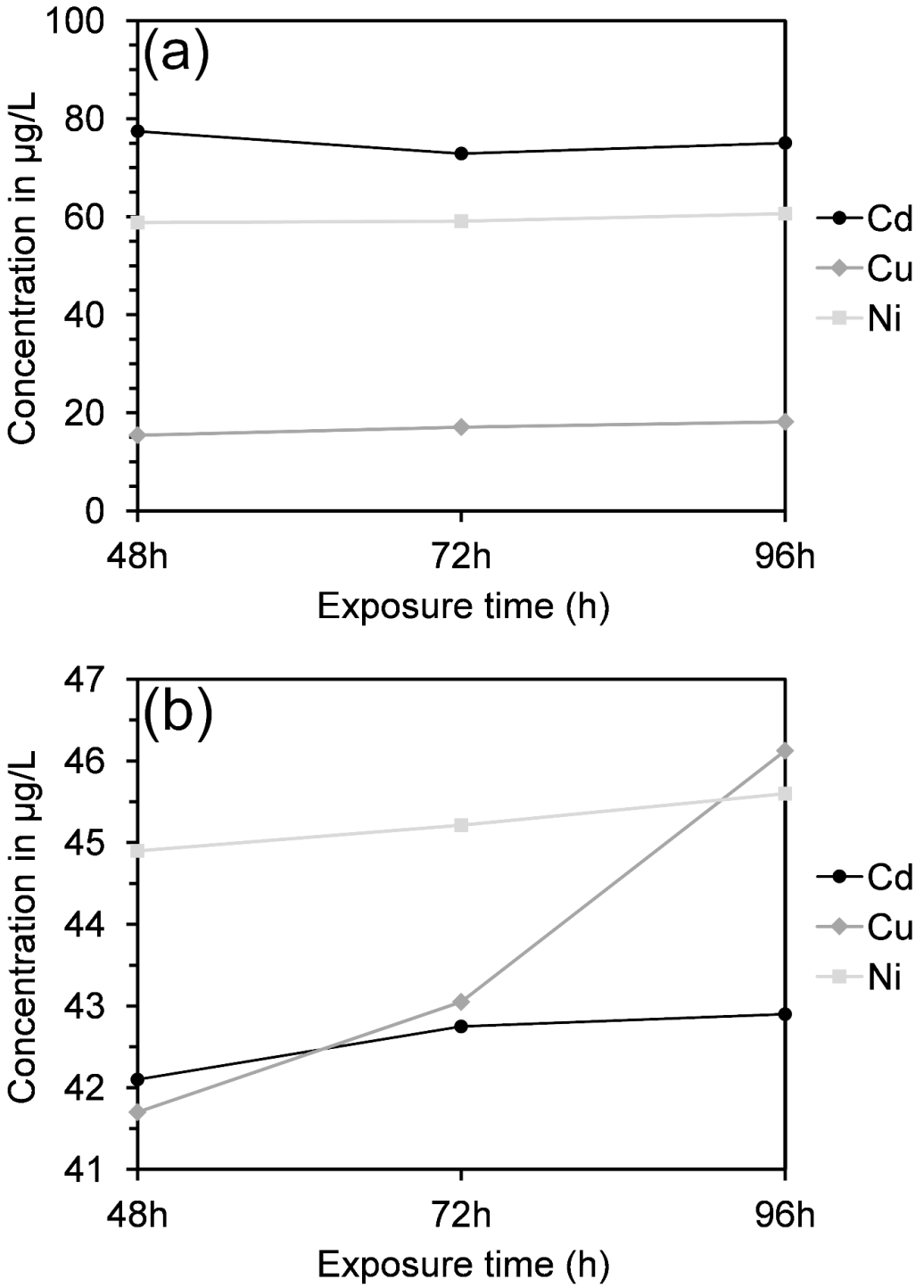

FIGURE 16. Concentrations of cadmium (Cd), copper (Cu) and nickel (Ni) in the water of the Mixfood condition during Experiment 1 (a) and Experiment 2 (b).

Results for Experiment 1 (**Fig. 7a**) indicate that all concentrations in the Mixfood beakers were quite steady throughout the whole experiment, with Cd and Ni being the highest compared to Cu. **Fig. 7b** shows the corresponding results for

Experiment 2. The concentration of all of the three tested metals in the water of the Mixfood beakers was increasing throughout the whole experiment, slightly for Cd and Ni but considerably for Cu, which presented the lowest concentration among the three at the beginning but became the highest after 96h of exposure.

4. DISCUSSION

4.1. Effects of metals on population growth of *Paracyclopina nana*

Total population growth

Overall results for Experiment 1 and for Experiment 2 showed that the total number of individuals was always higher in the control than in the treaments with metals. It is recognized that trace metals have toxic effects on marine organisms and can even affect their survival (Bryan, 1971; Calabrese et al., 1977; Florence et al., 1994; Zyadah & Abdel-Baky, 2000; Berasategui et al., 2018). Here, the decreasing populations observed among the different conditions of the experiments were due to mortality that occurred during *P. nana* life cycle.

Results of Experiment 1 showed that the lowest final population size among all conditions was obtained when copepods were exposed to Cd alone, whereas the mixture led to an increasing and higher final population density. Zidour et al. (2019) also found that the metal mixture composed of sublethal concentrations of Cd, Cu, and Ni did not present additive or synergistic effect in terms of mortality to the calanoid copepod *E. affinis*, whereas Cu alone induced a very high mortality rate. Brand et al. (2004) also showed that the total population size of the copepod

Tisbe holothuriae was always reduced when exposed to Cd only, even at low concentrations.

All conditions from Experiment 2 provided increasing population densities after 96h of exposure, except the populations from the unfed controls (Cnofood). The highest final populations were even obtained from the Mixfood beakers, being higher than all the control populations. The exposure to the mixture, therefore, did not affect the growth of the copepod population used for Experiment 2. This initial copepod cultures were grown for several months in 15 psu seawater made of the classic seawater used in the laboratory and tap water containing above average concentrations of Cu. Therefore, copepods growing in this culture were chronically exposed to the metal and already accumulated it in an abnormally high concentration, and were even already saturated in Cu before the beginning of the experiment. This led to the fact that copepods were not able to bioaccumulate more Cd, Cu, and Ni used at sublethal concentrations in the Mix beakers, and they were also not able to release their accumulated contaminants due to lack of uncontaminated water. These results may shed light on a phenomenon of tolerance to metals, and particularly Cu, that appeared in the *P. nana* population before the experiment. Moraitou-Apostolopoulou et al. (1983) observed a tendency for higher tolerance to Cu over generations of the marine copepod *Tisbe holothuriae* due to acclimation. The delay in maturation time induced by Cu in the F2 generation appeared less pronounced in F3 and similar to that of untreated individuals. The initial 48h-LC50 also presented an increase in the F3 and F5 generations. The *P. nana* population used for our experiment may have developed a metal tolerance over generations during its several months of acclimation. In addition, the Mixfood beakers eventually turned out to be those offering a most similar environment to

the one where copepods were adapted to, containing metals and food. Therefore, were not affected by these conditions but developed at these particular treatments. Another hypothesis proposed by Kadiene et al. (2019) was followed here as well. The authors showed that copepods are able to take in water orally thanks to a dyed medium and that the dye concentrated into the gut epithelium of the copepods. This phenomenon supported the idea that oral water intake may be involved in the higher metal body accumulation observed in copepods exposed to dissolved metals compared to those exposed to dietary metals. Feeding would then reduce the stress associated with exposure to dissolved metals in copepods by filling the guts, and preventing it from additional intake of metal-loaded water.

In both experiments, the unfed control Cnofood presented a decreasing population whereas the population for the fed control Cfood was always increasing to become one of the highest. In the Cfood beakers, these already contaminated copepods were fed and able to eliminate a part of their accumulated metals to the ambient water. It became obvious that starved copepods had decreasing population densities due to mortality (Cowey & Corner, 1963; Threlkeld, 1976). The mortality rate of *Calanus helgolandicus* was 5.0-6.5% per day for starved copepods and below 1% for fed ones in the starvation experiment performed by Cowey & Corner (1963). But this effect was reinforced when subjects are exposed to contaminants or additional stress such as non-optimal salinity (Devreker et al., 2004). Results of Experiment 2 showed that copepod populations of the Mixnofood beakers were lowest. The exposure to metals coupled to a lack of food amplified copepod mortality and population decrease. Pedroso et al. (2007) exposed the euryhaline copepod *Acartia tonsa* to silver and proved that the mortality observed in starved

copepods was explained by a decrease in the body concentration of Mg^{2+} due to the inhibition of the associated enzymes by increasing silver concentrations.

Comparing results from Experiment 1 and Experiment 2 demonstrated higher population densities in *P. nana* of Experiment 1 using the LC50 concentrations of *E. affinis*. This proves that LC50 concentrations for the same metals were higher than for *P. nana* than those for *E. affinis*, and so that *P. nana* appears as a less sensitive copepod species to metals compared to *E. affinis* (Dahms et al., 2016).

Stage-specific composition

Our results for Experiment 1 showed an abundance of nauplii in all treatments with an increasing density over time, particularly in the conditions exposed to metals, Cd and Mix. Zidour et al. (2019) also demonstrated an increasing number of nauplii of *E. affinis* in the Cd treatment. In their study, the copepodite density also increased at the end of the experiment particularly with Cd. Increased copepodites likely correspond to the high number of resisting nauplii who turned into copepodites. The increasing densities of larval and juvenile stages despite the exposure to metals may also be explained by the concentrations used for this experiment corresponded to the respective LC50 values of *E. affinis* and were too low for *P. nana*, which appeared as less sensitive to metals. Pre-adult individuals (C5 males and females) appeared to be much affected by metal exposure as their densities decreased over time in the metal treatments. The same pattern was found by Zidour et al. (2019), the number of pre-adults of *E. affinis* decreased after exposure to Cd, Cu, and Ni, studied alone and in mixture. Copepod C5 individuals,

therefore, appeared as a metal-sensitive life stage. This sensitivity could be related to the physiological requirements and the energetic cost of maturation from C5 to the final reproductive C6 stage. A slight decrease appeared in the densities of adult males at the end of the experiment in Cd and Mix conditions. Conversely, the number of adult females globally increased at all conditions, particularly the exposed ones. In copepods, male sensitivity was often described as higher than those of females. The male survival of the harpacticoid copepod *Amphiascus tenuiremis* exposed to Cu, Pb and Ni was significantly lower than that of female (Hagopian-Schlekat et al., 2001). Finally, our results showed that the densities of *P. nana* ovigerous females globally dropped at all the conditions of the experiment, and even more at metal exposure. The number of *E. affinis* ovigerous females decreased considerably when exposed both to Cd and Ni compared to the control, whereas no effect was observed in the combined treatment (Zidour et al., 2019). A similar phenomenon happened in our study as the population of ovigerous females in the Mix beakers decreased after 96h but appeared always higher than that of the Cd beakers and even reached one of the highest abundance, comparable to the controls. Ovigerous female densities were generally low indicating that this life stage was most sensitive to metals as shown earlier (Dur et al., 2009).

For Experiment 2, the densities of nauplii and copepodites started very low compared to Experiment 1 and had lower densities at all conditions. There was only a slight increase of population densities at 96h in some exposed beakers. It has been widely reported that nauplii are a particularly sensitive life stage when exposed to high metal concentrations (Kadiene et al., 2019). The most sensitive life stage of the copepod *Tisbe holothuriae* to both Cu and Cd was the one-day-old nauplius (Verriopoulos and Moraitou-Apostopoulou, 1982). Similarly, nauplii of

the marine copepod *Tigriopus brevicornis* appeared to be two to four times more sensitive than adults and copepodites for exposure to all tested contaminants, including Cd, arsenic (As), and four different pesticides (Forget et al., 1998). The Cd LC50 of the naupliar and copepodites stage of the asiatic copepod *Pseudodiaptomus annandalei* were very low, with 40.3 $\mu g.L^{-1}$ Cd and 120.4 $\mu g.L^{-1}$ Cd, respectively (Kadiene et al., 2019). Accordingly, in this study, the newly hatched nauplii showed a drastic decrease. The reduction of larval survival affected the recruitment potential of nauplii and resulted in a decreasing copepodites population. Conversely, the densities of C5 males and C5 females increased at all conditions during the experiment, particularly in the fed ones, with very high final densities for the Mixfood beakers. Moreover, a similar trend was observed for the adult stage: the numbers of adult males, females, and ovigerous females all increased overall at fed conditions, and particularly in the Mixfood treatment. This phenomenon can be explained by the fact that C5 individuals introduced in the experiment were already used to metal-contaminated water because of their prior acclimation. The pre-adult individuals from this population appeared to be tolerant to metals and thus developed within the experiment, especially in the exposed and fed beakers that correspond to their acclimation living conditions, and lead to thriving adults. The introduction of the food parameter in the experiment also confirmed this acclimation phenomenon in copepods since these individuals developed better under fed conditions than unfed and were, therefore, more affected by the lack of food than by exposure to metals. This can be explained by the necessity of energy supplies assisting in the energetic costs of detoxification. The occurrence of metal tolerance has already been reported in copepods. Kwok et al. (2009) studied the marine copepod *Tigriopus japonicus*

and showed that it can develop a Cu resistance through multigeneration acclimation to high Cu levels and that this resistance was increased from the very first generation of acclimation. Similarly, chronically exposed lines of *the copepod Tigriopus californicus* to sub-lethal Cu concentrations for 12 generations showed an increasing Cu tolerance over time from generation 3 onwards (Sun et al., 2014). Multigenerational exposure to Cu, therefore, generated a response consistent with the procedure of long-term acclimation in our study.

For both experiences, our results showed much lower adult male numbers than for females. Particularly in Experiment 2, which led to about half as many males as females. This parameter is affected by several factors such as species-specificity, but it was shown before that copepod males are more sensitive than females to contaminants. The study of the different lethal responses between male and female copepods to Cd toxicity revealed that males of *P. annandalei* were almost twice as sensitive as females when comparing their respective LC50 (120.6 $\mu g.L^{-1}$ Cd for males and 239.5 $\mu g.L^{-1}$ Cd for females) (Kadiene et al., 2017). The same conclusion was established for *E. affinis* before in terms of Cu and Ni toxicity as LC50 for males were 25.0 $mg.L^{-1}$ Cd and 90.0 $mg.L^{-1}$ Ni, whereas female LC50 were 38.0 $mg.L^{-1}$ Cd and 161.0 $mg.L^{-1}$ Ni (Zidour et al., 2019).

When comparing results from both experiments, we clearly saw that Experiment 2 gave far fewer individuals of each life stage in each condition than Experiment 1. The second experiment resulted in more than half as many adult males and females, and even more than ten time less ovigerous females than the first one. This explained by the lack of survival of newly hatched nauplii, resulting in low recruitment and low population growth.

4.2. Bioaccumulation of metals in *Paracyclopina nana*

Copepods from Experiment 1 accumulated high concentrations of metals at exposed conditions, and particularly in combined treatments as mixtures. This is explained by the fact that the population was originally grown in uncontaminated water without any acclimation to high metal concentrations. Results showed low initial concentrations of each metal in these copepods. We observed an upward trend with exposure time in the accumulation of the three tested metals. At the same time, a slight decrease of the three metal concentrations in water was noticed. All three metals were highly concentrated in copepods from the combined treatments, but Cu concentrations were always increasing over time and became particularly high after 96h. Besides, Cu concentrations in the water of the combined treatments were lowest compared to Cd and Ni which were highly concentrated, thus showing the opposite trend. However, Cu was the metal introduced at the lowest concentration at the beginning of the experiment. Cu therefore appeared as a more easily accumulable metal than Cd and Ni. This is explained by Cu being an essential trace metal and marine invertebrates present the ability to accumulate dissolved Cu from water directly by absorption through body surfaces (Pinho et al., 2007). The calanoid copepods *E. affinis* and *P. annandalei* both demonstrated a higher uptake of Cu than Cd and Ni from the dissolved phase, whereas it was the lowest concentrated in the dissolved metal mixture at the beginning of the experiment (Kadiene et al., 2019). This may also introduce the idea of a competition for the absorption between the metals present in a mixture, as found for the same three metals with *E. affinis* (Zidour et al., 2019). The metal presenting the highest affinity with the metal-binding sites in the body

of copepods may thus saturate these sites, preventing other metals to fix themselves, and resulting in being the most accumulated metal among the combined treatments. This idea is confirmed when comparing the respective Cd bioaccumulation levels in both the treatment with Cd alone and with the mixture. Higher Cd concentrations were found in copepods from the Cd alone beakers than in copepods from the mixture. Copepods were not able to accumulate as much Cd in the mixture as the diversity of metals present introduced a competition for binding sites and their rapid saturation preventing a higher Cd absorption.

In Experiment 2, copepods also accumulated metals more at the exposed conditions than the controls, but to a lesser extent. Cu was the most detected metal in copepod samples but lower concentrations of each metal were found in copepods from Mixfood than in Experiment 1. Cd was even half as accumulated as in the first experiment. Copepods from the second experiment were growing for months in water containing sub-lethal concentrations of metals and, therefore, had acclimated to metal exposure. Initial concentrations showed that individuals from this population had already concentrated metal levels, particularly in Cu, and so their bioaccumulation capacity against the exposure conditions of the experiment was not as high as the population used in Experiment 1. That explains the three tested metals had high concentrations (more than 40 $\mu g.L^{-1}$) in the water of all the Mix beakers of the second experiment. Interestingly, Cu concentrations were always decreasing over time in copepods from Mixfood. It was also the lower concentrated metal in water of these beakers at the beginning of the experiment. However, it constantly increased over time to concentrations more than twice as high as that of Experiment 1. At the beginning of this experiment, copepods were actually more concentrated in metals than the water they were transferred to

where they depurated and released their accumulated metals into the experimental water, particularly so for Cu. Copepod depuration experiments with *Acartia tonsa* showed that PCB-contaminated copepods rapidly eliminated the chemical once they were transferred into PCB-free water (McManus et al., 1983). However, Cailleaud et al. (2009) exposed *E. affinis* in large microcosms during 86h after a depuration and acclimation period of 3 days and showed that PHA were more easily eliminated than PCB. The origin of the population sampled in the field compared to our experiment using only mass culture of copepods as well as the mixture of PCB profile used in the experiment may explain those changes. Such rapid uptake and depuration rates of contaminants suggest that copepods are able to quickly respond to changing concentrations in their environment, confirming their strong potential as bioindicators in aquatic environments.

When comparing overall results for the feeding parameter, metal concentrations in copepods were mainly higher for the fed conditions than the unfed ones, and for both experiments. Each of the three metals was more concentrated in copepods from the fed controls than in the unfed ones for the two experiments. Looking at exposed conditions, Cd and Ni were also higher in copepods from Mixfood than Mixnofood of Experiment 2. Copepods therefore, accumulate more metals when they are fed, for the same exposure concentrations. Many studies already proved the existence of an increased effect of food presence on the bioaccumulation of contaminants. The bioaccumulation capacity of the calanoid copepod *Acartia clausi* was tested with three different PCBs and PBDE either by contaminated water or by feeding with a contaminated microalgae, *Thalassiosira weissflogii* (Magnusson & Tiselius, 2010). The tested bioaccumulation factors for all four contaminants were significantly higher

in *copepods* fed with contaminated phytoplankton than in those only exposed to dissolved contaminants. However, these organic contaminants were hydrophobic and needed solvents to obtain a dissolved phase in the water. Solvents can be a confounding factor as they themselves induce some toxicity. But for metals, a dissolved phase can be easily obtained. It has also been demonstrated that food-borne contamination is a major factor to consider for the body concentrations of several elements (Kadiene et al., 2019). Assimilation efficiency of trace elements is expected to increase with longer gut passage time, therefore corresponding to feeding and digestive intervals.

5. CONCLUSIONS

The contrasting results from the two different populations revealed the plastic survival and bioaccumulation capacity of *P. nana* for metals. The first experiment based on a regular population showed that metal exposure induces reduction in population growth and increased bioaccumulation in individuals. Cd was even more accumulated when it was treated separately than when in combined mixture. This unveils a phenomenon of competition between metals in mixture for the absorption in copepods. The second experiment started with a population already pre-exposed to metals over several generations and revealed a population growth that was less affected by metals and a lower bioaccumulation in exposed individuals. The overall metal concentrations decreased in the copepods and increased in experimental waters, demonstrating a depuration phenomenon in this population that was already loaded with metals and acclimated to metal exposure. The overall results also showed that food attenuated the effects of metal exposure

on population growth but increased the bioaccumulation capacity of metals. This study sheds light on the parameters that affect the bioaccumulation of metals of copepods in contaminated aquatic ecosystems.

CHAPTER IV

GENERAL DISCUSSION

It is only since the early 2000s that experimental research on copepods is interested in *Paracyclopina nana*. Its small size first aroused interest in aquaculture in order to feed fish larvae.

A study first investigated its culture parameters and showed that *P. nana* is a copepod that supports very high culture densities. It still presents a survival rate of 18.4% for a density of 300 nauplii/mL. Its optimal culture densities are also particularly high: 7 females/mL in order to obtain nauplii and 200 nauplii/mL to obtain adults (Lee & Park, 2005). In our routine mass culture, the density of *P. nana* reached 12 ind/ml (Souissi unpub. data) but the feeding of the 300 L tank was done only two times per week. Consequently, we believe that the species can reach much higher density when optimal feeding protocol will be applied. The sex-ratio was slightly biased towards females (on average ~44% of males) which may favour reproduction and nauplii recruitment.

P. nana has also been the subject of some work concerning its food preferences. Lee et al. (2012a) showed that the increased ingestion rate correlated with an increased algal concentration that is generally observed in copepods is also found in *P. nana* at all development stages. The increased algal concentration also increased the daily production rate of nauplii and the general growth rate of the individuals, so that the authors have been able to define an optimal algal concentration for each of the life stages of *P. nana*: nauplius, copepodite, adult male and adult female. Two studies conducted on *P. nana* by Min et al. (2006 & 2007) testing different microalgae species (*Tetraselmis suecica*, *Isochrysis galbana*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*) led to similar conclusions. First, the grazing rate of *P. nana* per hour varied with the four different microalgae species and concentrations tested, and the maximum rate was found with 0.63 ng chl

a/h when *P. nana* was fed with *T. suecica* at 39.3 ng chl a/ml. The other study testing fifteen microalgal species showed that total and daily nauplii production were highest in *P. nana* fed *T. suecica.* Similarly, looking at the fatty acid composition of *P. nana*, its eicosapentaenoic acid (EPA) content was the highest when fed *T. suecica*, reaching 5.4%, and its docosahexaenoic acid (DHA) content was also high, at 24.3%. Results jointly concluded that *T. suecica* was the best microalgae species among the tested ones here for the mass culture of *P. nana* (Min et al., 2006; Min et al., 2007). Lee et al. (2006) also investigated the effects of different microalgae diets on *P. nana* by testing 5 monospecific diets (*Phaeodactylum tricornutum* (PHA), *Isochrysis galbana* (ISO), *Tetraselmis suecica* (TET), marine *Chlorella* (MCH), condensed freshwater *Chlorella* (FCH)), and 2 mixed diets (TET + ISO, TET + PHA). The authors showed that females of *P. nana* present a higher fertility when fed mixed diets. In addition, the *Isochrysis* and *Tetraselmis* microalgae diets were those that induced the highest female fecundity and the highest growth rate of nauplii. These two microalgae species therefore appeared as particularly effective to feed *P. nana* in culture, and even more when they are presented together in a mixed diet. The genera *Chlorella* and *Phaeodactylum* were much less effective.

Concerning its potential as a living prey in aquaculture, Lee et al. (2013) showed that *P. nana* is the copepod species with the highest growth and productivity in mass monoculture in comparison with *Apocyclops royi* and *Tigriopus japonicus*. A study by Lee & Choi (2016) even demonstrated a successful mass production system providing a continuous production of *P. nana.* Two successive production methods were used in this study. A nauplius production method consisted in a 500 L tank filled with a copepod population whose adult females were concentrated at 5 ind/mL and maintained at this density during

production. Population was daily fed with 30 L of *Tetraselmis suecica*. Produced nauplii were daily filtered and removed. Daily mean nauplius production of two trials for 15 days were 6.9×10^6 and 7.2×10^6 individuals, respectively. In parallel, these same nauplii were directly used for the C4-adult production method, divided into two 20 L tanks for 15 days. The feeding amount supplied was gradually increased. Daily mean nauplius production of the two trials for the total 16 days were 8.2×10^5 and 9.0×10^5 individuals, respectively. A continuous and stable feeding based on *P. nana* for fish larvae in aquaculture would therefore be possible given its population growth capacity. Moreover, a recent work (Lee, 2018) investigated the food value of *P. nana* for a fish of commercial interest, *Paralichthys olivaceus*. Fish larvae fed with *P. nana* at their normally rotifer feeding stage showed a higher survival and growth. Similarly, when fed with *P. nana* at their *Artemia* feeding stage, fish larvae also showed a higher growth. Results of this study actually demonstrated the effectiveness of *P. nana* as a live food for fish larval production.

Experiments conducted in the present thesis tested different microalgae species, never tested before on *P. nana*: *Rhodomonas salina*, *Tisochrysis lutea* and *Pavlova lutheri*. Overall results are the first to show that *R. salina* is an effective microalga for a productive mass culture of *P. nana*, and even more in a mixed diet with *T. lutea*, as it led to the highest population growth and female reproductive investment in *P. nana*. The innovative study of the fatty acid and saccharide profiles of this copepod species also demonstrated that these same diets (R and R+T) induced a high level of internal fatty acid in *P. nana* and that its monosaccharide richness is a result of an additive effect of microalgae species within a diet. Among the different diets tested here, the R+T diet seemed to be the best microalgae combination to feed *P. nana* in mass culture. It induced an optimal

population growth and fecundity and also an interesting nutritive profile of *P. nana* for fish larve feeding, as *R. salina* and *T. lutea* seem to present complementary fatty acid and monosaccharide contents. Additionally, our results showed that *R. salina* was the microalgae tested presenting the lowest fatty acid content, whereas the diets R and R+T were those inducing the highest fatty acid content in copepods, therefore demonstrating a possible conversion capacity of fatty acids in *P. nana*. As similarly showed by Pan et al. (2018) with *Apocyclops royi*, *P. nana* may have the ability to synthesize long-chain polyunsaturated fatty acids (PUFA) from short-chain PUFA. Particularly, *P. nana* may be able to synthesize DHA by itself from short-chain fatty acids, just like already reported by Lee et al. (2006). This ability would explain the capacity of this copepod species to maintain a particularly high level of reproduction.

P. nana is also a copepod species with a high tolerance to wide ranges of salinity and temperature (Lee et al., 2006). But on the other hand, it also presents a high sensitivity to contaminants, which is a definite asset for ecotoxicology. Comparing the relative sensitivities of different copepods, *P. nana* turns out to be a very sensitive copepod to environmental stress of different types such as cadmium, copper, UV radiation, gamma radiation, or fluorene (PAH). *P. nana* LC50 values for copper, UV radiation and fluorene showed to be nearly three times lower than those found in *Tigriopus japonicus* (Won & Lee, 2014). Similarly, compared with other zooplankton species, *P. nana* showed a higher mortality rate in response to heavy metals (cadmium and copper), and UV and gamma radiation (Hwang et al., 2010; Won et al., 2014, Won et al., 2015). This high sensitivity allows to complete the acquired data concerning the tolerance ranges of copepods to

toxicity in the context of experimental tests. Copepod sensitivity to contaminants is influenced by several factors such as the species, but many studies also demonstrated that copepod males are often more sensitive to contaminants than copepod females. Our results for metal exposure notably showed much lower densities of adult males than adult females in *P. nana*. Kadiene et al. (2017) studied the different lethal responses between male and female of the copepod *P. annandalei* to Cd toxicity. Males were almost twice as sensitive as females when comparing their respective 96h-LC50 (120.6 $\mu g.L^{-1}$ Cd for males and 239.5 $\mu g.L^{-1}$ Cd for females). Zidour et al. (2019) made the same conclusion in *E. affinis* for Cu and Ni toxicity as male 96h-LC50 were 25.0 $\mu g.L^{-1}$ Cd and 90.0 $\mu g.L^{-1}$ Ni, whereas female 96h-LC50 were 38.0 $\mu g.L^{-1}$ Cd and 161.0 $\mu g.L^{-1}$ Ni. When comparing the general relative sensitivities of these same copepod species, our experimental observations showed globally slightly lower 96h-LC50 values for *P. nana* than for *E. affinis* (87 $\mu g.L^{-1}$ Cd for *P. nana* and 92 $\mu g.L^{-1}$ Cd for *E. affinis*, and 90 $\mu g.L^{-1}$ Ni for *P. nana* and 96 $\mu g.L^{-1}$ Ni for *E. affinis*). Only the Cu toxicity appeared much higher for *E. affinis* than for *P. nana*, with 21 $\mu g.L^{-1}$ Cu against 58 $\mu g.L^{-1}$ Cu, respectively. Zidour et al. (2019) similarly showed that *E. affinis* had relatively lower tolerance to Cu in both sexes than that of Cd and Ni. LC50 values of Cd from Kadiene et al. (2017) also showed *E. affinis* to be more sensitive to Cd than *P. annandalei*, with *E. affinis* females even 2.7 times more sensitive than the *P. annandalei* females. Overall results of the metal toxicity tests performed by the members of our research team on these three copepod species made it possible to establish an overall species-ranking for Cd sensitivity: *P. nana*, *E. affinis*, *P. annandalei*, classified from most sensitive to least sensitive. Our research therefore confirms the strong potential of *P. nana* in ecotoxicity testing.

Many characteristics of *P. nana* have also made it an interesting model for research in ecotoxicology. An important review bringed together (Dahms et al., 2016) all the findings and results acquired to date on *P. nana* to present its promising potential as a model species for contaminant toxicity testing on aquatic ecosystems. Indeed, *P. nana* has many characteristics making it an interesting candidate in this field: a small size, a very short generation time, a great ease of culture and manipulation, a key position within the food web of marine ecosystems, a high sensitivity to contaminants, and a large area of distribution complementing existing copepod models.

TABLE 8. Ecotoxicological studies with *Paracyclopina nana*, from Dahms et al., 2016.

Species	Stressors tested	Endpoint measured	Type of test	Reference
P. nana	UV	Mortality, reproductive parameters	life table	Won et al. (2014)
P. nana	Food (5 microalgae)	Fecundity, mortality, growth	diet	Lee et al. (2006)
P. nana	Temperature, salinity, density, LPS	Gene expression	mRNA expression	Jeong et al. (2015)
P. nana	Gamma radioisotope	Growth, fecundity	radiation	Won and Lee (2014)
P. nana	Heavy metals, EDCs	Molecular, Vg expression	EDCs, HM exposure	Hwang et al. (2010)
P. nana	UV	Clutch number, growth pattern, newly hatched nauplii, ingestion rate, assimilation of diet, DNA repair, heat shock protein	UV exposition	Won et al. (2015a,b)
P. nana	Light intensity	Survival, growth, productivity	light exposure	Lee et al. (2011)
P. nana	Density, antioxidants	Naupliar production, gene expression	culture density	Lee et al. (2012)

But the main asset of *P. nana* for ecotoxicology remains the fact that its complete genome has been recently sequenced, assembled and annotated. Its mitochondrial genome has been presented and detailed by Ki et al. (2009). It is 15,981 bp in length and consists of 37 genes (12 protein-coding genes, 2 rRNAs, and 23 tRNAs). It differs from all known copepod mitogenomes due to a rearranged gene order and high divergence (Jung et al 2006, Ki et al 2009), which reveals a particularly compact genetic structure. Indeed, to date, the known mitogenomes of

Tigriopus californicus and *Tigriopus japonicus* have respective lengths of 180 Mb and 196 Mb, whereas that of *P. nana* totals 85 Mb. Copepods with a short and compact genome offer more facilities in evaluating their promoter regions and their overall genetic structure. In this sense, it therefore seems advantageous to use *P. nana* for the transcriptomic assessment of the risks associated with marine contaminants.

Numerous sequences and gene expression profiles have thus been revealed in *P. nana*, encoding heat shock proteins, xenobiotics metabolizing enzymes, or antioxidants. These gene expression profiles are potential biomarkers that can be used in environmental pollution biomonitoring. Moreover, the application of gene expression techniques on these different biomarkers opened the way for studying the mechanisms of action of environmental stresses at the genome scale of a copepod. The induction of the gene encoding vitellogenin is conventionally used as a biomarker of exposure to trace metals and endocrine disruptors in aquatic organisms. Hwang et al. (2010) examined his expression in *P. nana*. It appeared that, in this copepod and under normal conditions, the expression of vitellogenin transcripts is detectable from copepodite stages 4 and 5 and that females express them nearly 200 times more than males. And under conditions of exposure to different metals (Cd, Cu, and As), these transcripts are strongly and increasingly induced over time, suggesting a high potential of this specific biomarker in trace metals ecotoxicology. Jeong et al. (2017) demonstrated by fluorescence that *P. nana* is capable of ingestion and excretion of nano- (0.05 µm) and micro- (0.5 and 6 µm) polystyrene beads. The smallest beads (0.05 µm) were the most strongly retained after 24 h and induced the most physiological effects: developmental delays and decreasing fecundity in a dose-dependent manner, and significant increase in

intracellular ROS (oxidative stress) and enzymatic expression of four antioxidant enzymes (GPx, GR, GST, and SOD). This study was the first to investigate the effects of microplastics on *P. nana*. In copepods, lipogenesis is a genetically induced process and modifications of expression of the genes involved in this function in link with possible biotic and abiotic factors changes have been widely studied. Lee et al. (2017) recently showed that the expression of mRNA involved in lipogenesis, lipid droplet surface, and fatty acid composition increased at low temperatures (15°C) and decreased at higher temperatures (25°C) in *P. nana*.

Concerning the ecotoxicological aspect, present thesis focused on a purely physiological approach in order to attest and understand the parameters affecting *P. nana* when exposed to contaminants, and particularly by testing its bioaccumulation capacity, which has never been done before. This innovative study for this copepod species has been supplemented by a population-level rather than individual-level approach. This allowed to explore the responses of a whole population of this copepod to the toxicity of its environment and thus to join the intention expressed for the aquaculture aspect to study the full potential of a mass culture of *P. nana* for current research. Present experiments conducted on *P. nana* subpopulations exposed to cadmium (Cd), copper (Cu), and nickel (Ni), confirmed the hypothesis of metal competition recently demonstrated in the calanoid copepod *Eurytemora affinis*, as well as the effect of food on the increased bioaccumulation capacity of metals in copepods. Results are also the first to show the capacity of *P. nana* to bioaccumulate metals and to demonstrate the occurrence of an acclimation phenomenon in *P. nana* when exposed to a high metal concentration for several generations, all of this supporting its undeniable potential as a model for ecotoxicological testing.

CHAPTER V

CONCLUSIONS AND PERSPECTIVES

The results obtained during this thesis confirmed the interesting potential of *P. nana* for its use in fish larval aquaculture. Microalgae diets intended to feed copepods must present a nutritional quality that ensures optimal population growth and female fecundity in order to obtain a thriving culture. Among the three tested microalgae species, *R. salina* was shown to be the most effective microalga on all the aspects leading to a high productivity of *P. nana*, and even more when combined with *T. lutea* in a mixed diet. It induced the highest population growth and for each copepod life stage, and the greatest individual size and clutch size in ovigerous females. This microalgae combination also led to the highest fatty acid content and a varied monosaccharide composition in copepods, resulting in a rich and interesting nutritive profile for the growth of fish larvae. In practice, a regular feeding composed of a mix of *R. salina* and *T. lutea* should be provided to *P. nana* for efficient aquaculture.

Innovative results have also been put forward to illustrate the potential of *P. nana* in ecotoxicology. Exposure to cadmium, copper and nickel was shown to induce a reduction in population growth and an increasing metal bioaccumulation in copepod individuals. A greater accumulation of cadmium than the other two metals also unveiled a phenomenon of competition between metals in mixture for the absorption in copepods organism. On another side, a pre-exposed population to copper over several generations revealed a population growth less affected by metals and a lower bioaccumulation in exposed individuals. A depuration and an acclimation phenomenon were shown in this population already loaded with metals for several months. This study shed light on the bioaccumulation capacity of *P. nana* and its place in contaminated aquatic ecosystems.

The whole work of this thesis on *P. nana* carried out on a large scale on

important culture and experimental volumes of copepods constituted an innovative approach. They made it possible to account for the potential of a *P. nana* mass culture in both studied fields and also for the effects of changes in the parameters of its environment at the population level.

For the aquaculture aspect, the study of the nutritive profile of copepods provides key information for culture optimization for use to feed fish larvae. Research on this topic should be continued and deepened to understand the culture parameters and different diets influencing the nutritional quality of copepods, especially their saccharide composition, which remains poorly studied. The nutritive profile of copepods should be optimized based on the energy requirements for good growth of fish larvae.

Further work is also needed in copepod ecotoxicology to understand the parameters governing the phenomena of competition and complexation of mixed metals in the dissolved phase. It is now an important parameter to consider in studying the bioaccumulation of contaminants in aquatic organisms. It presents direct consequences on the accumulation capacity and the incorporation of metals in trophic chains.

Furthermore, all the results of this thesis deserve to be supported by genetic studies on *P. nana*. The fact that its genome is sequenced is a real asset and some preliminary work in molecular biology was done at the end of the thesis. A training was followed at the PMOI laboratory of the Université du Littoral Côte d'Opale of Boulogne-sur-Mer and supervised by Odile Broux. After optimization of the protocol, *P. nana* RNA was extracted successfully from copepod samples of the metal exposure study. After different tests, beta-actin turned out to be a good reference gene. Some primers of interest related to the responses of copepods to

metal exposure (oxidative stress) were tested. The hsp90 gene gave interesting results on the samples from Experiment 2, the one whose copepods were already exposed to copper for several months. A Wilcoxon-Mann Whitney test indicated a significative difference of hsp90 gene expression between the control individuals Cfood and the exposed ones Mixfood. Expression levels of the hsp90 gene related to beta-actin were significantly higher in the exposed individuals compared to the controls, indicating the occurrence of an overexpression of this gene in copepods exposed to metals and confirming the acclimation and adaptation of this culture to copper exposure for several generations. These preliminary objective results on gene expression in *P. nana* need to be pursued and deepened as part of the study of its dual potential for copepod research. Particularly, it would be interesting to study the differences in expression of genes coding for lipogenesis in relation to the different microalgae diets ingested.

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Paracyclopina nana: a small copepod with a strong interest in ecotoxicology and aquaculture

The cyclopoid copepod *Paracyclopina nana* plays a key role in the trophic chains of the aquatic environments of Eastern Asia. It has a small adult size (600 µm), a short life cycle, a high fecundity, and can be easily cultured under medium salinity (15 psu) and a wide range of temperatures. Its whole genome has also been recently sequenced, assembled and annotated. All these assets give it a very interesting double potential for current research: as a test organism for risk assessment associated with aquatic pollutants (bioindicator), and as a live prey in mass culture for the feeding of fish larvae in aquaculture.

In the framework of this PhD project, we aimed: (i) to test the productive and qualitative potential of *P. nana* in aquaculture in relation to the nature of the ingested microalgae diet; (ii) to establish the profile of *P. nana* as an ecotoxicological model through metal contaminant exposure tests.

The effects of seven different microalgal diets constituted by *Rhodomonas salina* (R), *Tisochrysis lutea* (T), and *Pavlova lutheri* (P) on *P. nana* productivity in culture were explored. The R+T and R diets induced the highest population growth and the greatest reproductive investment in ovigerous females. Those same diets also generated the highest total fatty acid content in copepods, and the highest total monosaccharide content has been found in copepods fed R+T+P. Overall results demonstrated that all the diets including *R. salina* lead to an increasing productivity of *P. nana*, and particularly when combined with *T. lutea* in a mixed diet.

Another study examined the effects of cadmium (Cd), copper (Cu), and nickel (Ni), on two subpopulations of *P. nana*. A first experiment conducted on a regular *P. nana* culture showed a decreasing population growth but an increasing metal bioaccumulation in copepods. Cd was also more accumulated when it was alone than in the mixture with Cu and Ni, confirming the hypothesis of metal competition recently demonstrated in a calanoid copepod. A second experiment performed on a *P. nana* culture already exposed to a higher Cu concentration for several generations revealed a lesser impact on population growth and a lower metal accumulation in copepods. Increasing metal concentrations in the experimental water reflected the depuration happening in this metal-loaded population already acclimated to metal exposure.

Overall results are the first ones showing that *R. salina* is a suitable microalga for productive mass culture of *P. nana* for use as live food for marine fish larval aquaculture, and to investigate the parameters influencing the bioaccumulation capacity of *P. nana* in response to metals in contaminated aquatic ecosystems.

Keywords: **Aquaculture, bioaccumulation, copepod, ecotoxicology, microalgae diet, *Paracyclopina nana***

Paracyclopina nana : un petit copépode à fort intérêt en écotoxicologie et en aquaculture

Le copépode cyclopoïde *Paracyclopina nana* joue un rôle clé dans les chaînes trophiques des milieux aquatiques de l'Asie orientale. Il présente une petite taille au stade adulte (600 µm), un cycle de vie court, une fécondité élevée et peut être facilement cultivé sous une salinité moyenne (15 psu) et dans une large gamme de températures. Son génome entier a également été récemment séquencé, assemblé et annoté. Tous ces atouts lui confèrent un double potentiel très intéressant pour les recherches en cours : en tant qu'organisme d'essai pour l'évaluation des risques associés aux polluants aquatiques (bioindicateur), et en tant que proie vivante en culture de masse pour l'alimentation des larves de poissons en aquaculture.

Dans le cadre de ce projet de thèse, nous avions pour objectifs : (i) de tester le potentiel productif et qualitatif de *P. nana* en aquaculture en lien avec la nature du régime de micro-algues ingérées ; (ii) d'établir le profil de *P. nana* en tant que modèle écotoxicologique au moyen de tests d'exposition à des contaminants métalliques.

Les effets de sept régimes de micro-algues différents constitués de *Rhodomonas salina* (R), *Tisochrysis lutea* (T) et *Pavlova lutheri* (P) sur la productivité de *P. nana* en culture ont été explorés. Les régimes R+T et R ont induit la plus forte croissance de population et l'investissement reproductif des femelles ovigères le plus élevé. Ces mêmes régimes ont également généré la plus forte teneur en acides gras totaux chez les copépodes, et la plus forte teneur en monosaccharides totaux a été trouvée chez les copépodes nourris avec R+T+P. Les résultats globaux ont montré que tous les régimes incluant *R. salina* entraînaient une augmentation de la productivité de *P. nana*, en particulier en association avec *T. lutea* dans un régime mixte.

Une autre étude a examiné les effets du cadmium (Cd), du cuivre (Cu) et du nickel (Ni) sur deux sous-populations de *P. nana*. Une première expérience menée sur une culture classique de *P. nana* a montré une diminution de la croissance de population, mais une bioaccumulation croissante des métaux chez les copépodes. Le Cd était également accumulé davantage lorsqu'il était seul que dans le mélange avec Cu et Ni, confirmant l'hypothèse de la concurrence des métaux démontrée récemment chez un copépode calanoïde. Une seconde expérience réalisée sur une culture de *P. nana* déjà exposée à une concentration plus élevée de Cu depuis plusieurs générations a révélé un impact moindre sur la croissance de la population et une accumulation de métaux plus faible dans les copépodes. L'augmentation des concentrations de métaux dans l'eau expérimentale a reflété la dépuration se produisant chez cette population chargée en métaux et déjà acclimatée à leur exposition.

Ces résultats sont les premiers montrant que *R. salina* est une micro-algue appropriée à une culture de masse productive de *P. nana* pour son utilisation en tant que proie vivante pour l'aquaculture des larves de poissons marins, et investiguant les paramètres influant sur les capacités de bioaccumulation de *P. nana* en réponse aux métaux dans les écosystèmes aquatiques contaminés.

Mots-clés: **Aquaculture, bioaccumulation, copépode, écotoxicologie, régime de micro-algues, *Paracyclopina nana***