Filipa Maria Ribeiro Pinheiro

Nutritional effects of rotifers fed with microalgae blend on the growth and development of zebrafish

Faculdade de Ciências e Tecnologia

2022

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Supervisor:

Professor Doutor João Varela

Professor Doutor Paulo Gavaia

Dissertation Master of Aquaculture and Fisheries

Dissertação Mestrado em Aquacultura e Pescas com especialização em Aquacultura

UAlg
UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologias

2022

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Declaração de Autoria

Declaro ser a autora deste trabalho**,** que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Filipa Maria Ribeiro Pinheiro

Eles não sabem que o sonho
É uma constante da vida
Tão concreta e definida
Como outra coisa qualquer

António Gedeão – Pedra Filosofal

Acknowledgements

Ao Professor João Varela e ao Professor Paulo Gavaia, pela oportunidade, pela muita paciência e por todos os ensinamentos.

À Professora Luísa Barreira pela ajuda, paciência e palavras confortantes.

À Tamára por ser amiga e professora, por nunca me deixar sozinha mesmo quando eu lhe esgotava toda a paciência e por ter apagado tantos fogos.

À Inês Maia pela amizade e por toda ajuda na análise dos FAME. À Ana Gomes pela ajuda na estatística e nos longos sábados a fazer ácidos gordos. Ao Gil por toda a ajuda com os peixes, os protocolos relacionados e por corrigir a minha tese em plena Itália. À Daniela e à Katia por estarem sempre prontos para ajudar com tudo o que seja relacionado a peixes e rotíferos

À Sara Castanho por me salvar sempre que os rotíferos morriam disponibilizando os mesmos.

Ao Hugo e à Patrícia pelas dicas e por toda ajuda dada ao longo destes dois anos.

Ao Zé e à minha mega família do coração por estarem sempre a meu lado nos bons e nos maus momentos, por aturarem as minhas crises de ansiedade e por todo o amor e carinho que todos me dão diariamente.

À minha mãe, à Guida e ao meu pai, por estarem a 300 km, mas mesmo longe conseguem dar o amor e o carinho à maneira deles.

À Avó Augusta e à Avó Irene por serem dois pilares da minha vida e porque sem elas eu não estaria onde estou hoje.

Aos amigos Gabriel, Mélanie, Catarina, Marta, José, Inês, Vitor, Any, Lucas, Stefanie, Beatriz, Joana, a todos os que possa ter esquecido de mencionar e a todos os restantes colegas do MarBiotech e do Bioskel.

Agradeço a todos do fundo do coração, o amor, o carinho, a amizade que diariamente me dão.

Um Sincero Obrigada a Todos

Resumo

O peixe-zebra é o segundo animal modelo mais utilizado na investigação científica, tendo sido usado como modelo para aquacultura em áreas como o desenvolvimento ósseo, oncobiologia e toxicologia. Devido a estes estudos, foi possível, por exemplo, melhorar a utilização da anestesia. O peixe-zebra é um peixe de pequenas dimensões, que apresenta dimorfismo sexual, conseguindo as fêmeas de produzir até 200 ovos. É um peixe robusto e com curto ciclo de vida, com larvas translúcidas. Existe uma grande variedade de linhas mutantes que permite estudos genéticos e de biologia molecular neste organismo modelo. A dieta do peixe zebra no ambiente selvagem baseia-se em zooplâncton, nemátodos e pequenos insetos. Em cativeiro, varia entre comida inerte e alimento vivo, como os rotíferos ou a artémia. Os requisitos nutricionais do peixe zebra ainda não são totalmente conhecidos, mas sabe-se que este organismo modelo necessita aminoácidos essenciais, lípidos, mais propriamente ácidos gordos como os ácidos eicosapentenóico (EPA) e o docosahexenoico (DHA), minerais como o fósforo, o cálcio e o zinco, e vitaminas como as vitaminas A, B e D para que o seu desenvolvimento se faça de uma maneira correta. O desconhecimento e a complexidade das necessidades nutricionais do peixe zebra têm levado à utilização de uma elevada quantidade de protocolos que resultaram numa grande variabilidade biológica entre instituições. Os rotíferos são, assim, ideais para alimentar as larvas durante os seus primeiros dias de vida, devido às suas pequenas dimensões, natação lenta e filtração não seletiva de alimento. No entanto, apresentam um conteúdo nutricional pobre que não vai de encontro às necessidades das larvas de peixe-zebra. As microalgas, por sua vez, são ricas em lípidos, proteínas, minerais, antioxidantes e vitaminas, sendo, deste modo, boas candidatas para o enriquecimento de rotíferos para alimentação de larvas de *D. rerio*. Os valores nutricionais das microalgas podem ainda ser melhorados através de stress abiótico, como salinidade, alterações no meio nutricional, temperatura ou luminosidade. Assim sendo, o objetivo principal deste trabalho correspondeu à produção de microalgas com perfil bioquímico diferenciado de modo a poder satisfazer as necessidades nutricionais das larvas de peixe zebra. Com esse fim, foram testadas quatro espécies de microalgas (*Nannochloropsis oceanica, Tetraselmis verrucosa*, *Phaeodactylum tricornutum* e *Nanofrustulum shiloi)*, utilizando um meio de cultivo comercial Nutribloom Plus (NB+) com duas concentrações de nitratos (2 e 10 mM). As microalgas foram cultivadas à escala laboratorial em fotobiorreatores de 1 L com arejamento e luz constante, durante 9 dias. Ao final de 9 dias, metade das algas foi

centrifugada e a outra metade serviu para enriquecer rotíferos, que posteriormente foram também centrifugados e congelados para futuramente serem liofilizados para análise bioquímica. Foram analisados lípidos totais, conteúdo proteico, metilésteres de ácidos gordos (FAME) e, apenas no caso das microalgas, foram também analisados o seu conteúdo mineral e conteúdo inorgânico. A microalga *T. verrucosa* foi a que apresentou maior crescimento, atingindo 2 g/L de peso seco; já *N. oceanica* foi a que mostrou maior conteúdo lipídico, sendo *P. tricornutum* a que apresentou maior conteúdo proteico. Por sua vez, *N. shiloi* foi a microalga que apresentou maior conteúdo inorgânico. A estirpe selecionada para o passo seguinte foi a *T. verrucosa* por apresentar uma alta produtividade. Estas microalgas foram cultivadas da mesma maneira ao do teste anterior em que se usou NB+ com 2 (*T. verrucosa* 2 mM; TF2) ou 10 mM (*T. verrucosa* 10 mM; TF10) de nitratos, tendo sido usadas para enriquecer rotíferos, para serem posteriormente fornecidos a larvas de peixe-zebra durante 30 dias. Foram ainda testados durante este período rotíferos enriquecidos com Green Formula (GF) e como controlo uma ração comercial (ZF). As larvas foram alimentadas duas vezes por dia, sendo os tanques também limpos por sinfonação duas vezes por dia. Ao final dos 30 dias, as larvas foram eutanasiadas, tendo sido calculada a sobrevivência, medida as suas dimensões (comprimento total) e peso. Foram ainda analisadas as deformações esqueléticas e medidos as vilosidades do intestino médio. Os tratamentos em que foram utilizados rotíferos enriquecidos com microalgas aumentaram significativamente, para cerca de 89%, a sobrevivência das larvas, quando comparadas com a ração comercial com a qual a sobrevivência não foi acima dos 59%. Estes resultados indicam que as larvas apresentam alguma dificuldade na digestão de microdietas nos primeiros dias de vida. As larvas alimentadas com GF foram as que apresentaram maior crescimento. Larvas alimentadas com rotíferos enriquecidos com microalgas apresentaram menor incidência de deformações quando comparadas com o controlo, a ração comercial ZF. Tal resultado pode ser justificado pela biodisponibilidade nutricional, comparando rotíferos e ração. É ainda de realçar que a localização do maior número de deformações das larvas alimentadas com rotíferos foram nas vértebras da barbatana caudal. Larvas alimentadas com TF10 apresentaram vilosidades intestinais maiores. Acredita-se que haja uma relação entre sobrevivência, baixas deformações e tamanho das vilosidades com a saúde dos peixes. Por isso, conclui-se que para os primeiros dias de vida rotíferos enriquecidos com microalgas (*T. verrucosa*) crescidas em meio enriquecido com 10 mM de nitratos serão uma ótima opção caso se queira produzir larvas de peixe zebra saudáveis, já que este

último tratamento melhorou a sobrevivência, diminuiu a incidência de deformações e aumentou as vilosidades intestinais.

Palavras-chave: Peixe zebra; microalgas, *T. verrucosa, N. oceanica, P. tricornutum, N. shiloi,* rotíferos

Abstract

Zebrafish is the 2nd most used animal model in scientific research. Its nutritional requirements are yet to be fully understood and, as such, there are a variety of feeding protocols that result in biological variability between institutions. Generally, zebrafish feeding is based on commercial feed and/or live feed, as rotifers. Rotifers are small-sized, slow swimming, non-selective filter feeders, being thus ideal as live feed; however, they have poor nutritional content and therefore need to be enriched. Microalgae are good candidates for enriching rotifers because they are rich in proteins, lipids and minerals. The production of these nutrients can be enhanced with the induction of abiotic stress, as the microalgae produce them to protect themselves from detrimental conditions for growth. The objective of this work was to produce microalgae with different biochemical profiles and to evaluate their effect on zebrafish larvae growth, gut development and skeletal quality. Four species of microalgae (*Nannochloropsis oceanica, Tetraselmis verrucosa, Phaeodactylum tricornutum,* and *Nanofrustulum shiloi*) were cultivated with Nutribloom® (NB+) with two concentrations of nitrate (2 and 10 mM). *T. verrucosa* was the species showing the highest growth rates, being selected to enrich rotifers to be tested on larvae as compared to larvae fed with Green Formula and a commercial feed for 30 days. At the end of the trials, larvae were euthanized, and their weight and length measured. The incidence of deformities and body content were analysed, and the midgut villi length measured. The survival rate of larvae fed with rotifers improved up to 89%, while the survival rate of larvae fed with commercial feed was only 59%. Zebrafish larvae fed with rotifers enriched with *T. verrucosa* cultivated in NB+ with 10 mM nitrates showed the lowest incidence of skeletal deformities (40%), with minimal vertebral abnormalities. The same treatment promoted the development of the midgut villi, with larvae displaying a villi length of 60 µm in contrast to 40 µm of control-fed larvae. In conclusion, NB+ with 10 mM nitrates offers very promising results both in terms of nutrition and larval quality. *T. verrucosa* grown in this medium showed to be an effective microalga for rotifer enrichment to produce healthy zebrafish larvae.

Keywords: Zebrafish, microalgae, rotifers, larvae, *N. oceanica, T. verrucosa, P. tricornutum, N. shiloi*

Index

List of Figures

Figure 4.10 – Total length of zebrafish larvae at 15 and 30 dpf, fed with commercial feed (Zebrafeed; **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with 2 (**TF2**) or 10 (**TF10**) mM nitrates. Different lower- and uppercase letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=10).

Figure 4.11 - Weight of zebrafish larvae at15 and 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**) rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 mM (**TF2**) or 10 (**TF10**) mM nitrates. Different lower- and uppercase letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=10).

Figure 4.12 - Zebrafish larvae at 30 days post fertilization (dpf) fed with different treatments . **A**- Larvae fed rotifers enriched with *T. verrucosa* grown with Nutribloom (NB)+ at 2 mM of nitrates **B**- Larvae fed rotifers enriched with *T. verrucosa* grown with Nutribloom (NB)+ at 10 mM of nitrates. **C**- Larvae fed rotifers enrich with Green formula **D**- Larvae fed with commercial feed (ZF).

Figure 4.13 - Total lipids (%) of zebrafish larvae at 30 dpf fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 mM (**TF2**) and 10 (**TF10**) mM of nitrates. Different capital letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=3).

Figure 4.14 - Protein content (%) of zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 mM (**TF2**) or 10 (**TF10**) mM of nitrates. Capital letters denotes that no significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p > 0.05$, n=3).

Figure 4.15 - Incidence of malformations in zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 (**TF2**) and 10 (**TF10**) mM nitrates. Different letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=30).

Figure 4.16- Load of malformations in zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed; **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 (**TF2**) and 10 (**TF10**) mM nitrates. (One-way ANOVA followed by Tukey's post hoc test, $p > 0.05$ n=30).

Figure 4.17 - Distribution of malformations (number of malformations per region) in zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed;,**ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+at 2 (**TF2**) or 10 (**TF10**) mM of nitrates. Different capital letters denote significant differences between treatments were found (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=30).

Figure 4.18 – Examples of skeletal malformations exhibited by zebrafish larvae at 30 days post fertilization (dpf) such as malformation in the structure of vertebral centra (**a**) and fusion of vertebrae with malformed arches (28-29) (**b**).

Figure 4.19 – Length (µm) of mid gut villi in zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with NB+ at 2 mM (**TF2**) and10 (**TF10**) mM of nitrates. Different capital letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, $n=5$).

Figure 4.20 – Mid gut villi of larvae at 30 days post fertilization (dpf) fed with rotifers enriched with *T. verrucosa* grown with Nutribloom (NB+) at 2 mM nitrates.

List of Tables

List of Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DW	Dry weight
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
Fd	Ferrodoxin
FW	Final weight
GC-MS	Gas chromatography mass spectrometry
GF	Green formula
Glu	Glutamate
HUFA	Highly unsaturated fatty acids
IW	Initial weight
L	Litre
LA	Linoleic acid
LNA	Linolenic acid
LOD	Detection limit
min	Minute
MP-AES	Microwave plasma-atomic emission spectrometer
MUFAs	Monounsaturated fatty acids
NADP+	Nicotinamide-adenine dinucleotide phosphate (OXIDIZED)
NADPH	Nicotinamide-adenine dinucleotide phosphate (REDUCED)
NB+	Nutribloom plus
PFA	Paraformaldehyde
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
rots/mL	Rotifers per millilitre
s	Second
SFA	Saturated fatty acids
TAG	Triacylglycerol
TF10	Rotifers enriched with *T. verrucosa* 10 mm
TF2	Rotifers enriched with *T. verrucosa* 2 mm
TL	Total length
UALG	Universidade do algarve
v/v	Volume/volume
ZF	Zebrafeed

1. - Introduction

1.1. - Aquaculture

Aquaculture is the production of aquatic organisms such as molluscs, fish, and algae. In 2016, the aquaculture industry produced 80 million tons of fish, and 598 species such as carp, catfish, tilapia, salmon, and shrimp were grown. In 2016, Asia had a virtual monopoly of 89% of global aquaculture production (FAO, 2018).

With the growth of the world population, the need for higher food production has become urgent, and aquaculture is an industry undergoing rapid growth to meet this demand. From 2000 to 2016, the annual growth rate of aquaculture output was 5.6%, and it is estimated that, by 2030, 109 million tons of aquaculture products will be produced. Aquaculture provides the possibility of feeding the global population while decreasing the pressure on wild fish populations that suffer from intensive fishing (FAO 2018, Ahmed and Thompson 2019).

The production of aquatic organisms in aquaculture is not just for human consumption. The output of aquaculture can have other uses, such as the production of fish meal and oil, the production of raw materials to produce pharmaceuticals or cosmetics (Mehariya et al., 2021) and the production of fish for biomedical research such as zebrafish (*Danio rerio*) or other teleosts such as European sea bass (*Dicentrarchus labrax)* or Nile tilapia (*Oreochromis niloticus*) in order to study human and animal disease (Wafer et al., 2017).

1.2. - Biomedical research

Biomedical research has focused on the study of diseases such as cancer (Varela et al., 2021), osteoporosis (Rosa et al., 2021), Keutel Syndrome (Cancela et al., 2021), among others.

The use of animal models in scientific research has been responsible for notable advances, such as a better understanding of human and animal anatomy and physiology, pathology, and pharmacology. It is also worth noting that the use of animals was essential for understanding the role of the pancreas in digestion, the development of some vaccines as, for example, the poliovirus vaccine, and the development of new drugs, vaccines and

techniques used in surgery and anaesthesia (Lieschke and Currie 2007, Andersen and Winter 2019).

In scientific research, different animal models are used. Examples of them are insects (*Drosophila* sp.), nematodes (*Caenorhabditis elegans),* frogs (*Xenopus* sp.), some mammals such as rats, dogs, cats, pigs and monkeys (Institute of Medicine and National Research Council 1991) and fish such as zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), medaka (*Oryzias latipes*), alpine whiting (*Rutilus rutilus*), stickleback (*Gasterosteus aculeatus*), puffer fish (*Takifugu rubripes*) and swordfish *(Xiphophorus hellerii*) (Institute of Medicine and National Research Council 1991, Kinter et al., 2021). Among these, zebrafish is one of the most used animal models in biomedical research (Figure 1.1). Indeed, in recent years, there has been a growing demand for studies with this species, which resulted in a significant increase in publications that contained the keywords "zebrafish" and "biomedical" (Teame et al., 2019).

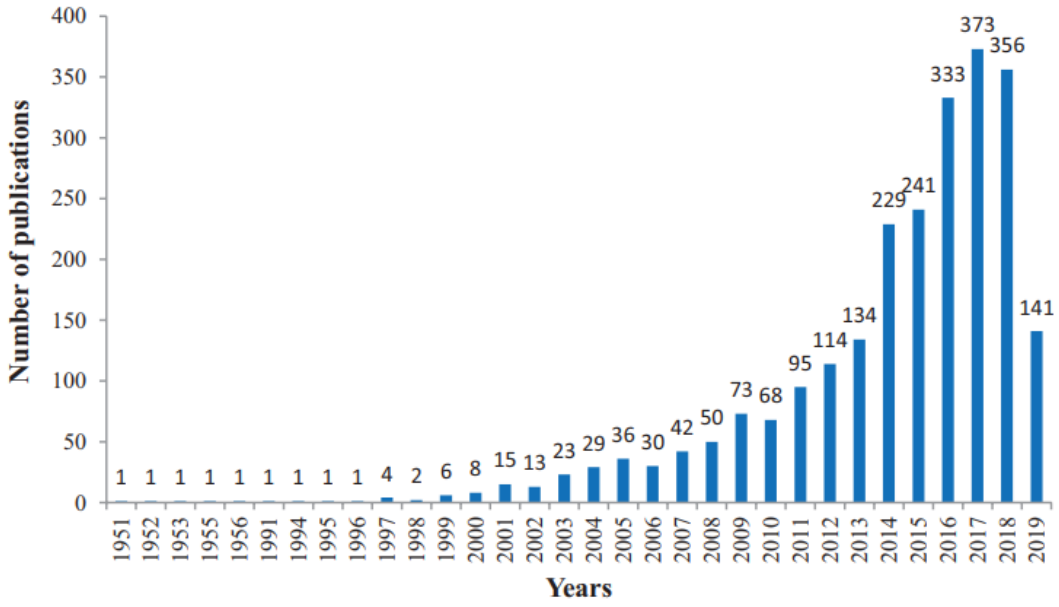

Figure 1.1 - Number of publications in scientific papers in the period between 1951 and 2019 matching the keyword's "zebrafish" and "biomedical" (Teame et al., 2019).

1.3. - Zebrafish (*Danio rerio*)

The zebrafish is a teleost fish (Engeszer et al., 2007; Meyers, 2018), belonging to the *Cyprinidae* family, originating in South Asia. It is a fish that lives in waters with low hydrodynamics (Engeszer et al., 2007; Meyers, 2018), and its length does not exceed 4 cm. In morphological terms, adults are sexually dimorphic: females are larger and have a

round belly, whereas males are smaller and slender (Figure 1.2) (Esmail et al., 2015; Teame et al., 2020).

Figura 1.2 - Zebrafish (*Danio rerio)* female and male (Teame et al., 2020).

In reproductive terms, zebrafish embryos are translucent and develop outside the female within a short time (Garcial et al., 2016). The female can lay about 200 eggs with a high fertilization rate (Kamel and Ninov, 2017). This fact, combined with the animal robustness and easy maintenance, made it a suitable animal model.

Also considered as a genetic model since 1981 (Streisinger et al., 1981), the zebrafish has anatomical, physiological, and developmental characteristics similar to those of higher vertebrates (Otte et al., 2017). Research concerning this organism enabled the first genetic screening to study mutations affecting organ development in vertebrates (Kamel and Ninov, 2017). This fact makes it a viable and simpler alternative to classical scientific models, such as rodents.

Used in other research areas such as pathology, zebrafish serves as a model to study some pathogens such as bacteria, parasites, and viruses (Meijer and Spaink, 2011; Crim and Riley, 2012; Torraca et al., 2014; Gratacap and Wheeler, 2014; Torraca and Mostowy, 2018). It is also used as a model to study immune system functions, ageing

system, toxicology, oncology, and behaviour (Aleström et al., 2006; Lin et al., 2012; Kent et al., 2012, Chiang and Tsai, 2016; Naert and Vleminckx, 2018; Kent et al. al., 2020).

As it is a fish widely studied and used as an experimental model, there are currently more than a thousand zebrafish laboratories worldwide (Alestrom et al., 2019; Hammer, 2020). However, there is still a high variability in the feeding protocols used between different institutions, which results in wide biological and experimental differences, becoming difficult to replicate the same results in different laboratories.

1.3.1. - Zebrafish diet

Zebrafish natural diet is based on insects, nematodes and other zooplankton species (McClure et al., 2006; Spence et al., 2008). However, the correct zebrafish diet in laboratory production is still unknown and not standardized among operators. To obtain consistent results, it is essential to optimize a feeding protocol. The most common strategy is the combination of live and inert food (Esmail et al., 2015).

Diets must contain the correct levels of essential nutrients, especially fatty acids and vitamins. It is also necessary to have the proper ratio of *n*-6:*n*-3 fatty acids to meet the zebrafish growth and development needs (Esmail et al., 2015).

Other nutrients are still needed, such as proteins and respective amino acids, which are involved in the growth of fish, since they are essential for protein synthesis and the lean body mass that constitutes several organs, including the red and white muscle (Guillaume, 2001; Fowler et al., 2019).

In zebrafish, hypertrophic growth begins with the addition of lean muscle mass, which can also be observed in higher mammals (Biga and Goetz, 2006; Fowler et al., 2019). In addition, amino acids are precursors for the synthesis of other substances such as nitrogen compounds, pigments, neurotransmitters, hormones, vitamins, and other compounds that regulate fish metabolism (Fowler et al., 2019).

In most fish species, lipids are the primary energy source from food used in the metabolism, development, growth and even reproduction of fish (Leaver et al., 2005). The amount of lipids in the diet will influence outcomes such as growth and body composition or other equally important factors for a healthy fish, such as vision, osmoregulation, immune response and even the composition of the gut microbiota (Arts

and Kohler, 2009; Watts et al., 2012; Falcinelli et al., 2017). In addition, lipids can also be components of membranes, pheromones, and hormones and even help the absorption of fat-soluble vitamins and pigments such as carotenoids (Meinelt et al., 2000; Guillaume, 2000, 2001).

1.4. - Live feed

Phytoplankton and zooplankton are the primary sources of nutrition for many species in aquaculture. Their nutritional content, although variable, is based on a set of macro- and micronutrients essential for the growth and development of fish larvae (Esmail et al., 2015). Although many planktonic species could be used as live feed, rotifers, artemia, copepods, moina, daphnia, and fairy shrimp are examples of zooplankton commonly used in aquaculture (Radhakrishnan et al., 2019).

Commonly known as "living capsules", live feed usually refers to organisms that swim freely in the water column. This group of organisms is described as beneficial for the early stages of fish development compared to inert food. Larvae are slow swimmers, and the use of swimming live feed stimulates their hunting behaviour, contributing to their swimming development (Bengtson, 2007; Radhakrishnan et al., 2019). Live food has other advantages, such as ease of ingestion and digestion (Kinne, 1997), without altering water quality (Neelakantan et al., 1988; Radhakrishnan et al., 2019).

Compared to inert feed, live feed has greater acceptability in fresh and saltwater fish (Koven et al., 2001); however, it has some disadvantages as, for example, its poor and variable nutritional content (Esmail et al., 2015; Radhakrishnan et al., 2019). Live feed is often composed of non-selective filter feeders (Radhakrishnan et al., 2019), allowing them to be fed with a diverse array of phytoplanktonic organisms that can be used for nutritional enrichment, thus counteracting their low nutritional value.

1.4.1 - Rotifers

Rotifers are one of the main preys used in aquaculture for fish and crustacean larvae (Radhakrishnan et al., 2019), being also the main constituents of zooplankton (Nordgreen et al., 2013). About 2,500 species of freshwater and saltwater rotifers are documented (Radhakrishnan et al., 2019), *Brachionus plicatilis* being an example of a

rotifer often used as live feed (Conceição et al., 2010; Hagiwara and Yoshinaga, 2017; Yanes-Roca et al., 2019).

Rotifers have a body size variation between 60 µm and 2 mm, with males being smaller than females (Radhakrishnan et al., 2019). The body of a rotifer is divided into three parts: i) head, ii) trunk, and iii) foot (Figure 1.3). The corona, mastax and lorica constitute the head. The corona is the organ that allows the locomotion of the rotifers and their movement in the water, and this organ also has the function of absorbing food particles. The mastax is a characteristic organ of rotifers and is based on a calcified structure that serves to grind the particles that the rotifers ingest. The trunk is formed by the digestive tract, the excretory system and the sexual organs. Finally, the foot is a ringed rectangular structure (FAO, 1996; Radhakrishnan et al., 2019; Gribble, 2021).

The lifespan of a rotifer is estimated between 3.4 and 4.4 days at 25 ºC, and generally, these organisms reach maturity between 0.5 and 1.5 days, and the females can start laying eggs after four hours. The reproduction of rotifers can be done in two ways, asexual reproduction or sexual reproduction (FAO, 1996; Gribble, 2021).

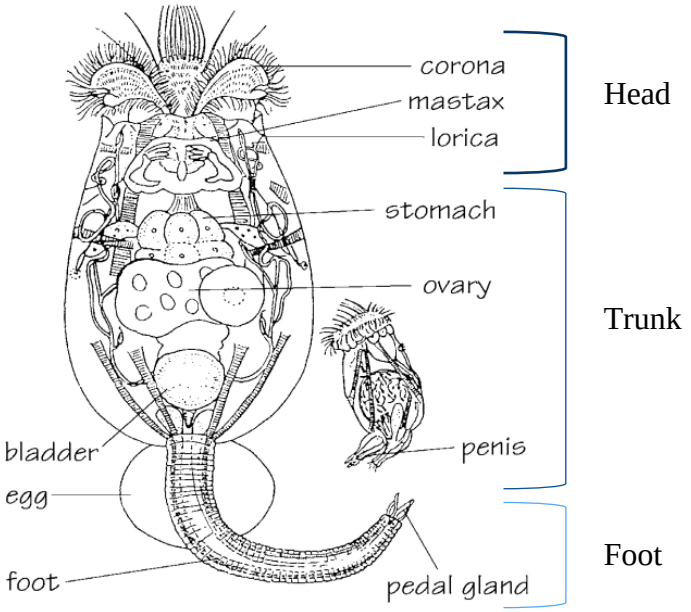

Figure 1.3 - Diagram of a rotifer with all the organs from the three divisions of the body: head (corona, mastax and lorica), trunk (digestive tract, excretory system, and sexual organs) and the foot (adapted from FAO, 1996).

The growing conditions of rotifers are essential for their successful production. Rotifers tolerate salinities between 1-97 ppt, with an optimal salinity of 35 ppt. However, certain predators used in aquaculture have lower optimal salinity levels, and rotifers can be adapted to these salinities as long as they are adequately acclimatized so that there are no sharp differences in this environmental parameter (FAO, 1996; Gribble, 2021).

The temperature has a substantial impact on the life cycle of rotifers. The optimal temperature for the growth of rotifers differs from the strain in question. For example, L-strain rotifers prefer lower temperatures than those of the S-strain. In addition, higher temperatures stimulate growth and increase the metabolism of rotifers, which results in greater food consumption. It is imperative to maintain water quality and avoid overfeeding and starvation periods, especially at higher temperatures, because at higher temperatures, the animals will consume faster their lipid and carbohydrate reserves (Fao, 1996; Gribble, 2021).

The optimal amount of dissolved oxygen in the water should be around 2 mg/L. However, this value can vary depending on other parameters such as temperature, salinity, population density and the type of food used. The aeration should not be too strong to

avoid physical damage to rotifers, such as the early release of eggs by females (FAO, 1996).

Certain species of bacteria stimulate the growth of rotifer cultures, such as *Pseudomonas* and *Acinetobacter*. *Pseudomonas* is a bacterial genus that synthesizes vitamin B12, a vitamin that may be a limiting factor in the growth of the rotifer population. On the other hand, some bacterial species have a negative impact, decreasing the growth rate of rotifers (FAO, 1996).

Fish larvae open their mouths when the yolk is absorbed or disappears and begin consuming exogenous food. The function of the larval gastrointestinal tract is not fully operational when they open their mouths, so they are not able to digest some nutrients. Larvae need certain nutrients for their development, such as phospholipids, polyunsaturated fatty acids (PUFA), highly unsaturated fatty acids (HUFA), taurine, vitamin C and other fat-soluble vitamins. Since rotifers are nutritionally poor, it is essential to enrich them to potentiate the development of fish larvae (Hagiwara and Yoshinaga, 2017).

1.5 – Microalgae

Microalgae are single-celled microorganisms responsible for producing 50% of atmospheric oxygen. They are generally photoautotrophs and transform light energy into chemical energy that will be stored in the form of compounds. As of 2017, about 30,000 algal compounds such as PUFAs, pigments, proteins, and essential amino acids (Plaza et al., 2009) have been isolated and purified (Ariede et al., 2020).

Each species can have different purposes, and these might depend on the nutritional content of a specific microalga. For example, species with higher lipid content are good candidates for biodiesel production, whereas species with high sugar concentrations can be used to produce bioethanol via yeast-promoted fermentation (Chen et al., 2009; Yen et al., 2015). Microalgae are also used in the cosmetic industry because they can be feedstocks for thickeners, binders, and antioxidant compounds (Ariede et al., 2020; Dionisio-Sese, 2010). They can also be used in the food industry to produce supplements and additives (Ariede et al., 2020).

In addition to compounds extracted from the microalgal biomass, they can also be used for animal and human consumption, such as *Chlorella* sp., which contains high protein content (up to 51 to 58%, Niccolai et al., 2019; Roy and Pal, 2015), or *Arthrospira platensis* with a protein content of up to 70% (Plaza et al., 2009). More recently authorized for human consumption, *Tetraselmis chui* may contain up to 52% protein, 15% carbohydrates and 16 to 45% lipids (Roy and Pal, 2015).

Despite all the possible applications, microalgal biomass is still mostly used in aquaculture for its ability to be nutritionally moulded and produce compounds of interest. They are used as a diet for live feed organisms (rotifers and brine shrimp), filtering organisms (oysters or mussels) and larvae and adult fish (Roy and Pal, 2015). These microorganisms are also used in aquaculture ponds to increase contrast, promoting prey hunting and decreasing cannibalism using the "green water" technique. The introduction of high concentrations of microalgae into the ponds also improves the maturation of the larval gastrointestinal tract (Cremen et al., 2007) and water quality through the consumption of excreted nitrogen-rich products by the microalgae (Sirakov et al., 2015).

1.5.1. – Nutritional profile of microalgae

Microalgae produce compounds through photosynthesis and transform light energy into chemical energy. Although this process is species-dependent, under conditions of abiotic stress, it is possible to induce the production of specific compounds (Ariede et al., 2020). For example, the production of lipids or proteins can be increased by altering the quality of light during microalgae growth (Schulze et al., 2017). Depending on the light intensity, it is also possible to improve the content of pigments, lutein or β-carotene (Schüler et al., 2020).

Another alternative for inducing the biochemical modulation of microalgae is temperature. For example, when comparing three temperatures, 10, 20 and 30 ºC, *Tetraselmis striata* CTP4 increases carotenoid production at 30 °C. Likewise, *Chlamydomonas* sp., grown at low temperatures, 8 °C, has a high protein and PUFA content (Schulze et al., 2019).

Furthermore, we can promote changes in the nutritional content of microalgae by manipulating the culture medium, nitrogen depletion being one of the most used and studied methods for lipid induction in different microalgal species (Table 1.1). Moreover,

this method is reported to affect the content and composition of fatty acids in microalgae. One of the reasons that might explain this metabolic change is that under normal growth conditions, adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are produced in photosynthesis, being consumed upon the generation of biomass, yielding ADP and NADP+, which become available again as acceptor molecules in photosynthesis. When cell growth and division are compromised due to a lack of nutrients, the primary electron acceptor in photosynthesis (NADP+) can become depleted, because this process is controlled by the abundance of light and cannot be completely turned off in obligate photoautotrophs. As NADPH is consumed in fatty acids biosynthesis, resulting in the production of NADP+ and triacylglycerols (TAGs), when under nitrogen-limiting conditions, cells often recycle this electron acceptor and store chemical energy in the form of TAGs. Nevertheless, high lipid production due to nitrogen stress can take 2 to 5 days, significantly affecting cell growth rate and resulting in lower cell concentrations (Sharma et al., 2012).

Nitrogen is a limiting reagent for the growth of all organisms. Microalgae transform inorganic nitrogen (ammonia, nitrites, nitrates) into organic nitrogen (proteins, peptides, enzymes, energy-carrying molecules such as ATP and ADP). The translocation of the inorganic nitrogen occurs across the plasma membrane, followed by the reduction of oxidized nitrogen and the incorporation of ammonium into amino acids. Nitrate and nitrite undergo reduction with the assistance of nitrate reductase and nitrite reductase, respectively. Nitrate reductase uses the reduced form of nicotinamide adenine dinucleotide (NADH) to transfer two electrons, resulting in the conversion of nitrate into nitrite. Nitrite is reduced to ammonium by nitrite reductase and ferredoxin (Fd), transferring a total of six electrons in the reaction. Thus, all forms of inorganic nitrogen are ultimately reduced to ammonium prior to being incorporated into amino acids in the cell. Finally, using glutamate (Glu) and ATP, glutamine synthase facilitates the incorporation ammonium into the amino acid glutamine (Maestrini et al., 1986; Cai et al., 2013). The more nitrogen offered to the microalgae, the more proteins can be produced (Cai et al., 2013). This nitrogen can be added in the form of nitrate powder or more recently as a nutrient present in wastewater, which can be treated using microalgae, promoting water reuse and the circular economy (Abdel-Raouf et al., 2012; Shanthi et al., 2021).

Table 1.1 - Examples of microalgae and the effect on the biochemical profile with specific induced stresses (adapted from Sharma et al., 2012).

Microalgae species or strains	Induced stress	Change in biochemical profile after induction	Reference
***Chaetoceros* sp.**	Phosphorus limitation	Increased total lipids with greater relevance to 16:0 and 18:1	Reitan et al., 1994
Chlamydomonas reinhardtii	Nitrogen limitation	Increased total lipids	Dean et al., 2010
Chlorella kessleri	Phosphorus limitation	Increased unsaturated fatty acids	El-Sheek et al., 1995
***Chlorella* sp.**	Nitrogen limitation	Increased lipid productivity	Praveenkumar et al., 2012
	Conditions devoid of nutrients (nitrogen, phosphorus, iron and the three combined)	Production of total lipids 49.16 ±1.36 $mg.L^{-1}$	Praveenkumar et al., 2012
Chlorella vulgaris	Nitrogen limitation	Total lipids increased by 16.41%	Converti et al., 2009
		Increased lipid productivity	Yen et al., 2011
		Increased TAG levels	Widjaja et al., 2009
***Coelastrella* sp.**	The combined effect of pH + nitrogen limitation	Increased TAGs	Gardner et al., 2011
Isochrysis galbana	Phosphorus limitation	Increased TAGs	Reitan et al., 1994
Monodus subterraneus	Phosphorus limitation	Increased total lipids	Goldberg et al., 2006
Nannochloropsis oculata	Nitrogen limitation	Total lipids increased by 15.31%	Converti et al., 2009
Phaeodactylum tricornutum	Nitrogen limitation	TAG levels increased from 69 to 75%	Alonso et al., 2000
	Phosphorus limitation	Increased total lipids with greater relevance to 16:0 and 18:1	Gardner et al., 2011
***Scenedesmus* sp.**	The combined effect of pH + nitrogen limitation	Increased TAGs	Gardner et al., 2011
	Depletion of nitrogen and phosphorus	Increased unsaturated fatty acids	Xin et al., 2010
Scenedesmus subspicatus	Nitrogen limitation	Increased total lipids	Converti et al., 2009

1.6. - Selected Microalgae species

1.6.1. - *Phaeodactylum tricornutum*

P. tricornutum is a diatom capable of growing with a wide variety of culture media, tolerant to high pH values and can grow under low light conditions (Butler et al., 2020). This species can be found in several habitats across the globe, being common in coastal areas with large fluctuations in salinity (Cui et al., 2019). *P. tricornutum* may present several morphotypes, such as fusiform, triradiate and oval-shaped cells. Its cell shape depends on the growth conditions. For example, low temperature and salinities stimulate the oval morphology, and the triradiate cell shape is the most sensitive to hyposaline conditions (Martino et al., 2007; Francius et al., 2008; Martino et al., 2011). The morphotype used in this work is a strain with fusiform cells (Figure 1.4).

Considered a species-rich in lipids and fucoxanthin, this species has about 14% of lipids of which 2.6 to 3.1% corresponds to the *n*-3 PUFA eicosapentaenoic acid (EPA; Branco-Vieira et al., 2020).

Figure 1.4 - Micrograph of *P. tricornutum* using DIC at a 400 × magnification. Scale bar = 5 μm.

1.6.2. - *Nannochloropsis oceanica*

N. oceanica has tiny cells with sizes ranging between 3 and 4 μm in diameter. Its cells have a spherical shape (Figure 1.5), being non-flagellated. It contains a cell wall with polysaccharides and has a single chloroplast (Ma et al., 2016). *Nannochloropsis* is a species known to produce high concentrations of lipids such as EPA. Ma *et al.* (2016) reported 12% DW of EPA in this species. This amount can also be stimulated by abiotic factors, as was the case reported by Xiao et al. (2015), who through light intensity managed to increase the amount of lipids from 7.41±0.06 to 18.53±0.00. In addition, *N. oceanica* has already been proposed for human consumption, as it is an excellent source of proteins, vitamins, acylglycerols, and pigments (chlorophyll, zeaxanthin, canthaxanthin and astaxanthin) (Liu et al., 2017; Chua et al., 2017).

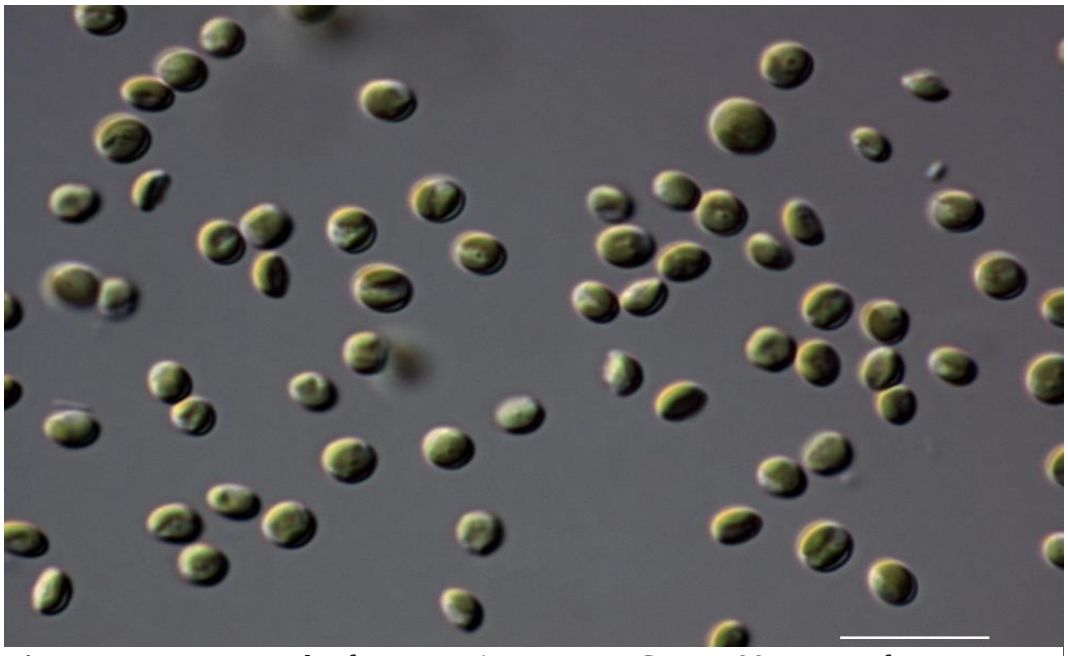

Figure 1.5 - Micrograph of *N. oceanica* using DIC at a 400 × magnification. Scale bar = 5 μm.

1.6.3. - *Tetraselmis verrucosa* f. *rubens*

Tetraselmis verrucosa f. rubens is a green alga (Figure 1.6) belonging to the *Chlorodendrophyceae*. *Tetraselmis* microalgae are used in aquaculture due to their nutritional interest (Cardoso et al., 2020). *T. verrucosa* is known for its robustness, resistance to abiotic stress, and ability to recover from damage caused by mechanical stress (Olsen et al., 2016). *T. verrucosa* is mainly used in aquaculture due to its pigments, such as lutein, violaxanthin, neoxanthin and *β*-carotene (Garrido et al., 2009; Pulz and Gross, 2004).

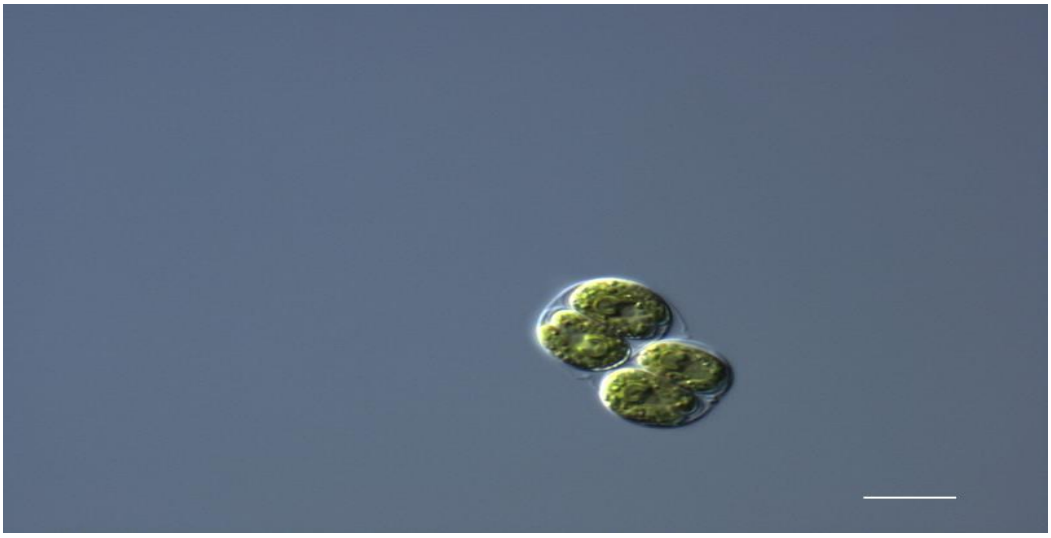

Figure 1.6 - *T. verrucosa* micrograph under DIC at a 400 × magnification scale bar = 5 μm.

1.6.4. - *Nanofrustulum shiloi*

N. shiloi is a benthic diatom (Figure 1.7) distributed along marine coasts and characterized by rectangular frustules, forming chains linked by spines (Ruocco et al., 2018). Despite showing some toxicity to marine invertebrates (Li et al., 2019), a symbiotic relationship between this diatom and photosynthetic sponges has recently been documented (Konstantinou et al., 2020). In addition, it is a species widely used in aquaculture to feed sea urchins (Ruocco et al., 2018).

Figure 1.7 - Micrograph of *N. shiloi* using DIC at a 400 × magnification. Scale bar = 5 μm.

Like any diatom, *N. shiloi* is rich in fucoxanthin, a compound with anti-cancer, anti-obesity, antioxidant, anti-inflammatory, and anti-diabetic properties. It can reach a lipid content of 37% and can produce nearly 8 mg/g of fucoxanthin (Sahin et al., 2019). Fucoxanthin has an inhibitory effect on ROS (reactive oxygen species) and is known to regulate lipid accumulation in zebrafish (Seo et al., 2016).

This microalgae species shows promising prospects for feeding zebrafish larvae.

2 – Objectives

The zebrafish, being one of the main animal models used by the scientific community, presents a great biological variability resulting from the feeding protocols used in different institutions. Rotifers have been shown to be good candidates for standardizing the zebrafish feeding protocol. However, their low nutritional value requires that they are enriched with microalgae that are rich in lipids, proteins, pigments

and vitamins that are ideal for the development of zebrafish. Thus, the final goal of this work is the establishment of a microalgal diet to enrich rotifers that meets the specific needs of zebrafish, and to contribute to standardize feeding protocols for this fish species. This will be accomplished by the following specific objectives:

1. Establish a protocol for inducing metabolites in the microalga selected to be used as live feed for zebra fish larvae.
2. Grow microalgae species with a differentiated biochemical profile to meet the needs of zebrafish larvae;
3. Determine the effect of the chosen microalgae to enrich rotifers to be used as live feed for zebrafish larvae

3 - Materials and Methods

3.1 - Serial scale-up of microalgal cultures

T. verrucosa and *N. shiloi* were provided by the Marine Biotechnology laboratory (MarBiotech) culture collection of the Centre of Marine Sciences (CCMAR), University of Algarve (UALG), whereas *N. oceanica* and *P. tricornutum* were provided by Necton S.A., Belamandil, Olhão.

All microalgal inoculation and handling processes were carried out in a laminar flow chamber (Microflow Laminar Flow Cabinet), and all material was previously decontaminated (autoclaved at 120 °C, 30 minutes, Tuttnawer 2540ML). Microalgal culture scale-up was carried out in order to produce sufficient inocula for the experiments. Scale-up started with a culture growing on 1.5% agar plate, from which a colony was removed and inoculated into a liquid culture medium formed by salt water and Nutribloom Plus®(NB+) diluted down to a 4 mM final nitrate concentration. Weekly, the culture volume was increased serially, using a 1:10 dilution of the inoculum (Figure 3.1).

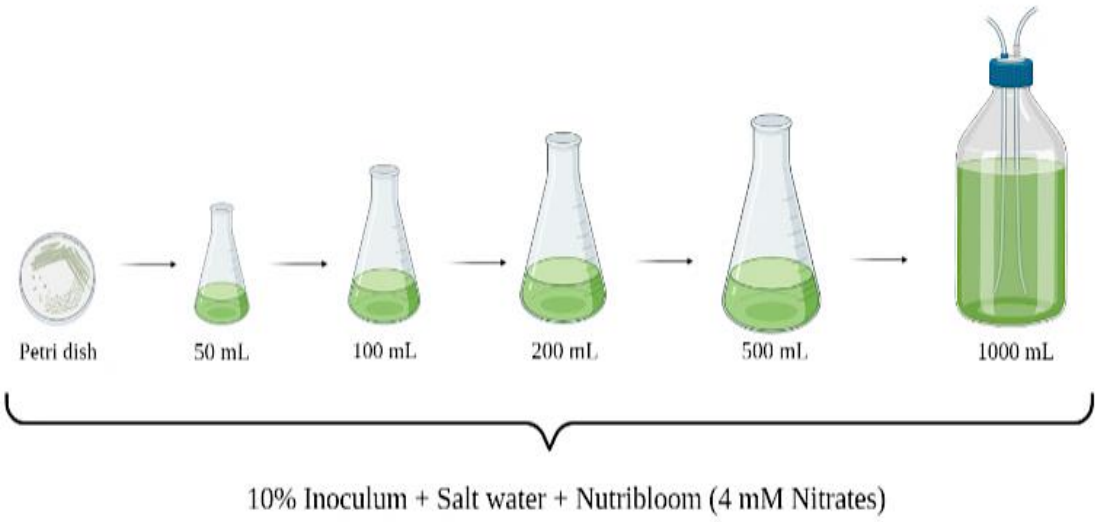

Figure 3.1 - Diagram of the culture scale-up pipeline used. Cells growing on solid medium were used to inoculate liquid media. The culture volume was increased serially using a 1:10 dilution of the inoculum. Dilution was achieved with the addition of salt water and concentrated nutrient media, in this case, Nutribloom Plus (NB+).

In the case of the diatom *N. shiloi* species, the medium was supplemented with a 0.4 mM silicate solution. The algae were kept in an orbital shaker in volumes less than or equal to 200 mL. If higher volumes were used, cells were transferred to containers with constant aeration. Microalgae were always cultivated with continuous light at room temperature.

3.2 - Induction of metabolites in algae

The experiment of induction of metabolites in microalgae was carried out with four species of microalgae: *T. verrucosa f. rubens*, *N. oceanica*, *P. tricornutum* and *N. shiloi.*

Twelve 1-L bubble columns made of transparent borosilicate glass (Normax, Lda.) were used to optimize the induction of metabolites in the aforementioned microalgae. Growth in the bubble columns was carried out at room temperature with light supplied by light-emitting diodes (LEDs, PrimeLux® lamps) at a constant photosynthetic flux density of 263 μmol photons m^{-2} s^{-1}, measured using a quantum sensor (Model US-MQS-B, Walz, Effeltrich, Germany). Air flow for bubbling the cultures was set at 800 mL min^{-1} using a pump (AIRLAB EV 120, Pondpro, Canada). Air was filtered through 0.22 μm Whatman PTFE filters.

To measure the effect of the nutrients on the cultures and determine the optimal conditions for metabolite induction, media were supplemented with Nutribloom® Plus (NB+) concentrated culture medium at a 2 and 10 mM final nitrate concentrations to test either nutrient depletion and nutrient repletion, respectively. The experiment lasted for 9 days. Cultures were monitored on alternate days to assess cellular concentration and nitrogen consumption by spectrophotometry (Figure 3.2). Half of the microalgae volume was centrifuged on the ninth day of growth and frozen at -20 ºC until further analysis. The remaining volume was used to enrich the rotifer cultures.

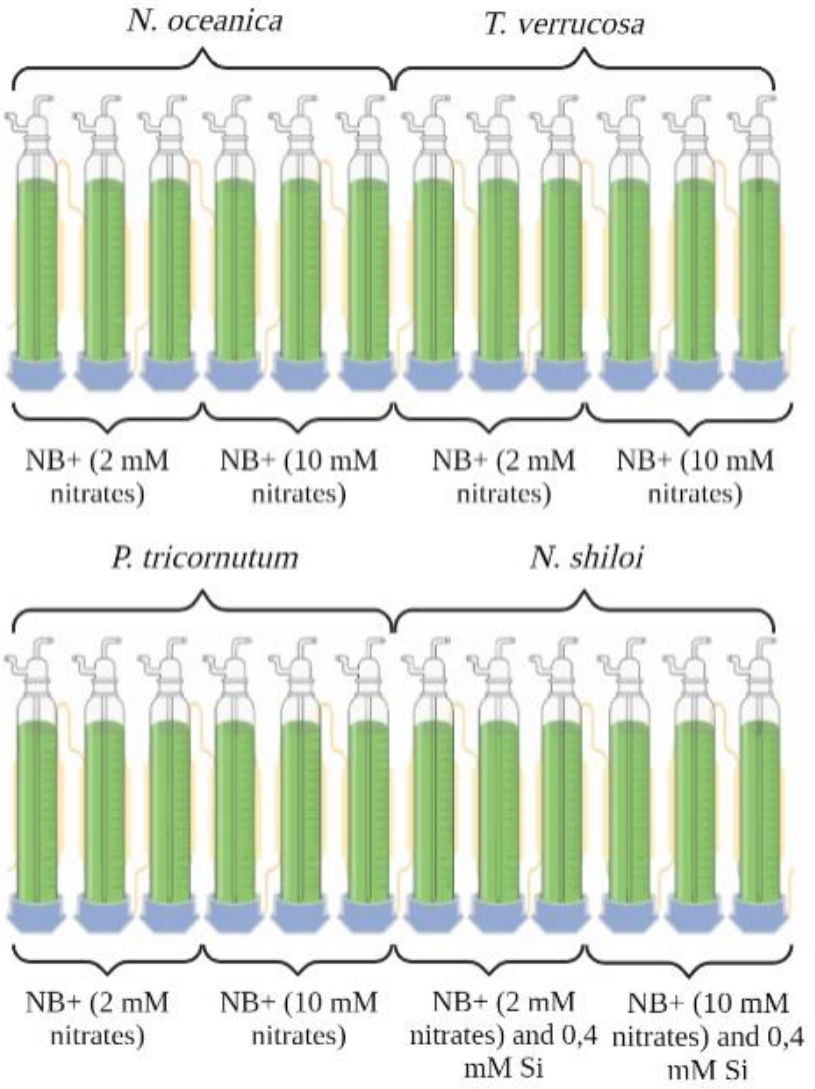

Figura 3.2 - Diagram of the 1-L bubble columns setup for the induction of metabolites in four species of microalgae, namely *N. oceanica*, *T. verrucosa*, *P. tricornutum* and *N. shiloi*. Growth was followed for nine days in NB+ medium with two concentrations of nitrates, 2 mM and 10 mM. *N. shiloi* growth media was supplemented with a 0.4 mM silicates (Si) solution.

3.3 - Calibration curves

Calibration curves were determined to obtain a correlation between the optical density with dry weight of the four microalgae species. For this purpose, serial dilutions of the microalgae were made so that the absorbance readings ranged between 0.1and 1.0.

using a microplate reader (Biotek Synergy 4). Absorbance was measured at 750 nm with triplicates for each sample.

The remaining cultures were filtered with a vacuum pump (VWR VCP 130 PUMP) and 0.7-µm glass microfiber filters (VWR I516-0345) in triplicate. Afterwards, the filter was treated with 10 mL of 31.5 g L^{-1} ammonium formate. The filters were placed in an incubator at 60 ºC until dryness and weighed on a precision balance.

Linear equations showing a correlation between dry weight and OD were obtained, namely $y = 1.139x - 0.1701$ with a correlation coefficient R^2 = 0.87 for *P. tricornutum*, $y = 0.612x + 0.329$ with a correlation coefficient R^2 = 0.64 for *N. oceanica*, $y = 1.137x - 0{,}170$ with a correlation coefficient R^2 = 0.91 for *N. shiloi* and $y = 1.744\,x - 0.033$ with a correlation coefficient R^2 =0.96 for *T. verrucosa* (Figure S1). All correlations were considered as significant ($p < 0.01$), enabling the estimation of DW for cultures through the measurement of absorbance (Figure S1).

3.4 - Nitrate consumption

The analysis of the consumption of nutrients, according to the standard ultraviolet spectrophotometric method (Armstrong, 1963), 10 mL samples from each culture were collected and centrifuged at 2700 g for 10 minutes. From the supernatant, 1 mL was collected to determine the respective nitrate concentration. Nitrate concentration was determined as follows: Falcon tubes were prepared with a stock solution with 9.8 mL of NaCl (35 g/L) and 0.2 mL of HCl. For each sample, duplicates were prepared with 9.3 mL of NaCl, 0.2 mL of HCl and 0.5 mL of supernatant. Absorbance was read in quartz cuvettes at 220 and 275 nm. The reading at the latter wavelength is required to detect whether the organic matter is also present, which might interfere with the correct determination of the nitrate concentration. NO_3^- concentration was calculated using a previously established calibration curve between known concentrations of this ion and respective absorbance values: $y = 6.793\,x - 0.013$ with a correlation coefficient R^2 = 0.99.

3.5 - Rotifers culture

Rotifers cultures were maintained in reactors with artificial salt water at 18 ppt prepared with reverse osmosis water and reef salts added (Tropic Marin Pro Reef Sea Salt, TMC Iberia, Spain), fed with 2.5 mL of Green Formula ® (Phytobloom, GF, concentrate of *N. oceanica*) per million rotifers, and the culture was supplemented daily with 0.5 mL/L of NB+ growth medium. Counting was made daily to know the concentration of rotifers, and partial water changes were carried out to maintain the ammonia levels at acceptable levels for the organisms in cultivation. Once a week, a complete water replacement was performed.

3.6 - Rotifers enrichment

Rotifers enrichment was performed using microalgae cultures at a concentration of 0.21 g DW/L to be comparable with the control rotifer enrichment product (Green Formula). The rotifers were enriched at a concentration of 450 rotifers/mL (rots/mL). Afterwards, they were sieved and washed at the end of the day, inoculated from the previously prepared microalgae, and were incubated overnight. The next morning, the water was changed, and more microalgae culture was added and left for another 2 hours.

The rotifers were sieved again and offered to the fish at a concentration of 100 rotifers/mL. For future biochemical analysis, rotifers were centrifuged at 8000 *g* for 10 minutes at room temperature, after which the samples were frozen at -20 °C for later lyophilization and characterization.

3.7 - Biochemical Analysis:

3.7.1 - Ash

Ash content was determined using the method as published by Widbom (1984). For ash determination, 50 mg of biomass was weighed in crucibles and then burned for 8 hours at 550 °C in a muffle furnace Naberthern Controller B170 (Naberthern, Germany).

The ash weight was subtracted from the initial biomass weight to calculate the percentage of inorganic matter (Equation 1).

$$1) - \ \% \text{ Ash} = \frac{(\text{IW} - \text{FW})}{\text{Sample weight}} \times 100$$

IW: Initial weight, FW: Final weight

3.7.2 - Total protein content

Protein content was determined by measuring nitrogen on a CHN elemental analyser (Vario EL III, Elementary). The total nitrogen value was multiplied by the ratio 4.78 (Lourenço et al., 2004) in the case of microalgae samples and 6.25 (Diniz et al., 2011) in the case of rotifer and larvae samples to obtain the total protein contents in our samples.

3.7.3 - Total Lipids

Total lipids were determined using a modified protocol of the Bligh & Dyer (1959) method adapted as described previously (Pereira et al., 2011).

Tubes for lipid determination were dried and weighed on an empty precision scale. Afterwards, 10-20 mg of lyophilized biomass was weighed in glass tubes, and to all samples 0.8 mL of distilled water, 2 mL of methanol and 1 mL of chloroform were added. The samples were homogenized for 60 s with a disperser (IKA Ultraturrax T18). A second 1-mL aliquot of chloroform was added and homogenized for another 30 s with the disperser. Then, 1 mL of distilled water was added and homogenized for another 30 s. Samples were centrifuged at 2000 *g* for 5 min. Upon the removal of the chloroform phase via evaporation using a dry bath at 60 °C overnight, 0.7 mL of resulting solution was transferred into the lipid determination tubes. The tubes were reweighed to obtain the percentage of total lipids using the following formula (Equation 2):

$$2) - \ \% \text{ total lipids } = \frac{\frac{[(\text{FW} - \text{IW}) \times \text{ total volume of chloroform}]}{\text{evaporated volume of chloroform}}}{\text{Sample weight}} \times 100$$

IW: initial weight, FW: final weight,

3.7.4 - Fatty acid methyl esters profile FAME

Fatty acid profiles were determined using a modified protocol of Lepage & Roy (1984), as described in Pereira et al. (2012). This method is based on direct transesterification and later extraction of the lipidic phase.

The profile of fatty acid methyl esters (FAME) was determined by gas chromatography in conjunction with mass spectrometry (GC-MS). Samples of 10-20 mg were weighed into derivatization tubes, homogenized in 1.5 mL of methanol/acetyl chloride (20:1, v/v), and homogenized for 90 s with a disperser. Then 1 mL of *n*-hexane was added, tubes were closed and placed in a water bath at 70 °C for 60 min. Then the samples were cooled, and 1 mL of distilled water and 4 mL of hexane were added. The mixture was vortexed at maximum speed in 2 cycles of 30 s, and the phases were separated using centrifugation (2000 *g* for 5 min). The organic phase was transferred to new tubes.

The extracts were washed with anhydrous sodium sulphate (Na_2SO_4) to remove any residual water, and then the samples were filtered using 0.45-µm syringe filters. Finally, the hexane phase was dried using a gentle stream of nitrogen. The samples were resuspended in 500 µL in gas chromatography-grade hexane and filtered again using 0.22 µm syringe filters. Fatty acid analysis was performed using an Agilent GC-MS instrument (6890 NetworkGC System with 5973 inert Mass selective detector, Agilent Technologies, USA) with an Agilent Tech DB-5MS column.

FAME identification was made by comparing the retention time of the standard samples (Supelco 37 FAME ix, Sigma-Aldrich) and the mass spectrum against the NIST library.

3.8.5 - Mineral analysis

Mineral analysis was done using the methods as described by Pereira et al. (2018).

About 90 mg of biomass were weighed and digested for mineral analysis with 65 % nitric acid in a digester (CEM, SPD 80 ML, Charlotte, North Carolina). Samples were analysed

for mineral content using a Microwave Plasma-Atomic Emission Spectrometer (MP-AES, Agilent 4200 MP-AES, Agilent Victoria, Australia). Different dilutions were prepared using certified standard solutions. All samples were analysed in duplicates. Quantification wavelengths and calibration curves were selected to obtain the highest signal ratio and the lowest interference for the target elements, spiking and recovery readings were carried out to assess the validity of the results.

3.8 - Zebrafish trials

3.8.1 - Broodstock maintenance

A breeding population of zebrafish wild-type AB strain (ZFIN ID: ZDB-GENO960809-7) maintained at the Centre of Marine Sciences (CCMAR, Portugal) was used to generate the specimens used in this work. The zebrafish rearing facilities had a controlled photoperiod in a 14:10 hour light: dark cycle, an independent air conditioning system (26 ± 1 °C), and an air extraction system to guarantee the air renewal in the room, maintaining the room air humidity close to 60 %. All animals were housed in 3.5-L tanks in a 980-L recirculating system ZBWTU0011 (ZebTEC® Tecniplast, Italy). The water quality was maintained automatically by partial water discharge (10% of total volume daily) and a filtration system: a mechanical filter (pleated cartridge filters, 50 µm), carbon filter (granular activated charcoal filter), and an ultraviolet sterilization system (180 000 µWs/cm^2). The system also contained a biological filter (ceramic beads) with nitrifying bacteria to remove toxic amounts of ammonia. The water conditions were the following: 28.0 ºC ± 0.5 ºC, pH 7.5 ± 0.1 and conductivity 750 ± 30 µS. Nitrogen compounds (ammonia, nitrite and nitrate) were monitored weekly, the levels of nitrates were being kept within the accepted limits throughout the experiment (50-100 mg per litre). Fish with 6-8-months were used, since previous studies showed that this is the optimal age for zebrafish reproduction (Diogo et al., 2019). Females (*n*=10) and males (*n*=10) were kept in separate tanks to stimulate breeding performance.

3.8.2 - Zebrafish reproduction and larval development

Adult fish were divided by sex in breeding tanks at a sex ratio of 1:1, where they were kept for 16 hours. In the first hour of light, the barrier that kept the fish separated was removed, and they were left to reproduce for 2 hours.

The eggs were all mixed in a single tank so that a random choice of eggs was made. A total of 200 eggs were placed in each tank (13 in total - 12 treatments plus one extra). After 24 hours, abortions were removed, and precisely 100 eggs were counted per tank, and 0.5 mL of methylene blue (0.5 mg/L) was added to the water to prevent fungal growth. Tanks were kept in a 28 °C water bath so that the temperature of the water in the tanks was constant.

On the fifth day, larvae of poor quality or that had not hatched were replaced by larvae from the supplementary tank.

On the fifth day after fertilization, 5 larvae were removed from the supplementary tank, measured and weighed for later calculation of the weight and initial length of the larvae.

From the fifth day onwards, the larvae began to be fed (Table 3.1, Figure 3.3). All tanks were cleaned twice a day. Partial water changes of 50 % were carried out, and in the first cleaning, the water volume was reduced by half.

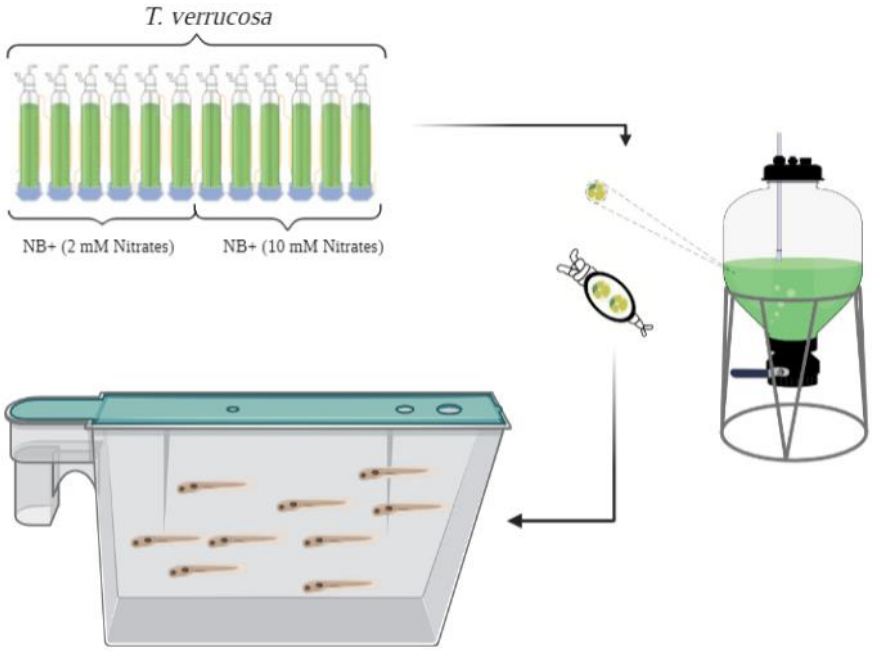

Figure 3.3 - Diagram of the implemented zebrafish feeding. Microalgae (*T. verrucosa*) were grown in NB+ medium with two concentrations of nitrates, 2 and 10 mM. The algae were used to enrich rotifers at a concentration of 450 rots/mL, which were given to the zebrafish larvae at a concentration of 100 rots/mL twice a day.

Table 3.1 - Representative diagram of the different treatments offered to zebrafish larvae.

Treatment	**Food**
GF	Rotifers (100 rot/mL) enriched with **Green Formula** ®
ZF	**Zebrafeed** ®
TF2	Rotifers (100 rot/mL) enriched with ***T. verrucosa* 2 mM**
TF10	Rotifers (100 rot/mL) enriched with ***T. verrucosa* 10 mM**

3.8.3 - Zebrafish sampling points

Two sampling points were performed, at 15 and 30 dpf-(days post fertilization), for which the fish were anaesthetized with a lethal dose of MS-222 (300 mg/mL).

At 15 dpf, it was measured the total length and weight. At 30 dpf, total length, weight, survival, histology, skeletal deformities, and biochemical profile were measured.

3.8.4 - Length, Weight and Survival

Total length (TL) was determined by taking photos using a Leica® stereomicroscope of 15 larvae from each replicate and measurements made using the software ImageJ. Total DW per fish was determined using15 larvae per replicate. Larvae were placed into pre-weighed dry Eppendorf tubes and then freeze-dried. Next, tubes were weighed and calculated using the equation below:

$$3) - \text{ DW per fish } = \frac{(\text{IW } - \text{FW})}{15}$$

IW: initial weight, FW: final weight,

Survival was calculated by counting all live fish remaining in tanks at 30 dpf.

3.8.5 - Skeletal deformities analysis

Staining of skeletal structures for assessing deformities were done as those

previously described (Gavaia et al., 2000). The skeletal deformities were assessed using 30 larvae per replicate (tank) sampled 30 dpf. After being lethally anaesthetized with dose of tricaine methanesulfonate (MS-222; Sigma-Aldrich), the larvae were fixed in 4% buffered paraformaldehyde solution at 4 °C for 24 hours. The staining of the cartilage was made by placing the larvae in Alcian blue 8GX at 70:30 (absolute alcohol: glacial acetic acid) for 45 min. The larvae were hydrated with decreasing concentrations of alcohol (100% - 25%), and then the larvae were placed in distilled water with increasing concentrations of KOH (0.5-1%). Alizarin red S (0.01 g/L) was used to stain the bone for 3 hours. The larvae were kept in KOH (1%) until they lost their scales and pigment. For conservation, the fish were placed in solutions of increasing glycerol concentrations (5%-70%). The analysis was performed according to the diagram described in Bird and Mabee (2003; Figure 3.4).

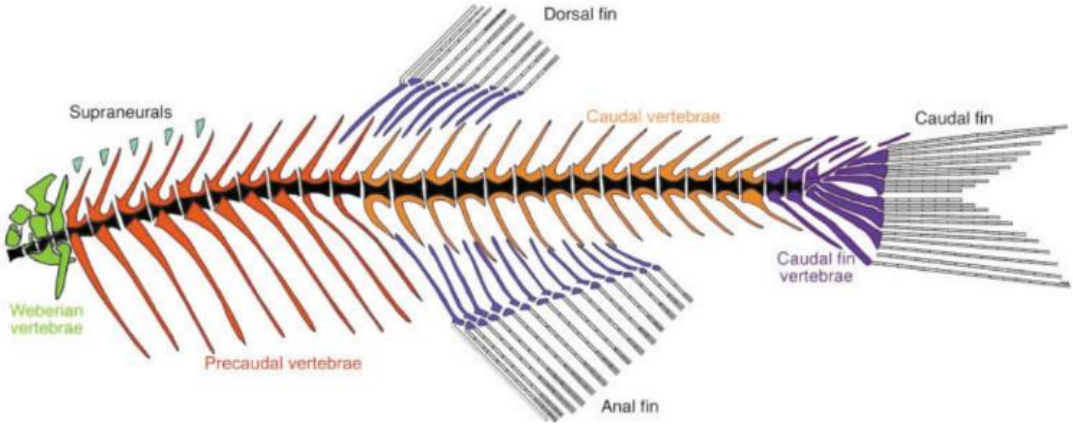

Figure 3.4 - Generalized diagram of zebrafish axial skeleton. Weberian vertebrae are green, supraneurals are light blue, precaudal vertebrae are red, caudal vertebrae are orange and caudal fin are purple (Bird and Mabee, 2003)

3.8.6 – Histology

Histological procedures were done as those previously described (Fischer et al., 2017). The larvae used for histology were fixed in buffered 4% paraformaldehyde (PFA), pH 7.4, overnight.

Samples were removed from 70% ethanol and 3 to 4 larvae were placed into a pre-labelled cassette with a blue sponge on the bottom. Another sponge was placed on-top,

the cassettes closed, and all cassettes were submerged into a container with 70% ethanol for 4 hours. Samples were placed into a Tissue TEK II Tissue Processor (Kedee Instruments Co., China). In the tissue processor, samples were mechanically passed through a series of baths. The first was 2 hours in an 75% ethanol treatment, followed by 90 min in 96% ethanol, 90 min in absolute ethanol, then two baths of 90 min in pure xylol, and finally three paraffin baths of 3 hours each. Cassettes were then brought to the KD-BM tissue embedding centre (Kedee Instruments Co., China) and placed in a heated tank with paraffin. Larvae were removed from the cassettes and 2-3 placed 2 mm apart in plastic moulds on a heated block with a slight amount of paraffin. Moulds were removed from heat and briefly cooled to fix larvae in place. A plastic cassette top was put onto the plastic moulds and filled with paraffin. The moulds were then transferred to a cooling block at -4 °C to chill. After 2 hours, the paraffin blocks could be removed from the metal moulds and placed into the refrigerator until processing. Sections were prepared at 6 µm using a microtome Microm HM 315 Rotary Microtome (Microm, USA) and stained using Harris haematoxylin and eosin as described by Fischer et al. (2017).

Intestinal villi length was measured using a microscope with the software Wavelimage. A photo was taken of the section, and villi measured with ImageJ®. From each section about 5 villi were chosen for measurement.

3.9 - Statistical analyses

The statistical treatment of the data was performed using the IBM-SPSS v.25 program. To compare the data obtained for the different variables between treatments of the same microalgae, a Student *t*-test was performed. To compare all treatments, a univariate ANOVA was performed, identifying the differences with a post-hoc SNK (Student Neuman Keuls) test ($p<0,05$).

4 – Results

4.1 – Laboratory-scale microalgae production

Growth curves of the four species under study, namely *N. oceanica*, *T. verrucosa*, *P. tricornutum* and *N. shiloi*, are shown in Figure 4.1. It can be observed that *N. oceanica* (Figure 4.1A) grew better with NB+ at 10 mM, reaching 1.2 g/L after nine days, while cells with NB+ at 2 mM reached only a concentration of 0.97 g/L. *T. verrucosa* showed the fastest growth of all the microalgae under study (Figure 4.1B). In this case, the microalgae cultivated with NB+ at 2 mM showed the highest growth, producing 1.86 g/L of biomass in dry weight. It was also possible to observe that the cells under treatment with NB+ at 10 mM reached the exponential phase between the third and the fifth day, producing 1.7 g/L of biomass in dry weight at the end of the experiment. The diatom *P. tricornutum* (Figure 4.1 C) was the one that presented the lowest growth when compared to all the others, with the microalga cultivated with NB+ at 10 mM producing 0.58 g/L of biomass in terms of dry weight, whereas, when grown in NB+ at 2 mM, it only reached a final concentration of 0.36 g/L. Finally, *N. shiloi* (Figure 4.1D) grew better with NB+ at 10 mM, achieving 1.18 g/L of biomass in dry weight at the end of the 9 days. The same microalgae, cultivated with NB+ at 2 mM reached a biomass concentration of 0.78 g/L dry weight.

4.2 – Nitrate consumption

Concerning the consumption of nitrates, it was possible to observe that *N. oceanica* (Figure 4.2A) and *T. verrucosa* (Figure 4.2B) consumed all the nitrates in the culture medium at 2 mM treatment after 3 days. In contrast, for *N. shiloi* (Figure 4.2D), under the same treatment, the exhaustion of nitrates is only reached on the fifth day. On the other hand, with *P. tricornutum* (Figure 4.2C) it was not possible to observe the total consumption of nitrates in any of the treatments, probably due to its low growth performance under the conditions used.

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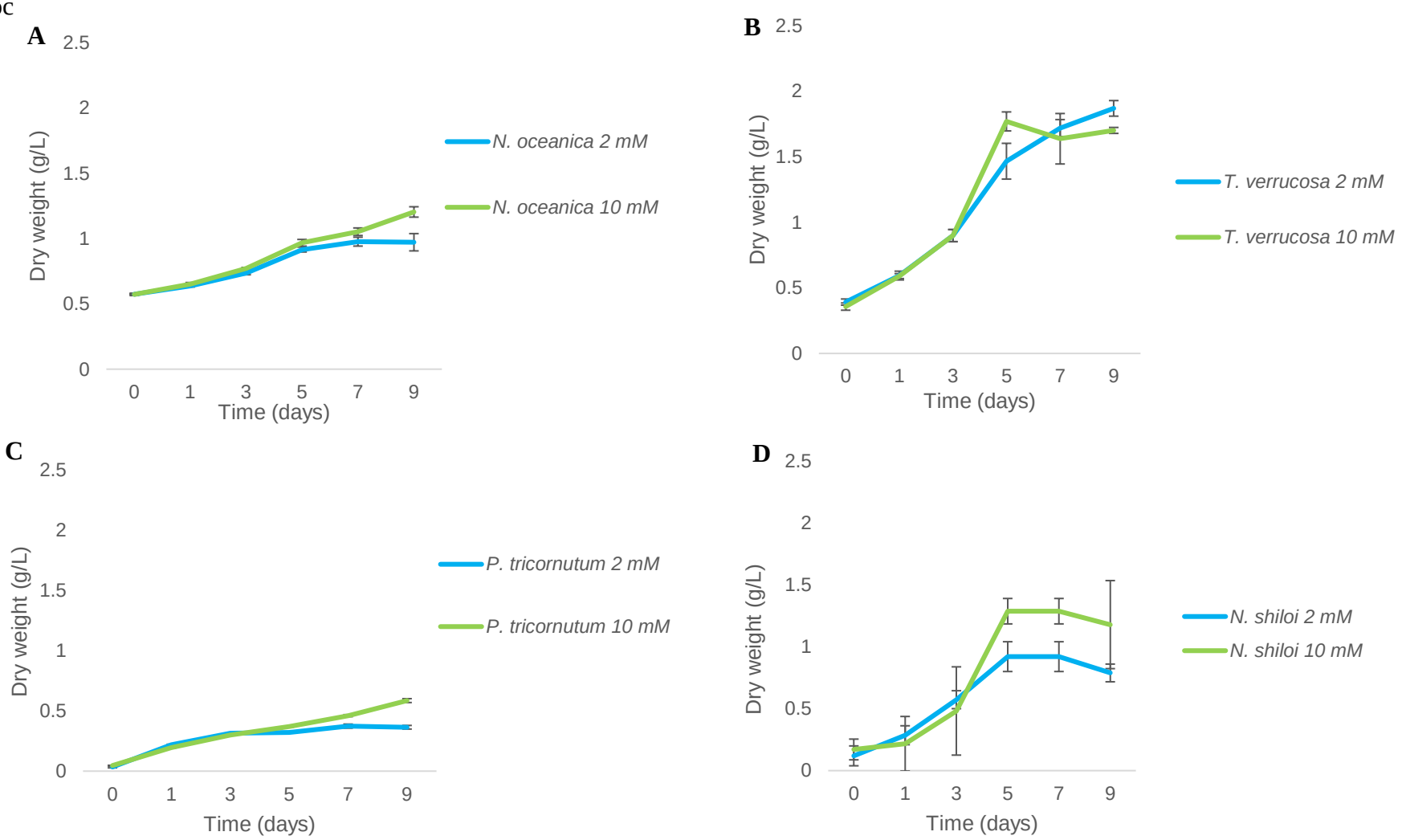

Figure 4.1 – Microalgal growth over 9 days with NB+ growth medium with 2 mM (blue), or 10 mM nitrates (green). **A** - *N. oceanica*, **B** - *T. verrucosa*, **C** - *P. tricornutum*, **D** - *N. shiloi* (*n*=3).

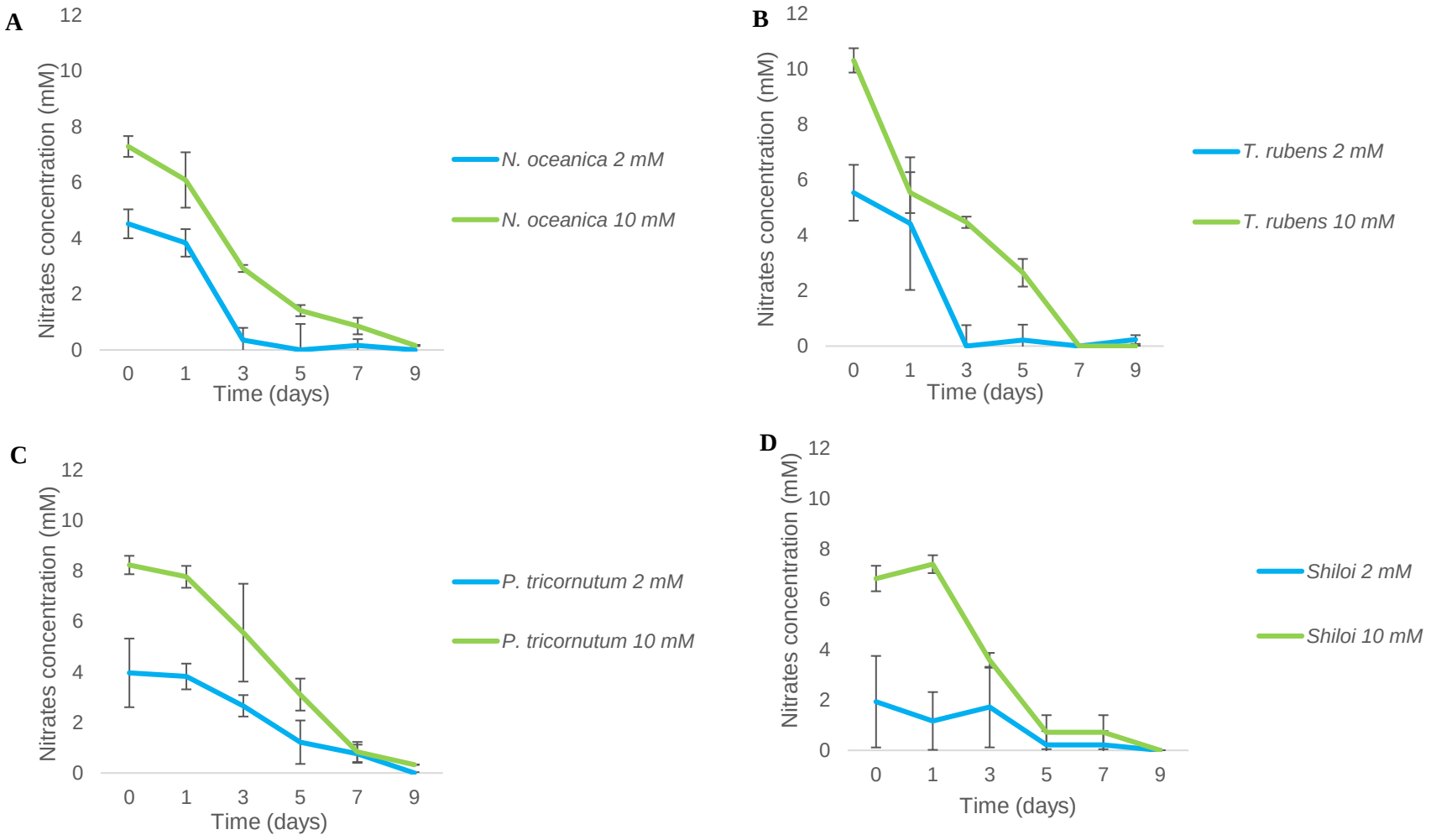

Figura 4.2- Nitrate consumption (mM) over 9 days culture with NB+ with 2 mM nitrates, shown in blue, and 10 mM nitrates, shown in green. **A** - *N. oceanica*, **B** - *T. verrucosa*, **C** - *P. tricornutum*, **D** - *N. shiloi* (*n*=3).

Regarding the microalgae cultured with NB+ at 10 mM nitrates, it is possible to observe that the total consumption of nitrates only happened on the seventh day in *T. verrucosa* cultures, while *N. oceanica* and *N. shiloi* cells were able to fully consume this nutrient on the ninth day. Once again, in *P. tricornutum* cultures, total consumption of nitrates was not detected.

4.3 – Biochemical composition of microalgae species

4.3.1 – Total lipids

The analysis of total lipids (%) of the four microalgae species (*N. oceanica, T. verrucosa, P. tricornutum* and *N. shiloi*) cultivated with NB+ at concentrations of 2 and 10 mM nitrates is presented in Figure 4.3. *N. oceanica* showed the highest lipid content, around 42 %, when cultivated with NB+ at 2 mM of nitrates and 35 % for the 10 mM treatment. Among the microalgae tested, *N. shiloi* was the species displaying the lowest amount of lipids in the treatment with NB+ at 10 mM reaching only 7.5% of total lipids. It should also be noted that only *N. oceanica* and *P. tricornutum* presented significant differences in lipid contents between different treatments. In both cases, the highest lipid

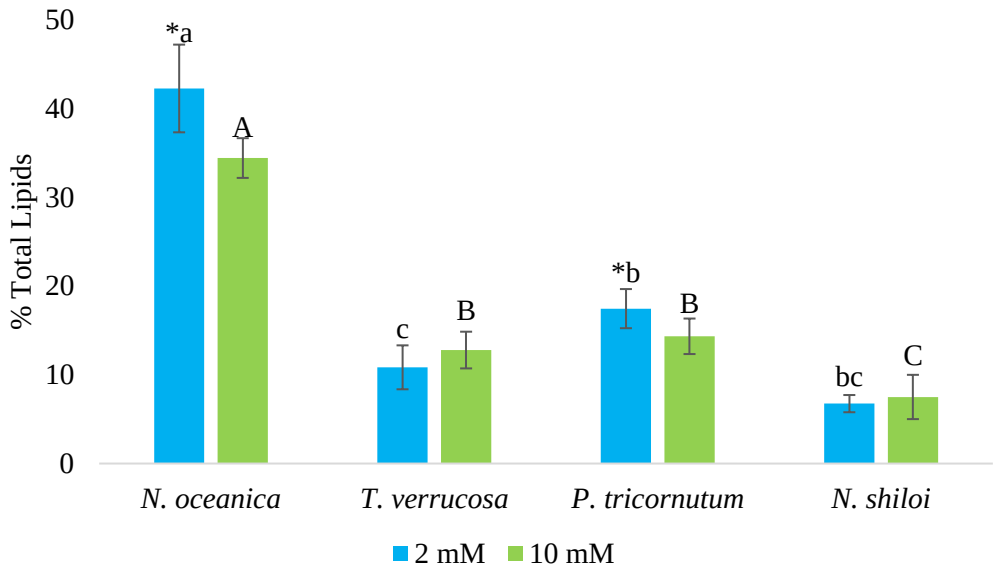

Figure 4.3 - Total lipids (%) of four microalgae species (*N. oceanica, T. verrucosa, P. tricornutum, N. shiloi)* cultivated with Nutribloom (NB)+ with two concentrations of nitrates (2 and 10 mM) for nine days. Different lower- and uppercase letters represent significant differences between microalgae grown with NB+ and 2 mM or 10 mM of nitrates, respectively (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$), * denotes significant differences between treatments in the same microalga (*t*-test, $p < 0.05$, $n=3$).

contents were achieved with the lowest (2 mM) concentration of nitrate assayed.

To ensure production of microalgae for the larval trial, it was necessary to produce microalgae for enrichment of rotifers for at least 25 days. Figure 4.4 shows the analysis of total lipids (%) of *T. verrucosa* cultivated for 30 days in a semi-continuous regime. These longer cultivation times apparently led to significant differences between *T. verrucosa* cells grown with NB+ at different nitrate levels. The treatment generating cells with the highest amount of lipids was the growth medium with 2 mM nitrates.

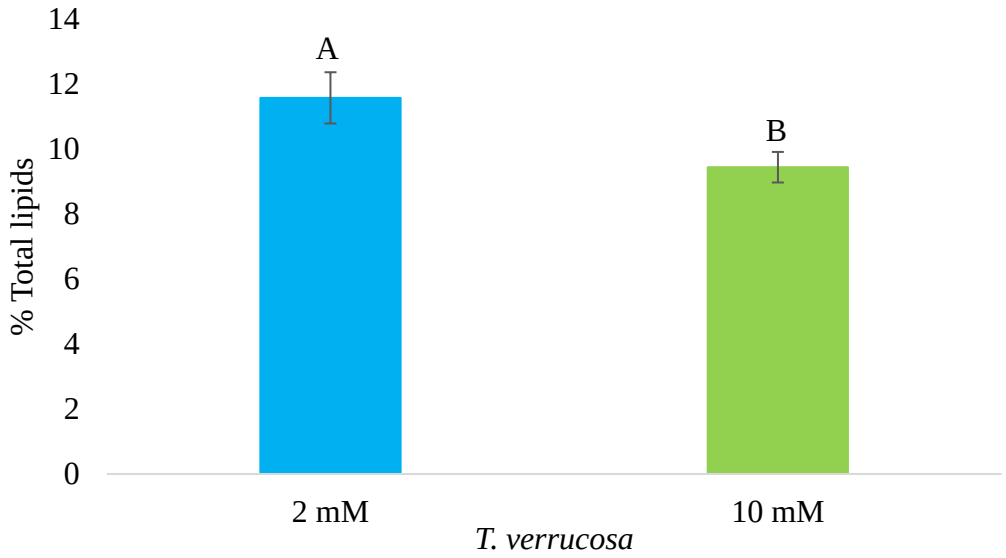

Figure 4.4 - Total lipids (%) of *T. verrucosa* cultivated in Nutribloom (NB)+ with two concentrations of nitrates (2 mM and 10 mM) for 30 days. Different letters represent significant differences between treatments (One-way ANOVA followed by a Tukey post hoc test, $p < 0.05$, $n=3$).

4.3.2 – Protein content

The protein contents of the four microalgal species cultivated with NB+ at the two concentrations of nitrates (2 and 10 mM) is shown in Figure 4.5. When the biomass of microalgae grown with NB+ at 2 mM of nitrates were analysed, *P. tricornutum* had the highest value (27.4%) followed by *N. oceanica* with 13.7% of protein content. Likewise, when the microalgae were grown with NB+ at 10 mM nitrates, significant differences were observed between the different microalgae, with the highest protein content

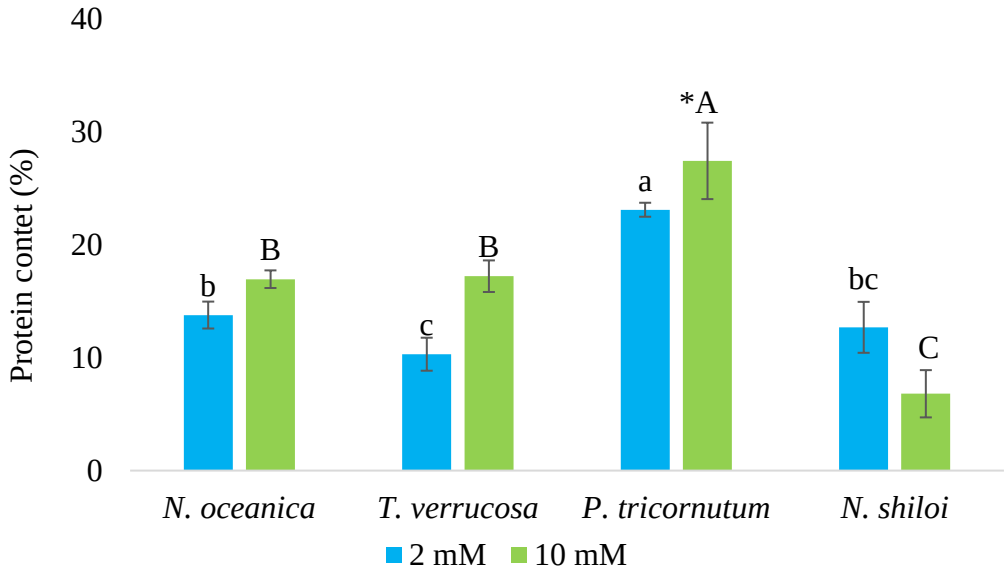

Figure 4.5 - Protein content (%) of four microalgae species (*N. oceanica, T. verrucosa, P. tricornutum, N. shiloi*) cultivated with Nutribloom (NB)+ at two concentrations of nitrates (2 and 10 mM) for nine days. Different lower- and uppercase letters represent significant differences between microalgae grown with 2 or 10 mM nitrates, respectively (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$), * denotes significant differences between treatments in the same microalga (*t*-test, $p < 0.05$, $n=3$).

observed in *P. tricornutum* with 23.1%. Only *P. tricornutum* showed significant differences in protein content between treatments, with NB+ at 10 mM of nitrates being the best treatment.

4.3.3 – Inorganic Content (% ash)

The inorganic content of the four species of microalgae under study was similar when samples of the same microalga were grown with different concentrations of nitrates (2 and 10 mM; Figure 4.6). However, when comparing microalgae at either 2 or 10 mM nitrates, the highest value was found for *N. shiloi* and the lowest one for *N. oceanica*.

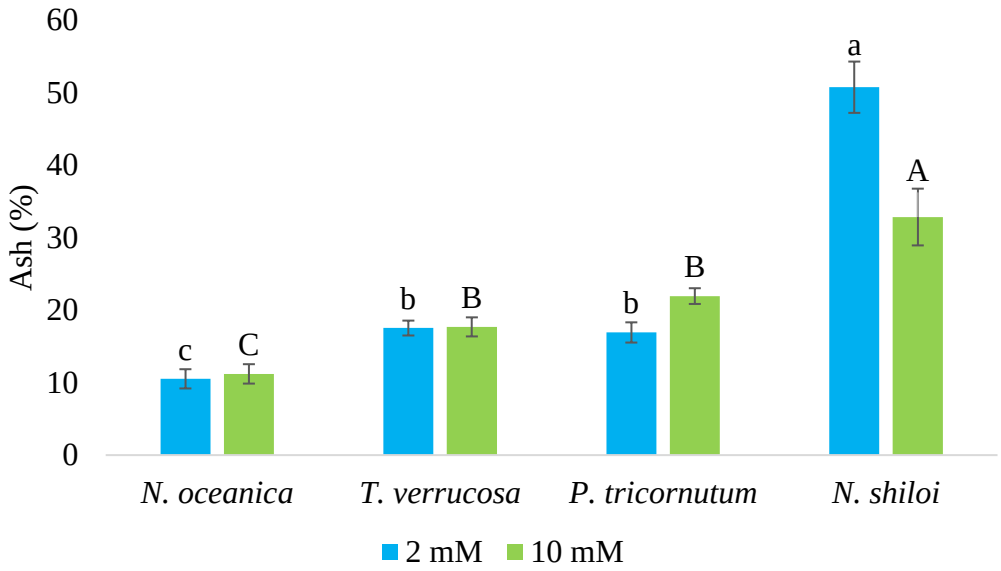

Figure 4.6 - Inorganic content of four microalgae species (*N. oceanica, T. verrucosa, P. tricornutum,* and *N. shiloi*) cultivated with Nutribloom (NB+) at two concentrations of nitrates (2 and 10 mM). Different lower- and uppercase letters represent significant differences between microalgae grown with 2 or 10 mM nitrates, respectively (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$), * denotes significant differences between treatments in the same microalga (*t*-test, $p < 0.05$, $n=3$).

4.3.4 – Mineral Content

Table 4.1 represent the mineral content of *N. oceanica* and *T. verrucosa* and Table 4.2 represent the mineral content of *P. tricornutum* and *N. shiloi* species under study cultivated with NB+ at two concentrations of nitrates (2 and 10 mM).

N. oceanica, cultivated with NB+ at 2 mM of nitrates, showed the highest accumulation of calcium, iron and zinc. This species was the only with detectable molybdenum content, and the same species cultivated with NB+ at 10 mM of nitrates had the highest percentage of potassium. *T. verrucosa* presented the highest concentration of copper and manganese when cultivated with NB+ at 10 mM of nitrates, while when cultivated with 2 mM presented the highest barium level. *P. tricornutum* presented the highest concentration of phosphorus with NB+ at 10 mM of nitrates, but with 2 mM presented the highest concentration of aluminium. *N. shiloi* cultivated with NB+ at 2 mM of nitrates was the only microalga with detectable amounts of antimony.

Table 4.1 – Mineral content of *N. oceanica* and *T. verrucosa* cultivated with NB+ at two concentrations of nitrates (2 and 10 mM) for nine days. The minerals whose values are presented as < LOD are bellow limit of detection. Different lower- and uppercase letters represent significant differences between algae grown with 2 mM or 10 mM nitrates, respectively (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$, n=3).

		N. oceanica						***T. rubens***					
Minerals	**Unit**	**2 mM**			**10 mM**			**2 mM**			**10 mM**		
P	%	2.716^{ab}	±	0.601	3.869	±	0.482	2.973^{a}	±	0.353	4.443	±	1.277
Ca	%	3.972^{a}	±	0.604	3.731^{A}	±	1.246	2.648^{b}	±	0.487	2.361^{B}	±	0.788
K	%	0.764^{ab}	±	0.050	0.776^{A}	±	0.165	0.746^{a}	±	0.071	0.626^{A}	±	0.113
Fe	mg/kg	0.558^{a}	±	0.053	0.053	±	0.013	0.288^{b}	±	0.035	0.052	±	0.008
Al	µg/kg	0.012^{ab}	±	0.002	0.062	±	0.003	0.043	±	0.010	0.080	±	0.087
Ba	µg/kg	<0.001	±	<0.001	< LOD			0.006	±	0.001	0.005	±	0.000
Cu	µg/kg	0.007^{ab}	±	0.000	0.008^{AB}	±	0,001	0.007^{a}	±	0.000	0.009^{AB}	±	0.000
Mn	µg/kg	0.015	±	0.001	0.085	±	0.008	0.031	±	0.006	0.089	±	0.007
Zn	µg/kg	43.952	±	4.236	0.194	±	0.012	0.058	±	0.004	0.202	±	0.015
Mo	µg/kg	2.057^{a}	±	0.663	< LOD			< LOD			0.930	±	0.263
Sb	µg/kg	< LOD			< LOD			< LOD			< LOD		

Table 4.2 – Mineral content of *P. tricornutum* and *N. shiloi* cultivated with Nutribloom (NB+) at two concentrations of nitrates for nine days. The minerals whose value are presented as < LOD are bellow limit of detection. Different lower- and uppercase letters represent significant differences between algae grown with 2 and 10 mM nitrates, respectively (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$), * denotes significant differences between treatments in the same microalga (*t*-test, $p < 0.05$, $n=3$).

		P. tricornutum						***N. shiloi***					
Minerals	**Unit**	**2 mM**			**10 mM**			**2 mM**			**10 mM**		
P	%	3.276^{b}	±	0.940	5.305	±	0.868	1.784^{a}	±	0.438	3.965	±	0.397
Ca	%	0.387^{ab}	±	0.421	0.389^{C}	±	0.202	1.665^{ab}	±	0.777	1.283^{C}	±	0.059
K	%	0.416^{b}	±	0.053	0.355^{B}	±	0.069	0.521^{b}	±	0.048	0.621^{A}	±	0.098
Fe	mg/kg	0.052^{b}	±	0.008	0.445	±	0.094	0.274^{c*}	±	0.321	0.060	±	0.012
Al	µg/kg	0.164^{b*}	±	0.109	0.191^{B*}	±	0.032	0.107^{ab}	±	0.076	0.100^{A}	±	0.015
Ba	µg/kg	0.005	±	0.001	< LOD			< LOD			< LOD		
Cu	µg/kg	0.001^{b}	±	0.000	0.007^{B}	±	0.001	0.005^{a}	±	0.001	0.008^{AB}	±	0.000
Mn	µg/kg	0.036	±	0.030	0.114	±	0.008	0.031	±	0.004	0.062	±	0.026
Zn	µg/kg	0.116	±	0.101	0.164	±	0.088	0.014^{*}	±	0.002	0.077^{*}	±	0.063
Mo	µg/kg	< LOD			1.542	±	0.245	2.391^{b*}	±	2.729	0.781^{*}	±	0304
Sb	µg/kg	< LOD			< LOD			9.088	±	3,344	< LOD		

Elements such as silver, arsenic, beryllium, cadmium, cobalt, chromium, nickel, lead, selenium, terbium, uranium and vanadium were not quantified, because the values obtained were lower than the LOD (limit of detection) of the equipment.

4.3.5 – FAME

Concerning the fatty acid methyl esters (FAME) profile (Table 4.3), *N. oceanica* showed the highest amount of saturated fatty acids (SFA) compared to all other species. *T. verrucosa* showed the highest percentage of polyunsaturated fatty acids (PUFAs), while *N. shiloi* had the highest percentage of monunsaturated fatty acids (MUFAs). *N. oceanica* displayed the highest production of SFA and polyunsaturated fatty acids (PUFAs) with NB+ at 10 mM of nitrates, while the production of MUFAs was higher when this microalga was cultivated with NB+ at 2 mM of nitrates. *T. verrucosa* showed higher production of SFA and MUFA with NB+ at 2 mM of nitrates, while the production of PUFAs was highest at 10 mM nitrates. *P. tricornutum* produced more SFA with NB+ at 2 mM of nitrates but more MUFAs and PUFAs with 10 mM nitrates. The other diatom under study, *N. shiloi*, produced SFA in greater amounts with NB+ at 2 mM of nitrates and MUFAs and PUFAs with 10 mM of nitrates.

The most abundant SFA in all species studied was C16:0 (palmitic acid), being more abundant in *N. oceanica* cultivated at 2 mM nitrates. On the other hand, in terms of MUFA, the most abundant fatty acid among species corresponded to C16:1 (palmitic acid) in *N. oceanica*, *P. tricornutum* and *N. shiloi*, while in *T. verrucosa* the most abundant was C18:1 (oleic acid). PUFA contents changed from species to species. In *N. oceanica* and *P. tricornutum*, the most abundant PUFA was C20:5 (eicosapentaenoic acid; EPA), whereas, in *T. verrucosa*, the most abundant PUFA was C18:2 (linoleic acid). Finally, in *N. shiloi* the most abundant PUFAs were arachidonic acid (C20:4) and EPA, respectively.

Although C18:2 was present in all species, it was in *T. verrucosa* that it was more abundant, reaching 12% with NB+ at 10 mM of nitrates. The *T. verrucosa* was the one that presented the highest percentage of C18:3, reaching 1% with the 2 mM treatment. *N. oceanica* presented the highest production of C20:4, reaching 3% with NB+ at 10 mM of nitrates treatment. Only *P. tricornutum* showed accumulation of C22:6, with the highest value in the treatment of NB+ at 10 mM of nitrates, reaching 0.6%.

Table 4.3 –Fatty acid profile (%) of *N. oceanica*, *T. verrucosa*, *P. tricornutum* and *N. shiloi* (*n*=6) cultivated with Nutribloom (NB+) at 2 mM and 10 mM of nitrates. Values presented as mean ± s.d., *n*=3

%FA	*N. oceanica*		*T. verrucosa*		*P. tricornutum*		*N. shiloi*	
	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM
C12:0	0.231 ± 0.036	0.272 ± 0.035	n.d.	n.d.	0.172 ± 0.120	0.340 ± 0.254	n.d. n.d.	n.d. n.d.
C14:0	5.508 ± 0.255	6.315 ± 0.166	1.076 ± 0.119	0.853 ± 0.061	0.626 ± 0.184	1.087 ± 1.099	1.105 ± 1.618	0.762 ± 0.778
C16:0	42.736 ± 0.732	43.257 ± 0.763	31.212 ± 0.831	25.347 ± 1.032	32.483 ± 1.060	27.726 ± 5.774	26.476 ± 3.611	27.135 ± 5.418
C17:0	0.354 ± 0.027	0.485 ± 0.042	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C18:0	1.387 ± 0.092	1.466 ± 0.194	0.881 ± 0.112	0.876 ± 0.288	0.265 ± 0.127	0.357 ± 0.248	0.768 ± 1.493	0.305 ± 0.749
∑ SFA	**50.217 ± 1.142**	**51.795 ± 1.200**	**33.169 ± 1.062**	**27.076 ± 1.381**	**33.546 ± 1.491**	**29.510 ± 7.375**	**28.350 ± 6.721**	**28.203 ± 6.946**
C14:1	0.087 ± 0.021	0.083 ± 0.013	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C16:1	31.013 ± 0.473	26.987 ± 0.175	6.718 ± 0.894	4.872 ± 0.753	62.164 ± 1,812	65.906 ± 4.650	64.060 ± 11.174	66.005 ± 4.682
C18:1	7.420 ± 0.280	6.014 ± 0.134	24.878 ± 1.043	24.466 ± 4.601	0.543 ± 0.231	0.625 ± 0,351	1.176 ± 2,143	0,582 ± 1.716
∑ MUFA	**38.519 ± 0.775**	**33.084 ± 0.323**	**31.596 ± 1.937**	**29.338 ± 5.354**	**62.707 ± 2.042**	**66.531 ± 5.001**	**65.236 ± 13.317**	**66.587 ± 6.398**
c16:2	n.d.	n.d.	2.432 ± 0.100	2.782 ± 0.165	n.d.	n.d.	n.d.	n.d.
C18:2	1.059 ± 0.082	1.449 ± 0.108	11.087 ± 0.742	12.142 ± 0.740	0.198 ± 0.088	0.329 ± 0.236	0.690 ± 1.134	0.310 ± 0.674
C16:3	n.d.	n.d.	3.583 ± 0.091	3.632 ± 0.118	n.d.	n.d.	0.656 ± 1.123	0.497 ± 0.415
C18:3	0.190 ± 0.034	0.330 ± 0.254	1.033 ± 0.082	0.950 ± 0.117	0.175 ± 0.087	0.288 ± 0.219	0.665 ± 1.176	0.285 ± 0.383
C20:3	n.d.	n.d.	0.778 ± 0.049	0.786 ± 0.120	n.d.	n.d.	0.516 ± 1.015	0.212 ± 0.366
C16:4	n.d.	n.d.	8.078 ± 0.406	12,767 ± 0,551	n.d.	n.d.	n.d.	n.d.
C18:4	n.d.	n.d.	2,510 ± 0,101	4,181 ± 0,417	n.d.	n.d.	0.599 ± 1.144	0.278 ± 0.538
C20:4	2.622 ± 0.206	3.207 ± 0.126	1.430 ± 0.060	1.572 ± 0.266	0.226 ± 0.109	0.969 ± 0.907	1.997 ± 1.647	1.507 ± 1.624
C20:5	7.393 ± 0.494	10.134 ± 0.733	4.305 ± 0.238	4.801 ± 0.727	2.669 ± 0.892	1.718 ± 0.501	1.293 ± 1.425	2.121 ± 2.391
C22:6	n.d.	n.d.	n.d.	n.d.	0.421 ± 0.210	0.654 ± 0.644	n.d. n.d.	n.d. n.d.
∑ PUFA	**11.264 ± 0.816**	**15.121 ± 1.221**	**35.235 ± 1.869**	**43.611 ± 3.221**	**3.690 ± 1.386**	**3.959 ± 2.507**	**6.415 ± 8.663**	**5.210 ± 6.391**

4.4 –Biochemical profile of rotifers

4.4.1 – Total lipids

The analysis of total lipids in rotifers enriched with microalgae cultivated with NB+ at two nitrate concentrations (2 and 10 mM) is presented in Figure 4.7. The rotifers enriched with *T. verrucosa* and *N. shiloi* cultivated with 2 mM of nitrates showed a higher lipid content than those fed with *N. oceanica* and *P. tricornutum*, However, there were no significant differences between microalgae grown with NB+ at 10 mM of nitrates. Only the rotifers enriched with *N. oceanica* showed significant differences between the NB+ at2 mM and 10 mM of nitrates.

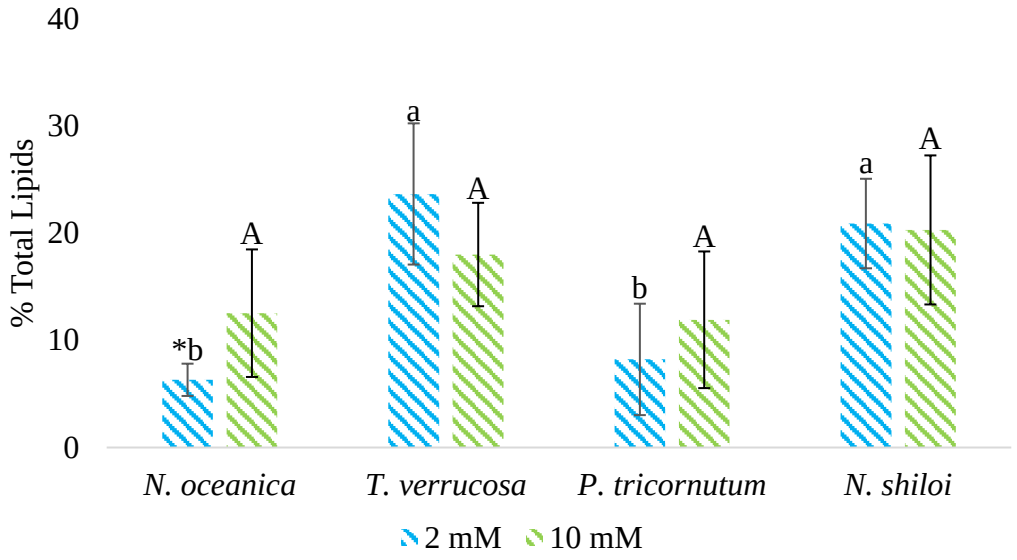

Figure 4.7 - Total lipids (%) of rotifers enriched with four species of microalgae (*N. oceanica*, *T. verrucosa*, *P. tricornutum* and *N. shiloi*) cultivated with Nutribloom (NB+) at two concentrations of nitrates (2 and 10 mM). Different lower- and uppercase letters represent significant differences between algae grown with 2 mM or 10 mM nitrates, respectively (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$), * denotes significant differences between treatments in the same microalga (*t*-test, $p < 0.05$, $n=3$).

4.4.2 - Protein Content

The protein content of rotifers enriched with the microalgae cultured with NB+ at 2 or 10 mM of nitrates under study is represented in Figure 4.8. Rotifers enriched with *P. tricornutum* cultivated with NB+ at 10 mM of nitrates yielded a significant higher protein

content when compared with the remaining microalgae used. For rotifers enriched with microalgae cultured with NB+ at 2 mM of nitrates, the highest protein content was observed in *P. tricornutum* and *T. verrucosa* treatments. Only the rotifers enriched with *N. shiloi* revealed statistical differences in response to the nitrate's concentration, where NB+ at 10mM of nitrates promoted an increase in the protein content compared to NB+ at 2mM of nitrates.

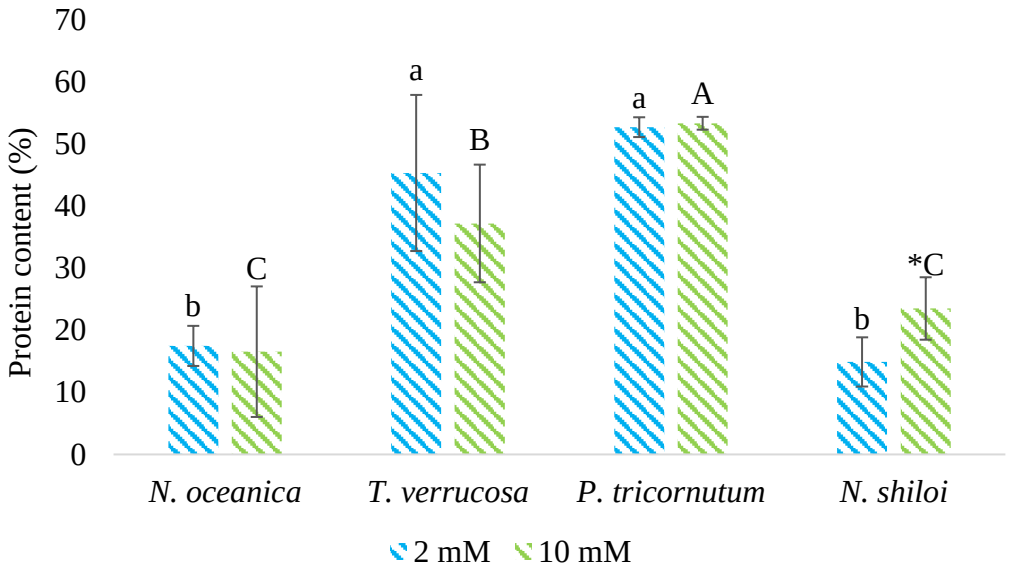

Figure 4.8 - Protein content (%) of rotifers enriched with four microalgae species (*N. oceanica, T. verrucosa, P. tricornutum* and *N. shiloi*) cultivated with Nutribloom (NB+) at two concentrations of nitrates (2 and 10 mM). Different lower- and uppercase letters represent significant differences between algae grown with 2 mM and 10 mM nitrates, respectively, * denotes significant differences treatments in the same microalga (One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$, $n=3$).

4.4.3 – FAME

Concerning the FAME profile (Table 4.4), rotifers enriched with *N. oceanica* showed the highest amount of SFA compared with other species. Rotifers enriched with *T. verrucosa* showed the highest concentration of PUFAs, while rotifers enriched with *P. tricornutum* had the highest MUFAs percentage.

Rotifers enriched with *N. oceanica* and *T. verrucosa* showed the highest production of SFA when cultivated with NB+ at 2 mM of nitrates, while the production of MUFAs and PUFAs was higher when cultivated with NB+ at 10 mM nitrates. Rotifers

enriched with *P. tricornutum* and *N. shiloi* had higher production of SFA and PUFA when cultivated with NB+ at 10 mM nitrates, while the production of MUFAs was higher when cultivated with 2 mM nitrates.

The most abundant SFA in all rotifers enriched with the studied microalgae was C16:0. For MUFAs, rotifers enriched with *N. oceanica*, *P. tricornutum* and *N. shiloi*, the most abundant was C16:1, while for *T. verrucosa* was C18:1. On the other hand, the most common PUFA in the rotifers enriched with *N. oceanica*, *P. tricornutum* or *N. shiloi* was C20:5. It is noteworthy that C18:2 was more abundant in rotifers enriched with *T. verrucosa,* reaching a production of 15% when cultivated with NB+ at 2 mM nitrates. Similarly, C18:3 showed the highest percentage in rotifers enriched with *T. verrucosa*, reaching 1.5% of production when cultured with NB+ at 2 mM nitrates. Likewise, C20:4 shows the highest percentage in the aforementioned species, reaching 6% when cultivated with NB+ at 2 mM nitrates. The C20:5 showed the highest value (13.7%) in rotifers enriched with *N. oceanica* grown with NB+ at 10 mM nitrates. It is noteworthy that C22:6 was only detected in rotifers enriched with *P. tricornutum* cultivated with NB+ at 2 mM nitrates.

Table 4.4 - Main fatty acids composition in percentage (%)of rotifers enriched with *N. oceanica, T. verrucosa, P. tricornutum* and *N. shiloi* cultivated with Nutribloom (NB)+ at 2 mM and10 mM of nitrates (*n*=6) Values presented as mean ± s.d. , n=3).

%FA	Rotifers enriched with *N. oceanica*		Rotifers enriched with *T. verrucosa*		Rotifers enriched with *P. tricornutum*		Rotifers enriched with *N. shiloi*	
	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM
C14:0	5.351 ± 0.857	5.451 ± 0.164	2.662 ± 0,196	2.720 ± 0.398	1.406 ± 0.347	4.170 ± 4.799	4.210 ± 1.671	4.420 ± 0.895
C16:0	32.392 ± 2.676	27.746 ± 0.953	27.626 ± 2.229	27.615 ± 2.271	19.184 ± 12.175	22.756 ± 11.051	24.956 ± 12.466	33.600 ± 6.998
C18:0	3.353 ± 0.289	2.991 ± 0.207	4.076 ± 0.513	3.598 ± 0.440	1.243 ± 0.309	4.794 ± 6.737	1.216 ± 0.222	2.365 ± 1.120
∑SFA	**13.699 ± 1.274**	**12.063 ± 0.441**	**11.455 ± 0.979**	**11.311 ± 1.036**	**7.278 ± 4.277**	**10.573 ± 7.529**	**10.127 ± 4.786**	**13.462 ± 3.004**
C16:1	27.527 ± 5.713	27.233 ± 0.886	10.681 ± 1.156	11.014 ± 3.356	64.238 ± 8.792	45.596 ± 28.238	46.394 ± 8.413	20.000 ± 13.610
c18:1	8.972 ± 5.434	10.718 ± 0.312	20.063 ± 3.403	23.935 ± 9.901	3.959 ± 0.832	12.294 ± 20.799	3.630 ± 0.996	18.910 ± 10.565
∑MUFA	**18.249 ± 5.573**	**18.976 ± 0.599**	**15.372 ± 2.279**	**17.475 ± 6.628**	**34.099 ± 4.812**	**28.945 ± 24.518**	**25.012 ± 4.705**	**19.455 ± 12.088**
C18:2	5.029 ± 2.370	4.740 ± 0.463	15.431 ± 0.911	12.454 ± 4.908	1.147 ± 0.265	1.301 ± 1.823	1.742 ± 0.508	6.515 ± 4.088
C18:3	0.511 ± 0.252	0.449 ± 0.073	1.535 ± 0.127	1.262 ± 0.728	0.392 ± 0.290	0.342 ± 0.450	0.842 ± 0.379	0.784 ± 0.542
C20:3	1.474 ± 1.106	1.001 ± 0.342	2.386 ± 2.510	3.143 ± 4.185	0.485 ± 0.138	0.040 ± 0.080	0.429 ± 0.107	0.822 ± 0.729
C20:4	5.641 ± 0.483	5.872 ± 0.279	6.065 ± 2.546	5.535 ± 1.606	0.897 ± 0.220	4.926 ± 3.517	5.555 ± 4.580	5.659 ± 5.304
C20:5	9.749 ± 4.846	13.799 ± 0.528	9.475 ± 4.375	8.723 ± 4.822	6.043 ± 0.845	3.790 ± 3.480	11.098 ± 4.000	6.923 ± 5.671
C22:6	n.d	n.d	n.d	n.d	1.005 ± 0.124	n.d	n.d	n.d
∑PUFA	**4.481 ± 1.811**	**5.172 ± 0.337**	**6.978 ± 2.094**	**6.224 ± 3.250**	**1.661 ± 0.314**	**2.080 ± 1.566**	**3.933 ± 1.915**	**4.141 ± 3.267**

4.5 – Development of zebrafish larvae

4.5.1 – Survival

The survival of zebrafish larvae in represent in Figure 4.9. After 25 days of treatment, larvae fed with GF, TF2 and TF10 treatments showed a significantly higher survival when compared to ZF. It is noteworthy that all treatments in which the larvae were fed with rotifers showed a survival rate higher than 90%, while the larvae fed with commercial feed (ZF) only reached 56%

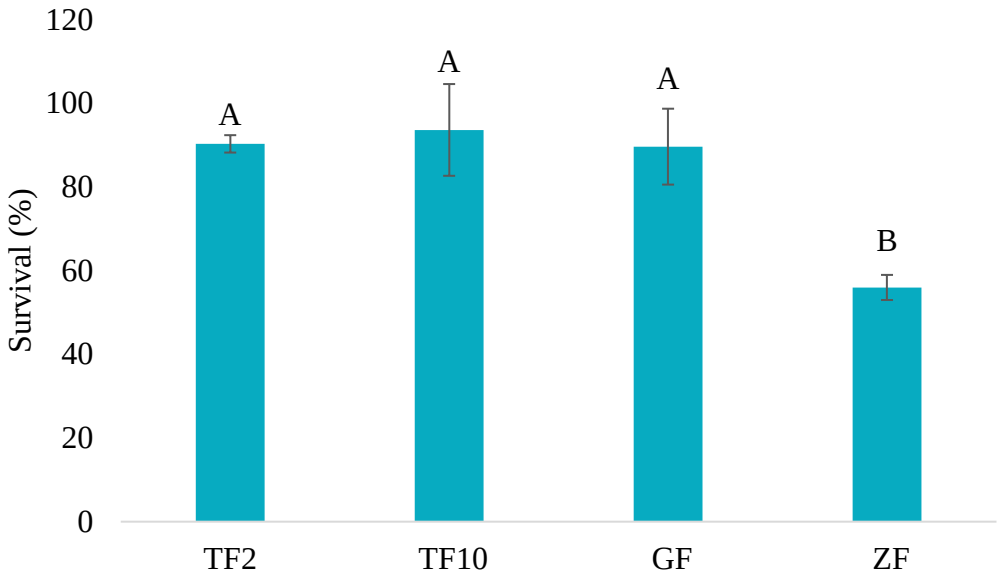

Figure 4.9 - Survival of zebrafish larvae at 30 dpf fed with commercial feed (Zebrafeed; **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with 2 (**TF2**) or 10 (**TF10**) mM of nitrates. Different letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=100).

4.5.2 – Length and weight

The total length of the larvae with 15 dpf and 30 dpf is shown in Figure 4.10. At 15 dpf, the largest larvae were from the TF2 and TF10 treatments, and the smallest were larvae fed with ZF. The larvae fed with GF reached the largest length 30 dpf, with 16 mm whereas the smallest larvae were those from the ZF group.

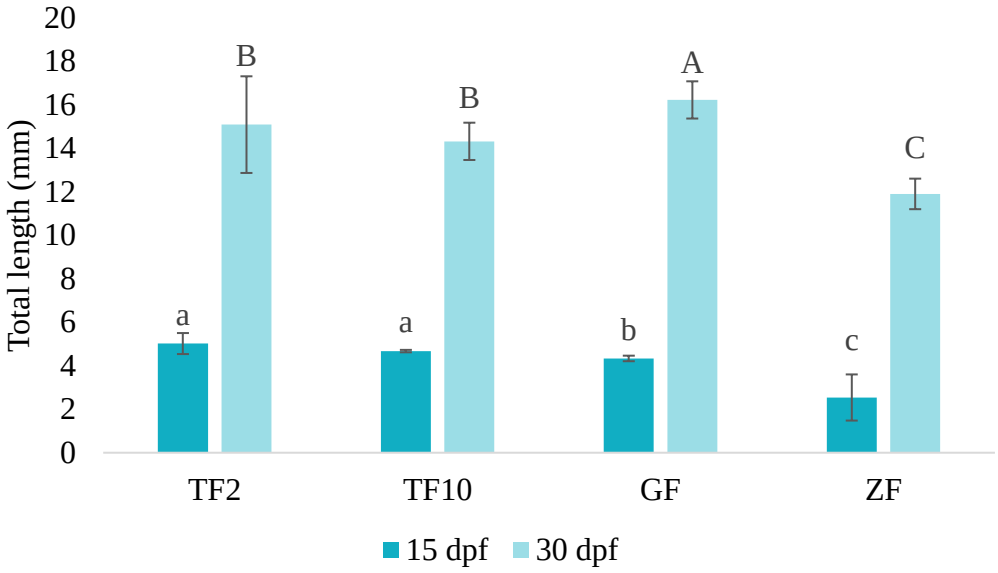

Figure 4.10 – Total length of zebrafish larvae 15 and 30 dpf, fed with commercial feed (Zebrafeed; **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with 2 (**TF2**) or 10 (**TF10**) mM nitrates. Different lower- and uppercase letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=10).

The weight of zebrafish larvae 15 and 30 dpf is shown in Figure 4.11. No significant differences were observed between treatments 15 dpf. However, 30 dpf, rotifers fed with GF presented a significantly higher weight (Figure 4.12).

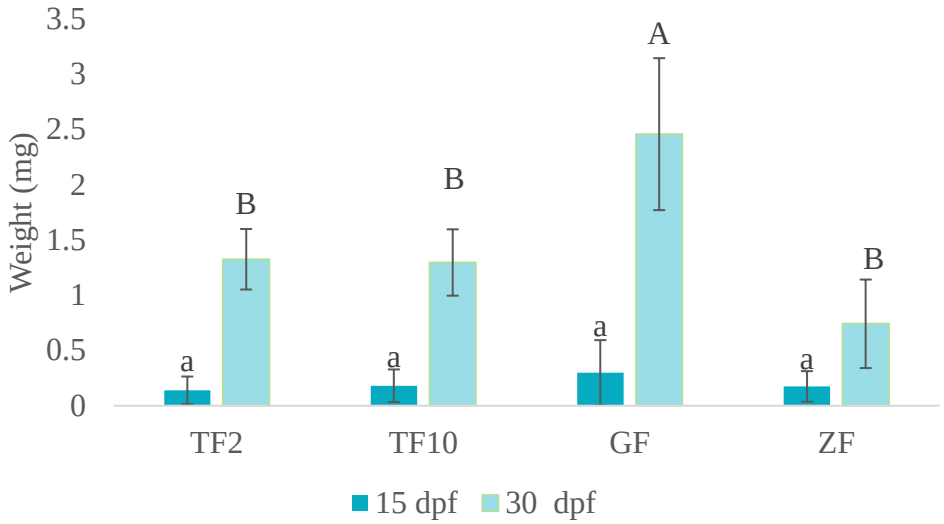

Figure 4.11 - Weight of zebrafish larvae 15 and 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**) rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 mM (**TF2**) or 10 (**TF10**) mM nitrates. Different lower- and uppercase letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=10).

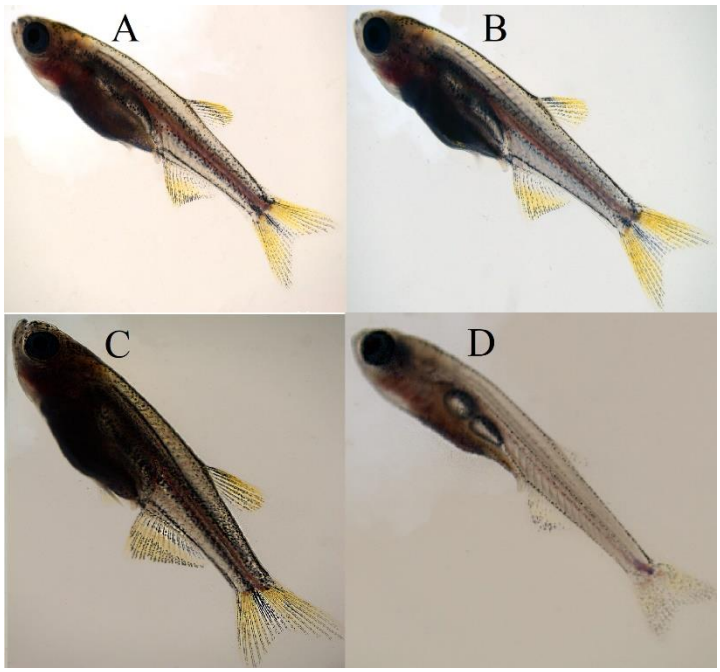

Figure 4.12 - Zebrafish larvae 30 days post fertilization (dpf) fed with different treatments. **A** - Larvae fed rotifers enriched with *T. verrucosa* grown with Nutribloom (NB)+ at 2 mM of nitrates **B** - Larvae fed rotifers enriched with *T. verrucosa* grown with Nutribloom (NB)+ at 10 mM of nitrates. **C** - Larvae fed rotifers enrich with Green formula **D** - Larvae fed with commercial feed (ZF).

4.5.3 – Biochemical profile of zebrafish larvae

4.5.3.1 - Total lipid contents

The biochemical analysis of total lipids (%) of zebrafish larvae at 30 dpf (Figure 4.13) no differences were found between treatmentswhereas TF10 (26,5%), TF2 (25%) and GF (20%) showed lower contents.

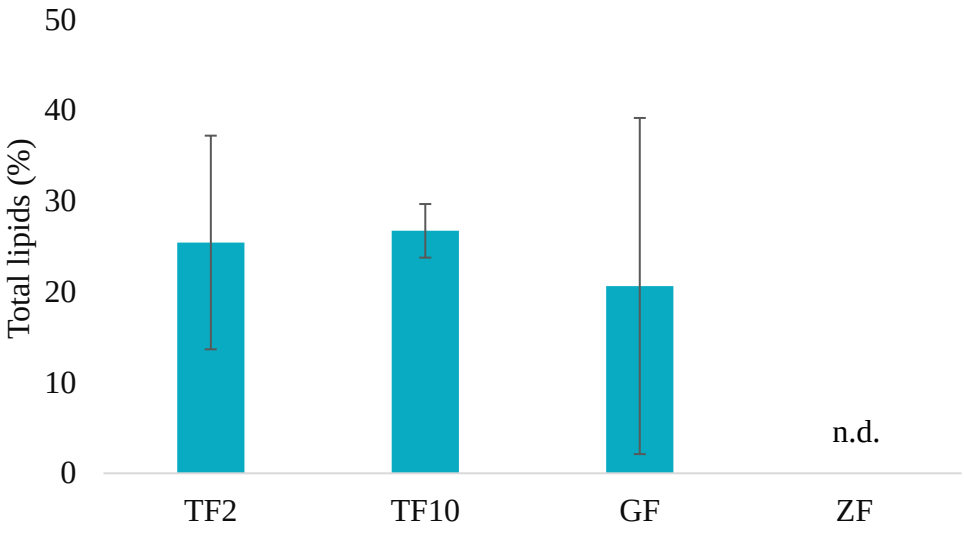

Figure 4.13 - Total lipids (%) of zebrafish larvae 30 dpf fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 mM (**TF2**) and 10 (**TF10**) mM of nitrates. N.d – not determinet. Different capital letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=10).

4.5.3.2 - Protein content

Figure 4.14 shows the protein content of zebrafish larvae at 30 dpf,, fed with ZF or with rotifers enriched with different microalgae treatments (TF2, TF10 and GF). No differences were found between treatments, where the protein content of the larvae varied between 61.2% and 69.4%.

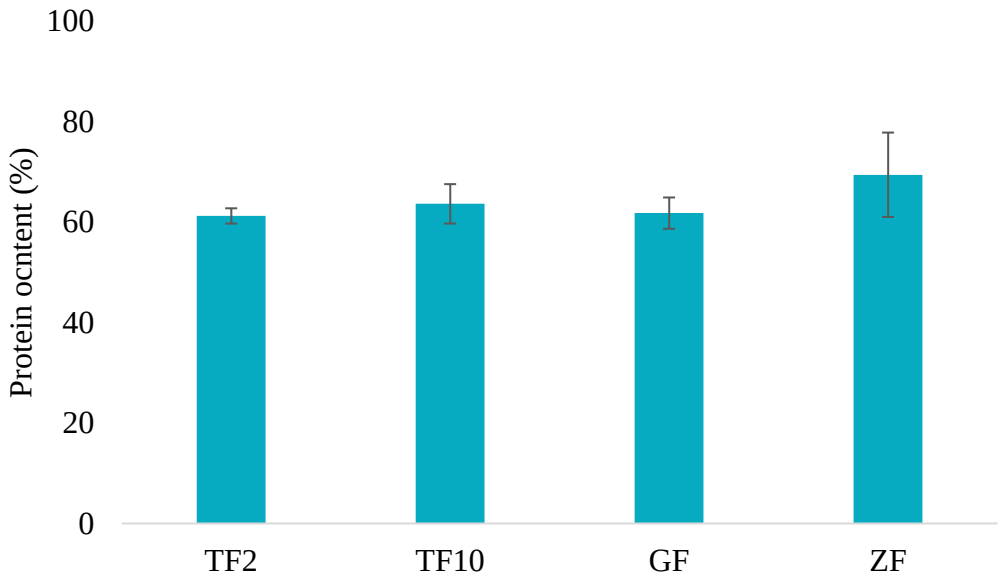

Figure 4.14 - Protein content (%) of zebrafish larvae 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 (**TF2**) or 10 (**TF10**) mM of nitrates. Capital letters denotes that no significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p > 0.05$, n=10).

4.5.4 – Skeletal malformations

4.5.4.1 – Incidence of malformations

Figure 4.15 shows the incidence of malformations in zebrafish larvae.No significant differences were found between the TF10 ZF, GF and TF2 treatments that ranged from 43 to 75%.

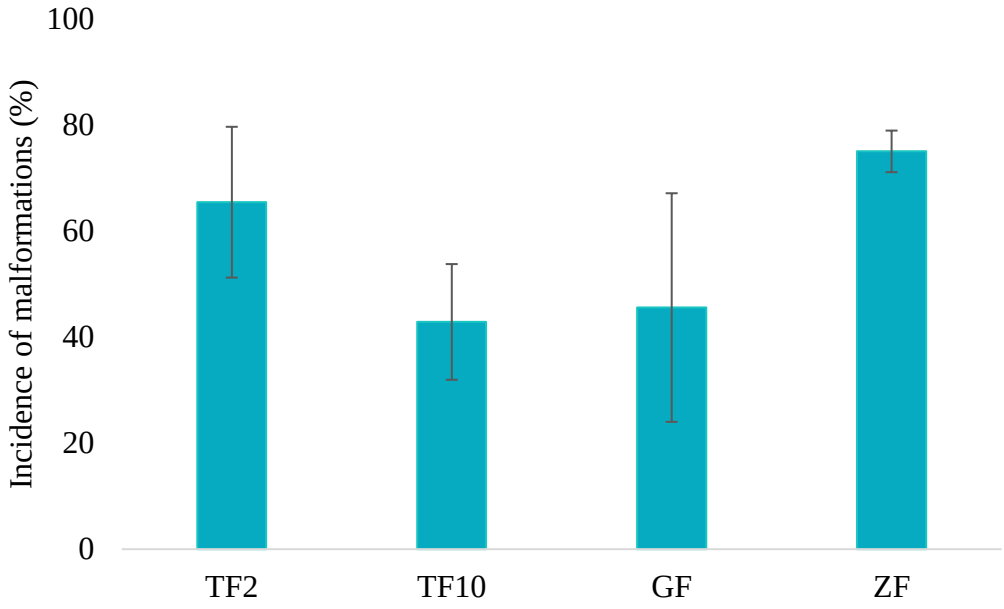

Figure 4.15 - Incidence of malformations in zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 (**TF2**) and 10 (**TF10**) mM nitrates. Different letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=30).

4.5.4.2 – Malformations Load

The load of skeletal malformations in zebrafish larvae at 30 dpf is shown in Figure 4.16. No significant differences were found between treatments. The malformations load ranged between 1.00 and 1.51.

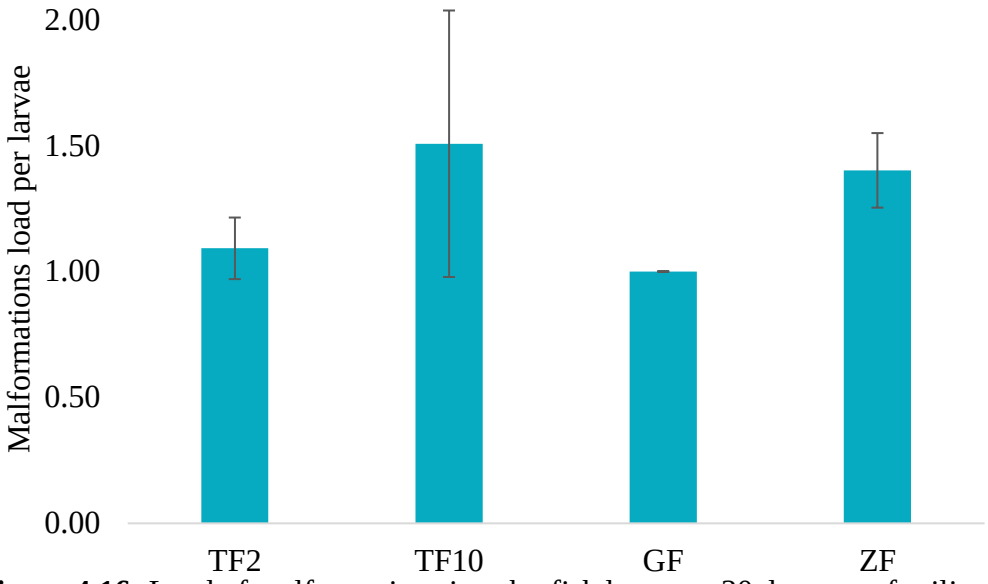

Figure 4.16- Load of malformations in zebrafish larvae at 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed; **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+ at 2 (**TF2**) and 10 (**TF10**) mM nitrates (One-way ANOVA followed by Tukey's post hoc test, $p > 0.05$, n=30)

4.5.4.3 –Distribution of malformations

Figure 4.17 shows the distribution of the malformations in zebrafish larvae at 30 dpf. Larvae fed with ZF had a significantly higher number of malformations in all the skeleton regions. The larvae fed with rotifers had the highest number of malformations in the caudal fin vertebrae. Figure 4.18 represents examples of malformations observed in larvae at 30 dpf, such as deformation in the structure of centra located in the pre caudal vertebra and fusion of vertebre (28-29) located in the caudal fin vertebrae.

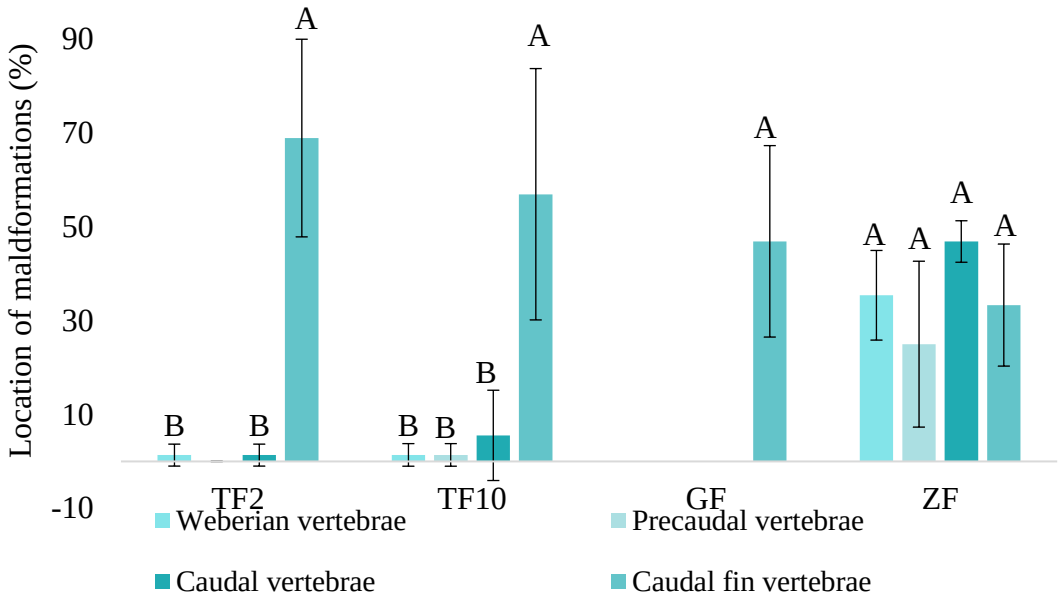

Figure 4.17 - Distribution of malformations (number of malformations per region) in zebrafish larvae 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed;,**ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with Nutribloom (NB)+at 2 (**TF2**) or 10 (**TF10**) mM of nitrates. Different capital letters denote significant differences between treatments were found (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, n=30).

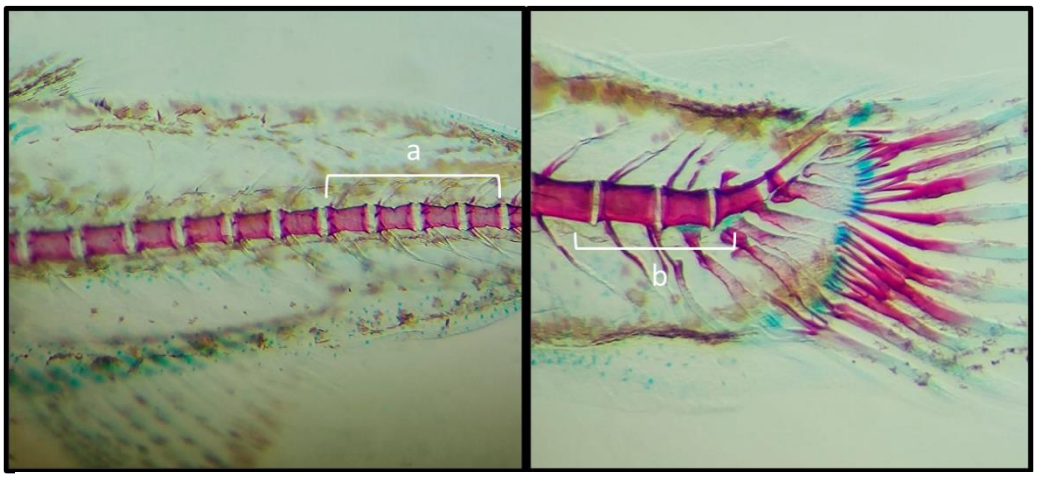

Figure 4.18 – Examples of skeletal malformations exhibited by zebrafish larvae 30 days post fertilization (dpf) such as malformation in the structure of vertebral centra (**a**) and fusion of vertebrae with malformed arches (28-29) (**b**).

4.5.5 – Histology

4.5.5.1 – Mid Gut villi

The mid gut villi were sampled of zebrafish larvae 30 dpf (Figures 4.19 and 4.20). The larvae fed with enriched rotifers showed longer villi (55-65 µm) compared with ZF (45 µm) that presented significantly smaller mid gut villi. Figure 31 shows the mid gut villi of a zebrafish larva 30 dpf fed with TF2.

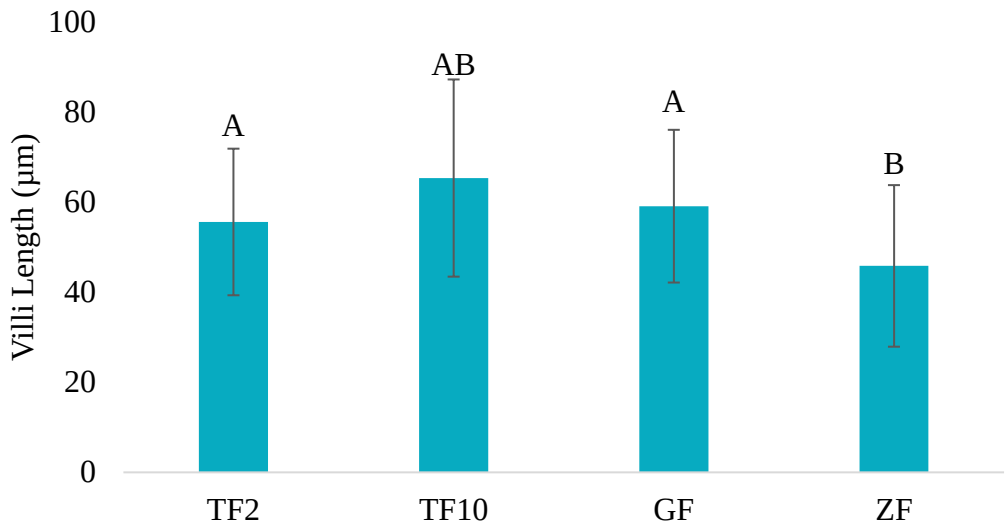

Figure 4.19 – Length (µm) of mid gut villi in zebrafish larvae 30 days post fertilization (dpf) fed with commercial feed (Zebrafeed, **ZF**), rotifers enriched with Green Formula (**GF**), rotifers enriched with *T. verrucosa* cultivated with NB+ at 2 mM (**TF2**) and10 (**TF10**) mM of nitrates. Different capital letters denote significant differences between treatments (One-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, $n=5$).

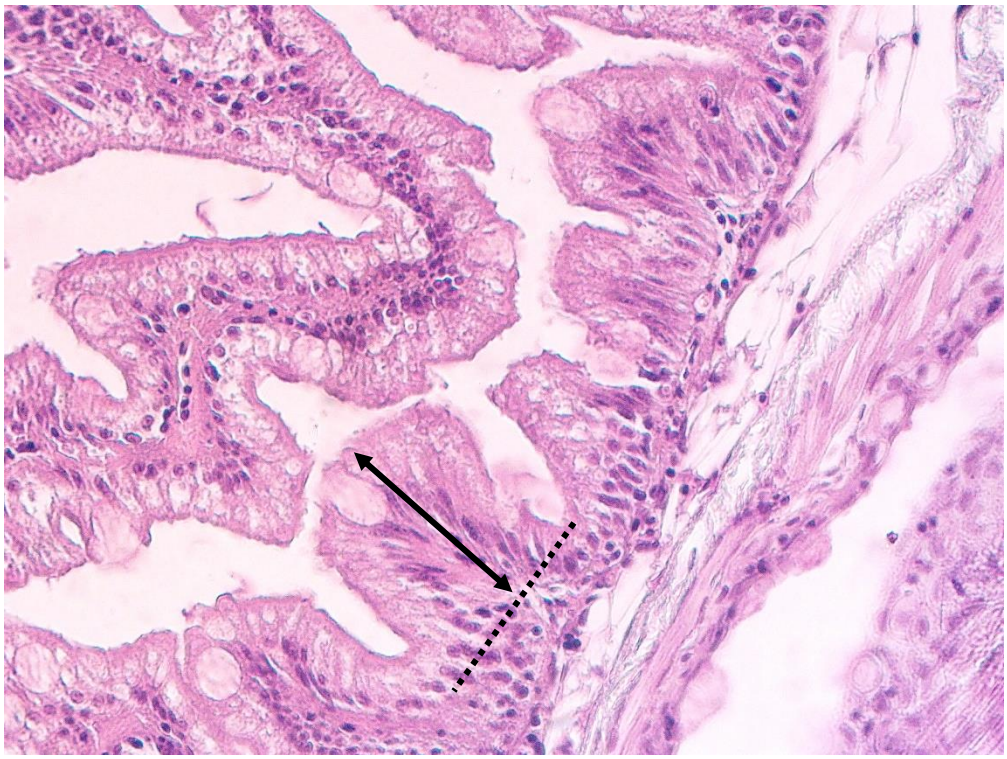

Figure 4.20 – Mid gut villi of larvae at 30 days post fertilization (dpf) fed with rotifers enriched with *T. verrucosa* grown with Nutribloom (NB+) at 2 mM nitrates.

5 – Discussion

5.1 - Laboratory scale microalgae production and nitrates consumption

Our results suggest that *T. verrucosa f. rubens* used in this study is a fast-growing, stress-resistant microalga, characteristics that it shares with other microalgae of the same genus (Pereira et al., 2018, Schüler et al., 2020). On the other hand, the microalga *P. tricornutum* displayed the lowest growth, most probably due to the low tolerance of this species to high temperatures. It was not possible to maintain the optimal growth temperature for this species, which is between 18 and 20 ºC (Nur et al., 2019). Another conclusion regarding microalgal cultivation suggests a correlation between lower concentration of nitrates with lower microalgal growth, as reported for other species such as *Chlorella* spp., *Scenedesmus* spp., *Microcystis ichthyoblabe and Microcystis aeruginosa* (Jin et al., 2006).

5.2 – Biochemical composition of microalgae species and rotifers

The microalga *N. oceanica* proved to be a species with a high lipid production. This microalga species shows potential to be an oleaginous model due to the high photosynthetic efficiency, high lipid productivity, established genetic toolbox, and relatively mature technology for outdoor cultivation systems on a large scale. Lipids play an essential role in cell structure and functions. Under optimal growth conditions, microalgae synthesize fatty acids primarily for esterification into glycerol-based membrane lipids, being responsible for 5%–20% of the cell dry weight (DW). Conversely, under stress conditions, microalgal lipid biosynthesis is channelled into neutral lipids, mainly in the form of TAGs (20%–50% DW). In this case, under nitrogen depletion, *N. oceanica* can reach 42% of lipids (Ma et al., 2014,2015). Indeed, *N. oceanica* and *P. tricornutum* showed significantly enhanced lipid contents upon nutrient depletion in growth media with lower 2 mM of nitrates (TF2). However, in this case, cell growth and division were not optimal, as nutrient depletion can cause the exhaustion of nitrogen and NADP+, which can have severe consequences for the cell. Nonetheless, the lack of these nutrients can be compensated by that fact that NADPH is consumed by the fatty acid biosynthesis, resulting in the production of NADP+ and TAGs. This can promote survival and even growth under nitrogen-limiting conditions because TAGs do not require nitrogen, unlike proteins, and as NADP+ is recycled, cells store the chemical energy in the form of TAGs (Sharma et al., 2012).

The results obtained for *N. oceanica e P. tricornutum* are in agreement with the literature, which shows that upon nitrogen depletion, there is an increase in the production of lipids in several microalgal species, chlorophytes in particular (Fenge et al., 2011; Go et al., 2012, Pedro et al., 2013, Sahin et al., 2019). Conversely, for the diatom *N. shiloi*, induction of stress by nitrogen depletion did not result in higher lipid levels. Schüler et al. (2021) have documented that microalgae such as *Pavlova* sp. require two main stress conditions: nutrient depletion and exposure to high temperatures. However, those authors observed an increase in the level of protein and decreased lipid content in the exponential phase (Schüler et al., 2021). Bearing in mind the results obtained for *T. verrucosa*, it is possible to observe that at the end of the 9thday of the growth trial, the microalgae had a lower amount of total lipids in NB+ at 2 mM nitrates than cells grown in same growth medium using 10 mM nitrates. This might have occurred because cells were still growing,

leading to a lower accumulation of lipids under both conditions for this microalga (Schüler et al., 2021). On the other hand, at the end of the 30th day of the trial, despite the small differences observed, the total lipids content were higher in the NB+ at 2 mM treatment, suggesting that the stress induced by the nitrogen depletion requires a longer period of time in order to promote lipid production (Sharma et al., 2012).

Fatty acids are essential for the biosynthesis of the main classes of lipids that can vary in chain length, from 4 to 36 carbons (Liang et al., 2006). Those are essential for the normal development of zebrafish and many other animals, contribute to a decrease in inflammation and prevent the development of cardiovascular diseases (Adarme-Vega et al., 2012). Among these, docosahexaenoic acid (DHA, C22:6) plays a vital role in maintaining the membrane fluidity of the brain and retina. Some of the DHA-derived mediators are involved in reducing inflammation and protecting against injury (Saini and Keum, 2018). The fatty acids profile of *N. shiloi* showed similar values in the amount of PUFAs under nitrogen depletion or nitrogen repletion treatments. However, the amounts of SFA and MUFAs were decreased. This work shows a higher percentage of MUFAs and a lower percentage of SFA than those reported in the literature, which can be justified by the type of culture medium used. NB+ medium was used rather than the F/2 medium, a considerably less rich nutrient medium and thus more adequate for growing diatoms (Sahin et al., 2019, Demirel et al., 2020). *N. oceanica* had the highest production of C20:5, an important fatty acid for zebrafish development. The values in the present work (7.3% in nutrient depletion) are slightly below the reported values in literature (Huerlimann et al., 2010; Gong et al., 2013; Xiao et al., 2013).; In all of these studies, it was shown that nutrient depletion may not be sufficient for induction of EPA production, with the values in these works ranging from 8.6% to 24.9%. *T. verrucosa* was the species selected for the next steps of this work since it showed the highest growth and best nutritional profile. Despite not being the highest producer of EPA, it was the one with the highest production of C18:2, which production is similar to that reported by Huang et al. (2013). In the aforementioned work, the lower the concentration of nitrates, the lower the production of this fatty acid was obtained. The absence of C22:6 in all species, except in *P. tricornutum*, was in agreement with the literature (Huerlimann et al., 2010, Gong et al., 2013; Xiao et al., 2013, Huang et al., 2013, Schüler et al., 2022). *T. verrucosa* cultivated in nitrogen depletion and repletion treatments obtained values for SFA, MUFAs, or PUFAs similar to those described in the literature, when standard cultivation conditions were used (4 mM

nitrates) (Cardoso et al., 2019). However, a species of the same genus, *T. subcordiformis*, showed higher values of PUFAs under nitrogen depletion. In general terms, the lower the nitrogen concentration the greater the amount of PUFAs produced by the cells (Huang et al., 2013). Literature related with *P. tricornutum* showed that SFA varies between 29-41%, while MUFA levels varies between 31-34%, and PUFAs varies between 25-38%. Contrary to these descriptions, our data showed a higher MUFA content and lower PUFA levels. However, this might be explained by the suboptimal conditions used in this study, where the growth of this microalga was not optimized, nor was it successful, due to thermal stress that these cells underwent during cultivation (Maia et al., 2022). *N. oceanica* contained SFA levels similar to those reported by Xiao et al. (2013), whereas PUFAs content were lower when this microalga was cultivated in F/2 growth medium with CO_2 injection. Huang and colleagues (2013) obtained similar results for MUFAs, lower SFAs, and higher values of PUFAs in another species of the same genus: *N. oculata.*

The increase in lipid production is not as linear as theoretically expected. There are factors such as the need for more than one stressor, certain culture media, or the growth phase at which the culture is.

Nitrogen is one of the limiting factors in the growth of microalgae. Microalgae transform inorganic nitrogen into organic to produce proteins, amino acids, peptides, and energy transfer molecules. The translocation of the inorganic nitrogen occurs across the plasma membrane, followed by the reduction of oxidized nitrogen and the incorporation of ammonium into amino acids. Nitrate and nitrite undergo reduction with the assistance of nitrate reductase and nitrite reductase, respectively. Nitrate reductase uses the reduced form of NADH to transfer two electrons, converting nitrate into nitrite. Nitrite is reduced to ammonium by nitrite reductase and ferredoxin (Fd), transferring six electrons in the reaction. Thus, all forms of inorganic nitrogen are ultimately reduced to ammonium before being incorporated into amino acids in the cell. Finally, using glutamate (Glu) and adenosine triphosphate (ATP), glutamine synthase catalyses the incorporation of ammonium into the amino acid glutamine (Cai et al., 2013). Greater availability of inorganic nitrogen usually translates into higher production of organic nitrogen, as observed in the results of this work for all microalgae, except for *N. shiloi* (Cai et al., 2013, Guldhe et al., 2017, Ansari et al., 2017 Ranadheer et al., 2019). Indeed, this diatom was an exception, presenting higher amounts of protein in the 2 mM nitrates treatment,

when compared to growth medium with 10 mM nitrates. Although this can be caused by the fact that the cells were still growing actively (Schüler et al., 2021), this could also be due to the fact that the response to nutritional stress is species-dependent (Schüler et al., 2017).

The levels of protein produced by the cell differ with several factors, such as whether the cell is in a particular growth phase, i.e., a culture in exponential phase will have higher protein content than a culture in stationary phase. Protein production can also be increased by the availability of nitrogen (nitrates), since proteins have an amine group, i.e., one nitrogen and two hydrogens.

Interestingly, *N. shiloi* cells also presented the highest amount of inorganic matter. This might be explained by the fact that *N. shiloi* is a diatom that forms chains by linking together their frustules, undergoing silicalization to produce a tough, mechanically resistant outer protection. This silicalization might be even further potentiated by silica supplementation of the culture medium (Reynolds, 2006, Sahin et al., 2019)

Minerals are inorganic compounds essential to all organisms. They present the most diverse functions in organisms, such as structural, functional muscle and nerves, fluid balance, carbohydrate, fat metabolism, among others (Underwood, 1981, Fox, 2018). Metals such as aluminium are not common in microalgae cultures. This element may present toxicity to zebrafish larvae in values higher than 0.001 mg/kg. The values found in this work were far below toxic levels to zebrafish (Ferrandino et al., 2022). This mineral may come from anthropogenic contamination in Ria Formosa, which was the water source used for the cultivation of all the microalgae species involved in this study (Silva et al., 2015; Pereira et al., 2019). Other elements such as Cu, Zn and Fe show a positive relationship with PUFAs levels (Batista et al., 2013). The results presented in this work are similar to those documented in the literature (Batista et al., 2013; Fox, 2018; Pereira et al., 2019).

When comparing the biochemical profile of rotifers and of microalgae that were used for enrichment, the disparity between the analytical results is clear. In theory, rotifers, being non-selective filterers, could have retained most nutrients of the microalgae they were enriched on and pass on those nutrients to the larvae. However, living organisms metabolize nutrients in unexpected ways, like any other animal over time, leading to variations between the composition of the microalgae and the larval live feed,

as observed in the case of rotifers (Fernandez-Reiriz and Labarta, 1995, Hagiwara and Yoshinaga, 2017). Therefore, it is clear that microalgae-based protocols for live feed enrichment must be designed in a case-by-case basis scenario.

5.4 – Development of zebrafish

The production of high-quality fish is one of the critical factors for the sustainable growth of the aquaculture industry sector. Large amounts of larvae of many fish species are already produced in aquaculture. However, survival rates are lower than expected causing large economic losses. This problem is caused by the existence of a nutritional gap knowledge in fish larvae (Conceição et al., 2009). An optimal diet must have nutritional stability and biosecurity, while maximizing animal performance (growth, survival, and reproduction). The current problem with all diets used in fish culture is the fact that they do not meet all these requirements (Lawrence, 2015).

Zebrafish larvae present some difficulties in the digestion of microdiets due to the immaturity of their gastrointestinal tract, especially in the first days of life (Ronnestad et al., 2013). Rotifers are small, slow-swimming animals, which are ideal for larvae at their early development stages as they stimulate the swimming ability of the latter through hunting without much effort (Lawrence, 2015). Bagatto et al. (2001) showed that fish swimming ability is directly involved with the survival and growth, since animals with better swimming ability will be able to better hunt and consequently absorb more nutrients.

The results of this work are in line with the literature, where it is shown that occurs an increase in the survival of larvae fed with live food (i.e., rotifers) when compared to larvae fed with inert food (Conceiçao et al., 2009, Harper and Lawrence, 2011, Castaldi, 2019). One of the contributing factors is the accumulation of feed debris at the bottom of the tank. Although, the tanks were cleaned twice a day, there may have been accumulation of residues that later, with their degradation, can reduce the quality of the water, increasing the levels of ammonia, which in turn may have decreased larval survival (Carvalho et al., 2006, Harper and Lawrence, 2011). Another contributing factor was the inert food ability to sink, since it also impacts larval survival. Since zebrafish inert food contains a small pellet size (<100 µm), it is difficult to break the surface tension, requiring larvae to swim towards inert food, which involves an extra expenditure of energy and larvae with reduced fitness can die of starvation (Martins et al. 2019).

The zebrafish is an experimental model of paramount importance in scientific research, as it is not only essential to produce fish in an efficient manner, but also fish without skeletal malformations, and with greater resistance to stress and pathogens (Best et al., 2010). The size of the fish attained in this work is in agreement with that found in the literature. Fish fed with rotifers were larger than those fed with inert food, and the size of the fish was around 14 mm 30 dpf (Best et., 2010, Kaushik et al., 2011, Castaldi, 2019). Nutritional content in essential fatty acids and proteins are key factors for proper muscle and bone development of fish (Guillaume, 2001, Fowler et al., 2020). The results obtained in this study regarding fish weight agree with previous studies, with fish fed either with inert food, live food or its combination. However, fish fed with live food yielded a higher length compared to those fed with the other types of feed used. The obtained length values present similarities with previous studies performed with zebrafish larvae (Best et al., 2010, Castaldi, 2019).

Diet, water contaminants, and water quality influence growth, healthy fish development, reproduction, gene expression, epigenetic markers, and cellular metabolism (Watts et al., 2016, Martins et al., 2020). In the present work, it was possible to reduce the incidence, load, and severity of deformations. This improvement can be justified by the nutritional quality of the microalgae used, levels of fatty acids, proteins and minerals that are essential for the good and correct development of zebrafish larvae. The presence of heavy metals reported by Castaldi (2019), which are absent in this work, have harmful effects on the development of zebrafish. Thus, the absence of elements such as lead, vanadium, and nickel may justify such results (Watts et al., 2016; Roberto et al., 2017; Castaldi, 2019; Martins et al., 2020; Tarasco et al., 2022).

Roberto et al. (2018) using supplementation with zinc showed results comparable to the ones in this work for the incidence of malformations, although there were differences in their location. Mainly, the highest incidence of malformations was found in the caudal fin region, unlike Roberto et al. (2018) who reported that the highest incidence took place in the Weberian apparatus region. Zinc has been shown to be an essential element for bone development, as supplementation with this element has led to the upregulation of osteogenic markers that are crucial for normal bone formation and mineralization, indicating that this element plays an important role in zebrafish bone metabolism, as previously described for other models (Ma and Yamaguchi, 2001, Seo et al., 2010, Kwun et al., 2010, Zheng et al., 2010a,b, Fukada et al., 2013).

Phosphorus is also an essential element for zebrafish and many other organisms. This mineral has an important role in cellular metabolism, in the composition of phospholipids in cell membranes and in nucleic acids. It is also crucial in the mineralization of the skeleton, bones and even dentin. Ca and P form the hydroxyapatite crystals that make up the mineral phase, which, together with fibrillar collagen type I that compose the organic matrix, form the extracellular matrix (Cotti et al., 2020). Phosphorus is a limiting element in the growth and development of zebrafish and other teleosts. In general, phosphorus deficiency causes compression and fusion of vertebrae (Boccaccio and Pappalettere, 2011, Weinans and Prendergast, 1996, Pauwels, 1960). Indeed, those malformities were found in larvae fed with ZF. However, Witten et al. (2019) stated that such correlation is not linear, showing that malformations such as vertebra fusions are not the consequence of a single cause of phosphorus deficiency, but related to multiple factors. The results of the present work are similar to those described by Castaldi (2019) for the quantification of phosphorus.

Adequate levels of amino acids for protein biosynthesis are essential for the proper growth of zebrafish. There is a correlation between the essential amino acids present in the diet and the amino acids present in the body composition of fish. The protein contents found on the body of zebrafish larvae in this work are in agreement with those found by Kaushik et al. (2011) for the presence of protein, both in the body and diet. Moreover, the fatty acid composition in terms of long chain *n*-3 PUFA are also important for bone mineralization and development. For example, EPA has been referred to as an inhibitor of extracellular matrix mineralization, and a high DHA content is required for bone formation by altering the cell phenotype, gene expression, and mineralization capacity (Viegas et al. 2012). The same relationship between DHA and correct bone formation was observed by Izquierdo et al. (2010) with a decrease in 50% in the number of skeletal malformations of red porgy with higher DHA supplementation in the diet. However, the EPA and DHA requirements differ between marine and freshwater species, and comparisons on the effects of these dietary factors in marine or freshwater fish development should be taken carefully (Izquierdo et al., 2010, Martins et al., 2018). The results of the incidence of malformations in this work are in agreement with the work of Martins et al. (2018), although there is an absence of DHA in the studied microalgae. There are other factors that favour a good mineralization of the skeleton of zebrafish, such as adequate amounts of minerals such as calcium, phosphorus and zinc (Ma and

Yamaguchi, 2001, Seo et al., 2010, Kwun et al., 2010, Zheng et al., 2010a,b, Fukada et al., 2013; Roberto et al., 2018). It is known that zebrafish have the ability to biosynthesize EPA and DHA through the essential fatty acids ALN (alpha-linolenic acid) and LA (linoleic acid). It is known that this synthesis depends on the activation of enzymes such as desaturases and elongases. This synthesis can occur in adults in reproductive phases such as oocyte maturation and ovulation (Hastings et al 2001, Ishak et al., 2008). Comparing the difference in the amount of lipids present in the body of larvae between the various feeding treatments it can be justified by the source of protein offered or other nutrients such as glycids. In addition, other non-nutritive compounds can also influence the metabolism of the fish or the gut microbiome, that influence the body composition between larvae fed with rotifers or inert food (Smith Jr et al., 2013). The body composition results of this work are also corroborated by previous studies carried out in other species, such as Golden fish and rainbow trout (Gatlin et al., 1987, Akiyama et al., 1997, Mambrini et al., 2007, Kaushik et al., 2011).

The enrichment of rotifers with microalgae have reported benefits for the most varied animals, either because they are non-toxic or because they have characteristics such as antioxidant or anti-inflammatory properties (Hernandez et al., 2018). For example, in piglets, the insertion of microalgae in the diet increases digestibility in the jejunum and helps to reduce inflammatory responses (Furbeyre et al., 2017, Martins et al., 2022). Moreover, microalgae help in the regulation of homeostatic abundance between probiotics and harmful bacteria, which will have a positive effect on the health and pathogen resistance of zebrafish (Sugita et al., 1991, Ramirez et al., 2018, Ma et al., 2022). In addition, the bioactive compounds present in microalgae, such as polyphenols, fatty acids, and proteins, decrease the inflammatory state and promote cell proliferation (Wallace et al., 2005, Pollares et al., 2011) which will result in larger gut villi, that in turn will have direct implications for the overall health of the animal (Pollares et al., 2012, Burgman, 2016, Ma et al., 2022). The villi length found in this work agree with what is documented in the literature, where fish fed with microalgae had larger villi than those fed with inert food (Castaldi, 2019, Monteiro et al., 2021, Ma et al., 2022).

Overall, there is a correlation between larval survival, villi size and incidence of malformations, in all the analyses carried out in this work. It is possible to observe that larvae fed with rotifers have a higher quality. Rotifers are small and slow swimmers, with this characteristics, live foodd encourages the fish to swim and hunt, improving the

animal's metabolism (Conceição et al., 2010; Hagiwara and Yoshinaga, 2017). Therefore, a beneficial cycle is promoted: the better the fish is, the more it moves and eats, the more nutrients it has and the more energy it has to swim, starting the cycle again (Bagatto et al. 2001). In addition, microalgae are organisms that contain bioactive molecules that are essential for fish, such as essential amino acids, lipids such as EPA and DHA, vitamins and minerals, such as potassium, iron, and zinc (Liang et al., 2006, Fox, 2018, Sahin et al., 2019, Cardoso et al., 2019, Demiren et al., 2020). These nutrients can be encapsulated into live food, such as rotifers. However, these organisms have their own metabolism, which can change significantly the nutritional profile relatively to the microalgae used for enrichment purposes. A possible advantage of rotifer enrichment, beside encapsulation and concentration of nutrients, is that nutrients can become more bioavailable and easier to absorb, because these organisms have their own enzymes leading to a pre-digestion and thus facilitate the process of nutrient assimilation (Gribble, 2021).

6.0 – Conclusions

Zebrafish is the second most used model animal in scientific research, being urgent to determine his nutritional needs and establish a standard feeding protocol to the initial stages of life, so that the biological variability observed in results from different institutions is minimized. In this work it was possible to observe a significant improvement in larval survival when larvae were fed with rotifers enriched with microalgae. Microalgae such as *T. verrucosa* (TF2 and TF10) and *N. oceanica* (GF) proved to be efficient in enriching rotifers to feed zebrafish larvae. These two species produced larger larvae, with less malformations and with larger villi than the larvae fed with inert food. It is thus possible to conclude that rotifers are suitable as live feed for the first stages of larval life and the enrichment of rotifers with *T. verrucosa* and *N. oceanica* are suitable for the production of healthy zebrafish larvae.

7.0 – Future perspectives

Taking into account the results obtained in the experiments described above, further studies are still needed to consolidate the results here presented:

1. Test the effect of *P. tricornutum* and *N. shiloi* on the growth performance of zebrafish larvae
2. Study the relationship between rotifer feeding and gene expression, such *as pparγ* that is involved in lipid metabolism.
3. Study the microbiota of the fish in order to try to understand the relationship between the microalgae used for enrichment and the gut microbiome of zebrafish
4. Apply the same diets to adult fish, in order to try to establish the effects on reproductive performance.

8.0- Bibliography

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9.0 – Supplementary data

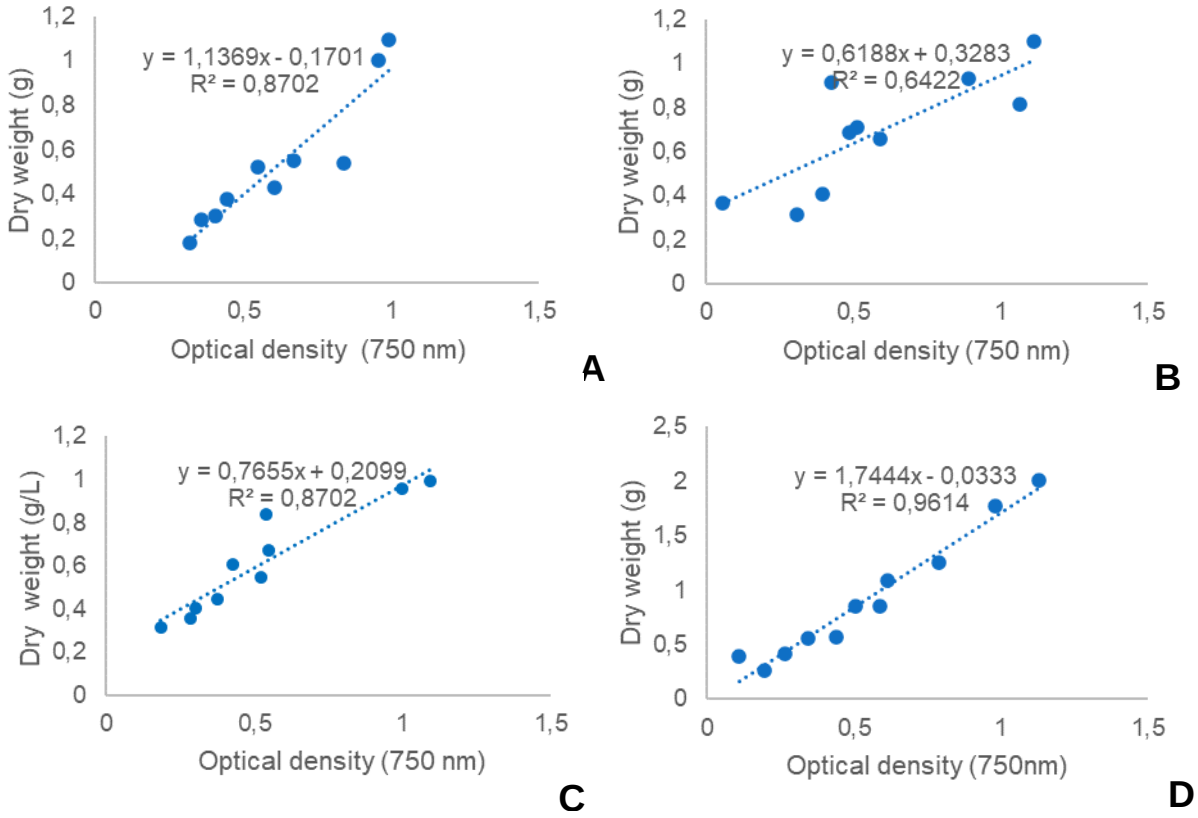

Figure S1 - Correlation estimated between DW and OD. A - *P. tricornutum*, B - *N. oceanica*, C - *N. shiloi* and D - *T. verrucosa*.