Universidade do Algarve

Faculdade de Ciências e Tecnologia

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Matthew Bernt Castaldi

Supervisors

Prof. Dr. Paulo Gavaia

Centro de Ciências do Mar

Universidade do Algarve, Faro, Portugal

&

Prof. Dr. João Varela

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Dissertation

Master of Aquaculture and Fisheries

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Declaração de autoria de trabalho

Declaro ser o (a) autor (a) 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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Resumo

O peixe zebra (*Danio rerio*) é uma espécie modelo usado em institutos de investigação mundialmente devido às suas inumeras vantagens. Esta espécie tem a capacidade de viver em diversos tipos de ambientes. Estes foram encontrados em temperaturas que variam entre 6.7 - 41.7° C e em salinidades tão baixas entre 5 partes por milhar (ppt). Esta espécie é usada numa larga variedade de campos incluindo toxicologia, aquacultura, evolução, nutrição e doenças ósseas.

Um dos grandes problemas associado ao peixe zebra remete para as suas necessidades nutricionais não são ainda conhecidas. Em investigação tais como para outras espécies modelos e importante reportar a dieta usada sendo que este não e o caso para o peixe zebra. Estes são alimentados com uma variedade de dietas de diversas origens o consequentemente afeta os resultados experimentais. Para uma confiança nos resultados científicos é importante investigar as necessidades nutricionais para esta espécie.

Usualmente no início, os rotíferos são usados na alimentação do peixe zebra sendo depois efetuada transição para a alimentação seca. Os rotíferos são uma presa adequada por causa do seu pequeno tamanho, velocidade lenta, e capacidade de dispersão na coluna de água. O valor nutricional dos rotíferos depende de tipo de enriquecimento usado, sendo, as microalgas comumente usadas para tal efeito. As microalgas têm um adequado valor nutricional, rico em proteínas, lípidos, minerais e perfil de ácidos gordos e aminos ácidos. Para maximizar o valor nutricional o ‘blending’ e uma técnica que visa uma mistura de microalgas para se proceder ao enriquecimento de rotíferos. Uma mistura de microalgas promove um balanço de ácidos gordos e amino ácidos que se visa melhorar as taxas de cumprimento, peso e sobrevivência. Este estudo teve como objectivo devolver um novo produto de enriquecimento para peixe zebra de forma a maximizar as taxas de cumprimento, peso, sobrevivência e minimizar a incidência de deformações esqueléticas.

Para tal, um conjunto de três desenhos experimentais foram elaborados. No primeiro e segundo desenho experimental um conjunto de 8 tratamentos foram usados, para o terceiro desenho experimental um conjunto de 6 tratamentos foram usados. Em cada desenho experimental uma microdieta - Zebrafeed®- foi utilizada como controlo. Aos 15 e 30 dpf, as taxas de comprimento, peso, sobrevivência foi analizadas. No primeiro desenho experimental,

análises histológicas do trato digestivo foram elaboradas aos 15 e 30 dpf. Em todos os desenhos experimentais a incidência de deformações esqueléticas foi feita com indivíduos colhidos aos 30 dpf. Foi feita a análise bioquímica das proteínas totais, lipídos totais, carbohidratos totais, cinzas totais e ester metílico de ácidos gordos (FAME), para as microalgas e rotíferos enriquecidos de todos os desenhos experimentais. Para o segundo e terceiro desenhos experimentais, além da análise bioquímica, o conteúdo em minerais foi analisado para os rotíferos e larvas de peixe zebra.

O primeiro desenho experimental visou a determinação de qual tipo de enriquecimento, pasta ou liofilizados, no enriquecimento de rotíferos e a performance de larvas de peixe zebra. Três espécies de microalgas foram avaliadas: *Nannochloropsis* sp., *Isochrysis* sp., e *Tetraselmis* sp. Foram um tratamento que usou rotiforos enriquecidos com *Nannochloropsis* sp. em salinidade de 10 ppt. O tratamento com a pasta de *Nannochloropsis* sp. apresentou os melhores resultados em termos de taxas de cumprimento, peso e sobrevivência. Devido ao comportamento e dissolução da pasta comparativamente ao liofilizado, o segundo e terceiro desenhos experimentais foram feitas somente com pasta de microalgas.

O segundo desenho experimental investigou quais as microalgas como melhor interesse para inclusão numa mistura de microalgas. Um total de 8 tratamentos foram compreendo o uso de 7 microalgas: as microalgas foram *Nannochloropsis* sp, *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp., *Skeletonema* sp., *Phaeodactylum* sp. and *Chaetoceros* sp. Neste desenho experimental, as microalgas *Isochrysis* sp. e *Tetraselmis* sp. diferiram de espécie. No final do desenho experimental, os peixes alimentados com rotíferos enriquecidos com *Nannochloropsis* sp. mostraram melhores taxas de comprimento, peso, sobrevivência e com menos deformações esqueléticas. Os grupos alimentados com rotíferos enriquecidos com *Skeletonema* sp. tive menos deformidades esqueleticas mas nao era significativo menos que o grupo que foram alimentados com rotiferos enriquecidos com *Nannochloropsis* sp.. Os grupos alimentados com rotíferos enriquecidos com *Isochrysis* sp., *Tetraselmis* sp. e *Spirulina* sp., obtiveram um bom desempenho e com um aumento do valor nutricional.

No terceiro desenho experimental, além da microdieta Zebrafeed® e *Nannochloropsis* sp., três misturas de microalgas e um tratamento de alimentação combinada de rotíferos e microdieta. A mistura combinada compreendeu a alimentação de larvas com rotíferos enriquecidos com

Nannochloropsis sp. e Zebrafeed® entre os 5-8 dpf e depois somente com microdieta Zebrafeed®. Os tratamentos com rotíferos enriquecidos com as misturas de microalgas obtiveram bons despenhos em termos de taxa de comprimento, peso, sobrevivência e deformações esqueléticas. No entanto, sem diferenças comparativamente ao grupo enriquecido com *Nannochloropsis* sp. Os rotíferos enriquecidos com misturas de microalgas obtiveram um bom valor nutricional relativamente aos ácidos gordos essenciais e importantes minerais, como Ca e Sr.

O tratamento de co-feeding tive problemas em termos de deformidades esqueléticas. Neste grupo, os peixes exhibiu muitas deformidades incluindo 'scoliosis' e 'kyphosis'. É uma possibilidade que o peixe zebra deve ser alimentado com rotíferos até pelo menos 15 dpf para minimizar deformidades esqueléticas.

Este trabalho indica que o enriquecimento com uma mistura de microalga é mais apropriado para um melhor desenvolvimento do peixe zebra, sendo por isso um avanço na compreensão das necessidades nutricionais. Uma mistura boa mistura deve ter uma relação de *n-3:n-6* PUFAs entre 0.42-0.81 com um perfil bem de ácidos gordos rico em arachidonic acid e eicosapentaenoic acid. Esse enriquecimento deve ter uma quantidade de proteína entre 34-59% e ser alto em todos os aminoácidos essenciais. O perfil meneral também é importante, essa mistura deve ter uma relação de Ca:P baixa e ser rica em Sr. Numa próxima abordagem, seria importante investigar outras espécies de microalgas, com varias quantidades na mistura e investigar o perfil de aminoácidos.

Este trabalho contribui com avanços para a determinação dos requisitos nutricionais do peixe zebra. Os resultados obtidos evidenciam que o enriquecimento com mistura de microalgas apresenta benefícios no desenvolvimento do peixe zebra. Futuros estudos deverão avaliar o potencial de outras espécies de microalgas, como a sua contribuição para a formulação de misturas de microalgas.

Palavras-chave: Peixe zebra; Microalga; Rotíferos; Nutrição; Deformidades esqueléticas; Alimento vivo; FAME

Abstract

Zebrafish (*Danio rerio*) is a model species used in research facilities worldwide because of its many advantageous qualities. Although this species is used in many labs, the research community has not implemented a strict dietary standard, as done with other model species. This dietary variation effects experimental results and impedes the replicability of studies. The aim of this research was to develop a microalgae enrichment blend for live feeds to maximize growth and skeletal development while gaining a better understanding of zebrafish larvae nutritional needs. To achieve these goals, three experimental trials were performed. The first investigated whether a microalgal paste or powder had an effect on zebrafish growth. The second was done to identify promising microalgae species to include in an enrichment blend. The third study incorporated five microalgae species combined into blended enrichments to see which blends maximized growth parameters and minimized skeletal deformities. Frozen microalgae pastes were chosen, as they exhibited better growth and are cheaper to produce. In trial two, five microalgae *Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp. and *Skeletonema* sp. were selected due to their proximal compositions, results in growth trials and effect on skeletal development. In the first two trials, zebrafish fed rotifers enriched with *Nannochloropsis* sp. alone or combined performed better than all other treatments in length, weight, survival and skeletal deformities In trial three, rotifers enriched with microalgae blends had a more complete nutritional profile than those enriched with single microalga. Diet A which combined *Nannochloropsis* sp., *Isochrysis* sp. and *Tetraselmis* sp. had the lowest incidence of severe deformities, and all fish fed rotifers enriched with microalgae blends preformed similarly in terms of length, weight and survival. Zebrafish given rotifers with microalgae blends had lower standard deviation between quadruplicates regarding length and performed as well as those given *Nannochloropsis* sp. enriched rotifers. Although fish given rotifers enriched with microalgae blends did not significantly outperform those given *Nannochloropsis* sp. in terms of length, weight, survival and skeletal deformities, it provides a starting point for further experiments with altered blend recipes.

Keywords: zebrafish; rotifers; microalgae; skeletal deformities; nutrition; live feeds; FAME

List of Abbreviations

Kg - kilogram

g - gram

L - litre

ml – milliliter

µl – microliter

m - meter

cm - centimeter

mm – millimeter

µm - micrometer

h - hour

min - minutes

s- second

ppt – parts per thousand

DW – dry weight

°C – temperature measurement in Celsius degrees

dpf – days post fertilization

TL – total length

SL – standard length

SFA – saturated fatty acid

UFA – unsaturated fatty acid

MUFA – monounsaturated fatty acid

PUFA – polyunsaturated fatty acid

FA – Fatty acid

n – omega

LA – linoleic acid

LNA – linolenic acid

EFA – essential fatty acid

EPA – eicosapentaenoic acid

ARA - arachidonic acid

DHA – docosahexaenoic acid

FAD – fatty acid desaturase

Z-FAD – zebrafish fatty acid desaturase

AA – amino acid

IDAA – indispensable amino acid

DAA – dispensable amino acid

FAA – free amino acid

HUFA – highly unsaturated fatty acid

dpf – days post fertilization

PFA – paraformaldehyde

PBS – phosphate-buffered saline

EDTA – ethylenediaminetetraacetic acid

KOH – potassium hydrozide

GC-MS gas chromatography with mass spectra

FAME – Fatty Acid Methyl Ester

TFA – total fatty acid

CHO – carbohydrates

NPA – *Nannochloropsis* sp. paste

NPO – *Nannochloropsis* sp. powder

IPO – *Isochrysis* sp. powder

IPA – *Isochrysis* sp. paste

TPO – *Tetraselmis* sp. powder

TPA – *Tetraselmis* sp. paste

IE2 – *Isochrysis* sp. experiment 2

TE2 – *Tetraselmis* sp. experiment 2

SPIRULI – *Spirulina* sp.

SKEL – *Skeletonema* sp.

PHAEO – *Phaeodactylum* sp.

CHC – *Chaetoceros* sp.

A – enrichment blend A

B – enrichment blend B

C – enrichment blend C

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Figure 33 - Charge of deformities in deformed fish at 30 days post fertilization (dpf) for trial 3 (n = 40). Letters indicate significant difference between regions using one-way ANOVA with Tukey's test ($p \leq 0.05$). NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B – fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.). Colors indicate number

of deformities in fish. Peach – 0 deformities; Lime green – 1 deformity; Dark green – 2 deformities; Blue – 3 deformities; Orange – 4 deformities; Red – 5 deformities.

Figure 34 - Location of deformities in deformed fish for trial 3 ($n = 40$). Letters indicate significant difference between regions using one-way ANOVA with Tukey's test ($p \leq 0.05$). NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B – fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.). ABD – Abdominal Vertebrae; CV - Caudal Vertebrae; CFV – Caudal Fin Vertebrae; CFN – Caudal Fin.

Figure 35: A. Compressed caudal fin vertebrae centra in fish fed rotifers with blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.). B. Compressed abdominal centra in fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.). C. Double neural arch and missing caudal fin vertebrae centra of fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.).

Figure 36 - A. CF (co-feeding) individual with severe scoliosis in caudal fin vertebrae and caudal fin. B. CF (co-feeding) individual with severe abdominal kyphosis.

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Powder; IPA ROTS - Rotifers enriched with *Isochrysis* sp. Paste; IPO ROTS – Rotifers enriched with *Isochrysis* sp. Powder; TPA ROTS – Rotifers enriched with *Tetraselmis* sp. Paste; TPO ROTS – Rotifers enriched with *Tetraselmis* sp. Powder.

Table 6: Zebrafish condition factor in Trial 1 at 15 and 30 days post fertilization (dpf) (n = 10). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF-Zebrafeed®; NPA – Fish fed rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 PPT – Fish fed rotifers enriched with *Nannochloropsis* sp. reared at 10 parts per thousand (ppt); NPO – Fish fed rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Fish fed rotifers enriched with *Isochrysis* sp. Paste; IPO – Fish fed rotifers enriched with *Isochrysis* sp. Powder; TPA – Fish fed rotifers enriched with *Tetraselmis* sp. Paste; TPO – Fish fed rotifers enriched with *Tetraselmis* sp. Powder.

Table 7 - Proximal composition of microalgae (n = 3). NPA - *Nannochloropsis* sp.; IE2 - *Isochrysis* sp. Experiment 2; TE2 - *Tetraselmis* sp. Experiment 2; SPIRULI – *Spirulina* sp.; SKEL – *Skeletonema* sp.; PHAEO – *Phaeodactylum* sp.; CHC – *Chaetoceros* sp.

Table 8 - Proximal composition of Zebrafeed® and enriched rotifers (n = 3). ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp.; IE2 ROTS - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 ROTS - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI ROTS – Rotifers enriched with *Spirulina* sp.; SKEL ROTS – Rotifers enriched with *Skeletonema* sp.; PHAEO ROTS – *Phaeodactylum* sp.; CHC ROTS – *Chaetoceros* sp.

Table 9 - Main fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae. NPA - *Nannochloropsis* sp.; IE2 - *Isochrysis* sp. Experiment 2; TE2 - *Tetraselmis* sp. Experiment 2; SPIRULI – *Spirulina* sp.; SKEL – *Skeletonema* sp.; PHAEO – *Phaeodactylum* sp.; CHC – *Chaetoceros* sp.

Table 10 - Main fatty acid composition % Total fatty acid (%TFA) ± S.D of Zebrafeed® and enriched rotifers. ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp.; IE2 ROTS - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 ROTS - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI ROTS – Rotifers enriched with *Spirulina* sp.; SKEL ROTS – Rotifers enriched with *Skeletonema* sp.; PHAEO ROTS – Rotifers enriched with *Phaeodactylum* sp.; CHC ROTS – Rotifers enriched with *Chaetoceros* sp.

Table 11 - Mineral content (mg/kg) ± S.D. of Zebrafeed® and enriched rotifers (*n* = 2). ZF- Zebrafeed®; NPA – Rotifers enriched with *Nannochloropsis* sp.; IE2 - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI – Rotifers enriched with *Spirulina* sp.; SKEL – Rotifers enriched with *Skeletonema* sp.; PHAEO – Rotifers enriched with *Phaeodactylum* sp.; CHC – Rotifers enriched with *Chaetoceros* sp..

Table 12 - Mineral content (mg/kg) ± S.D. of zebrafish (*n* = 2). ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

Table 13 - Zebrafish condition factor at 15 and 30 days post fertilization (dpf). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$) ($n = 10$). ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

Table 14 - Proximal composition of microalgae used in trial 3 (*n* = 3). ZF - Zebrafeed®; NPA – rotifers enriched with *Nannochloropsis sp.*; CF – co-feeding using rotifers enriched with *Nannochloropsis sp.* until 8 DPF and Zebrafeed®; A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.), B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.) and C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Table 15 - Proximal composition of Zebrafeed® and enriched rotifers used in trial 3 (*n* = 3). ZF - Zebrafeed®; NPA –rotifers enriched with *Nannochloropsis sp.*; CF – co-feeding using rotifers enriched with *Nannochloropsis sp.* until 8 DPF and Zebrafeed®; A – Rotifers enriched with blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.), B - Rotifers enriched with blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C - Rotifers enriched with blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Table 16 Main fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae, Zebrafeed® and enriched rotifers for trial 3 (n = 3). NPA – *Nannochloropsis* sp. microalgae; NPA ROTS – rotifers enriched with *Nannochloropsis* sp.; ZF - Zebrafeed®; Blend A – Microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); Blend A ROTS – rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); Blend B – Microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); B ROTS – rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); Blend C – microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.); C ROTS – rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Table 17 - Mineral content (mg/kg) ± S.D. of Zebrafeed® and enriched rotifers in trial 3 (n = 2). ZF - Zebrafeed®; NPA ROTS – rotifers enriched with *Nannochloropsis sp.*; CF – co-feeding using rotifers enriched with *Nannochloropsis sp.* until 8 DPF and Zebrafeed®; A ROTS – rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B ROTS – rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C ROTS – rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Table 18 - Mineral content (mg/kg) ± S.D. of zebrafish from trial 3 (n = 2). ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B – fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Table 19 - Trial 3 zebrafish condition factor for 15 and 30 days post fertilization (dpf) (n = 10). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp.,

Tetraselmis sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Annex 1:

Table A - Complete fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae in trial 1 (n = 3). NPA - *Nannochloropsis* sp. Paste; NPA 10 ppt – *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO – *Nannochloropsis* sp. Powder; IPA - *Isochrysis* sp. Paste; IPO – *Isochrysis* sp. Powder; TPA – *Tetraselmis* sp. Paste; TPO – *Tetraselmis* sp. Powder.

Table B - Complete fatty acid composition % Total fatty acid (%TFA) ± S.D of Zebrafeed® and enriched rotifers in trial 1 (n = 3). ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt ROTS – *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO ROTS – Rotifers enriched with *Nannochloropsis* sp. Powder; IPA ROTS - Rotifers enriched with *Isochrysis* sp. Paste; IPO ROTS – Rotifers enriched with *Isochrysis* sp. Powder; TPA ROTS – Rotifers enriched with *Tetraselmis* sp. Paste; TPO ROTS – Rotifers enriched with *Tetraselmis* sp. Powder.

Table C - Complete fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae. NPA - *Nannochloropsis* sp.; IE2 - *Isochrysis* sp. Experiment 2; TE2 - *Tetraselmis* sp. Experiment 2; SPIRULI – *Spirulina* sp.; SKEL – *Skeletonema* sp.; PHAEO – *Phaeodactylum* sp.; CHC – *Chaetoceros* sp.

Table D - Complete fatty acid composition % Total fatty acid (%TFA) ± S.D of Zebrafeed® and enriched rotifers. ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp.; IE2 ROTS - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 ROTS - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI ROTS – Rotifers enriched with *Spirulina* sp.; SKEL ROTS – Rotifers enriched with *Skeletonema* sp.; PHAEO ROTS – Rotifers enriched with *Phaeodactylum* sp.; CHC ROTS – Rotifers enriched with *Chaetoceros* sp.

Table E - Complete fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae, Zebrafeed® and enriched rotifers for trial 3 (n = 3). NPA – *Nannochloropsis* sp. microalgae; NPA ROTS – rotifers enriched with *Nannochloropsis* sp.; ZF - Zebrafeed®; Blend A – Microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); Blend A ROTS – rotifers enriched

with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); Blend B – Microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); B ROTS – rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); Blend C – microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.); C ROTS – rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

1. Introduction

1.1 Zebrafish

Zebrafish, *Danio* rerio, is a member of the *Cyprinidae* family. Native to south Asia, which has a dramatic difference between wet and dry seasons, zebrafish are able to adapt to changes in environmental conditions including temperature and salinity (Best et al., 2010). Due to its robustness and adaptability to captivity (Lawrence, 2007), zebrafish has become a widely used research model. Not only because they are easy to rear, but also the many traits shared with other organisms. Moreover, zebrafish develop rapidly, reaching sexual maturity at 10-14 weeks post fertilization (Ulloa et al., 2011). Continuous spawning can result in hundreds of eggs per week (Wixon, 2000) and embryo development occurs in 48-96 hours (Wixon, 2000; Kimmel et al., 1995). Embryos are translucent making them easy to observe and manipulate (Wixon, 2000). Zebrafish also display 'shoaling' behavior (Ruhl et al., 2009; Tsang et al., 2017), which allows larger numbers to be housed together, saving space.

Figure 1 - Zebrafish (*Danio rerio*) (socmucimm.org, 2014).

Transgenic lines were found to be capable of living in temperatures ranging from 6.7-41.7° C (Cortemeglia and Beitinger, 2011). Zebrafish are known to tolerate brackish conditions, however the process of osmoregulation has a high energy demand (Lawrence, 2007). Best *et al*. (2010) observed high growth and survival rates at salinities up to 5 ppt for 96 hours and slightly elevated salinities may minimize the growth of harmful bacteria (Boisen et al., 2003).

Several fields of research such as drug screening, toxicology, aquaculture, evolution, developmental biology, human disease (Lawrence et al., 2012), growth, nutrition (Cascio et al., 2018; Ulloa et al., 2011) and vertebral bone disease (Bruneel and Witten, 2015; Pasqualetti et al.,

2013; Roberto et al., 2018; Siccardi et al., 2010) have adopted zebrafish as a model organism. Human and zebrafish genes have functional and structural similarities (Carnevali et al., 2013) with 71.4% of human genes sharing a zebrafish ortholog (Howe et al., 2013). The zebrafish central nervous system is similar to humans (Bakthavatsalam et al., 2014). This genetic homology with humans and the short generation time allows for manipulation to study diseases such as cancer, heart disease, Alzheimer's, diabetes and osteoporosis (Siccardi et al., 2010).

With a similar bone plasticity to other research models including rats, monkeys and mice (Siccardi et al., 2010), zebrafish is now considered an acceptable model for studying vertebrae osteogenesis (Roberto et al., 2018) Zebrafish has a mineralized bone matrix, osteoblasts (bone forming cells), osteoclasts (bone remodeling cells) as well as endochondral and intramembranous ossification processes similar to mammals (Bruneel and Witten, 2015; Pasqualetti et al., 2013; Roberto et al., 2018). The biomineralization and microstructures are similar to human Haversian bone (Siccardi et al., 2010). In addition, the lamellar structure is the primary feature of human osteons (Siccardi et al., 2010) and zebrafish lamellar bone formation and histology is similar to human compact bone (Pasqualetti et al., 2013).

Many factors, especially nutrition, contribute to skeletal development (Nguyen et al., 2008). Deformities commonly occur during early life stages such as metamorphosis and organ development (Nguyen et al., 2008). Nutrient supplementation during skeletal formation and ossification can affect deformities and growth (Nguyen et al., 2008).

1.1.2 Zebrafish Nutrition

Although zebrafish is used in many fields of research worldwide, nutritional requirements are poorly understood unlike other model organisms (Lawrence et al., 2012; Siccardi et al., 2009; Smith et al., 2013; Martins et al., 2019). Since zebrafish is being discussed as a model for growth and nutrition (Ulloa et al., 2011) a standard diet must exist to understand how diet composition effects normal growth (Smith et al., 2013). Researchers using rodents must report diet composition, as variation can significantly influence experimental results (Siccardi et al., 2009). In addition, diet is known to alter gene and protein expression as well as metabolism in mice lungs and liver (Kozul et al., 2008). Therefore, in order to compare different experimental data, dietary requirements must be established for this model organism (Siccardi et al., 2009).

Zebrafish are reared on a variety of diets, many of which are without nutritional value and designed for ornamentals (Fernandes et al., 2016). Many laboratory diets use ingredients only known to the manufacturer, the total protein and lipid contents may be the same, but the sources differ depending on market availability and price fluctuations (Kozul et al., 2008). Common ingredients in fish feed have been found to affect physiology and behavior (Siccardi et al., 2009). Plant based nutrient sources have been used as alternative feed ingredients (Francis et al., 2001). Rapeseed, lupin, pea seed, sunflower oil and mustard oil among others, are common plant based ingredients but have many adverse effects such as protease inhibitors and antivitamin properties (Francis et al., 2001). In zebrafish, protein source is known to affect growth and body composition (Smith et al., 2013). To optimize experimental results and zebrafish rearing, nutritional requirements must be established for each life stage and physiological state (Wixon, 2000).

Currently, the two diets most used for rearing zebrafish are Gemma Micro® from Skretting and Zebrafeed® from Sparos, but these diets have shown different effects on larval growth and reproductive performance (Diogo et al., 2018; Farias and Certal, 2016; Martins et al., 2018; Monteiro et al., 2018). Enriched live feeds, particularly saltwater rotifers, *Brachionus plicatilis*, are commonly used in zebrafish facilities as they contain high amounts of nutrients suitable for all life stages (Lawrence et al., 2015)

1.2 Nutrients

1.2.1 Lipids

Lipids are the major organic component of fish (Tocher, 2003) and have many functions including energy reserves, electron carriers, membrane components and hormones (Mcdonald et al., 2011; Meinelt 1999; Ulloa 2011). Fats are derived from fatty acids (FA) which can be either saturated or unsaturated (Brett and Muller-Navarra, 1997; Mcdonald et al., 2011). Saturated fatty acids (SFA) have no double bond between carbon atoms and are solid at room temperature, while unsaturated fatty acids (UFA) have double bonds, are liquid at room temperature and are more chemically reactive (Brett and Muller-Navarra, 1997; Mcdonald et al., 2011). For example, diets high in saturated fats were found to be more nutritious because the energy could be released more efficiently than from unsaturated fats in oyster larvae (Brown et al., 1997). Unsaturated fats with one double bond are monounsaturated fatty acids (MUFA), those with multiple double bonds are polyunsaturated fatty acids (PUFA). Highly unsaturated fatty acids (HUFAs) are PUFAs with more than 20 carbon atoms (Brett and Muller-Navarra, 1997). The FA are named according to the

number of carbon atoms and position of double bonds. The methyl carbon at the distal end of a fatty acid chain is known as the omega (*n*) carbon (McDonald et al., 2011), such that omega 3 (*n*-3) fatty acids have the first double bond at the number 3 carbon from the distal end of the fatty acid (McDonald et al., 2011).

The PUFAs are important in reproduction, development, gene regulation, membrane maintenance, membrane fluidity and as eicosanoid precursors (Brett and Muller-Navarra, 1997**;** Jaya-ram et al. 2008; Mcdonald et al., 2011). Eicosanoids are hormone-like biochemicals that regulate functions such as blood clotting, blood pressure, immunity, inflammatory response, reproduction, neural functions and smooth muscle contraction (Halver 2002; Tocher et al., 2008); McDonald et al. 2011). Eicosanoids derived from *n-3* PUFAs have an anti-inflammatory effect, while *n-6* eicosanoids have a pro-inflammatory effect (Lebold et al., 2011). Linoleic acid (18:2 *n*-6, LA) and linolenic acid (18:3 *n*-3 LNA) are essential fatty acids (EFAs), fatty acids which must be obtained in the diet, and precursors of the PUFAs eicosapentaenoic (20:5 *n*-3, EPA), arachidonic (20:4 *n*-6, ARA) and docosahexaenoic (22:6 *n*-3, DHA) acids (Chen et al., 2013; Meinelt et al., 2000).

LA and LNA are converted to EPA, DHA and ARA through a series of elongation and desaturation steps, see Figure 2. In mammals, fatty acid desaturation involves Δ-5 and Δ-6 fatty acid desaturases (FAD) (Chen et al., 2013). In zebrafish, one universal FAD gene has been identified, this zebrafish fatty acid desaturase (Z-FAD) has similar activity to Δ-5 and Δ-6 desaturase in mammals (Chen et al., 2013)**.** Zebrafish has the ability to desaturate LA and LNA into EPA, DHA and ARA (Brett and Muller-Navarra, 1997; Brown et al., 1997; Chen et al., 2013; Jaya-ram et al., 2008; Lawrence, 2007). Larvae have a higher dietary PUFA requirement as demand exceeds conversion ability (Brett and Muller-Navarra, 1997), which must be taken into account when developing a product for larval zebrafish.

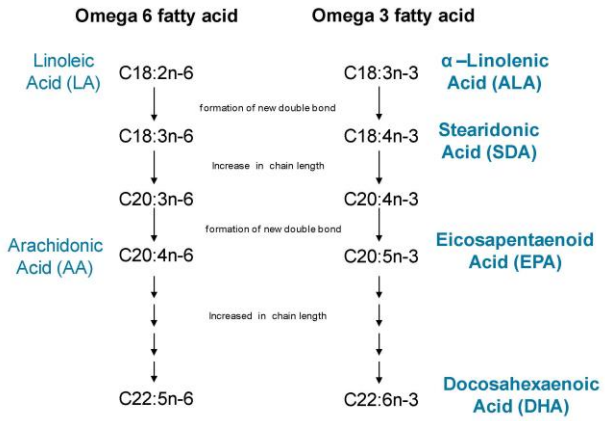


Figure 2 - Schematic representation of conversion process of Linoleic acid (LA) and α-Linolenic acid (ALA) to arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docasahexaenoic acid (DHA) (Extension, 2012).

Cold water species have a higher *n*-3:*n*-6 PUFA ratio than warm water species. Zebrafish, a warm water species, require more *n*-6 than *n*-3 PUFA (Lawrence 2007; Meinelt et al., 2000, 1999). Studies on *n*-3 and *n*-6 PUFAs in zebrafish suggest there is an optimal dietary ratio that could improve growth (Jaya-ram et al., 2008; Kaushik et al., 2011; Lawrence, 2007; Meinelt et al., 2000, 1999). Spawning in zebrafish is affected by broodstock *n*-6 levels (Meinelt et al., 1999), as reported by Jaya-Ram *et al*. (2008) that found highest egg production at low *n*-3:*n*-6 ratios, and by Meinelt *et al*. (1999) who observed increased fertilization rates. While varying *n*-3:*n*-6 ratio led to different amounts present in reproductive organs as well as a variation of fertilization rates, indicating that *n*-3:*n*-6 PUFA ratios are important in reproduction (Meinelt et al., 1999).

1.2.2 Protein

Proteins are a major fish component whose quantity and quality are related to larval growth and development (Conceição et al., 2003; Halver and Hardy, 2002). Amino acids (AA) are the building blocks of proteins which are digested and distributed to blood, organs and tissues for protein synthesis (Halver and Hardy, 2002; Li et al., 2009). AA must be obtained from the diet and are required for growth and development, metamorphosis, feed intake, reproduction, immunity, metabolic pathway regulation and stress resistance (Li et al., 2009). Supplementation may be beneficial to suppress aggression, increase larval development and survival, optimize spawning and increase the chemo-attractiveness of feed low in fish meal (Li et al., 2009). More AA functions are shown below in Figure 3. Low AA intake can lead to reduced growth and weight loss (Halver

and Hardy, 2002), while elevated AA intake can cause AA catabolism, decreased protein synthesis, reduced growth (Zhang et al., 2006) and increase ammonia excretion (Fernandes et al., 2016).

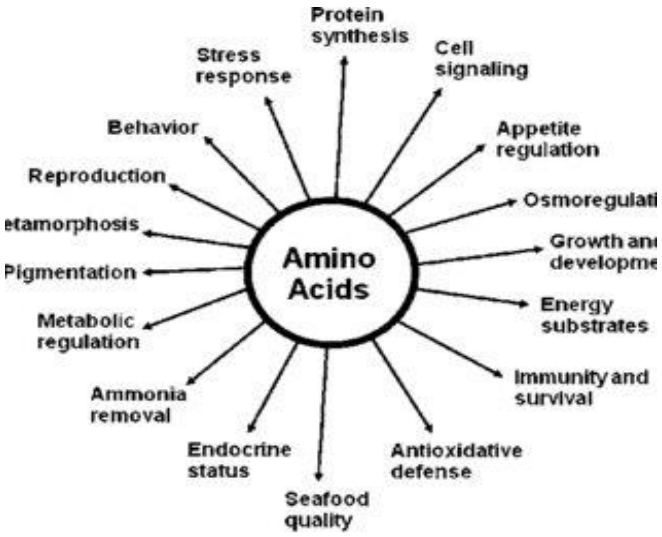


Figure 3 - Functions of amino acids in fish nutrition from Li *et al*. (2009).

AA are classified as indispensable amino acids (IDAA), which must be obtained in the diet, and dispensable amino acids (DAA), which are synthesized from IDAA (Li et al., 2009). Both IDAA and DAA must be supplied at the proper ratio in diet to meet dietary demands and maximize growth (Halver and Hardy, 2002). Fish species do not have a specific protein requirement but rather require an appropriate balance of both IDAA and DAA which must be determined in order to optimize larval performance (Abidi and Khan, 2004). The first limiting IDAA, the IDAA in lowest supply, sets the limit for protein synthesis (Conceição et al., 2003). When formulating a diet, it is important to understand the target species IDAA requirement and the mechanisms these nutrients are involved in.

Fast growing larvae need to be supplied proper amounts of AA, especially IDAA, to avoid loss of feed conversion efficiency and maintain growth (Conceição et al., 2003). Free amino acids (FAA) are amino acids not bound to proteins (Conceição et al., 2003). Larvae have difficulty digesting complex proteins when switching from endogenous to exogenous feeding so a large FAA supply is important (Aragão et al., 2004; Conceição et al., 2003). FAA constitute an important energy source for first feeding larvae and feed high in FAA increases the amino acid availability for growth and protein synthesis (Aragão et al., 2004). It is also suggested that different FAA stimulate feeding in different species (Aragão et al., 2004). Protein supplemented diets in cod

(*Gads morhua)* increased survival however, dietary protein too high above the requirements was shown to have detrimental effects in both gilthead seabream (*Sparus aurata*) and carp (*Cyprinus carpio*) (Rønnestad et al., 2013). There has been very little research into the protein requirement of zebrafish. The available information focuses on juveniles (Fernandes et al., 2016) and adults (O'Brine et al., 2015; Smith et al., 2013). As zebrafish mature, growth rate decreases, lowering the protein requirement (Fernandes et al., 2016).

Global aquaculture production is increasing along with the demand for fish meal and fish oil (Bravo-Tello et al., 2017). The increased cost and diminishing availability of these products has pressed the search for an alternative product that is sustainable and economically viable (Bravo-Tello et al., 2017). The previous study by Fernandes *et al.* (2016) on juvenile zebrafish used fish meal as a protein source. Dietary protein source has a significant impact on growth, length, weight and body composition (Smith et al., 2013). When four protein sources, wheat gluten, casein, fish and soy were tested, soy protein and the mixed source (combination of all four), had the best results (Smith et al., 2013). Soy protein has a limitation in sulfur containing AA which would be expected to reduce growth rate (Smith et al., 2013). However, the diet was high in betaine which could act as a methyl donor to assist in methionine production (Smith et al., 2013). The fact that soy is low in sulfur containing AA along with the other negative side effects (e.g., antinutritional factors) associated with soy indicates that a mixed protein source could be the best option. In this context, many microalgae strains are rich in protein and can help offset intestinal inflammation caused by soy, making it a good addition to feeds containing soy (Bravo-Tello et al., 2017). Larvae are fast growing and have a high protein demand (Zhang et al., 2006), proper AA inclusion and appropriate protein source ensures maximum feed conversion and growth (Conceição et al., 2003).

1.2.3 Minerals

Minerals are required for normal fish processes and are found naturally in the water or provided in diet (Watanabe et al., 1997). Although requirements are generally low for fish, they are an essential nutrient (Watanabe et al., 1997). Trace elements are important in bone health, acting directly on cells or as part of the extracellular matrix (Roberto et al., 2018). Minerals are responsible for skeletal formation, maintaining colloidal systems, regulating acid base equilibriums, hormones and part of metalo-enzyme complexes (Watanabe et al., 1997). Mineral deficiencies can cause biochemical, structural and pathological issues (Watanabe et al., 1997).

1.2.4 Vitamins

The vitamin requirements for zebrafish are not fully understood, but are assumed to be similar to other fish (Watts et al., 2012). Vitamins can be water or fat soluble and both are important for fish development and physiology (Halver and Hardy, 2002). Although some of these nutrients are required in small amounts they play crucial roles in regulating gene expression, as coenzymes (Yossa et al., 2011), endocrinol functions, osteoblast activity, osteoclast formation, cell proliferation, regulating intracellular mineral uptake (Lock et al., 2010), growth, reproduction and survival (Watts et al., 2012). Deficiencies can cause decreased growth, increased mortality, decreased food consumption and compromise immune system functions (Halver and Hardy, 2002; Watts et al., 2012; Yossa et al., 2011).

1.2.5 Zebrafish Diet

Zebrafish are euryphagous omnivores (Lawrence et al. 2012). In the wild, D. *rerio* is known to feed on a variety of insects, invertebrate eggs, arachnids, worms and phytoplankton (Lawrence, 2007; Ulloa et al., 2011). This variety suggests a good diet could be composed of either plant or animal proteins (Ulloa et al., 2011). Typical feeding regimes amongst zebrafish facilities include the use of live feed (*Artemia sp.* and rotifers) followed by weaning to microdiets (Kaushik et al., 2011; Lawrence, 2007; Siccardi et al., 2009; Ulloa et al., 2011; Wixon, 2000). Live feeds are both chemically and visually attractive to zebrafish, and it is believed that when enriched can be used throughout life as they contain a well-balanced nutritional profile (Lawrence, 2007). The development of an enrichment product based in microalgae that can be easily used within the zebrafish research community will be beneficial for the standardization of feeding procedures ensuring confidence in experimental outcomes.

1.3 Rotifers

Rotifers, *Brachionus plicatilis,* are the most common live feed at first feeding and possess many qualities desired for rearing larvae (Conceição et al., 2010). The small size and slow movement makes rotifers easy prey allowing greater energy to be expended on growth (Best et al., 2010; Lawrence et al., 2015). Gut flora and bacteria found in rotifers aid in larval digestive functions (Lawrence et al., 2016). Unlike formulated feed, which sink to the bottom or remain on the surface, rotifer movement within the water column stimulates larvae to attack (Conceição et al., 2010). Consuming live prey has been shown to increase digestive enzyme activity and using formulated diets during early larval stages can delay gut development (Eryalcin, 2018).

Continuous production of rotifer culture is both easy and cost effective (Best et al., 2010). In addition, rotifers have a high growth rate and tolerate a wide range of conditions (Conceição et al., 2010). Once stock cultures are established, rotifers can be maintained for continuous generations and do not rely on wild captures like *Artemia* sp. (Conceição et al., 2010).

Rotifers are able to tolerate a wide range of salinities 1-97 parts per thousand (ppt) (Best et al., 2010). This tolerance allows cultivation at low salinities (10-15 ppt) which minimizes osmotic stress and increase survival when fed to zebrafish reared at 0-4 ppt (Lawrence et al., 2012). Breeding at low salinities reduces the risk of normal pathogens associated with saltwater rotifer production as they are unable to survive (Henry et al., 2017). Unlike other organisms which feed on bacteria, rotifers feed on microalgae, which lowers the risk of pathogen exposure and provides nutritional benefits (Martins et al., 2016). Rotifers have two structures aiding in algae consumption see figure 4. The corona is a ciliated structure used in swimming and to sweep prey into the mouth (Lawrence et al., 2012; Henry et al., 2017). The mastax is an internal chewing mechanism responsible for the disruption of hard algae cell walls, allowing for easy digestion (Henry et al., 2017; Lawrence et al., 2012).

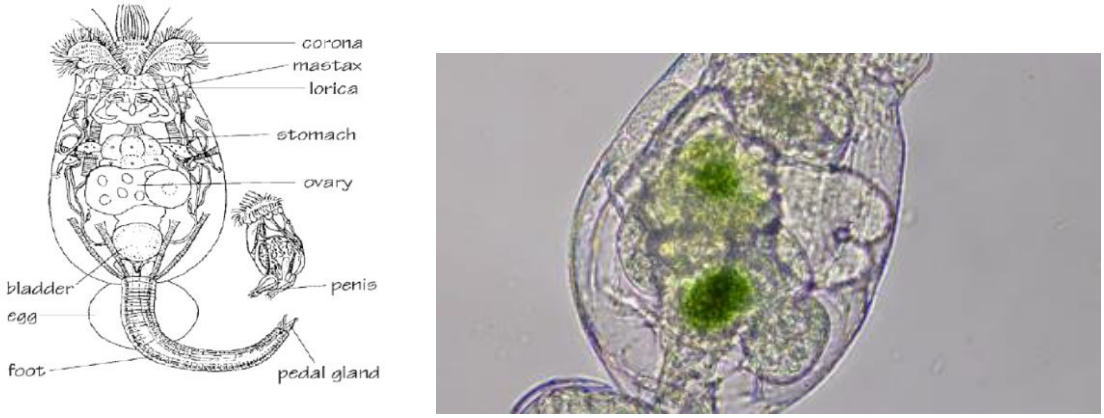


Figure 4 - Left: Saltwater rotifer *Brachionus plicatilis* diagram from Henry *et al*., (2016). Right: Rotifer with microalgae enrichment (Easy Reefs, 2019).

Alone, rotifers possess an incomplete nutritional profile (Conceição et al., 2010; Eryalcin, 2018; Lawrence et al., 2015; Nordgreen et al., 2012; Thépot et al., 2016) however, this is easily overcome as the nutritional value can be boosted using enrichments (Hagiwara and Yoshinaga, 2017). Although rotifers have high vitamin C, E, B1 and B2 contents, they are deficient in a

number of other nutrients. Trace elements such as iodine (Conceição et al., 2010), manganese, copper, zinc and selenium were all found to be too low to meet marine larval nutrient requirements (Nordgreen et al., 2012). In addition to possessing an unbalanced amino acid profile for most larval species (Conceição et al., 2010), rotifers are low in *n*-3 HUFAs. Nutrient deficiencies in rotifers cause low growth, increased mortality and skeletal deformities (Ma and Qin, 2014).

Rotifer nutritional value depends mainly on the feed used, proper enrichments maximize both rotifer production and meet larvae nutritional demands (Lawrence et al., 2012). Food typically passes through the rotifer gut in about 45 minutes (Henry et al., 2017). At high feed concentrations ingestion occurs rapidly and feed can be pushed out of the gut before digestion occurs (Lawrence et al., 2012). This allows nutrients to quickly be packed into rotifers prior to feeding (Lawrence et al., 2012). Rotifer enrichment can be performed using microalgae as well as a variety of emulsions such as, DHA culture SELCO®, protein SELCO® and Red pepper ®, however, emulsified oils are not always affective (Hagiwara and Yoshinaga, 2017).

1.4 Microalgae

Microalgae, shown below in Figure 5, are the first link in the aquatic food chain (Conceição et al., 2010) and commonly used as an enrichment to increase the nutritional profile of live feed (Conceição et al., 2010; Thépot et al., 2016) as they are the primary prey for zooplankton (Brown et al., 1997). Microalgae species are known to be high in protein, lipids and carbohydrates, as well as significant quantities of carotenoids and vitamins (Bravo-Tello et al., 2017). In addition, they also contain high contents of PUFAs (Conceição et al., 2010; Tocher, 2003) as well as of indispensable amino acids (Brown et al., 1997).

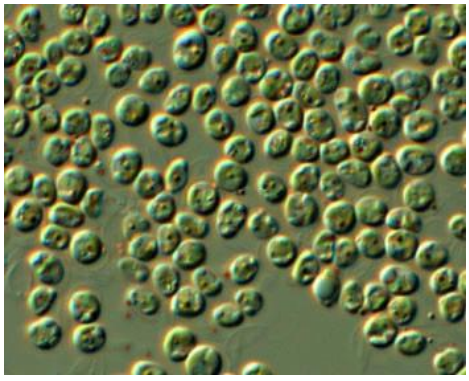


Figure 5: *Nannochloropsis* sp. microalgae. (Algae Research Supply, 2019)

Along with being a rich source of nutrients, microalgae have shown to increase nutrient retention, increase survival against pathogens, decrease mortality and lower intestinal inflammation in both fresh and saltwater species (Bravo-Tello et al., 2017). However, individual microalgae have different nutritional profiles (Thépot et al., 2016), nutritional value changes according to culture conditions (Bravo-tello et al., 2017; Muchlisin, 2012) and the growth stage at which harvest occurs (Brown et al., 1997).

1.4.1 Microalgae as Enrichments

The FA composition of rotifers is affected by the microalgal species used (Hagiwara and Yoshinaga, 2017). Microalgae have varying amounts of PUFAs which can be used to increase the rotifer FA profile (Sorgeloos, 1996). Total lipids amongst microalgae species range from 1-40% with some species having a dry weight composed of up to 85% lipids (Becker, 2004).

Blending of different microalgal strains provides a balanced FA, AA, vitamin and mineral profiles and is preferred over single microalga enrichments (Muchlisin, 2012; Thépot et al., 2016). Chlorophytes such as *Chlorella* spp. have a low nutritional value and are thus not suitable as a single alga enrichment (Brown et al., 1997). However a 50-50% blend of *Nannochloropsis oculata* and *Chlorella vulgaris* yielded improved growth, development and stress resistance in barramundi, (*Lates calcarifer),* larvae when compared to other blends and single microalga enrichments (Thépot et al., 2016). The improved nutritional profile associated with microalgae blends increases growth as different microalgal species compensate for the lack of nutrients in other species (Conceição et al., 2010).

On-site microalgal production is expensive (Conceição et al., 2010; Thépot et al., 2016), time-consuming and require specific conditions and trained operators. Microalgal concentrates are favored as they are easy to use, cost effective and yield good results (Conceição et al., 2010; Thépot et al., 2016). Pastes and powders are commonly used to modify rotifer nutritional profiles (Conceição et al., 2010). In addition, microalgal pastes can be stored for long periods without significant loss of FA (Hagiwara and Yoshinaga, 2017).

The green water technique, a common strategy used in saltwater aquaculture, refers to the addition of phytoplankton to the rearing tank (Conceição et al., 2010; Muchlisin, 2012; Thépot et al., 2016). Phytoplankton stabilize and possibly improve the culture medium (Muchlisin, 2012). This has been found to improve larval growth, survival, feed intake and larval gut microbiota

(Conceição et al., 2010) as well as maintain the nutritional quality of live prey in the tank (Conceição et al., 2010; Thépot et al., 2016). These benefits are possibly due to increased oxygen production, pH stability, bacterial regulation and increased immunity (Muchlisin, 2012).

2. Objectives

This works aims to determine the effects of microalgae enrichment in larval and juvenile zebrafish growth, survival and skeletal development. In addition, we propose to formulate a blended microalgae rotifer enrichment which fulfills zebrafish larval nutritional requirements and optimize larval skeletal development.

3. Materials and Methods

3.1 Live Microalgae Culture

At the beginning of each week, 2, 5-L jugs of saltwater at 20 parts per thousand (ppt) were prepared using synthetic salt from Tropic Marin® (TMC Iberia, Portugal) and Milli-Q water (Merck, Germany). Next, 7 mL of sodium hypochlorite (bleach) were added and left under strong aeration for 24 hours. After 24 hours, 7 mL of sodium thiosulfate were added to neutralize the sodium hypochlorite and after 2 hours 1 mL of phosphates, 1 mL of metals, 2 mL of nitrates and 0.1mL of vitamins were nutrients were added to each 5 L jug. If microalgae were needed in less than 2 weeks, 4 mL of nitrates were added. Finally, 100 mL of live *Tetraselmis chui* culture were added and left under strong aeration with constant light.

3.2 Microalgae Enrichment

In total, 12 individual microalgae enrichments were tested plus 3 microalgae blends, the microalgae used and blended recipes are presented in Tables 1a and 1b**.** Enrichments were prepared by combining 30 mL of Milli-Q water in a 50 mL Falcon tube with 900 mg of synthetic salt from Tropic Marin® (TMC Iberia, Portugal) for a final salinity of 30 ppt. Next, 5.4 g of each desired microalgae powder or frozen paste obtained from Necton S.A® (Olhão, Portugal) were added to the saltwater to reach a concentration of 18%. The Falcon tube was than vortexed to homogenize the mixture. Enrichments were stored in the refrigerator at 3 ˚C.

Table 1a - Microalgae strains used in Trials 1 and 2. 5.4 g of indicated microalgae were used. Total microalgae used based on 18% microalgae mixture. Note: * indicates different species than trial 1. ** indicates powder.

Trial 1	Trial 2
Nannochloropsis sp. Paste (NPA)	*Nannochloropsis* sp. (NPA)
Nannochloropsis sp. Powder (NPO)	*Isochrysis* sp.* (IE2)
Nannochloropsis sp. Paste at 10 ppt (NPA 10 ppt)	*Tetraselmis* sp.* (TE2)
Tetraselmis sp. Paste (TPA)	*Spirulina* sp.** (SPIRULI)
Tetraselmis sp. Powder (TPO)	*Skeletonema* sp. (SKEL)
Isochrysis sp. Paste (IPA)	*Phaeodactylum* sp. (PHAEO)
Isochrysis sp. Powder (IPO)	*Chaetoceros* sp. (CHC)

Table 1b - Microalgae strains used in trial 3. Total amount in grams (g) and percent (%) are given, all mixes used a total of 5.4 g microalgae. Total microalgae used based on 18% microalgae mixture. Note: * indicates different species than trial 1. ** indicates powder.

Microalgae	Blend A	Blend B	Blend C
Nannochloropsis sp.	4.32 g (80%)	2.16 g (40%)	3.73 g (69%)
Isochrysis sp.*	0.54 g (10%)	0.27 g (5%)	1.03 g (19%)
Tetraselmis sp.*	0.54 g (10%)	0.27 g (5%)	0.54 g (10%)
Spirulina sp.	-	2.7 g (50%)	-
Skeletonema sp.	-	-	0.11 g (2%)

3.3 Rotifer Culture

Saltwater was prepared using synthetic salt (Tropic Marin®,Tropical Marine Centre) added to 5 L jugs and Milli-Q water according to the manufacture procedure. Rotifers were cultured at 20 ppt under slight aeration.

Initially, rotifer stocks were obtained from LEOA facilities in the Centre of Marine Sciences (CCMAR, Portugal) and diluted into a volume of 3 L, using 2 L of salt water at 20 ppt and 1 L of live *T. chui* to achieve a final concentration between 50-70 rotifers/mL. Each morning, rotifer counts were performed under a dissecting microscope by stirring the culture vigorously to homogenize and removing 1 mL with a glass pipette. As the cultures grew, rotifers were harvested

and transferred into new 5 L culture bottles with a density between 290-360 rotifers/mL. Rotifers were fed 1.2mL/L/day Phytobloom Green Formula®. Feedings were done 3 times per day with 1.6 mL of from Necton S.A® given daily, for a total of 4.8 mL/day. Every other day, rotifers were fed with 300 mL of live *T. chui*.

Throughout the experiment, 8 rotifer cultures were maintained in 5 L bottles and 4 harvested each day, allowing the harvested cultures to recover. From the harvested cultures, 25-50% of the rotifers were removed to maintain stocks between 290-360 rotifers/mL with 50% water renewal. Harvesting was performed by emptying the bottles into a bucket through a 250 µm sieve to remove debris. The desired volume of rotifers was than collected and put into saltwater containing no microalgae for enrichment later. The remaining rotifers were then concentrated using a 55 µm sieve. Saltwater and 300 mL of *T. chui* were added to return volumes to 4 L. All rotifers were fed throughout the day using Phytobloom® as previously described.

3.3.1 Enrichments

Figure 6 - Rotifers in 1.5 L enrichment bottles for trial 3. From left to right enrichments using *Nannochloropsis* sp., blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.), blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.) and blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Harvested rotifers were concentrated using a 55 µm sieve and, fresh saltwater was then added to increase the volume to the desired volume for enrichment. The rotifers were mixed, and 1 L was poured into a 1.5 L enrichment bottle at an initial concentration between 400-500

rotifers/mL. Microalgae used for enrichments at 2 mL/L of water were added in the afternoon and left under slight aeration overnight, enrichment bottles are shown above in Figure 6. In the morning 2 mL of microalgal suspensions used for enrichment were again added and left for 2 hours prior to larval first feeding. At this time, rotifer concentrations were checked to ensure the proper amount would be administered over the two feedings (200-230 rots/mL).

The necessary volume of rotifers was then removed into a plastic graduated beaker and concentrated using a 55 µm sieve to 100 mL. These rotifers were put into the properly labeled container for feeding. A general schematic of the rotifer culture is shown below in Figure 7.

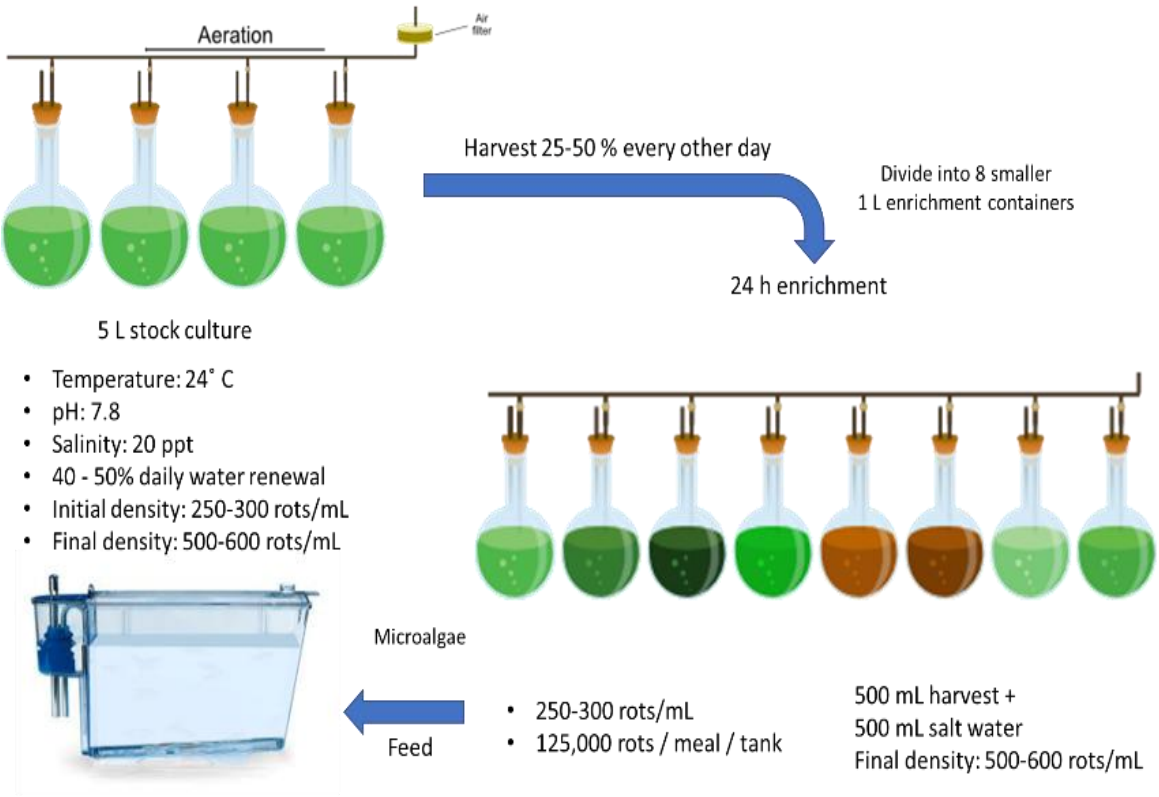


Figure 7 - General scheme of rotifer production and enrichment used in experiments.

3.3.2 Rotifer Collection for Biochemical Analysis

To determine rotifer biological value, it was necessary to increase rotifer production. Rotifers were enriched as done prior in the experiment, but in 4 L jugs at a concentration between 400-500 rotifers/mL and given microalgal suspensions used for enrichment at 2 mL/L water in the afternoon. In the morning, rotifers were again enriched for 2 hours. The rotifers were harvested,

rinsed to remove microalgae and concentrated, using a 55 µm sieve, into 50 mL Falcon tubes. The remaining microalgae settled to the bottom of the Falcon tubes and rotifers could be removed from the top. The collected rotifers were again rinsed on a 55 µm sieve to remove any remaining microalgae in the water. Next, 50 mL of saltwater were added to the Falcon tubes with microalgae and the solution mixed. This was allowed to again settle and the process of rotifer collection and rinsing repeated. This fraction was combined with the first collection from each sample and concentrated down to 40 mL. Samples were then centrifuged for 3 minutes at 8000 *g*. After centrifuging, rotifers settled to the bottom and water was removed from the top until the volume was below 15 mL. Falcon tubes were then placed into the freezer at -80° C until freeze-drying.

3.4 Zebrafish

This research was carried out using 3 trials. The first 2 trials utilized triplicates for each condition, while the 3rd trial had quadruplicates. Zebrafeed® (ZF) and rotifers enriched with *Nannochloropsis* sp. (NPA) were used as controls in all 3 trials. In trial 3 a co-feeding group (CF) was introduced. This group was co-fed rotifers enriched with *Nannochloropsis* sp. and Zebrafeed® from days 5-8 dpf and fed only Zebrafeed® until 30 dpf, the end of the trial.

Zebrafish were spawned naturally and embryos transferred into 1 L tanks with 50 ppt methylene blue embryo medium to prevent fungal growth. At 5 dpf, larvae were divided randomly into triplicate groups of 100 and placed into 1-L larval rearing tanks. Tanks were filled with 800 mL system water and 200 mL saltwater at 20 ppt for a final salinity of 4 ppt. Tanks were kept in plastic bins half-filled with water able to hold 8 tanks and a heater to maintain temperature at 28 ˚C, pH 7.6 and a photoperiod of 14:10 light:dark. The experimental set up with tanks in bins is shown below in Figure 8. Each experimental group was labeled and subjected to a different rotifer enrichment.

In the morning, tanks were cleaned to remove dead rotifers and debris. This was done by carefully pouring larvae through a 250 µm sieve in a small plastic container. Next, the rearing tank was rinsed and 400 mL system water added. Larvae were then transferred back into the container; 15 mL of microalgal suspensions used for enrichment were combined with 85 mL saltwater at 20 ppt and added to the container for a final salinity of 4 ppt. Remaining rotifers were put into the refrigerator at 3 ˚C until the afternoon feeding. In the afternoon, 400 mL system water, 15 mL of

microalgal suspensions used for enrichment and 85 mL saltwater at 20 ppt were combined and added to the containers for a final volume of 1 L at 4 ppt.

Figure 8 - 3 L larval rearing tanks set up in plastic bins with heater (not seen).

At 15 dpf survival rate was calculated. From each tank, 15 larvae were collected and euthanized using tricaine (Sigma-Aldrich), from which, 10 were used for length, weight and condition factor and 5 used for histology. At this point, larvae were transferred into 3 L tanks, containing 2400 mL system water combined with 600 mL saltwater at 20 ppt for a final volume of 3 L at 4 ppt. The daily feeding and cleaning schedule remained the same however 1.5 L of saltwater in the morning and afternoon were added and a greater number of rotifers given to maintain the rotifer density between 200-230 rots/mL.

At 30 dpf, the total survival was calculated with all remaining larvae. A group of 10 larvae were used for length, weight, condition factor and mineral analysis, another 5 larvae were used for histology and 40 larvae observed for skeletal deformities. A schematic of the experimental procedure is shown below in Figure 9.

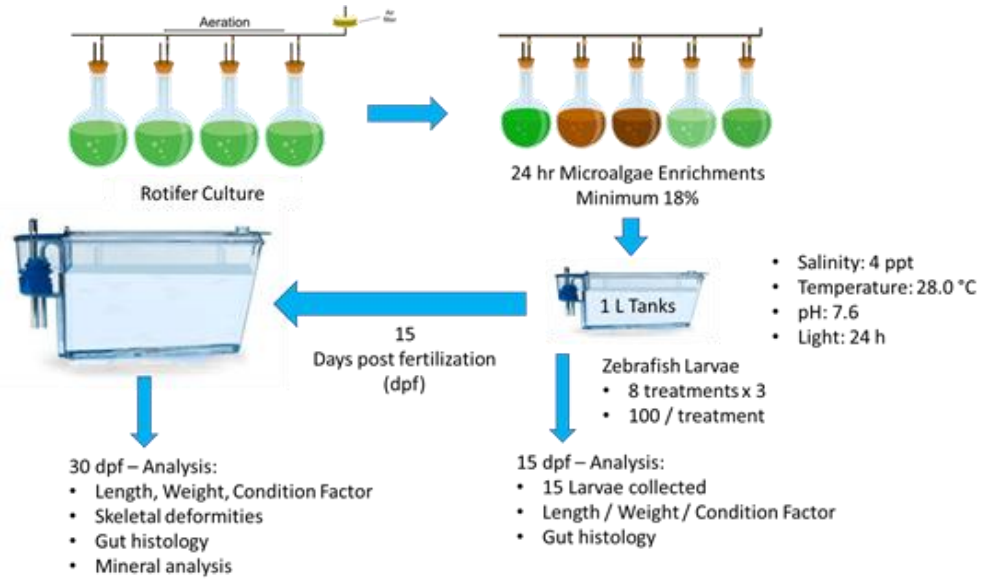


Figure 9 - General scheme of rotifer and zebrafish culture conditions as well as analysis done throughout experiment.

3.5 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. The following morning, larvae were briefly rinsed with 1x phosphate-buffered saline (PBS) and placed into increasing ethanol baths for 30 minutes (25, 50 and 70%), then stored at 70% until decalcification.

Ethanol was removed and Eppendorf tubes filled with 2 mL of ethylenediaminetetraacetic acid (EDTA) 10% and containing PFA 1%. After 24 hours, EDTA was replaced and larvae allowed to sit for another 24 hours. After 48 hours, 15 dpf larvae were removed from EDTA. These larvae were washed 2 times in 1x PBS for 30 minutes each, then placed into 35% ethanol for 30 minutes and finally transferred to 70% ethanol for storage until paraffin inclusion.

In the case of 30 dpf larvae, after 48 hours in EDTA, the solution was again replaced and allowed to sit for 48 hours. Next, EDTA was replaced and allowed to sit for 120 hours, at which point larvae were removed from the EDTA solution as described previously for the 15 dpf larvae.

Samples were removed from 70% ethanol and 3 to 4 larvae were placed into a pre-labeled 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 96% ethanol for 4 hours. Samples were

placed into the Tissue TEK II Tissue Processor (Kedee Instruments Co., China). In the processor, samples were mechanically brought through a series of baths. The first was 6 hours in an absolute ethanol treatment, followed by 2 hours in absolute ethanol xylol 1:1 mix, then 2 hours in pure xylol, another 2 hours in pure xylol and finally two paraffin baths of 2 hours each.

Cassettes were then brought to the KD-BM tissue embedding center (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 metal molds on a heated block with a slight amount of paraffin. Molds were removed from heat and briefly cooled to fix larvae in place. A plastic cassette top was put onto the metal molds and filled with paraffin. The molds were then transferred to a cooling block at -4 °C to chill. After 2 hours, the paraffin blocks could be removed from the metal molds 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 hematoxylin and eosin as described by Fischer *et al.* (2017).

Intestinal villi length was measured using a microscope with an iPad (Apple Inc, USA) attached. A photo was taken of each section, see Figure 10, and villi measured. From each section about 5 villi were chosen for measurement.

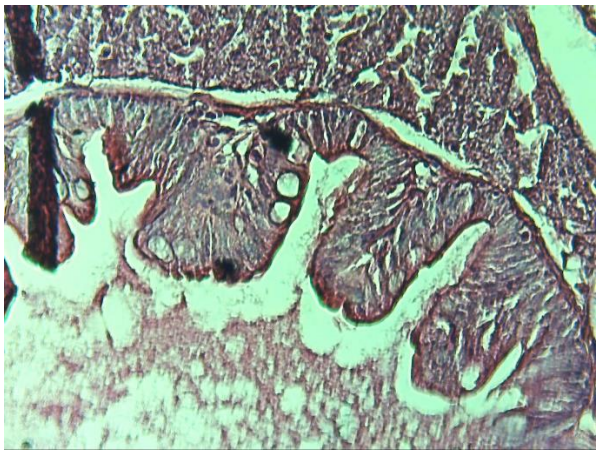


Figure 10 - Anterior gut section of zebrafish given rotifers enriched with *Nannochloropsis* sp. using hematoxylin and eosin (40x).

3.6 Length, Weight, Survival and Condition Factor

Total length (TL) was determined by taking photos using a Leica® stereomicroscope of 10 larvae from each replicate and measurements made using Image J®.

Total dry weight (DW) per fish was determined using10 larvae per replicate. Larvae were placed into pre-weighed dry Eppendorf tubes and freeze dried. Next, tubes were weighed and calculated using equation below.

$$DW\ per\ fish = \frac{(Initial\ weight - final\ weight)}{10}$$

Survival was calculated by counting all live fish still in tanks at 15 and 30 dpf.

Condition factor was analyzed using methods described by Siccardi *et al*, (2009).

$$Condition\ Factor = \frac{(wt * 100)}{(Length^3)}$$

3.7 Alcian blue and Alizarin red S Double Staining:

Alcian blue and alizarin red S double staining was done using a modified protocol described by Gavaia *et* al., (2000). Larvae were fixed in 4% PFA overnight. The next morning, larvae were washed in increasing ethanol solutions (25-50-75%) for 30 minutes each. Larvae were stored at room temperature in 75% ethanol. Prior to processing, larvae were washed in decreasing ethanol baths (75-50-25%) followed by distilled water for 15 minutes each, followed by 2 mL of 0.1% alcian blue 8GX (Sigma-Aldrich, Spain) staining for 20-30 minutes. Samples were rinsed briefly with 2 mL of 96% ethanol. After alcian blue staining the remaining acidity in the samples was neutralized in 100% ethanol with 300 µL of 8% potassium hydroxide (KOH) for one hour. Next, decreasing ethanol baths were done (96-75-50-25%) followed by distilled water, for 30 minutes each. Finally, samples were stained using a 0.01% alizarin red S (Sigma-Aldrich, Spain) solution overnight. The next morning, the alizarin red S stain was removed and final clearing performed using 10 mL of 1% potassium hydroxide KOH for 24 hours. Samples were left in partial light as UV exposure accelerates the bleaching process. After 24 hours, the larvae were put through a series of increasing water glycerol baths (3:1-1:1-1:3) for 2 hours each and stored in pure glycerol until further analysis. A stained zebrafish is shown below in Figure 11.

Figure 11 - 30 days post fertilization (dpf) zebrafish stained using alcian blue and alizarin red.

3.8 Skeletal Analysis

For analysis of skeletal deformities, a group of 40 fish from each triplicate (n = 40) were analyzed using a Leica MZ 7.5 stereomicroscope (Leica, Germany). Each fish was observed individually for abnormalities in the head, abdominal vertebrae, caudal vertebrae and caudal fin regions, see Figure 12 below, following the nomenclature by Bird and Mabee 2003.

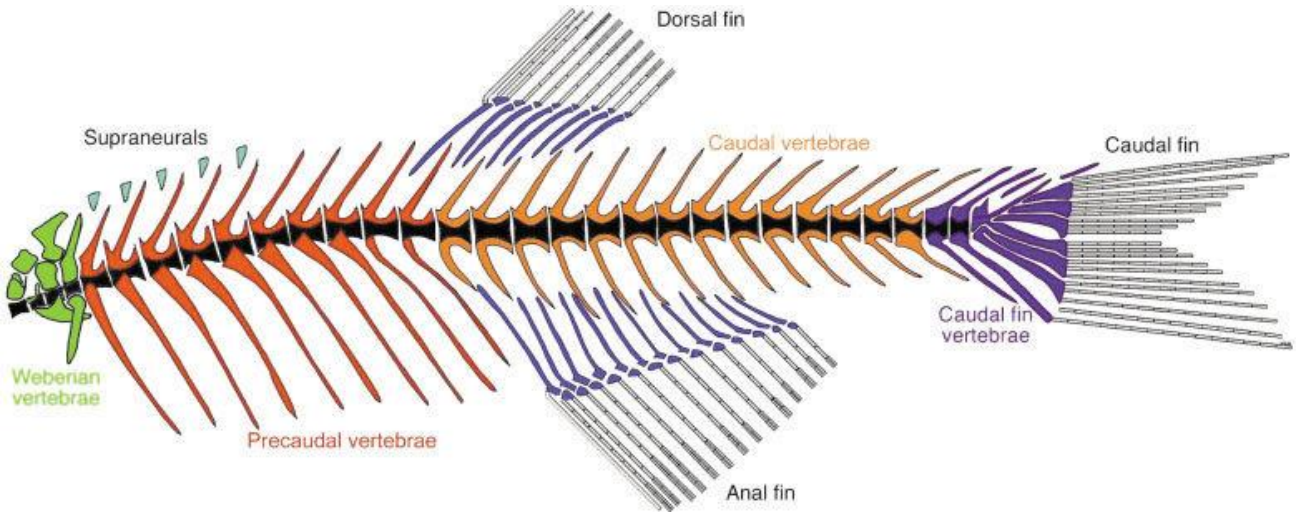


Figure 12 - Zebrafish axial skeleton diagram. Centra are in black, the Weberian apparatus is green, supraneurals are light blue, precaudal vertebrae are red, dorsal and anal fin endoskeletons are blue, caudal vertebrae are orange and caudal fin vertebrae is purple (Bird and Mabee, 2003).

3.9 Biochemical Analysis

3.9.1 Proteins (CHN)

Samples were freeze dried for 24 hours and ground into a fine powder. Using a precision balance, 1 mg of sample was weighed into small foil bins. Next, samples were placed into the oven and burned to determine percent nitrogen. Average percent protein was calculated below using a 6.25 conversion factor (Diniz et al., 2011). The variation coefficient was used to detect abnormalities in results, if the variation coefficient was greater than 10 the experiment was repeated.

$$Average\ Protein = \%\ N * 6.25$$

$$Variation\ Coefficient = \frac{Standard\ Deviation}{Average} * 100$$

3.9.2 Total Lipids (Bligh and Dyer, 1959)

Total lipids were determined using a modified protocol from Bligh and Dyer (1959), described in Pereira *et al.* (2018). Lipid tubes were labeled and dried at 60 °C in the oven for 3 hours, then transferred to the desiccator for 3 hours. Tubes were labeled and weighed using the precision balance and set aside. Thereafter, 15-16 mg of freeze-dried samples were weighed into glass tubes, with duplicates for each sample, and 0.8 mL distilled water added. Next, 2 mL of methanol and 1 mL of chloroform were added to each tube and homogenized for 60 seconds using an Ultraturrax (IKA) disperser. Samples were homogenized while in an ice bath to prevent temperature increase. After homogenization, 1 mL of chloroform was added to each sample and the homogenization procedure repeated for 30 seconds. Next, 1 mL of distilled water was added to each sample and homogenized for 30 seconds. Following homogenization, samples were transferred to centrifuge tubes using a pipette. Samples were centrifuged for 10 min at 10 °C at 2000/5000 *g*, and the chloroform phase was transferred from the bottom of the tube into a new test tube. Extracted chloroform phase is visible below in Figure 13. Next, 0.7 mL of the chloroform sample was transferred into the pre-weighed tubes and placed in a dry bath at 60° C overnight to completely evaporate the chloroform. Once dry, the tubes were weighed on the precision balance and percent total lipids calculated using the equation below.

$$\%\ Total\ Lipid = \left[\frac{(final\ tube\ weight - initial\ tube\ weight) * total\ volume\ chloroform}{\frac{volume\ evaporated\ chloroform}{sample\ dry\ weight}}\right] * 100$$

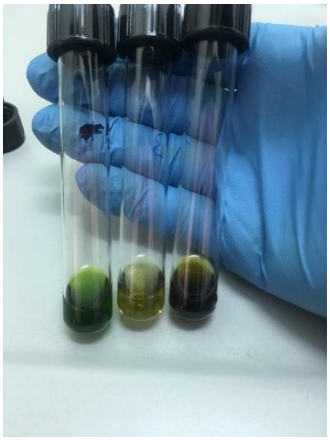


Figure 13 - Chloroform extracted phase during total lipid determination.

3.9.3 Fatty acid methyl esters (FAME) (Lepage and Roy, 1984; Pereira et al., 2012)

First, 15-17 mg of sample were weighed into derivatization vessels. Thereafter, 1.5 mL of methanol (20:1 v/v) were added and samples were homogenized in ice using the Ultraturrax for 60 seconds, followed by a brief pause and then 30 seconds of further homogenization. Next, 1 mL of n-hexane was added to the derivatization vessel. Vessels were closed and sealed using Teflon tape to prevent opening and placed in a water bath at 70 °C for 60 minutes. Samples were removed from the water bath and placed on ice to cool for 10 minutes. After cooling, samples were transferred to centrifuge tubes, 1 mL of distilled water was added to the original tube to ensure all material was transferred, and 4 mL of n-hexane was added to the centrifuge tube. Samples were vortexed at maximum speed in 2 cycles of 30 seconds then centrifuged for 5 minutes at room temperature at 2000 *g*. The hexane (top liquid phase) was removed using a Pasteur pipette and transferred to a new glass tube. Next, 4 mL of n-hexane was again added to the centrifuge tube and the vortex, centrifuge and collection process was again repeated. Afterwards, anhydrous sodium sulfate (Na_2SO_4) was added in excess to extracts in order to remove any remaining water. Samples were then filtered into new tubes using a syringe with a 0.22 µm filter. Tubes were later placed under a gentle nitrogen gas flow to evaporate the hexane. Once dry, resuspension was

performed using 0.5 mL chromatography-grade hexane which was filtered through a 0.22 µm filter and placed into a spectrometry jar for analysis. Samples were run in triplicates.

Analysis was done using an Agilent GC-MS (6890 Network GC System with a 5973 inert Mass Selective Detector, Agilent Technologies, USA) with Agilent Tech DB-5MS column. Identification was done by comparing the retention times of standard samples (Supelco 37 FAME Mix, Sigma-Aldrich) and the mass spectra compared to the NIST library. FAME determination was done using the calibration curves for all FAME detected using four dilutions of the initial standard.

3.9.4 Ash (Widbom, 1984)

Crucibles were first labeled and weighed then, approximately 50 mg of sample were weighed into each crucible. Crucibles were placed into the oven, Naberthern Controller B170 (Naberthern, Germany), inside a fume hood. Samples were left overnight at 525° C to burn. The following day, crucibles were removed and weighed.

$$Total\ Ash = \frac{(Final\ weight - Initial\ weight) * 1000}{sample\ weight * 100}$$

3.9.5 Minerals

Mineral analysis was done using the methods described by Pereira *et al.* (2018). Freeze dried samples were digested in 4 mL of nitric acid ($NHO_{3)}$ and 1 mL of hydrogen peroxide (H_2O_2). Hydrogen peroxide was used to catalyze the reaction and remove pigments to ensure all material was digested. Zebrafish pre and post digestion are shown below in Figure 14.

Samples were analyzed for mineral content using a Microwave Plasma-Atomic Emission Spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia). Standards of different concentrations were prepared using certified standard solutions. Results were corrected by subtracting a blank from the analyzed metal concentrations. All samples were analyzed in triplicates.

Quantification wavelengths and calibration curves were selected to obtain the highest signal to ratio and the lowest interference for the targets elements; spiking and recovery readings were carried out to assess validity of the results.

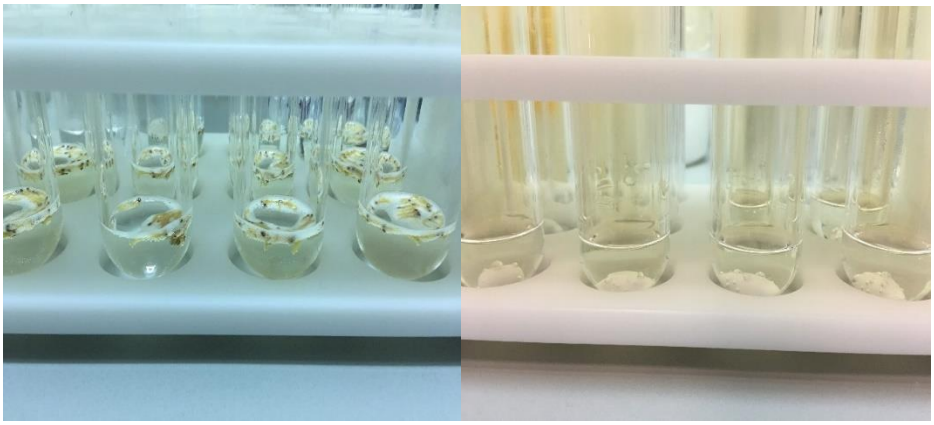


Figure 14 - Zebrafish pre (left) and post (right) digestion using nitric acid (NHO_3) and hydrogen peroxide (H_2O_2).

4. Results

4.1. Trial 1: Discrepancies Between Paste and Powder Enrichments.

4.1.1 Trial 1 Microalgae and Rotifer Proximal Composition

Proximal composition of microalgae are presented below in Table 2. Amongst microalgae, the total lipid content was highest in *Isochrysis* sp. paste (IPA) and powder (IPO), reaching 31.9% and 27.5% of biomass dry weight (DW) respectively. On the other hand, only 14.08% DW of lipids were registered in *Nannochloropsis* sp. powder (NPO). Regarding proteins, IPA displayed the highest content, 51.7% DW, while *Tetraselmis* sp. paste (TPA; 34.62% DW) displayed the lowest content. The ash content was highest in TPA (26.59% DW) and lowest in NPO (8.83% DW). Total carbohydrates (CHO) were highest in NPO (39.42% DW) and lowest in IPA (3.59% DW).

Amongst enriched rotifers a different pattern was observed (Table 3). A higher content of total lipids was detected in rotifers enriched with *Nannochloropsis* sp. paste (NPA ROTS), 14.73% DW, while the lowest value, 6.48% DW, was observed in IPO ROTS. The rotifers enriched with NPA at 10 ppt (NPA 10 ppt ROTS) displayed the highest protein content (42.59%), while only 21.94% DW of protein was obtained in rotifers enriched with *Nannochloropsis* powder (NPO ROTS). The ash content was highest in rotifers enriched using *Nannochloropsis* powder (NPO ROTS) (54.52% DW) and lowest in the rotifers enriched with NPA at 10 ppt (29.18% DW). Total carbohydrates were highest in the rotifers enriched with TPA (22.79% DW) and lowest in those enriched with NPA at 10 ppt (13.59% DW).

Table 2- Proximal composition of microalgae (*n* = 3).

Treatment	% Lipids	% Protein	% ASH	% CHO
NPA	17.63	44.61	12.71	25.05
NPA 10 ppt	17.63	44.61	12.71	25.05
NPO	14.08	37.66	8.83	39.42
IPA	31.90	51.72	12.79	3.59
IPO	27.45	46.04	12.06	14.45
TPA	15.82	34.62	26.59	22.97
TPO	20.99	45.33	18.72	14.96

NPA – *Nannochloropsis* sp. Paste; NPA 10 ppt – *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO – *Nannochloropsis* sp. Powder; IPA - *Isochrysis* sp. Paste; IPO – *Isochrysis* sp. Powder; TPA – *Tetraselmis* sp. Paste; TPO – *Tetraselmis* sp. Powder.

Table 3 - Proximal composition of Zebrafeed® and enriched rotifers (*n* = 3).

Treatment	% Lipids	% Protein	% ASH	% CHO
ZF	15.41	66.21	13.00	5.38
NPA ROTS	14.73	34.38	36.07	14.82
NPA 10 ppt ROTS	14.64	42.59	29.18	13.59
NPO ROTS	6.93	21.94	54.52	16.61
IPA ROTS	10.43	39.84	33.00	16.72
IPO ROTS	6.48	30.85	42.88	19.78
TPA ROTS	9.76	32.37	35.08	22.79
TPO ROTS	9.76	40.28	32.01	17.95

ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt ROTS – Rotifers enriched with *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO ROTS – Rotifers enriched with *Nannochloropsis* sp. Powder; IPA ROTS - Rotifers enriched with *Isochrysis* sp. Paste; IPO ROTS – Rotifers enriched with *Isochrysis* sp. Powder; TPA ROTS – Rotifers enriched with *Tetraselmis* sp. Paste; TPO ROTS – Rotifers enriched with *Tetraselmis* sp. Powder.

4.1.2 Trial 1 Fatty Acids Methyl Esters (FAME) of Microalgae, Diet and Enriched Rotifers.

The fatty acid composition of microalgae (Table 4) is presented below, while the complete FAME profiles can be found in Annex 1, Table A. For microalgae, C16:0, C16:1, C18:2 *n*-6 (LA) and C20:5 *n*-3 (EPA) were present in the highest levels. IPA and IPO had low levels of EPA, but

high levels of C22:6 *n*-3 (DHA). The *n*-3:*n*-6 PUFA ratios varied between 0.97 (TPO) and 13.29 (IPO). PUFA/SFA levels ranged from 0.29 (TPO) and 1.33 (NPO).

The FAME profile of enriched rotifers and diet (Table 5) is shown below and the complete FAME profile can be found in ANNEX 1, Table B. In enriched rotifers, C16:0, C16:1 and C18:1c were found in the highest amounts. Levels of C18:2 *n-6* (LA) were lowest in the rotifers enriched with IPA (0.51% of total fatty acids; TFA) and highest in those enriched with TPA (10.64% of TFA). The amount of C20:4 *n*-6 (ARA) varied between 2.00% (TPO) and 4.8% (IPA) among the different microalgae enrichments. Regarding EPA percentages of TFA ranged between 6.52% (NPA 10 ppt) and 1.90% (IPO). The rotifers enriched with NPO (0.19) had the lowest *n*-3*:n*-6 PUFA ratio, while IPA enrichment (1.00) led to the highest value. The PUFA/SFA ratio ranged between 0.25 and 0.46 for the enrichments with NPO and NPA at 10 ppt, respectively. The commercial diet displayed a highest content of C16:0 (28%) and LA (32.54%)

Table 4- Main fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae in trial 1 (*n* = 3).

Fatty Acid %	NPA	NPO	IPA	IPO	TPA	TPO
C14:0			13.26 ± 0.07	14.34 ± 0.11		
C16:0	29.23 ± 0.71	24.56 ± 0.22	21.10 ± 0.25	20.40 ± 0.22	71.54 ± 2.93	42.20 ± 0.74
C14:1	6.17 ± 0.16	5.54 ± 0.05				
C16:1	28.02 ± 0.31	31.00 ± 0.94	10.57 ± 0.21	12.57 ± 0.07		7.16 ± 0.17
C18:1c	4.09 ± 0.10	2.70 ± 2.54	14.19 ± 0.07	14.30 ± 0.13	3.34 ± 0.10	33.86 ± 0.81
C18:2n-6c	2.54 ± 0.00	4.68 ± 0.15	7.27 ± 0.02	1.28 ± 0.07	12.87 ± 1.05	6.17 ± 0.11
C20:4n-6	4.48 ± 3.98		0.82 ± 0.05	1.03 ± 0.00		
C20:5n-3	23.82 ± 2.30	29.14 ± 1.25	1.08 ± 0.02	1.17 ± 0.00	12.24 ± 3.88	5.99 ± 0.17
C22:6n-3			26.46 ± 0.17	29.48 ± 0.13		
Σn-3 : Σn-6	3.31	6.34	3.40	13.29	0.95	0.97
PUFA / SFA	1.06	1.33	0.96	0.91	0.35	0.29

NPA - *Nannochloropsis* sp. Paste; NPA 10 ppt – *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO – *Nannochloropsis* sp. Powder; IPA - *Isochrysis* sp. Paste; IPO – *Isochrysis* sp. Powder; TPA – *Tetraselmis* sp. Paste; TPO – *Tetraselmis* sp. Powder.

Table 5- Main fatty acid composition % Total fatty acid (%TFA) ± S.D of Zebrafeed® and enriched rotifers in trial 1 (n = 3).

Fatty Acid %	ZF	NPA ROTS	NPA 10 ppt ROTS	NPO ROTS	IPA ROTS	IPO ROTS	TPA ROTS	TPO ROTS
C16:0	28.12 ± 0.71	33.31 ± 1.04	33.09 ± 0.02	35.81 ± 2.02	33.77 ± 0.19	37.11 ± 0.81	34.61 ± 1.66	32.46 ± 0.46
C18:0	7.67 ± 0.10	3.61 ± 0.05	4.28 ± 0.73	6.21 ± 0.29	4.37 ± 0.13	4.71 ± 0.78	5.81 ± 0.42	4.46 ± 0.04
C16:1	3.06 ± 0.22	24.04 ± 0.55	23.23 ± 0.47	18.78 ± 1.10	13.89 ± 0.02	20.10 ± 3.23	14.15 ± 0.12	13.50 ± 0.05
C18:1c	10.52 ± 0.12	9.26 ± 0.15	8.88 ± 0.23	13.31 ± 0.05	16.65 ± 0.32	10.46 ± 0.40	17.29 ± 1.31	20.40 ± 0.13
C18:1t	2.29 ± 0.13	4.94 ± 0.01	4.61 ± 0.05	6.10 ± 0.90	6.17 ± 0.00	6.03 ± 0.80	4.13 ± 0.30	5.05 ± 0.12
C20:1	1.03 ± 0.03	1.82 ± 0.18	1.85 ± 0.14	2.27 ± 0.64	3.70 ± 0.15	1.77 ± 0.65	3.04 ± 0.22	3.27 ± 0.17
C22:1	2.54 ± 0.17	0.65 ± 0.10	0.59 ± 0.03	0.57 ± 0.28	1.05 ± 0.00		0.80 ± 0.12	1.15 ± 0.02
C24:1	0.40 ± 0.03	0.23 ± 0.08	0.20 ± 0.13	0.88 ± 0.49	0.63 ± 0.07	2.16 ± 0.07	1.06 ± 0.70	0.44 ± 0.43
C18:2n-6c	32.54 ± 0.12	5.81 ± 0.05	7.43 ± 0.11	8.97 ± 1.93	0.51 ± 0.06	6.79 ± 0.73	10.64 ± 0.43	9.56 ± 0.08
C20:4n-6	0.12 ± 0.02	4.15 ± 0.09	4.55 ± 0.43	0.85 ± 0.72	4.81 ± 0.00	2.23 ± 0.97	2.00 ± 0.01	2.57 ± 0.10
C20:5n-3	3.15 ± 0.08	6.16 ± 0.28	6.52 ± 0.77	1.91 ± 1.16	6.09 ± 0.05	1.90 ± 1.33	2.87 ± 0.03	4.88 ± 0.19
C22:6n-3	6.18 ± 0.10				0.96 ± 0.27			
Σn-3 : Σn-6	0.29	0.66	0.58	0.19	1.00	0.21	0.21	0.40
PUFA / SFA	1.10	0.39	0.46	0.25	0.32	0.22	0.39	0.44
EPA : ARA	26.25	1.48	1.43	2.25	1.27	0.85	1.44	1.90

ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt ROTS – *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO ROTS – Rotifers enriched with *Nannochloropsis* sp. Powder; IPA ROTS - Rotifers enriched with *Isochrysis* sp. Paste; IPO ROTS – Rotifers enriched with *Isochrysis* sp. Powder; TPA ROTS – Rotifers enriched with *Tetraselmis* sp. Paste; TPO ROTS – Rotifers enriched with *Tetraselmis* sp. Powder.

4.1.3 Trial 1 Length, Weight, Survival and Condition Factor

The total length (TL) of fish were determined at both 15 and 30 dpf to monitor growth over time (Figure 15). At 15 dpf, fish fed with NPA (6.38 mm) were the largest and significantly larger than IPO (5.66 mm) and ZF (4.92 mm), which displayed the lowest TL. No significant differences were observed between the fish fed with rotifers enriched with IPA (6.25 mm), NPA 10 ppt (6.15 mm), NPO (6.12 mm), TPA (6.21 mm) and TPO. The fish from the ZF group were significantly smaller than all other treatments. At 30 dpf, zebrafish fed with the NPA 10 ppt treatment displayed the highest TL, 9.79 mm, significantly larger than the TPO (8.89), NPO (8.60 mm), TPA (8.45 mm), IPA (8.21 mm), IPO (8.13 mm) and ZF (6.68 mm) treatments. The NPA (9.49 mm) treatment were the second largest group of zebrafish and significantly greater than all treatments, except NPA 10 ppt and TPO. The group fed with Zebrafeed® (ZF) were significantly smaller than all other treatments.

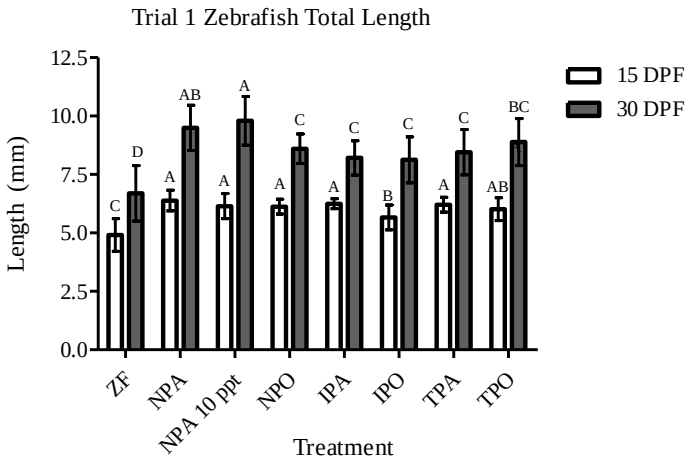


Figure 15 - Zebrafish total length at 15 and 30 day post fertilization (dpf). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$) ($n = 10$). ZF- Zebrafeed®; NPA – Fish fed rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt – Fish fed rotifers enriched with *Nannochloropsis* sp. reared at 10 parts per thousand (ppt); NPO – Fish fed rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Fish fed rotifers enriched with *Isochrysis* sp. Paste; IPO – Fish fed rotifers enriched with *Isochrysis* sp. Powder; TPA – Fish fed rotifers enriched with *Tetraselmis* sp. Paste; TPO – Fish fed rotifers enriched with *Tetraselmis* sp. Powder.

Fish dry weight (DW) was determined at 15 and 30 dpf (Figure 16). At 15 dpf, the NPA (0.33 mg) and NPA 10 ppt (0.33 mg) fed groups displayed the highest weight, significantly heavier than those fed with IPO (0.23 mg) and ZF (0.12 mg), which were the lightest. While groups fed rotifers enriched with TPO (0.29 mg), IPA (0.29 mg), TPA (0.27 mg) and NPO (0.25 mg) were significantly larger than those give ZF. At 30 dpf, fish fed with NPA 10ppt displayed a weight of 0.99 mg, significantly heavier than those fed with NPO (0.53 mg), TPA (0.53 mg), IPA (0.46 mg), IPO (0.43 mg), and ZF (0.29).

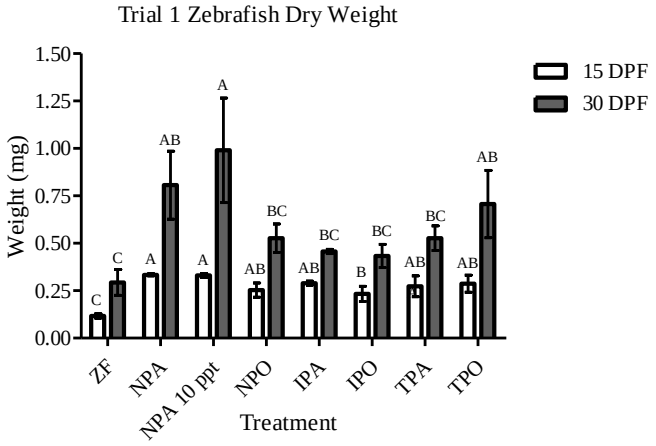


Figure 16 - Dry weight of fish from trial 1, at 15 and 30 dpf. Letters indicate significant difference using Chi-Square test ($p \leq 0.05$) (n =10). ZF- Zebrafeed®; NPA – Fish fed rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt – Fish fed rotifers enriched with *Nannochloropsis* sp. reared at 10 parts per thousand (ppt); NPO – Fish fed rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Fish fed rotifers enriched with *Isochrysis* sp. Paste; IPO – Fish fed rotifers enriched with *Isochrysis* sp. Powder; TPA – Fish fed rotifers enriched with *Tetraselmis* sp. Paste; TPO – Fish fed rotifers enriched with *Tetraselmis* sp. Powder.

Zebrafish survival was determined at 15 and 30 dpf (Figure 17). At 15 dpf no significant difference in fish survival was observed between treatments. Survival ranged from 88.6% (TPA) to 98.3% (NPA 10 ppt). At 30 dpf, fish fed with NPA 10 ppt had the highest survival, 79.3%, while the ZF group displayed the lowest survival (38.3%). Amongst the rotifer fed treatment groups, no significant differences in zebrafish survival were observed, however all rotifer fed groups had significantly higher survival at 30 dpf than the ZF group.

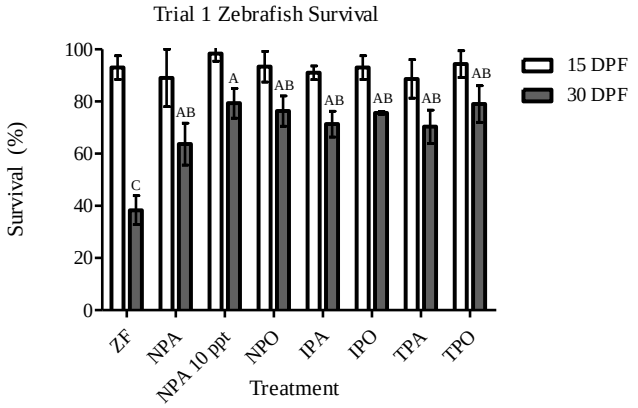


Figure 17: Zebrafish survival in Trial 1, at 15 and 30 dpf (*n* = 100). Letters indicate significant differences using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF- Zebrafeed®; NPA – Fish fed rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt – Fish fed rotifers enriched with *Nannochloropsis* sp. reared at 10 parts per thousand (ppt); NPO – Fish fed rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Fish fed rotifers enriched with *Isochrysis* sp. Paste; IPO – Fish fed rotifers enriched with *Isochrysis* sp. Powder; TPA – Fish fed rotifers enriched with *Tetraselmis* sp. Paste; TPO – Fish fed rotifers enriched with *Tetraselmis* sp. Powder.

The condition factor of zebrafish from trial 1 were significantly different between all treatments at 15 dpf with values ranging from 0.99 in ZF to 1.42 in NPA 10 ppt (Table 6). At 30 dpf those fed with NPO, IPA and IPO did not show significant differences while all other treatments did. The lowest condition factor was found in fish fed rotifers enriched with NPO and IPA at 0.83, while the highest was in those fed with NPA 10 ppt at 1.05.

Table 6: Zebrafish condition factor in Trial 1 at 15 and 30 days post fertilization (dpf) (*n* = 10). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$).

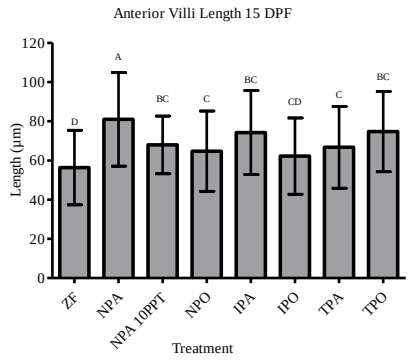
Treatment	15 dpf	30 dpf
ZF	0.99^{G}	0.98^{C}
NPA	1.28^{C}	0.94^{D}
NPA 10 ppt	1.42^{A}	1.05^{A}
NPO	1.10^{F}	0.83^{F}
IPA	1.19^{D}	0.83^{F}
IPO	1.29^{C}	0.81^{F}
TPA	1.14^{E}	0.87^{E}
TPO	1.32^{B}	1.01^{B}

ZF- Zebrafeed®; NPA – Fish fed rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 PPT – Fish fed rotifers enriched with *Nannochloropsis* sp. reared at 10 parts per thousand (ppt); NPO – Fish fed rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Fish fed rotifers enriched with *Isochrysis* sp. Paste; IPO – Fish fed rotifers enriched with *Isochrysis* sp. Powder; TPA – Fish fed rotifers enriched with *Tetraselmis* sp. Paste; TPO – Fish fed rotifers enriched with *Tetraselmis* sp. Powder.

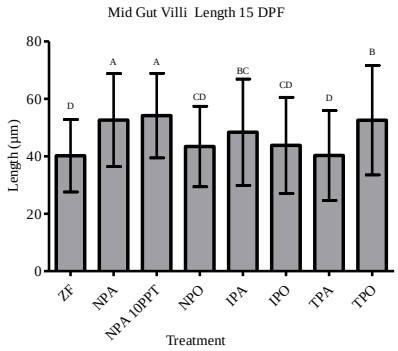
4.1.4 Histology

Intestinal villi were sampled at 15 and 30 dpf, (Figure 18). Anterior villi at 15 dpf (18A) were longest in the NPA treatment (80.96 µm) and shortest in the fish fed with ZF (56.36 µm). Similarly, the NPA 10 ppt group displayed the highest mid gut villi length (18B), 54.16 µm, while fish of the ZF group had the lowest (40.15 µm). At 30 dpf, anterior villi (18C) was longest in fish fed with NPA (99.36 µm), while the IPA group (82.41 µ) were the shortest. Mid gut villi at 30 dpf were longest in NPA (74.41 µm) and shortest in TPO (58.33 µm) (18D).

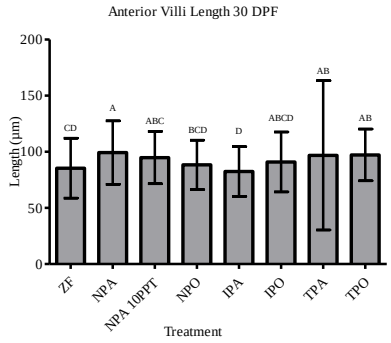
A.



B.



C.



D.

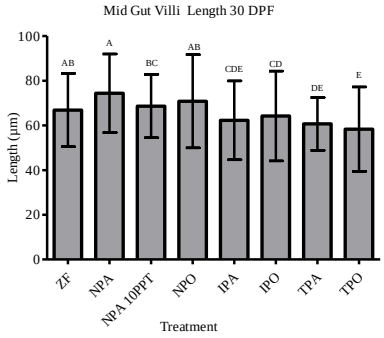


Figure 18: Zebrafish gut villi length in Trial 1. **A.** Anterior gut villi length at 15 dpf. **B.** Mid gut villi length at 15 dpf. **C.** Anterior gut villi length at 30 dpf. **D.** Mid gut length at 30 dpf. Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$ ZF- Zebrafeed®; NPA – Fish fed rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt – Fish fed rotifers enriched with *Nannochloropsis* sp. reared at 10 parts per thousand (ppt); NPO – Fish fed rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Fish fed rotifers enriched with *Isochrysis* sp. Paste; IPO – Fish fed rotifers enriched with *Isochrysis* sp. Powder; TPA – Fish fed rotifers enriched with *Tetraselmis* sp. Paste; TPO – Fish fed rotifers enriched with *Tetraselmis* sp. Powder.

The anterior gut villi of a zebrafish fed rotifers enriched with *Nannochloropsis* sp. is presented below in Figure 19. The villi amongst treatments had normal development.

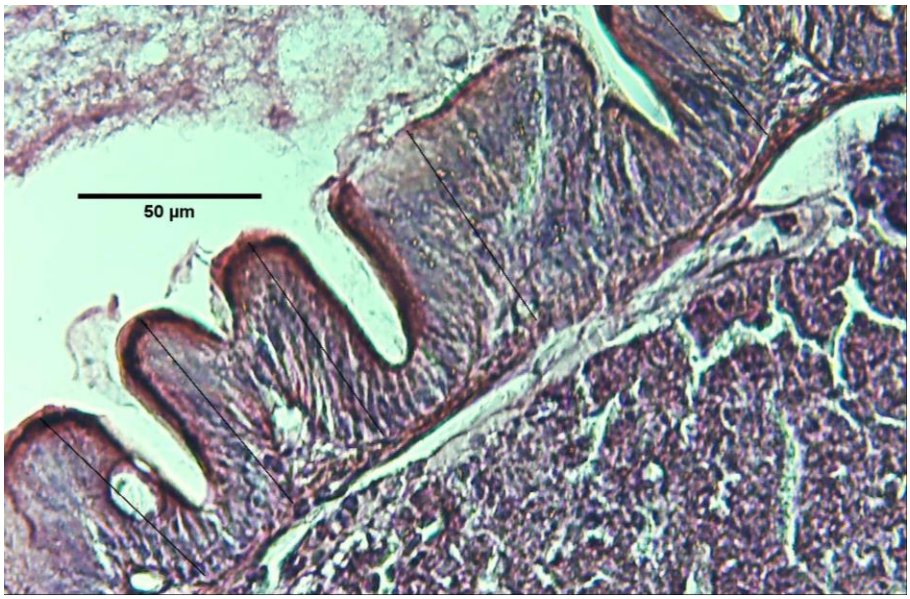


Figure 19: Mid gut villi of zebrafish fed rotifers enriched with *Nannochloropsis* sp. at 30 dpf. Stained with hematoxylin and eosin (40x).

4.2 Trial 2 Investigation of Promising Microalgae Species for Use with Zebrafish

4.2.1 Microalgae and Rotifer Proximal Composition

The proximal composition of microalgae are presented below in Table 7. Amongst microalgae, the biomass of *Isochrysis* sp. (IE2) displayed the highest total lipid content, 26.7% DW, while *Skeletonema* sp. (SKEL) displayed the lowest (8.6% of DW). *Spirulina* sp. (SPIRULI) displayed the highest content of proteins, (65.7% DW) and *Chaetoceros* sp. (CHC) the lowest (37.0% DW). On the other hand, SKEL had the highest ash content (35.9% DW) and SPIRULI the lowest (8.2% DW). Total CHO were highest in NPA (25.1% DW) and lowest in SPIRULI (5.6% DW).

Proximal compositions were determined for the commercial diet and enriched rotifers (Table 8). Amongst enriched rotifers, total lipids were highest in NPA ROTS (14.7% DW) and lowest in IE2 ROTS (8.2% DW). Total proteins were highest in SPIRULI ROTS at 46.0% DW and lowest in CHC ROTS at 31.2% DW. Regarding ashes, the highest content was detected in CHC ROTS (45.4% DW) and lowest in SPIRULI (30.9% DW). Total CHO were highest in SKEL ROTS (19.8% DW) and lowest in SPIRULI ROTS (9.4% DW).

Table 7 - Proximal composition of microalgae (*n* = 3).

Treatment	% Lipids	% Protein	% ASH	% CHO
NPA	17.63	44.61	12.71	25.05
IE2	26.66	43.95	15.00	14.39
TE2	16.82	40.76	26.22	16.20
SPIRULI	20.58	65.70	8.16	5.56
SKEL	8.57	38.08	35.92	17.43
PHAEO	18.78	45.81	21.77	13.64
CHC	15.87	37.03	32.52	14.57

NPA - *Nannochloropsis* sp.; IE2 - *Isochrysis* sp. Experiment 2; TE2 - *Tetraselmis* sp. Experiment 2; SPIRULI – *Spirulina* sp.; SKEL – *Skeletonema* sp.; PHAEO – *Phaeodactylum* sp.; CHC – *Chaetoceros* sp.

Table 8 - Proximal composition of Zebrafeed® and enriched rotifers (*n* = 3).

Treatment	% Lipids	% Protein	% ASH	% CHO
ZF	15.41	66.21	13.00	5.38
NPA ROTS	14.73	34.38	36.07	14.82
IE2 ROTS	8.21	37.82	41.96	12.01
TE2 ROTS	8.6	37.76	35.64	18.00
SPIRULI ROTS	13.75	46.02	30.87	9.35
SKEL ROTS	7.93	33.93	38.34	19.80
PHAEO ROTS	13.8	43.40	31.82	10.98
CHC ROTS	12.02	31.22	45.39	11.37

ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp.; IE2 ROTS - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 ROTS - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI ROTS – Rotifers enriched with *Spirulina* sp.; SKEL ROTS – Rotifers enriched with *Skeletonema* sp.; PHAEO ROTS – *Phaeodactylum* sp.; CHC ROTS – *Chaetoceros* sp.

4.2.2 Fatty Acids Methyl Esters (FAME) of Microalgae, Diet and Enriched Rotifers.

The main fatty acid composition of microalgae are presented below in Table 9, and the complete FAME profiles can be found in Annex 1, Table C. The main fatty acid present in NPA, IE2, TE2 and SPIRULI was C16:0, while C16:1 was the most abundant in SKEL, PHAEO and

CHC. DHA was present in IE2 (18.4% of TFA), PHAEO (1.2% of TFA) and CHC (0.75% of TFA). The highest percentage of ARA was found in NPA (4.5% of TFA).EPA was found in highest amounts in PHAEO (33.2% of TFA) however, none was detected in SPIRULI. CHC (12.9) had the highest *n*-3*:n*-6 PUFA ratio of 12.9 and SPIRULI, 0.00, had the lowest.

In regards to enriched rotifers, the main fatty acid profile is shown in Table 10, while complete FAME profiles are shown in Annex 1, Table D. Amongst enriched rotifers, DHA was only detected in IE2 ROTS (2.4% of TFA). In regards to ARA, the greatest percentage was in IE2 ROTS (4.2% of TFA). Amongst enriched rotifers, EPA was highest in PHAEO ROTS (14.4% of TFA) and lowest in TE2 ROTS (2.8% of TFA). The lowest amount of LA in enriched rotifers was observed in NPA ROTS (5.8% of TFA). The highest *n*-3*:n*-6 PUFA ratio was observed in PHAEO ROTS (0.88% of TFA) and lowest in SPIRULI ROTS (0.18% of TFA). The commercial diet had the lowest amounts of ARA (0.12% of TFA and the highest content of LA (32.5% of TFA) amongst all tested.

Looking at Tables 9 and 10, it is evident that the percentage of LA increased from microalgae to enriched rotifers in SKEL ROTS (11.5% of TFA), PHAEO ROTS (12.4% of TFA) and CHC ROTS (16.0% of TFA).

Table 9 - Main fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae.

Fatty Acid %	NPA	IE2	TE2	SPIRULI	SKEL	PHAEO	CHC
C14:0	-	12.80 ± 0.27	0.54 ± 0.19		26.05 ± 0.40	5.75 ± 0.05	6.97 ± 0.02
C16:0	29.23 ± 0.71	19.71 ± 0.19	58.22 ± 0.41	56.60 ± 0.60	21.38 ± 0.74	19.97 ± 0.01	34.84 ± 0.81
C16:1	28.02 ± 0.31	12.18 ± 0.05	5.68 ± 0.20	6.08 ± 0.28	36.61 ± 0.43	34.63 ± 0.26	36.34 ± 1.34
C18:1c	4.09 ± 0.10	13.28 ± 0.00	2.63 ± 0.83	1.19 ± 1.07	4.29 ± 0.14	0.71 ± 0.08	1.96 ± 0.17
C18:1t	-	3.31 ± 0.13	-	-	-	0.47 ± 0.14	4.93 ± 0.00
C20:1	-	-	0.74 ± 0.26	-	-	-	-
C22:1	-	-	-	-	-	-	-
C24:1	-	-	-	-	-	0.13 ± 0.00	-
C18:3n-6	0.26 ± 0.06		7.90 ± 0.14	15.60 ± 0.19	3.75 ± 1.12	0.10 ± 0.03	1.03 ± 0.53
C18:2n-6c	2.54 ± 0.00	13.18 ± 0.21	7.00 ± 0.13	20.10 ± 0.06	2.15 ± 0.04	2.74 ± 0.02	-
C20:4n-6	4.48 ± 3.98	1.55 ± 0.04	1.12 ± 0.10	-	-	0.66 ± 0.01	-
C20:5n-3	23.82 ± 2.30	1.87 ± 0.03	13.10 ± 0.26	-	0.78 ± 0.07	33.19 ± 0.32	12.42 ± 0.30
C22:6n-3	-	18.44 ± 1.56	-	-	-	1.22 ± 0.35	0.75 ± 0.67
Σn-3 : Σn-6	3.31	1.38	0.82	0.00	0.13	9.86	12.79
PUFA / SFA	1.06	0.99	0.47	0.63	0.13	1.45	0.32

NPA - *Nannochloropsis* sp.; IE2 - *Isochrysis* sp. Experiment 2; TE2 - *Tetraselmis* sp. Experiment 2; SPIRULI – *Spirulina* sp.; SKEL – *Skeletonema* sp.; PHAEO – *Phaeodactylum* sp.; CHC – *Chaetoceros* sp.

Table 10 - Main fatty acid composition % Total fatty acid (%TFA) ± S.D of Zebrafeed® and enriched rotifers.

Fatty Acid %	ZF	NPA ROTS	IE2 ROTS	TE2 ROTS	SPIRULI ROTS	SKEL ROTS	PHAEO ROTS	CHC ROTS
C16:0	28.12 ± 0.71	33.31 ± 1.04	20.91 ± 1.28	43.21 ± 3.11	34.05 ± 1.59	35.69 ± 0.44	26.70 ± 0.06	30.40 ± 0.92
C18:0	7.67 ± 0.10	3.61 ± 0.05	8.97 ± 0.13	8.20 ± 0.66	13.63 ± 9.72	4.83 ± 0.54	4.90 ± 0.11	6.07 ± 0.01
C16:1	3.06 ± 0.22	24.04 ± 0.55	5.72 ± 0.25	7.77 ± 0.10	8.87 ± 1.77	20.12 ± 0.28	17.51 ± 0.26	16.42 ± 0.30
C18:1c	10.52 ± 0.12	9.26 ± 0.15	10.05 ± 0.13	12.69 ± 0.44	5.43 ± 0.32	8.86 ± 0.54	6.78 ± 0.10	9.25 ± 0.22
C18:1t	2.29 ± 0.13	4.94 ± 0.01	4.69 ± 0.09	5.73 ± 0.54	2.08 ± 0.03	4.76 ± 0.13	4.28 ± 0.01	3.26 ± 0.25
C20:1	1.03 ± 0.03	1.82 ± 0.18	4.23 ± 0.05	2.84 ± 0.08	1.11 ± 0.04	1.57 ± 0.04	2.11 ± 0.11	2.15 ± 0.02
C22:1	2.54 ± 0.17	0.65 ± 0.10	3.84 ± 0.24	0.41 ± 0.08	-	-	0.39 ± 0.02	0.14 ± 0.01
C24:1	0.40 ± 0.03	0.23 ± 0.08	4.44 ± 0.30	1.54 ± 1.27	-	2.44 ± 0.16	0.44 ± 0.02	0.49 ± 0.05
C18:2n-6c	32.54 ± 0.12	5.81 ± 0.05	6.20 ± 0.35	8.99 ± 0.02	18.85 ± 4.07	11.45 ± 0.46	12.40 ± 0.08	16.09 ± 0.19
C20:4n-6	0.12 ± 0.02	4.15 ± 0.09	4.17 ± 0.08	1.38 ± 0.33	2.97 ± 0.40	1.88 ± 0.01	3.08 ± 0.07	2.72 ± 0.16
C20:5n-3	3.15 ± 0.08	6.16 ± 0.28	2.92 ± 0.02	2.75 ± 0.31	2.20 ± 0.18	5.49 ± 0.06	14.40 ± 0.12	4.59 ± 0.45
C22:6n-3	6.18 ± 0.10	-	2.36 ± 0.16	-	-	-	-	-
Σn-3 : Σn-6	0.29	0.66	0.46	0.20	0.18	0.42	0.88	0.24
PUFA / SFA	1.10	0.39	0.63	0.31	0.61	0.43	0.92	0.53
EPA : ARA	26.25	1.48	0.70	1.99	0.74	2.92	4.68	1.69

ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp.; IE2 ROTS - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 ROTS - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI ROTS – Rotifers enriched with *Spirulina* sp.; SKEL ROTS – Rotifers enriched with *Skeletonema* sp.; PHAEO ROTS – Rotifers enriched with *Phaeodactylum* sp.; CHC ROTS – Rotifers enriched with *Chaetoceros* sp.

4.2.3 Mineral Analysis of Enriched Rotifers and Zebrafish

Mineral profiles of enriched rotifers in trial 2 are presented in Table 11. K was the most abundant mineral and ranged from 6,420.25 mg/kg in ZF to 31,810.81 mg/kg in NPA ROTS. Another abundant mineral, Ca ranged from 3674.68 mg/kg in ZF to 12,301.62 mg/kg in NPA ROTS. Fe levels were highest in rotifers enriched with the commercial diet, 180.34 mg/kg, and lowest in NPA ROTS, 390.41 mg/kg. P was lowest in CHC ROTS (449.46 mg/kg) and highest in ZF (1080.41 mg/kg). Sr amounts ranged from 47.71 mg/kg to 88.40 mg/kg in PHAEO and SKEL respectively. Zn was not found in TE2 ROTS, SPIRULI ROTS, SKEL ROTS, PHAEO ROTS and CHC ROTS and was highest in ZF (21.04 mg/kg).

Table 11 - Mineral content (mg/kg) ± S.D. of Zebrafeed® and enriched rotifers (n = 3).

	ZF	NPA ROTS	IE2 ROTS	TE2 ROTS	SPIRULI ROTS	SKEL ROTS	PHAEO ROTS	CHC ROTS
Al	62.33 ± 0.09	10.05 ± 0.06	3.64 ± 0.11	9.35 ± 0.32	6.48 ± 0.22	-	6.03 ± 0.13	4.88 ± 0.06
Ba	0.82 ± 0.00	0.45 ± 0.00	0.40 ± 0.01	-	-	-	-	-
Ca	11337.12 ± 145.52	12301.62 ± 278.13	5470.17 ± 61.78	4425.86 ± 24.05	3492.00 ± 27.87	5703.23 ± 149.75	3674.78 ± 36.91	5879.04 ± 84.58
Cr	-	4.18 ± 0.06	-	6.29 ± 0.12	-	-	-	-
Cu	-	4.81 ± 0.04	2.74 ± 0.04	-	-	-	-	-
Fe	180.34 2.77	390.41 ± 2.08	144.30 ± 3.97	206.89 ± 8.53	108.77 ± 2.57	246.44 ± 9.29	118.74 ± 2.93	119.60 ± 0.67
K	6420.25 ± 141.15	31810.81 ± 704.41	13441.04 ± 397.43	11206.78 ± 280.96	11820.43 ± 37.32	13348.32 ± 462.33	13199.69 ± 304.77	13389.93 ± 115.32
Mg	1500.06 ± 17.33	37167.86 ± 971.15	15375.78 ± 257.39	12308.42 ± 263.74	10379.84 ± 146.53	12388.69 ± 226.13	10809.78 ± 105.43	16097.73 ± 93.43
Mn	7.50 ± 0.15	9.87 ± 0.07	5.31 0.07	5.05 ± 0.05	4.98 ± 0.18	5.84 ± 0.10	5.76 ± 0.04	4.99 ± 0.06
Na	6521.65 ± 23.58	59973.84 ± 412.22	25482.30 ± 197.76	76646.15 ± 102.84	56450.81 ± 125.24	119922.94 ± 89.48	22528.55 ± 282.16	30375.19 ± 353.74
Ni	-	0.65 ± 0.02	-	-	-	-	-	-
P	1080.41 ± 3.24	870.57 ± 8.39	451.88 ± 3.88	614.33 ± 5.27	702.51 ± 11.81	711.71 ± 11.01	691.53 ± 3.36	449.46 ± 1.78
Sb	-	19.84 ± 2.49	10.26 ± 0.96	-	-	-	-	-
Sr	69.71 ± 0.41	86.51 ± 0.71	61.56 ± 1.00	67.43 ± 1.38	49.32 ± 0.75	88.40 ± 1.54	47.71 ± 0.64	58.60 ± 0.69
V	6.53 ± 0.08	69.29 ± 1.16	26.25 ± 0.40	-	-	-	-	25.08 ± 0.33
Zn	21.09 ± 0.23	16.10 ± 0.28	10.14	-	-	-	-	-
Ca: P	10.49	14.13	12.11	7.20	4.97	8.01	5.31	13.08

ZF- Zebrafeed®; NPA – Rotifers enriched with *Nannochloropsis* sp.; IE2 - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI – Rotifers enriched with *Spirulina* sp.; SKEL – Rotifers enriched with *Skeletonema* sp.; PHAEO – Rotifers enriched with *Phaeodactylum* sp.; CHC – Rotifers enriched with *Chaetoceros* sp..

Mineral profiles of zebrafish in trial 2 are presented in Table 12. Ca was the most abundant mineral and ranged from 10,289.26 mg/kg in ZF to 21,265.53 mg/kg in NPA. Lower amounts of Fe were found and ranged from 31.21 mg/kg in ZF to 89.92 mg/kg in SPIRULI. P was lowest in ZF (1,339.93 mg/kg) and highest in NPA (2033.98 mg/kg). ZF contained the lowest amount of Sr, 80.00 mg/kg, while IE2 had the highest amount with 167.48 mg/kg. It was determined that at 87.52 mg/kg, SPIRULI had the lowest amount of Zn, while ZF, with 238.51 mg/kg had the most.

Table 12 - Mineral content (mg/kg) ± S.D. of zebrafish (n = 3).

	ZF	NPA	IE2	TE2	SPIRULI	SKEL	PHAEO	CHC
Al	62.21 ± 0.19	11.92 ± 0.39	-	-	-	-	-	-
Ba	-	-	-	-	-	0.74 ± 0.02	-	-
Ca	10289.26 ± 120.05	21165.53 ± 77.87	20792.26 ± 450.11	15363.47 ± 647.13	18982.30 ± 404.53	18874.67 ± 475.64	17722.82 ± 1.38	20133.23 ± 569.74
Cr	-	-	-	-	-	-	-	-
Cu	-	-	-	-	-	-	-	-
Fe	31.21 ± 3.33	25.95 ± 0.61	88.90 ± 4.67	67.53 1.84	89.92 ± 1.75	114.87 ± 1.71	88.32 ± 2.28	89.07 ± 2.77
K	6381.27 ± 290.84	2065.34 ± 35.12	6959.57 ± 113.18	7778.70 ± 168.51	8602.51 ± 132.02	8348.21 ± 170.04	11535.27 ± 273.03	9818.10 ± 141.15
Mg	1273.77 ± 144.84	432.21 ± 2.31	1547.16 ± 18.15	1289.62 ± 5.72	1577.57 ± 25.49	1543.03 ± 28.25	1521.86 ± 6.86	1637.36 ± 13.45
Mn	1.55 ± 0.18	3.34 ± 0.02	3.42 ± 0.06	2.94 ± 0.10	2.73 ± 0.11	3.05 ± 0.07	3.68 ± 0.09	4.12 ± 0.03
Na	5501.00 ± 540.93	4909.92 ± 107.07	4057.31 ± 107.07	4335.68 ± 90.57	4269.95 ± 18.41	4461.33 ± 34.66	5492.27 ± 114.59	5634.93 ± 55.25
Ni	-	-	-	-	-	-	-	-
P	1339.93 ± 2.79	2033.98 ± 42.11	2027.35 ± 52.40	1614.76 ± 59.10	2053.69 ± 24.07	1947.60 ± 29.48	1952.25 ± 38.21	2076.74 ± 10.61
Sr	80.00 ± 0.20	162.23 ± 3.75	167.48 ± 4.59	123.34 ± 3.63	161.18 ± 1.00	143.30 ± 1.49	138.01 ± 0.80	149.67 ± 3.20
V	-	-	-	-	-	-	-	-
Zn	238.51 ± 0.29	121.47 ± 1.27	132.69 ± 0.23	103.53 ± 0.89	87.52 ± 2.73	158.81 ± 2.58	107.42 ± 1.23	116.85 ± 3.62
Ca: P	7.68	10.41	10.26	9.51	9.24	9.69	9.08	9.69

ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

4.2.4 Length, Weight, Survival and Condition Factor

TL was determined at 15 and 30 dpf . At 15 dpf, there was no significant difference amongst rotifer fed treatments (Figure 20), with the exception of fish from the ZF treatment (4.87 mm) that were significantly smaller than all other treatments. At 30 dpf, fish fed with rotifers enriched using *Nannochloropsis* sp. (NPA) were significantly larger than all groups, reaching 13.32 mm, with the exception of the ISO treatment (12.70 mm). Those fed rotifers enriched with ISO were significantly larger than TETRA (11.65 mm), PHAEO (11.59 mm), CHC (11.36 mm) and ZF (6 mm), while the ZF group was significantly shorter than all other treatments.

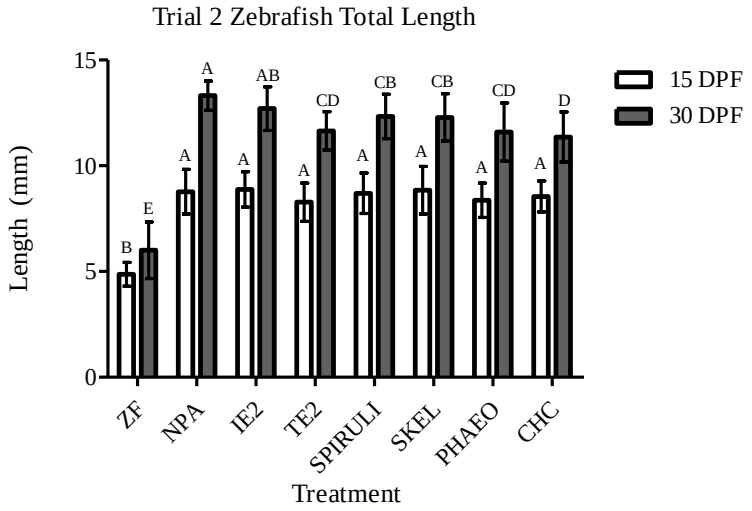


Figure 20 - Total length of zebrafish at 15 and 30 dpf. Letters indicate significant differences using one-way ANOVA with Tukey's Test ($p \leq 0.05$) ($n = 10$). ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

The average DW of fish was determined at 15 and 30 DPF. At 15 dpf no statistical differences were observed between rotifer fed treatment groups, however, ZF with an average dry weight of 0.15 mg were significantly lighter (Figure 21). At 30 dpf the fish fed with NPA had an average dry weight of 2.83 mg and were significantly heavier than those fed CHC and ZF, whose dry weights were 1.66 mg and 0.21 mg respectively. No statistical differences were observed between those given SPIRULI (2.40 mg), ISO (2.33 mg), SKEL (2.09 mg), PHAEO (1.92 mg), TETRA (1.75 mg) and CHC, while ZF was lighter than all other groups.

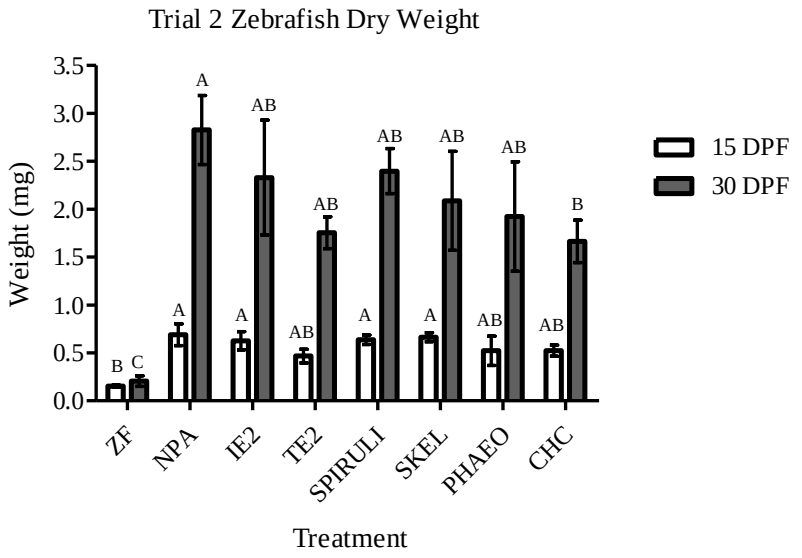


Figure 21: Dry weight (mg) of zebrafish at 15 and 30 DPF . Letters indicate significant differences using Chi-Square test ($p \leq 0.05$) ($n = 10$). ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

Average survival at 15 dpf amongst treatments were not statistically different (Figure 22), with the exception of fish fed with NPA enrichment (93%), which were significantly greater than the SPIRULI group (70 %). At 30 DPF a similar result was observed, the NPA group had the highest survival rate of all treatments, 89%, but was only significantly greater than SPIRULI (62 %) and ZF (27%). The ZF treatment exhibited significantly lower survival than all other groups.

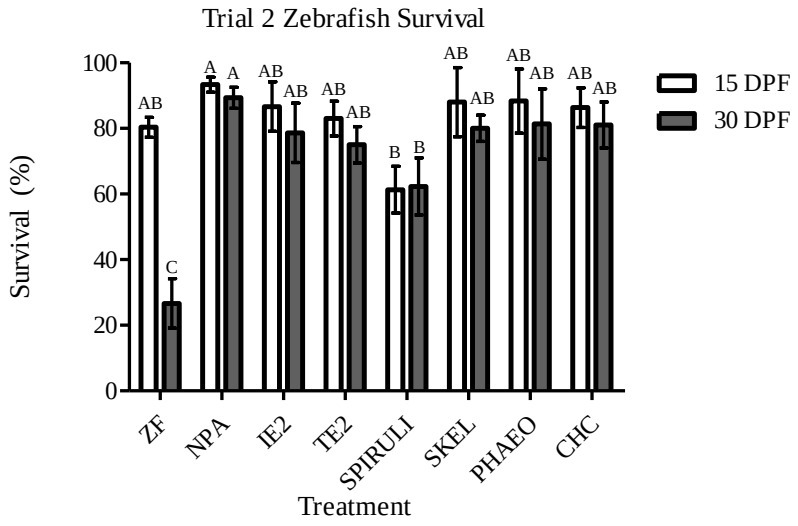


Figure 22 - Zebrafish survival at 15 and 30 days post fertilization (dpf). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$) (n = 100). ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

At 15 dpf, the ZF group was shown to have the highest condition factor (1.32) and TE2 group the lowest (0.82) and was not significantly different than those fed rotifers enriched with CHC with a condition factor of 0.84 (Table 13). At 30 dpf, condition factor ranged between 0.96 in ZF to 1.28 in fish fed rotifers enriched with SPIRULI. TE2, SKEL and CHC were not significantly different from each other at 30 dpf, while those fed rotifers enriched with IE2 were not significantly different from TE2 and CHC.

Table 13 - Zebrafish condition factor at 15 and 30 days post fertilization (dpf). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$) ($n = 10$).

Treatment	15 dpf	30 dpf
ZF	1.32^{A}	0.96^{F}
NPA	1.02^{B}	1.20^{C}
IE2	0.89^{D}	1.14^{D}
TE2	0.82^{E}	1.11^{E}
SPIRULI	0.97^{C}	1.28^{A}
SKEL	0.96^{C}	1.12^{DE}
PHAEO	0.89^{D}	1.24^{B}
CHC	0.84^{E}	1.13^{DE}

ZF- Fish fed with Zebrafeed®; NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

4.2.5 Skeletal Deformities

At 30 dpf skeletal deformities were present in all groups (Figure 23). The lowest incidence of deformities was observed in those fish fed rotifers enriched with *Nannochloropsis* sp. (NPA) (69%) which was significantly lower than those fed with *Skeletonema* sp. enriched rotifers (SKEL) (93%) and *Phaeodactylum* sp. enriched rotifers (PHAEO) (92%). There were no significant differences observed between treatments SKEL, PHAEO, CHC (91%), TE2 (89%), IE2 (86%) and SPIRULI (85%).

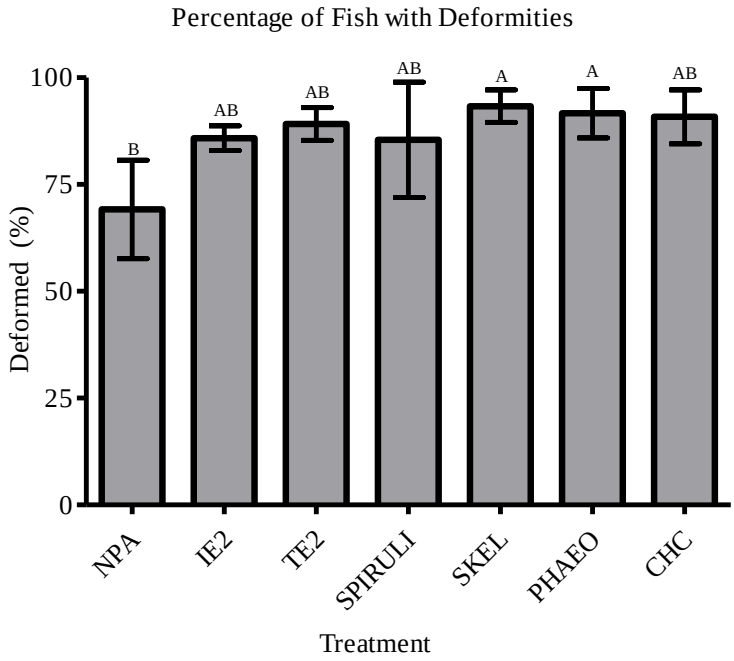


Figure 23 - Incidence of deformities in zebrafish from Trial 2 (n = 40). Letters indicate significant differences using Chi-Square test ($p \leq 0.05$). NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

Fish were considered severely deformed if they had 3 or more regions affected by deformities, 5 or more structures affected by deformities or a deformity which altered the physical appearance such as scoliosis, lordosis or kyphosis. NPA and SKEL had significantly lower incidences of severe deformities when compared with all other groups, 14 and 22%, respectively. CHC had the highest percentage of severe deformities, with up to 37.3% of deformed fish exhibiting severe deformities (Figure 24).

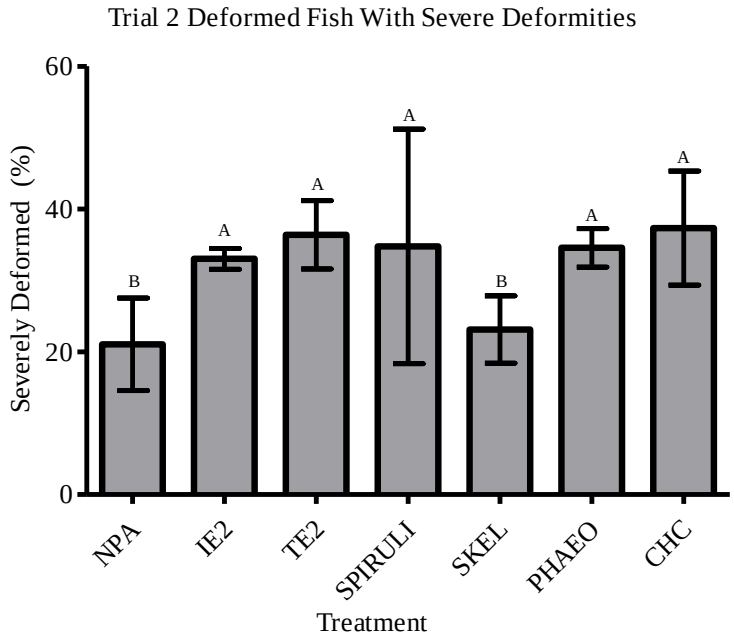


Figure 24 - Percent severe deformities in zebrafish from Trial 2 (n = 40). Letters indicate significant differences using Chi-Square test ($p \leq 0.05$). NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers.

The charge of deformities, the number of deformities in deformed fish, at 30 DPF is shown in Figure 25. The experimental group given rotifers enriched with *Nannochloropsis* sp. (NPA) had the highest percentage of fish with no deformities, 30.33%, while the group SKEL had the lowest (7.5%). The NPA treatment had the highest percentage of fish with 1 deformity with 23.33% being affected, whereas those fed rotifers enriched with CHC had the lowest percentage with 13.33%. In the case of fish exhibiting 2 deformities, SKEL with 30.00% had the highest percentage affected while NPA, with 22.50%, had the lowest. SPIRULI (15.83%) had the highest percentage of fish with 3 deformities, while SKEL (13%) had the lowest percentage. Fish with 4 deformities were found in the highest percentage of those fed with *Chaetoceros* sp. enriched rotifers (CHC) (15%) and in the lowest percentage with those given NPA enriched rotifers (4.17%). A similar result was seen in fish with 5 or more deformities, CHC had the highest percentage, 29.17%, while NPA, 7.5%, had the lowest.

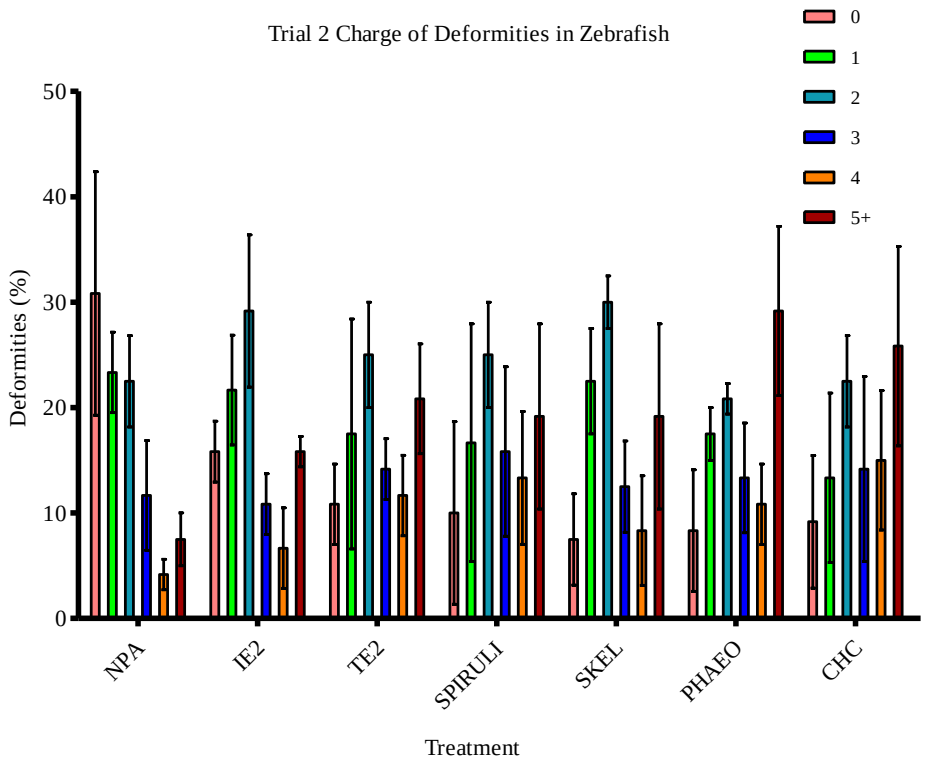


Figure 25 - Charge of deformities (%) in zebrafish from trial 2 (n = 40). Letters indicate significant difference using Chi-Square test ($p \leq 0.05$). NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers. Colors indicate number of deformities in fish. Peach – 0 deformities; Lime green – 1 deformity; Dark green – 2 deformities; Blue – 3 deformities; Orange – 4 deformities; Red – 5 deformities.

Deformity location (Figure 26) was determined in 30 dpf larvae to verify which structures were most affected by deformities. In all treatments the regions most affected were the caudal vertebrae and caudal fin vertebrae. In the caudal vertebrae region, a significant difference in the percentage of deformities was observed between all treatment groups. The CHC group had the highest percentage of deformities in the caudal vertebrae, 57.07%, while those fed rotifers enriched with *Spirulina* sp. (SPIRULI) had the lowest, with 24.56% affected. In the caudal fin vertebrae region, a significant difference in the amount of deformities was present between all groups. The

NPA group, 65.78%, had the highest percentage of deformities in the caudal fin vertebrae region while CHC exhibited 35.70% of deformities in this region and was the lowest. The head was the region least affected by deformities, fish fed rotifers enriched with NPA (2.81%) had the highest percentage of deformities while SKEL (0.33%) had the lowest. In the abdominal region, the highest average percentage of deformities was seen in SKEL which had 7.39% of fish affected, while PHAEO (0.50%) had the lowest. SPIRULI (6.14%) had the highest average percentage of deformities in the caudal fin, while CHC (2.99%) had the lowest.

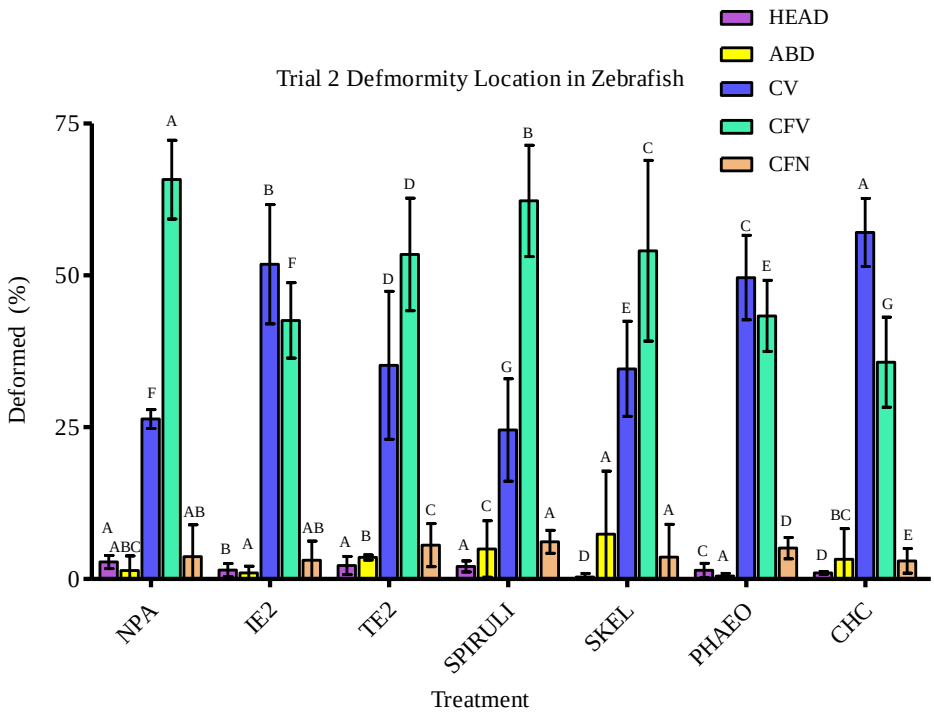


Figure 26 - Location of deformities in deformed fish for trial 2 (n = 40). Letters indicate significant difference between regions using Chi-Square test ($p \leq 0.05$). NPA - Fish fed with *Nannochloropsis* sp. enriched rotifers; IE2 - Fish fed with *Isochrysis* sp. Experiment 2 enriched rotifers; TE2 - Fish fed with *Tetraselmis* sp. Experiment 2 enriched rotifers; SPIRULI – Fish fed with *Spirulina* sp. enriched rotifers; SKEL – Fish fed with *Skeletonema* sp. enriched rotifers; PHAEO – Fish fed with *Phaeodactylum* sp. enriched rotifers; CHC – Fish fed with *Chaetoceros* sp. enriched rotifers. Head – Head; ABD – Abdominal Vertebrae; CV - Caudal Vertebrae; CFV – Caudal Fin Vertebrae; CFN – Caudal Fin.

The majority of deformities in the caudal vertebrae and caudal fin vertebrae were fused vertebrae and double arches, minor deformities which do not affect the physical appearance (Figure 27-A). Scoliosis, a severe deformity, was found in a few individuals sampled in the caudal

vertebrae and caudal fin vertebrae (Figure 27-B). In the abdominal region, deformities tended to affect the centra, producing an hour-glass appearance (Figure 27-C).

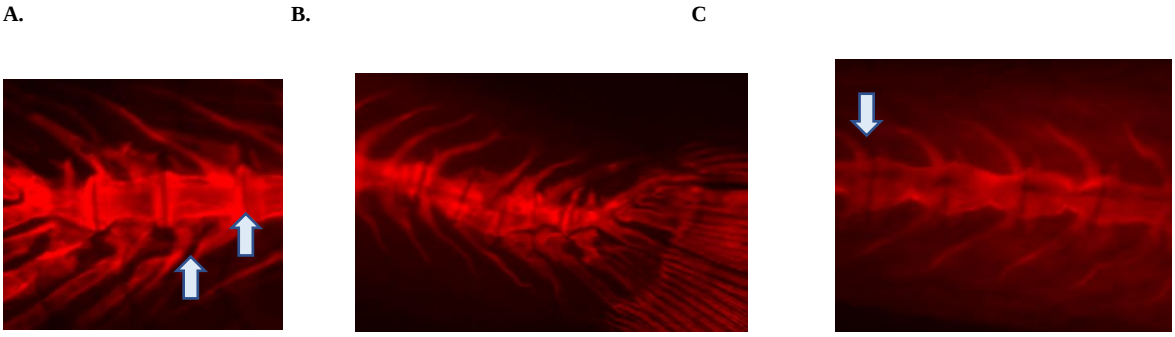


Figure 27 - A**.** Double haemal arch and fused vertebrae between caudal vertebrae and caudal fin vertebrae in fish fed rotifers enriched with IE2. B**.** Lateral view of fish fed rotifers enriched with SPIRULI with scoliosis effecting caudal vertebrae and caudal fin vertebrae region. C**.** Deformed abdominal centra in SKEL treatment.

Due to their potential, four microalgae from trial 2, *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp. and *Skeletonema* sp. were selected for incorporation into the blended formula. Groups fed rotifers enriched with IE2, SPIRULI and SKEL all exhibited high length and weight at 30 dpf. Those fed rotifers enriched with SKEL had the second lowest percentage of fish with severe deformities and TE2, SPIRULI and SKEL have high percentages of LA making these microalgae promising components of a live feed enrichment.

4.3 Trial 3: Development of Blended Enrichment Formulas

4.3.1 Microalgae and Rotifer Proximal Composition

The proximal composition of microalgae were determined in trial 3 (Table 14). Amongst microalgae, blend C with 19.49% DW lipids had the highest total lipid content, while B had the lowest with 11.73% DW. The percent protein was determined to be highest in blend B, at 46.29% DW and lowest in A, 25.10% DW. With a total percent ash of 40.51% DW, blend C had the highest amount, while the lowest was found in NPA with 12.71% DW. Regarding, percent carbohydrates, NPA had the highest percentage while blend C had the lowest with 25.05% and 14.81% DW respectively.

Enriched rotifers exhibited a different proximal composition than microalgae (Table 15). Rotifers enriched with *Nannochloropsis* sp. (NPA), had the highest lipid content at 14.73% DW, while rotifers enriched with blend B exhibited the lowest lipid amount, 11.23% DW. Contrary to

the microalgae, rotifers enriched with blend A were found to have the highest protein content at 59.35% DW, while NPA at 34.38% DW had the lowest. NPA was composed of the highest percent ash at 36.07% DW while rotifers enriched using blend A, with 36.07% DW, contained the least. Total carbohydrates were determined to be highest in A at 22.1 % DW and lowest in NPA, 14.82%DW.

The commercial diet had the highest total percent lipid (15.41%), total protein (66.21%) and lowest total percent CHO (5.38%) of all feeds measured.

Table 14 - Proximal composition of microalgae used in trial 3 (*n* = 3).

Treatment	% Lipids	% Protein	% ASH	% CHO
NPA	17.63	44.61	12.71	25.05
A	15.61	25.10	35.83	23.45
B	11.73	46.29	24.35	17.64
C	19.49	25.19	40.51	14.81

ZF - Zebrafeed®; NPA – rotifers enriched with *Nannochloropsis sp.*; CF –co-feeding using rotifers enriched with *Nannochloropsis sp.* until 8 DPF and Zebrafeed®; A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.), B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.) and C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Table 15 - Proximal composition of Zebrafeed® and enriched rotifers used in trial 3 (*n* = 3).

Treatment	% Lipids	% Protein	% ASH	% CHO
ZF	15.41	66.21	13.00	5.38
NPA	14.73	34.38	36.07	14.82
A	14.27	59.35	4.29	22.10
B	11.23	56.20	13.42	19.15
C	13.00	58.10	8.40	20.50

ZF - Zebrafeed®; NPA –rotifers enriched with *Nannochloropsis sp.*; CF – co-feeding using rotifers enriched with *Nannochloropsis sp.* until 8 DPF and Zebrafeed®; A – Rotifers enriched with blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.), B - Rotifers enriched with blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C - Rotifers enriched with blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

4.3.2 Fatty Acids Methyl Esters (FAME) of Microalgae, Zebrafeed® and Enriched Rotifers.

The main fatty acid composition is presented below in Table 16 and complete FAME profiles can be found in Annex 1 Table E. In all samples C16:0 was the main FAME present, except in the commercial diet, which was highest in LA (32.5% of TFA). DHA was determined to be highest in the diet (6.2% of TFA), amongst microalgae it was present in Blend C at 1.6% of TFA and in enriched rotifers, was found in equal amounts in B ROTS and C ROTS (1.33% of TFA). EPA was present in the greatest amount in blend C at 14.38% of TFA, while in enriched rotifers it was most abundant in A ROTS at 8.81% of TFA. In regards to ARA, Zebrafeed® had the lowest amounts of ARA (0.12% of TFA) while blend A ROTS (7.49% of TFA) had the most.

Table 16 Main fatty acid composition % Total fatty acid (%TFA) ± S.D of microalgae, Zebrafeed® and enriched rotifers for trial 3 (n = 3).

Fatty Acid %	NPA	NPA ROTS	ZF	Blend A	A ROTS	Blend B	B ROTS	Blend C	C ROTS
C16:0	29.23 ± 0.71	33.31 ± 1.04	28.12 ± 0.71	36.55 ± 0.12	26.60 ± 0.38	39.26 ± 0.18	25.03 ± 0.27	27.42 ± 0.41	25.55 ± 0.55
C16:1	28.02 ± 0.31	24.04 ± 0.55	3.06 ± 0.22	10.19 ± 0.24	17.06 ± 0.21	12.32 ± 0.37	14.44 ± 0.35	20.86 ± 0.20	15.84 ± 0.60
C18:1c	4.09 ± 0.10	9.26 ± 0.15	10.52 ± 0.12	8.33 ± 0.05	6.98 ± 0.03	5.30 ± 0.17	6.28 ± 0.23	6.77 ± 0.19	6.88 ± 0.05
C20:1	-	1.82 ± 0.18	1.03 ± 0.03		2.12 ± 0.01		2.40 ± 0.28		2.27 ± 0.09
C22:1	-	0.65 ± 0.10	2.54 ± 0.17		1.71 ± 0.01		1.73 ± 0.13		1.75 ± 0.03
C24:1	-	0.23 ± 0.08	0.40 ± 0.03		1.76 ± 0.02		2.08 ± 0.08		1.86 ± 0.02
C18:2n-6c	2.54 ± 0.00	5.81 ± 0.05	32.54 ± 0.12	15.71 ± 0.03	5.39 ± 0.04	11.88 ± 0.06	7.38 ± 0.03	4.27 ± 0.19	5.41 ± 0.01
C20:4n-6	4.48 ± 3.98	4.15 ± 0.09	0.12 ± 0.02	4.72 ± 0.13	7.49 ± 0.11	4.92 ± 0.05	6.68 ± 0.22	9.06 ± 0.06	7.27 ± 0.19
C20:5n-3	23.82 ± 2.30	6.16 ± 0.28	3.15 ± 0.08	7.57 ± 0.18	8.81 ± 0.13	6.82 ± 0.11	7.61 ± 0.57	14.38 ± 0.16	8.36 ± 0.33
C22:6n-3	-	-	6.18 ± 0.10	0.59 ± 0.02	1.22 ± 0.01	1.17 ± 0.10	1.33 ± 0.10	1.60 ± 0.02	1.33 ± 0.01
Σn-3 : Σn-6	3.31	0.66	0.29	0.72	0.81	0.36	0.59	1.11	0.72
PUFA / SFA	1.06	0.39	1.10	0.62	0.69	0.77	0.80	0.83	0.69
EPA : ARA		1.48	26.25		1.18		1.14		1.15

NPA – *Nannochloropsis* sp. microalgae; NPA ROTS –rotifers enriched with *Nannochloropsis* sp.; ZF - Zebrafeed®; Blend A – Microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); Blend A ROTS – rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); Blend B – Microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); B ROTS – rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); Blend C – microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.); C ROTS – rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

4.3.3 Mineral Analysis of Enriched Rotifers and Zebrafish

Mineral profiles of enriched rotifers in trial 3 are presented in Table 17. Aside from Na, K was the most abundant mineral, ranging from 6,420.25 mg/kg in ZF, to 31,810.81 mg/kg in NPA ROTS. Ca was determined to be in lowest amounts in A ROTS, 2456.86 mg/kg, and highest amounts in NPA ROTS, 12,301.62 mg/kg. The lowest amount of Fe at 180.34 mg/kg was found in ZF while 390.41 mg/kg was the highest in NPA ROTS. In regards to P, A ROTS, 801.29 mg/kg, had the lowest levels while ZF, 1080.41 mg/kg, had the highest. Sr contents

ranged from 26.38 mg/kg to 86.51 mg/kg in A ROTS and NPA ROTS respectively. Zn totals were lowest in NPA ROTS (16.10 mg/kg) and highest in B ROTS (21.29 mg/kg).

Table 17 - Mineral content (mg/kg) ± S.D. of Zebrafeed® and enriched rotifers in trial 3 (*n* = 3).

	NPA ROTS	ZF	A ROTS	B ROTS	C ROTS
Al	10.05 ± 0.06	62.33 ± 0.09	3.11 ± 0.05	6.14 ± 0.20	3.67 ± 0.19
Ba	0.45 ± 0.00	0.82 ± 0.00	0.36 ± 0.00	0.31 ± 0.01	0.52 ± 0.01
Ca	12301.62 ± 278.13	11337.12 ± 145.52	2456.86 ± 69.44	2669.31 ± 41.39	3328.44 ± 120.05
Cr	4.18 ± 0.06	-	1.22 ± 0.00	0.31 ± 0.00	0.88 ± 0.01
Cu	4.81 ± 0.04	-	3.54 ± 0.04	2.68 ± 0.08	3.54 ± 0.07
Fe	390.41 ± 2.08	180.34 2.77	214.63 ± 3.58	200.43 ± 2.65	331.79 ± 3.33
K	31810.81 ± 704.41	6420.25 ± 141.15	11549.40 ± 51.81	11566.69 ± 141.23	12179.75 ± 290.84
Mg	37167.86 ± 971.15	1500.06 ± 17.33	6158.91 ± 20.87	6173.65 ± 105.28	7582.43 ± 144.84
Mn	9.87 ± 0.07	7.50 ± 0.15	5.64 ± 0.13	5.47 ± 0.06	6.59 ± 0.18
Na	59973.84 ± 412.22	6521.65 ± 23.58	19545.95 ± 831.92	28629.17 ± 195.78	23732.80 ± 540.93
Ni	0.65 ± 0.02	-	0.48 ± 0.03	0.30 ± 0.02	0.28 ± 0.02
P	870.57 ± 8.39	1080.41 ± 3.24	801.29 ± 16.61	906.95 ± 5.90	782.39 ± 2.79
Sb	19.84 ± 2.49	-	-	-	-
Sr	86.51 ± 0.71	69.71 ± 0.41	26.38 ± 0.34	28.02 ± 0.13	34.73 0.20
V	69.29 ± 1.16	6.53 ± 0.08	7.82 ± 0.24	8.49 ± 0.26	11.46 ± 0.26
Zn	16.10 ± 0.28	21.09 ± 0.23	19.53 ± 0.51	21.29 ± 0.45	20.75 ± 0.29
Ca: P	14.13	10.49	3.07	2.94	4.25

ZF - Zebrafeed®; NPA ROTS – rotifers enriched with *Nannochloropsis sp.*; CF – co-feeding using rotifers enriched with *Nannochloropsis sp.* until 8 DPF and Zebrafeed®; A ROTS – rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B ROTS – rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C ROTS – rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

From the mineral profiles of zebrafish in trial 3 (Table 18) Ca was present in higher amounts than any other mineral and ranged from 10,289.26 mg/kg in ZF to 21,866.03 mg/kg in NPA. Fe levels varied between 25.95 mg/kg and 107.95 in NPA and C respectively. P was lowest in ZF (1339.93 mg/kg) and highest in B (2156.78 mg/kg). While Sr levels were determined to be lowest in ZF at 80.00 mg/kg and highest in B 173.36 mg/kg. Regarding Zn, ZF was found to have the greatest amont with 238/51 mg/kg and NPA had the least with 121.47 mg/kg.

Table 18 - Mineral content (mg/kg) ± S.D. of zebrafish from trial 3 (*n* = 3).

	ZF	NPA	CF	A	B	C
Al	62.21 ± 0.19	11.92 ± 0.39	9.89 ± 0.33	21.21 ± 0.51	-	-
Ba	-	-	-	1.17 ± 0.03	0.90 ± 0.02	-
Ca	10289.26 ± 120.05	21165.53 ± 77.87	16467.23 ± 346.49	19919.22 ± 459.81	20630.47 ± 329.67	21866.03± 642.36
Cr	-	-	-	-	-	-
Cu	-	-	-	-	-	-
Fe	31.21 ± 3.33	25.95 ± 0.61	60.40 ± 3.19	93.52 ± 1.41	98.67 ± 3.85	107.86 ± 4.27
K	6381.27 ± 290.84	2065.34 ± 35.12	10127.82 ± 236.54	8850.38 ± 74.33	11706.61 ± 200.75	8127.99 ± 129.39
Mg	1273.77 ± 144.84	432.21 ± 2.31	1244.41 ± 9.33	1531.99 ± 39.39	1597.42 ± 12.39	1692.81 ± 32.16
Mn	1.55 ± 0.18	3.34 ± 0.02	1.25 0.04	5.32 ± 0.08	4.36 ± 0.03	4.55 ± 0.03
Na	5501.00 ± 540.93	4909.92 ± 107.07	5179.46 ± 117.49	5172.75 ± 72.80	5817.65 ± 42.15	5284.51 ± 112.15
Ni	-	-	-	-	-	-
P	1339.93 ± 2.79	2033.98 ± 42.11	1736.39 ± 29.41	2008.23 ± 35.07	2156.78 ± 17.97	2110.61 ± 10.68
Sr	80.00 ± 0.20	162.23 ± 3.75	102.38 ± 1.36	170.63 ± 1.60	173.36 ± 1.62	-
V	-	-	-	-	-	-
Zn	238.51 ± 0.29	121.47 ± 1.27	149.79 ± 2.22	155.60 ± 3.08	168.04 ± 3.01	199.62 ± 2.93
Ca: P	7.68	10.41	9.48	9.92	9.57	10.36

ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

4.3.4 Length, Weight, Survival and Condition Factor

At 15 dpf, no statistical differences were observed in TL amongst rotifer fed treatment groups. However, a significant difference existed between ZF (5.86 mm), CF (8.59 mm) and all rotifer fed treatments (Figure 28). At 30 dpf, no statistical differences were observed between rotifer fed groups. ZF and CF at 8.05 mm and 10.62 mm respectively, were significantly shorter than all rotifer fed treatment groups. With a TL of 9.27 mm, fish fed rotifers enriched with blend A were the largest at 15 dpf, and those given blend C were the largest at 30 dpf, 11.48 mm, while ZF were the shortest at both 15 dpf and 30 dpf.

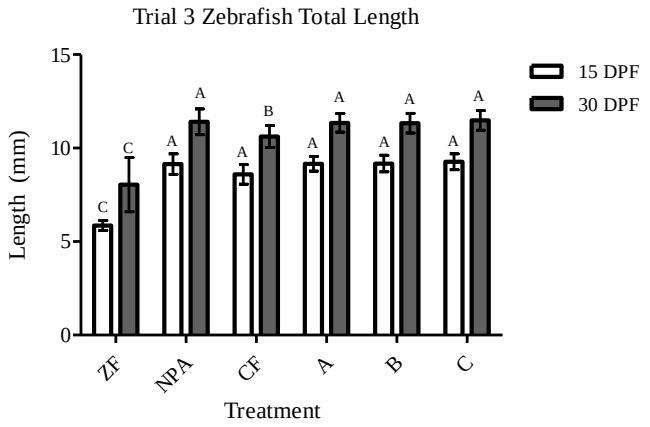


Figure 28 - Average length (mm) of zebrafish at 15 and 30 days post fertilization (dpf) for trial 3 (n = 40). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

. In regards to weight, at 15 dpf, fish fed with ZF (0.13 mg) and CF (0.44 mg) were significantly different than each other and weighed significantly less than all rotifer fed treatment groups (Figure 29). At 15 dpf, the fish fed rotifers enriched using NPA had reached a weight of 0.66 mg and were the heaviest group. At 30 dpf, the fish fed with ZF were 0.45 mg and significantly lighter than all other treatment groups.

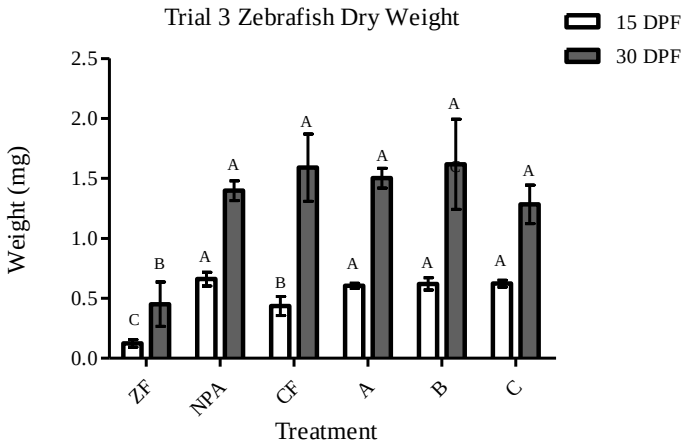


Figure 29 - Average dry weight (mg) of zebrafish at 15 and 30 days post fertilization dpf for trial 3 (n = 40). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

For trial 3, no significant differences were observed between groups at 15 dpf (Figure 30). At 30 dpf no significant differences were observed between rotifer fed groups and CF however, ZF, with a survival of 46.75% were found to have significantly lower survival than all other treatments. Treatment C (98.50%) had the highest average survival at 30 dpf.

The blended microalgae groups had higher survival rates at 15 and 30 dpf than all groups in both trials 1 and 2.

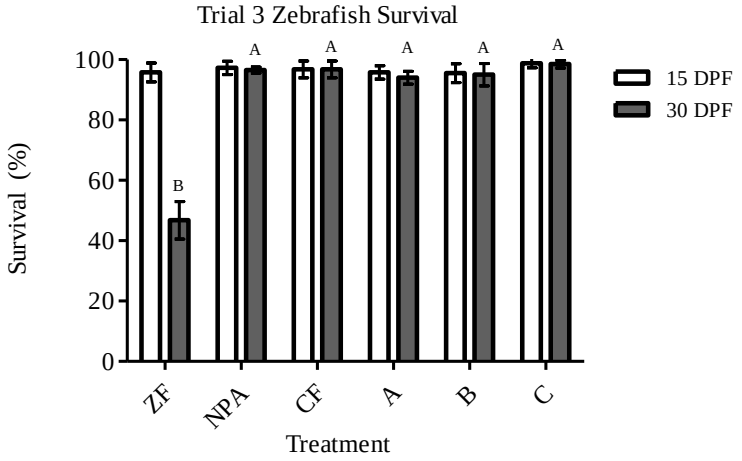


Figure 30 - Average survival (%) of zebrafish at 15 and 30 days post fertilization (dpf) for trial 3 (n = 100). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). **Figure 29 -** Average dry weight (mg) of zebrafish at 15 and 30 days post fertilization dpf for trial 3 (n = 40). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

Condition factor was calculated for zebrafish at 15 and 30 dpf (Table 19). At 15 dpf, the highest condition factor of 0.86 was found in those fed rotifers enriched with NPA and the lowest of 0.55 found in fish from group C. A significant difference in condition factor was found between all treatments except for A and B. At 30 dpf, the condition factor ranged from 0.87 in ZF to 1.33 in CF with significant difference being observed between all treatments.

Table 19: Trial 3 zebrafish condition factor for 15 and 30 days post fertilization (dpf) (n = 10).

Treatment	15 dpf	30 dpf
ZF	0.65^{D}	0.87^{F}
NPA	0.86^{A}	0.94^{E}
CF	0.69^{C}	1.33^{A}
A	0.79^{B}	1.03^{D}
B	0.80^{B}	1.11^{B}
C	0.55^{E}	1.07^{C}

Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). ZF - Zebrafeed®; NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

4.3.5 Skeletal Deformities

Deformities were present in all experimental groups at 30 DPF, see Figure 31 with CF having significantly more skeletal deformities than those fed rotifers enriched with NPA. Fish fed with the CF enrichment had the highest average percentage of deformed fish (96.88%), while NPA had the lowest, 89.38%.

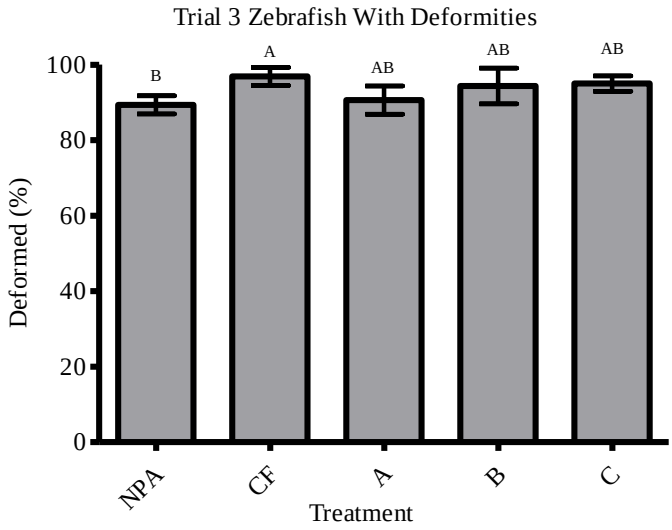


Figure 31 - Incidence of deformities at 30 days post fertilization (dpf) in zebrafish from Trial 3 (n = 40). Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

The percentage of deformed fish with severe deformities are presented in Figure 32. No statistical differences were observed between rotifer fed treatments, however the lowest percentage of deformed fish with severe deformities was found in the group fed blend A with 18.57%. The CF group had significantly more severely deformed fish than all other treatments, with 76.12% affected. Individuals with severe deformities tended to have 5+ deformities, however in the CF group a high percentage were affected by scoliosis and kyphosis.

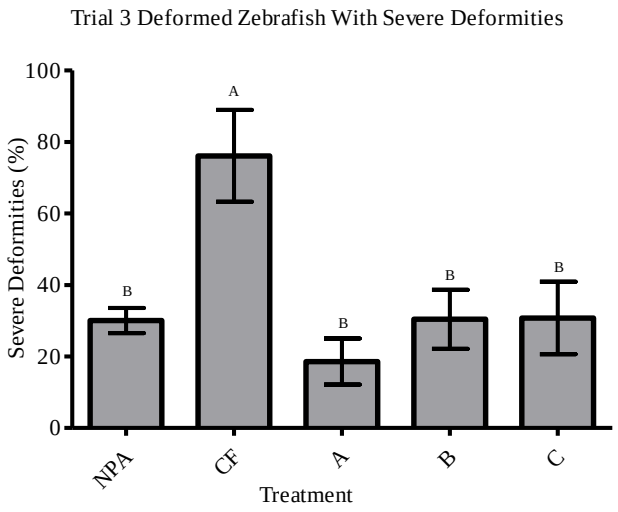


Figure 32 - Percentage (%) of deformed zebrafish with severe deformities at 30 days post fertilization (dpf) (n = 40). Severe deformities classified as three or more regions affected by deformity, five or more deformities or any deformity affecting the physical appearance. Letters indicate significant difference using one-way ANOVA with Tukey's Test ($p \leq 0.05$). NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.).

The charge of deformities, the number of deformities in fish, is presented in Figure 33**.** The NPA treated fish had the highest percentage of individuals with no deformities, 11.25%, and the CF group had the lowest, 3.13%. Fish fed rotifers enriched with blend A led to the highest percentage of fish with 1 deformity, 20.63% and CF, 4.38%, the lowest. The highest percentage of fish with 2 deformities were those given blend A, 28.75%, compared to the CF group which had the lowest percentage affected (4.38%). In regards to those with 3 deformities, fish fed rotifers enriched with blend B had the highest amount and CF the lowest, 29.38% and 6.25% respectively. The highest percentage of fish with 4 deformities was found in those given blend C, 20%, while CF yielded, had the lowest incidence (6.88%). Conversely, the CF group had the highest percentage of fish with 5 or more deformities, 73.13%, while B had the lowest, 9.38%.

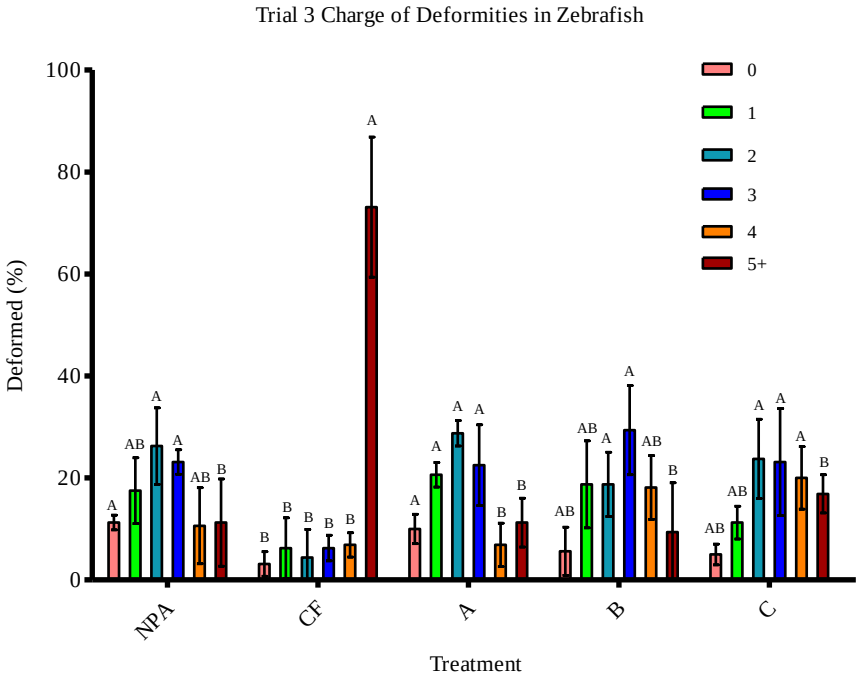


Figure 33 - Charge of deformities in deformed fish at 30 days post fertilization (dpf) for trial 3 (n = 40). Letters indicate significant difference between regions using one-way ANOVA with Tukey's test ($p \leq 0.05$). NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.). Colors indicate number of deformities in fish. Peach – 0 deformities; Lime green – 1 deformity; Dark green – 2 deformities; Blue – 3 deformities; Orange – 4 deformities; Red – 5 deformities.

Deformity location is presented in Figure 34. The highest percentage of deformities in the head, abdominal and caudal fin region were found in CF, 3.4, 16.3 and 23.0% respectively, deformities in this treatment were significantly higher than in all other treatments. The highest percentage of deformities in the caudal vertebrae were found in fish fed rotifers enriched with NPA (37.4%) and the lowest percentage in CF (16.3%) which was significantly lower than all other treatments. In the caudal fin vertebrae, fish fed rotifers enriched with blend B with 69.3% had the highest percentage of deformities and CF (41.0%) had the lowest.

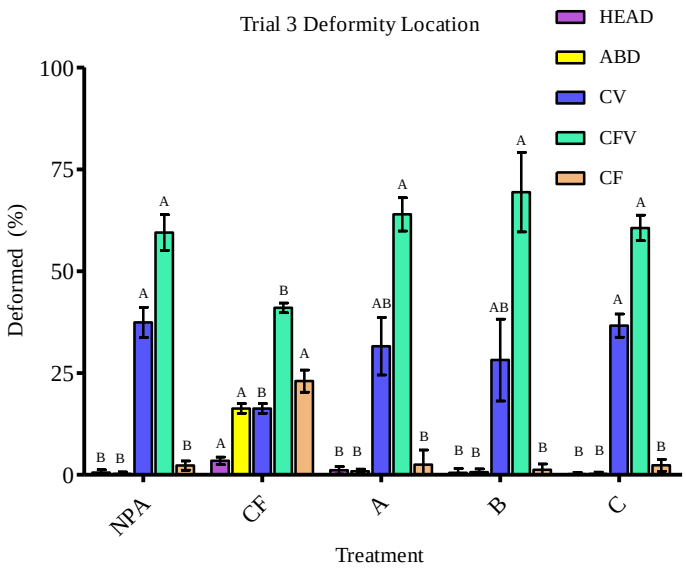


Figure 34 - Location of deformities in deformed fish for trial 3 (*n* = 40). Letters indicate significant difference between regions using one-way ANOVA with Tukey's test ($p \leq 0.05$). NPA – fish fed rotifers enriched with *Nannochloropsis* sp.; CF – fish under co-feeding regime using rotifers enriched with *Nannochloropsis* sp. until 8 DPF and Zebrafeed®; A – fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.); B –fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.); C – fish fed rotifers enriched with microalgae blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.). ABD – Abdominal Vertebrae; CV - Caudal Vertebrae; CFV – Caudal Fin Vertebrae; CFN – Caudal Fin.

Deformities tended to be minor, including hour-glass shaped centra (Figure 35-A) compressed centra (Figure 35-B) and double arches (Figure 35-C).

A. **B.** **C.**

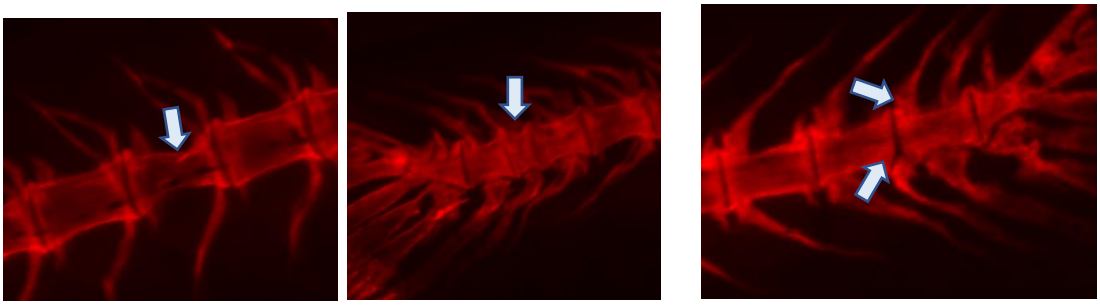


Figure 35 - A. Compressed caudal fin vertebrae centra in fish fed rotifers with blend C (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Skeletonema* sp.). B. Compressed abdominal centra in fish fed rotifers enriched with microalgae blend B (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp.). C. Double neural arch and missing caudal fin vertebrae centra of fish fed rotifers enriched with microalgae blend A (*Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp.).

5. Discussion

5.1 Diet and Live Feed

The rearing of zebrafish larvae on artificial diet alone is difficult (Harper and Lawrence, 2011), since young larvae have difficulty digesting micro-diets. As larvae grow the stomach develops and the digestion process becomes easier (Rønnestad et al., 2013). In this study, larvae fed Zebrafeed® had significantly poorer results in terms of length, weight at 15 and 30 dpf as well as survival at 30 dpf. Although zebrafish can be reared on artificial diet alone (Carvalho et al., 2006; Martins et al., 2018), previous studies using zebrafish have found significantly reduced growth when comparing artificial diets with live feeds (*Artemia* sp.) (Harper and Lawrence, 2011). Carvalho *et al.* (2006) achieved 84% survival, similar to the survival rate of those raised on *Artemia nauplii*, but with shorter length. Similar results of reduced weight, length and survival when rearing larvae on artificial feed are found in other species as well, such as rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) (Akbary et al., 2010; Sharma, 1999). When fish are overfed, a build-up of artificial feed can lead to fouling in tanks. Attempts to successfully rear zebrafish larvae without live feeds have been done when using conditions not typical to zebrafish facilities such as large tanks and continuous feeders which can be laborious and time consuming (Harper and Lawrence, 2011).

Artificial diets must be highly stable in water as microparticles need to be available to the larvae for an extended period of time (Harper and Lawrence, 2011). Water soluble vitamins are not preserved well, and leaching occurs quickly due to the high surface to volume ratio (Rønnestad et al., 2013). Shortly after addition to rearing tanks, diets can lose 50-95% of free amino acids and protein as well as up to 90% of water soluble vitamins and minerals (Rønnestad et al., 2013). This creates a nutritionally empty food which causes fouling to tanks and reduced water quality (Harper and Lawrence, 2011) making zebrafish cultivation difficult.

5.2 Microalgal Paste vs Powder

Trial 1 was conducted to see if a difference exists in larval zebrafish development when fed diet, and rotifers enriched with microalgae biomass in two different formulations, paste and powder as well as a difference in zebrafish growth between microalgae strains used for enrichments. Amongst rotifer groups using *Isochrysis* sp. and *Tetraselmis* sp. no significant differences in length, weight and survival were observed between paste and powder enrichments. However, amongst fish given rotifers enriched with *Nannochloropsis* sp., those given the paste, NPA and NPA 10 ppt, showed better results in length, weight and survival than those fish fed rotifers enriched with NPO.

The nutritional content of microalgae can be affected by the processing method as, for example, drying can alter the nutritional value (Becker, 2004). From Tables 2 and 3 it is possible to observe the differences in the proximal composition between microalgae pastes and powders. These differences are also observed in the enriched rotifers themselves with the exception of the percent total lipids of TPA and TPO enriched rotifers. Microalgae powders did not dissolve as easily when added to the enrichment containers which could have made the nutrients less available to the rotifers. In addition, the production of microalgae pastes is cheaper for commercial producers. Our results demonstrate that the groups given rotifers enriched with *Nannochloropsis* sp. paste performed better than all other groups. In addition, no significant differences were observed between paste and powder amongst the other microalgae species. Based on these findings trials 2 and 3 were conducted using microalgae pastes when available.

Several zebrafish facilities use 10 ppt rotifer culture medium to save money on salt and minimize osmotic shock when rotifers are introduced to larval tanks (Lawrence et al., 2007). Type – L *Brachionus plicatilis* populations adapt to temperature and salinity changes, this adaption has

an effect on the populations sexual reproduction patterns (Hagiwara and Yoshinaga, 2017). An experimental group NPA 10 ppt was used to evaluate if any difference existed when rotifers were maintained at a salinity of 10 ppt. The particular strain used in this experiment was from a culture which has been reared for generations at ocean salinity, making culture at 10 ppt difficult. Other facilities have found optimal rotifer production at salinities between 20-26 ppt (Dabbagh et al., 2011; Kobayashi et al., 2008). Since this strain of rotifers was easier to culture at increased salinities, 20 ppt was used for trials 2 and 3.

Diet composition plays an important role in fish health. Siccardi *et al.* (2009) showed that nutrient sources effect growth as well as the physiological, cellular and molecular process involved in weight gain in zebrafish. We observed that, the proximal composition between microalgal paste and powder as well as enriched rotifers varied greatly. Fish given rotifers under these enrichment conditions had different results in terms of length and survival. In trial 2, rotifers enriched with NPA, SPIRULI, PHAEO and CHC all had similar proximal compositions however, zebrafish larvae fed these rotifers yielded different results in terms of length, weight and skeletal deformities. These differences can be related with the presence of secondary metabolites that were not analysed in this work. In trial 3, proximal composition and nutrient sources of rotifer diets were similar amongst rotifer treatments with the exception of blend B which was high in *Spirulina* sp. (50%). It was expected that rotifers enriched with *Spirulina* sp. would have a higher total percent protein. However, as this was not the case, it is possible these rotifers had a much higher free amino acid content. Deeper investigation into the AA profile of these rotifers is needed to see the effect of high amounts of *Spirulina* sp. on the AA profile.

The groups fed microalgae blends all had similar results in terms of length, weight, survival and skeletal deformities. *Nannochloropsis* sp., *Isochrysis* sp. and *Tetraselmis* sp. were present in all blends indicating that the feed source is important in the development of zebrafish larvae.

5.3 Microalgae Selection

Although zebrafish fed with rotifers enriched with *Nannochloropsis* sp. outperformed all other treatments, many groups exhibited properties which, when combined with *Nannochloropsis* sp. could improve the nutritional value of the enrichment and maximize zebrafish development.

In the first two trials zebrafish fed on rotifers enriched with *Nannochloropsis* sp. had better results in terms of length, weight, survival and skeletal deformities. Rotifers enriched

using this microalga had a well-balanced fatty acid profile and a *n*-3*:n*-6 PUFA ratio similar to that suggested by Meinelt *et al.* (1999, 2000). *Nannochloropsis* spp. have been found to have a well-balanced AA profile, containing all EAA except tryptophan (Krishna et al., 2019) and higher amounts of glutamate and proline (Brown, 1991; Brown et al., 1993). Tryptophan is converted into the neurotransmitter and anti-oxidant serotonin and melatonin respectively (Li et al., 2009). Serotonin has been shown to reduce aggression in rainbow trout, reduce feed intake in European sea bass and lower cannibalism rates as well as stress induced anorexia in grouper (Li et al., 2009). Serotonin reduces cortisol levels which if elevated lower immunity, feed conversion and growth (Li et al., 2009). Tryptophan deficiency in rainbow trout has led to scoliosis, lordosis, caudal fin erosion, cataracts and short gill opercula (Halver and Hardy, 2002). Glutamate acts as a neurotransmitter involved in pituitary hormone release in fish (Trudeau et al., 2000) being decarboxylated to the neurotransmitter gamma-aminobutyric acid (GABA) (Li et al., 2009). High amounts of dietary GABA have been shown to inhibit food intake in Japanese flounder (Li et al., 2009). Proline levels in rainbow trout were found to be dependent on dietary levels indicating this must be provided in the diet especially due to the importance in early larval stages and biosynthesis from glutamate being unable to meet dietary demands (Li et al., 2009). Hydroxyproline is a derivative of proline and was found to have a positive correlation with growth and alter bone composition in salmon when added as a dietary supplement (Li et al., 2009). Other microalgae chosen as ingredients in the blend have been shown to contain some amount of tryptophan (Brown, 1991; Brown et al., 1993) in order to produce a more complete amino acid profile of the enrichment.

In most zebrafish facilities, rotifers are enriched using *Nannochloropsis* spp. (Best et al., 2010). For this reason it was chosen as the main ingredient in two of the three microalgae blends.

The *Isochrysis* sp. enriched rotifers (IE2), had a well-balanced fatty acid profile (Annex 1, Table D), and were the only enriched rotifer group in trial 2 to contain DHA. This microalgae contains all EAA and is high in branch chain AA, isoleucine, leucine and lysine, as well as glutamate, proline and glycine (Brown, 1991; Brown et al., 1993). *Isochrysis* sp. exhibits a good vitamin profile, high in vitamins A, and C (Fabregas and Herrero, 1990). Zebrafish larvae fed with *Isochrysis* sp. enriched rotifers performed well in terms of growth, survival and exhibited lower percentage of severe deformities than those fed with rotifers enriched with *Tetraselmis* sp.,

Spirulina sp., *Phaeodactylum* sp., *and Chaetoceros* sp. The addition of *Isochrysis sp.* to an enrichment blend provides a source of DHA necessary for fish growth and survival as well as eye, brain and nervous system development (Halver and Hardy, 2002) and increases the overall nutritional profile.

When incorporated into teleost feed, *Spirulina* sp. has been shown to enhance growth, improve protein digestibility and increase immunity, since this microalga has a high protein and vitamin content (Geffroy and Simon, 2013; Senroy and Pal, 2014). *Spirulina* sp. has been observed to have a similar AA profile to chicken eggs (Senroy and Pal, 2014) and contain up to 60% protein (Geffroy and Simon, 2013). In addition, *Spirulina* sp. contains all EAA as well as thiamine, riboflavin, ascorbic acid and carotenoids (Alvarenga et al., 2011). *Spirulina* sp. is high in many B vitamins, of particular importance is B_{12} (Geffroy and Simon, 2013), a vitamin vital for rotifer growth (Hagiwara and Yoshinaga, 2017). Although high in proteins, vitamins and *n*-6 PUFAs, when given alone, zebrafish females exhibited weight loss and larvae had reduced growth and survival, most likely due to the high level of HUFA not suitable for zebrafish development (Geffroy and Simon, 2013) and possibly the presence of cyanotoxins (Roy-Lachapelle et al., 2017). The diet provided in these trials dissolved quickly and was not very attractive to zebrafish, indicating the low survival could be due to the inability of fish to feed. In trial 2, zebrafish given the S*pirulina* sp. enrichment exhibited the lowest survival (62%) amongst rotifer fed treatments but were only significantly shorter than NPA at 30 dpf and were the second heaviest (2.40 mg) at 30 dpf. A possible reason for the low survival rate could be the low *n*-3*:n*-6 PUFA ratio and EPA:ARA ratio. *Spirulina* sp. is a popular nutritional supplement that is affordable to produce and yields good results in terms of growth making it a good option as a main ingredient of an enrichment formula. Although when used alone *Spirulina* sp. has detrimental effects on survival, the beneficial growth effects make it a useful ingredient when incorporated into a blended product as the presence of other microalgae decreases the levels of harmful compounds.

Tetraselmis sp. is high in vitamins A, C and B (Fabregas and Herrero, 1990) and contains all EAA as well as arginine, glutamate and aspartate (Brown, 1991). When cultured at high concentrations, rotifers have shown good growth rates when fed this microalga (Kobayashi et al., 2008) indicating the addition of *Tetraselmis* sp. would improve rotifer production and aid in zebrafish growth. Our results showed that enriched rotifers had high levels of LA and total protein

contents similar to those proposed by Fernandes *et al.* (2016). Although the fish fed on *Tetraselmis* sp. (TE2) enriched rotifers were statistically similar to all rotifer-fed groups in weight and survival, they had higher a incidence of severe skeletal deformities possibly due to the low amounts of EPA and ARA.

Zebrafish given rotifers enriched with *Skeletonema* sp, had a performance statistically similar in terms of length, weight and survival. Interestingly, this group had the second lowest rate of severe deformities, with only slightly more than fish given rotifers enriched with *Nannochloropsis* sp. This result may be explained by the balanced nutritional profile of *Skeletonema* sp. which contains all EAA and is high in aspartate, glutamate and arginine (Brown, 1991). Enriched rotifers, displayed a well-balanced fatty acid profile, containing high amounts of LA and an *n*-3:*n*-6 PUFA ratio similar to that suggested by Meinelt *et al.* (1999, 2000). These results suggest that when incorporated into a microalgae blend, *Skeletonema* sp. will aid in minimizing the incidences of severe skeletal deformities in larval zebrafish.

5.4 Fatty Acids Methyl Esters (FAME)

The results of FAME analysis for trials 1 (Annex 1, Tables A and B**)** and 2 (Annex 1, Tables C and D) showed microalgae fatty acid profiles compared with those obtained in previous studies (Berge et al., 1995; Custódio et al., 2013; Liang et al., 2006; Ohse et al., 2015; Patil et al., 2005; Wijffels et al., 2017) indicating the results obtained were accurate.

Rotifers have the ability to synthesize FA according to their dietary needs (Fernandez-Reiriz and Labarta, 1996). In this study, enriched rotifers exhibited a more diverse fatty acid profile than microalgae alone indicating they are able to synthesize fatty acids. MUFAs C20:1, C22:1 and C24:1 were not present in microalgae but were found in enriched rotifers during all three trials indicating the ability to synthesize these fatty acids.

Prior research has shown rotifers possibly have the ability to synthesize ARA (Hagiwara and Yoshinaga, 2017). In trial 1, ARA was only found in NPO, IPA and IPO, though it was present in all enriched rotifer groups. In trial 2, ARA was not found in SPIRULI, SKEL or CHC microalgae, however all rotifers enriched with these microalgae were found to have some amounts. In trial 3, with the exception of NPA ROTS and blend C ROTS, there was an increase in the amount of ARA present between microalgae blends and enriched rotifers. In SPIRULI and SKEL microalgae, trace amounts of EPA were present, however SPIRULI ROTS and SKEL ROTS

showed increased amounts, 2.2 and 5.49% of TFA respectively. A similar result was seen in trial 3, EPA levels increased from microalgae to rotifers in blend A (7.57 to 8.81% of TFA) and blend B (6.82 to 7.61% of TFA). These increased amounts indicate rotifers may have the ability to synthesize EPA and agree with previous studies which suggest rotifers have the limited ability to synthesize *n-3* PUFAs from dietary precursors (Fernandez-Reiriz and Labarta, 1996). This is a problem for the zebrafish community as live feed enriched with microalgae may have slightly different fatty acid profile than expected which could alter experimental results.

Meinelt *et al.* (1999, 2000), showed that low *n*-3*:n*-6 PUFA ratios resulted in high growth rates. In trial 1, The *n*-3*:n-6* PUFA ratio of enriched rotifers varied between 0.19 (NPO) and 1.00 (IPA), the longest and heaviest fish were found in groups NPA 10 ppt, NPA and TPO, with *n*-3*: n*-6 PUFA ratios of 0.58, 0.66 and 0.40 respectively. All other treatments with the exception of IPA ROTS had *n*-3*:n*-6 PUFA ratios of 0.21 or below.

The largest fish at 30 dpf in trial 2 were the NPA treatment which had an *n*-3*:n*-6 PUFA ratio of 0.66. The IE2 group, which were not significantly shorter than NPA, had an *n*-3*:n*-6 PUFA ratio of 0.46, similar to that suggested by Meinelt *et al.* (1999, 2000). The SKEL treatment, with an *n*-3*:n*-6 PUFA ratio of 0.42, had weight and survival rate statistically similar to all other rotifer treatments, and length similar to that of the IE2 group. IE2 and SKEL had the 2nd and 3rd lowest rate of severe deformities behind NPA. These *n*-3*:n*-6 PUFA ratios, although slightly lower than NPA, are closer than all other treatments.

In trial 3, rotifer fed groups showed no statistical differences in length, weight and survival from each other and NPA. These *n*-3*:n*-6 PUFA ratios varied between 0.59 (B) and 0.81 (A). Zebrafeed® (0.29), had the lowest *n*-3*:n*-6 PUFA ratio, shorter fish than all rotifer fed groups and the highest amount of severe deformities. Treatment A with the highest *n*-3*:n*-6 PUFA ratio also had the lowest percentage of severe deformities (18.57%). These results indicate larval zebrafish could require a higher *n-3:n-6* PUFA ratio than those suggested for proper skeletal development. An optimal diet for zebrafish larvae skeletal development could have a greater *n-3:n-6* PUFA ratio than that suggested by Meinelt *et al.* (1999, 2000) (0.45) and Kaushik *et al.* (2011) (0.32) however, more research into larval *n-3:n-6* PUFA ratio requirements is needed.

Alterations of the fatty acid composition in mammals effects bone formation (Berge et al., 2009). In rats, an increase in the *n-3:n-6* PUFA ratio had a negative effect on rat bone formation

(Berge et al., 2009). In juvenile Nile tilapia (*Oreochromis niloticus*), *n-3:n-6* PUFA ratio did not have an effect on growth rate (Mufatto et al., 2019). The direct role of *n-3:n-6* ratio on bone metabolism is still poorly understood but EFA play an important role in bone development (Boglione et al., 2013).

Common carp given a diet with no EFA had deformed vertebral columns however, this could be prevented when given a diet with 1% LA (Boglione et al., 2013). In marine fish, high amounts of *n*-3 PUFAs early in development accelerates osteoblast differentiation which can cause supernumerary vertebrae (Boglione et al., 2013). When gilthead seabream (Sparus aurata) osteoblastic cells were exposed to ARA, EPA and DHA, gene expression and mineralization capacity were altered (Boglione et al., 2013). ARA and EPA were found to inhibit extracellular bone mineralization while DHA stimulates this process (Boglione et al., 2013). DHA enriched diets given to milkfish (*Chanos chanos*) reduced operculum deformities and reduced overall skeletal deformities in red porgy (*Pagrus pagrus*) by 50% (Boglione et al., 2013). European seabass (*Dicentrarchus labrax*) given diets high in EPA and DHA saw increased amounts of vertebral column diseases (Boglione et al., 2013). In Senegalese sole (*Solea senegalensis*) larvae given diets enriched with ARA, no effect was seen on skeletal anomalies however, larvae skeletons were more calcified (Boglione et al., 2013).

In fish development, the amount of individual PUFAs is not as important as the DHA:EPA:ARA ratio (Sargent et al., 1999). Eicosanoids that are important in biological processes are derived from these fatty acids and compete with each other (Sargent et al., 1999). Prostaglandins are a group of eicosanoids derived from EPA and ARA which have an essential role in vertebrae development (Jaya-ram et al., 2008). Prostaglandin E2 is known to regulate osteoblast and bone metabolism, whose levels are affected by the EPA:ARA ratio (Boglione et al., 2013). Bone formation is influenced by prostaglandin E2 in a concentration dependent manner (Berge et al., 2009). Sufficient DHA amounts are important in minimizing skeletal deformities in some species (Nguyen et al., 2008). A study on European seabass (Dicentrarchus labrax) larvae found a correlation between EPA/DHA ratios and vertebral deformities (Jaya-Ram et al., 2008). Excess amounts of PUFAs accelerated osteoblast differentiation (Nguyen et al., 2008).

Zebrafish have the ability to convert LA and ALA to EPA, DHA and ARA (Brett and Muller-Navarra, 1997; Brown et al., 1997; Chen et al., 2013; Jaya-ram et al., 2008; Lawrence,

2007). However, this process expends energy (Chen et al., 2013) and could perhaps be avoided if EPA, DHA and ARA were provided at higher amounts in the diet as these fatty acids are preferentially incorporated into fish tissue when available (Brown et al., 1997).

The commercial diet had the highest LA levels but was low in ARA and EPA while NPA ROTS were low in LA and higher in ARA and EPA. Fish fed rotifers enriched with NPA had better results in terms of length, weight, survival and skeletal deformities than all other treatments.

NPA ROTS, NPA 10 ppt ROTS and IPA ROTS had similar EPA:ARA ratios, but fish fed rotifers enriched with IPA were found to be shorter and weigh less. Interestingly, IPA ROTS were also the only enriched rotifers in trial 2 to contain any DHA. TPO ROTS had lower EPA and ARA levels than the previously mentioned groups, but a similar EPA:ARA ratio and had higher amounts of LA. The fish given this diet had the third highest length and weight amongst treatments. NPA ROTS had less LA than NPA 10 ppt ROTS, the individuals given this treatment were slightly shorter and weighed less, although not statistically.

In trial 2, IE2 ROTS had a lower EPA:ARA ratio than NPA ROTS but fish subject to this treatment were statistically similar in terms of length and weight. IE2 ROTS had nearly the same percentage of ARA as NPA ROTS. CHC ROTS had an EPA:ARA ratio most similar to NPA ROTS but much lower percent ARA and had poorer results in length, weight and skeletal deformities.

Trial 3 enriched rotifers had similar EPA:ARA ratios. The fish fed these enrichments all preformed similarly in terms of length, weight, survival and skeletal deformities.

Zebrafish from trial 3 were given enriched rotifers with a well-balanced fatty acid profile, with all EFA in similar amounts. These experimental groups had nearly identical results in terms of length, weight, survival and skeletal deformities indicating the importance of *n*-3:*n*-6 PUFA ratio, EPA:ARA ratio and presence of EFA in zebrafish larval growth and development.

5.5 Proteins

Good growth results were found in fish fed enriched rotifers with varying percent protein content. Amongst groups that performed well in regard to length, weight, survival and skeletal development, NPA ROTS had the lowest percent protein (34.38% DW) and A ROTS (59.35% DW) had the highest. Fernandes *et al.* (2016) suggests 37.6% as an ideal percent protein for

zebrafish development. Although the total percent protein can be determined, the amino acid profile is still lacking. This study could be improved by determining the AA profile of enriched rotifers, to understand the effect on zebrafish development.

Best *et al.* (2010), found free AA contents higher in rotifers maintained at higher salinities. The NPA 10 ppt ROTS had a higher total percent protein than NPA ROTS, it is possible that these rotifers had greater amounts of free AA, thus having a lower protein content.

Free AA are more easily absorbed than complete proteins by some larval species (Aragão et al., 2004; Conceição et al., 2003), and are important in physiological processes and development. Histidine is involved in DNA and protein synthesis (Li et al., 2009). Leucine, isoleucine and valine are branched chain amino acids (BCAA) (Ahmed and Khan, 2006). Deficiencies in BCAAs in Indian major carp (*Labeo rohita*) have resulted in weight loss and poor feed conversion (Ahmed and Khan, 2006). Unlike other AA which are metabolized in the liver, BCAAs are mainly oxidized in skeletal muscle (Ahmed and Khan, 2006). In human skeletal muscle, BCAAs make up 14% of total AA (Ahmed and Khan, 2006). Leucine was found to be a limiting AA for turbot (*Scophthalmus maximus*) and seabream (*Sparidae*) larvae, while supplementation in Senegalese sole increased AA retention (Aragão et al., 2004). Carp fed diets high in isoleucine showed reduced growth which was reversed by providing low isoleucine diets (Halver and Hardy, 2002). Excess valine in Indian major carp caused reduced growth and feed conversion efficiency which was also observed in common carp (*catla*) (Abidi and Khan, 2004). Lysine is involved in carnitine synthesis, which transports long chain fatty acids to the mitochondria for oxidation and has been associated with increased growth, immunity, reproduction, acclimation to temperature as well as aiding in stress resistance in Jian carp (*Cyprinus carpio 'jian'*) (Li et al., 2009). Tryptophan is converted into serotonin, a neurotransmitter, and melatonin, an anti-oxidant (Li et al., 2009). In zebrafish, melatonin is involved in basic physiological process such as sleep, development, nutrition, appetite and reproduction (Lima-Cabello et al., 2014). In zebrafish embryos, melatonin induces cell proliferation and accelerates development (Lima-Cabello et al., 2014). Further studies should investigate the amino acid profile of enriched rotifers as a diet higher in free amino acids could be beneficial to larval zebrafish.

5.6 Minerals

Sr is a bone seeking mineral with chemical and physical similarities to Ca (Cabrera et al., 1999; Roberto et al., 2018; Siccardi et al., 2010). These similarities allow Sr to enter cells through Ca channels, and when present at high amounts, replace Ca in bone, decreasing overall bone calcium content, disrupting bone mineralization (Cabrera et al., 1999) and lowering bone mass density (Pasqualetti et al., 2013).

This trace element is involved in bone metabolism (Pasqualetti et al., 2013; Roberto et al., 2018) and dietary supplementation at proper rates has been shown to increase bone mass density in mice, rats, humans and zebrafish (Siccardi et al., 2010). When added to zebrafish diet in amounts up to 2586 mg/kg, Roberto *et al.* (2018) showed bioencapsulated Sr reduced skeletal deformities in larval zebrafish. Pasqualetti *et al.* (2013), found skeletal mineralization during embryonic osteogenesis was influenced by Sr:Ca ratio and not by total Sr. Proper Sr: Ca ratio in cod has shown reduced skeletal deformities (Pasqualetti et al., 2013).

In trial 2, NPA ROTS and SKEL ROTS had the highest amounts of Sr, 86.51 mg/kg and 88.40 mg/kg respectively. These two treatments also had the lowest amount of severe skeletal deformities agreeing with Roberto *et al.* (2018) that higher Sr levels decrease skeletal deformities. The mixed microalgae enriched rotifers had lower amounts of Sr than NPA ROTS and SKEL ROTS but fish fed mixed microalgae rotifers had lower levels of severe deformities as well. The obtained results indicate that increasing the amount of Sr in the blended formulas could further decrease the instances of severe deformities.

During skeletal development, Zn has a stimulatory effect on bone formation and mineralization (Roberto et al. 2018; Nguyen 2008). Zn activates aminoacyl-tRNA synthase in osteoblastic cells (Nguyen et al. 2008), stimulating osteoblastogenesis (Roberto et al. 2018). Osteoclastogenesis is suppressed (Roberto et al. 2018) by the inhibition of osteoclast-like cell formation from marrow cells (Nguyen et al. 2008). In addition, Zn is a collagenase cofactor involved in bone health and supports extra cellular matrix (ECM) mineralization (Roberto et al., 2018). Zn promotes bone formation by activating genes involved with the process, for example, Roberto *et al*. (2018), found deficiencies in zebrafish down regulated important genes associated with bone formation. When given supplements, increased osteoblastic activity was observed (Roberto et al., (2018). Nguyen et al. (2008) suggest Zn supplementation in red sea bream (*Pagrus*

major) may promote normal skeletal development as less deformities were observed. Zn must be supplemented with Mn to avoid a decrease in whole body Mn.

In fish, deficiencies have been shown to cause growth retardation, cataracts, fin and skin erosion, reduced immune response in trout (Watanabe et al. 1997; Nguyen et al. 2008), low appetite and reduced bone Zn and Ca levels in catfish (*Siluriformes*) (Watanabe et al. 1997).

Zn requirements must be established at different life stages (Roberto et al. 2018). Zebrafish skeletogenesis occurs between 5-30 dpf (Roberto et al. 2018). In this study, fish with the lowest incidences of severe deformities were fed enriched rotifers with total Zn amounts ranging between 16.10 mg/kg in NPA ROTS to 19.53 mg/kg in A ROTS. The group fed SKEL ROTS in trial 2 had a low incidence of severe deformities but no zinc present, possible supplementation with Zn in this treatment could have reduced severe deformities. Roberto *et al*. (2018) found concentrations of 30-120 mg/kg did not increase deformities in larvae. Zheng *et al.* (2010) found 223 mg/kg was sufficient in juvenile zebrafish, indicating lower amounts are needed at the larval stage and higher for juvenile-adults. Zn is important in zebrafish bone metabolism and the requirement must be determined (Roberto et al. 2018).

Low Mn intake lowers skeletal Mn levels (Watanabe et al., 1997) leading to skeletal abnormalities and poor skeletal growth (Davis and Gatlin, 1996). In trial 2, Mn amounts were present in highest amounts in NPA ROTS and SKEL ROTS, 9.87 mg/kg and 5.84 mg/kg respectively. In trial 3, Mn amounts ranged between 5.47 mg/kg in B ROTS and 6.59 mg/kg in C ROTS. These groups had the highest amounts of Mn of any treatments in trials 2 and 3 and the lowest rate of severe deformities.

In red sea bream, deficiency produced short, thick bones, while supplementation improved growth of red sea bream larvae (Nguyen et al., (2008). Nguyen *et al*., (2008) suggested that dietary supplementation during rapid ossification (20-30 dpf) increases growth performance and skeletal development. Mn is an essential mineral in fish with dietary requirements varying by species (Davis and Gatlin, 1996). In common carp and rainbow trout, 12-15 mg mg/kg is recommended (Davis and Gatlin, 1996; Nguyen et al., 2008). In channel catfish 2.4 mg mg/kg was shown to be sufficient (Davis and Gatlin, 1996). Consequently, the dietary requirement in zebrafish should be determined to ensure sufficient quantities are given.

Ca is the main component in human and teleost bone, 98% and 99% respectively (Hussein, 2014). Ca interacts with P, Mg and Zn, but is especially important in regards to P as Ca-binding protein is a carrier for both Ca and P (Hussein, 2014). P is required for bone development (Costa et al., 2018). Fish maintain a constant Ca:P ratio in bone and blood (Hussein, 2014) as bone is used as Ca and P reservoir for blood and fluids (Costa et al., 2018). In carp, excess Ca prevents P from being absorbed in the intestine (Hussein 2014). P deficiencies are linked to low bone mineralization and skeletal deformities, particularly in the caudal and caudal fin regions, it has been suggested that these areas require greater amounts of P for mineralization (Costa et al., 2018).

In this study, Ca: P ratios of enriched rotifers in trial 3 ranged from 2.94 (B ROTS) to 14.13 (NPA ROTS). Fish fed with these rotiefers performed better in regards to the overall instance of severe deformities and had a similar percentage of deformities in the caudal fin region. More research into the Ca: P ratio requirement for zebrafish needs to be done, however a low Ca: P ratio could be beneficial.

In trial 2 a high amount of skeletal deformities was found in all treatments however, no experimental group had more than 40% of individuals displaying severe deformities. The majority of deformities were present in the caudal vertebrae and caudal fin vertebrae, which is common amongst zebrafish (Fazenda et al., 2018; Martins et al., 2018). Caudal region deformities have been found in 86% of wild and 100% of cultured zebrafish (Costa et al., 2018). Treatments given rotifers enriched with *Spirulina* sp. and *Skeletonema* sp. displayed higher rates of deformities in the abdominal regions.

5.7 Co-fed (CF)

In the co-feeding (CF) group, no statistical differences were observed in survival or weight; however, this group was significantly shorter than the rotifer fed groups with significantly greater amounts of severe deformities. Regarding skeletal deformities, 76.12% of those observed exhibited severe deformities. The severe deformities consisted mainly of scoliosis of the caudal vertebrae and caudal fin vertebrae, between vertebrae 26-31 with some individuals displaying scoliosis in the caudal fin region (Figure 36-A as well as kyphosis of the abdominal vertebrae region effecting vertebrae 1-9 (Figure 36-B).

The commercial diet had a very low percentage of ARA while enriched rotifer treatments had between 4.15% (NPA) – 7.49% (A ROTS). It is possible the reduced amounts of ARA given

to the CF group during skeletal development had an impact on the number of severe deformities present.

A. **B.**

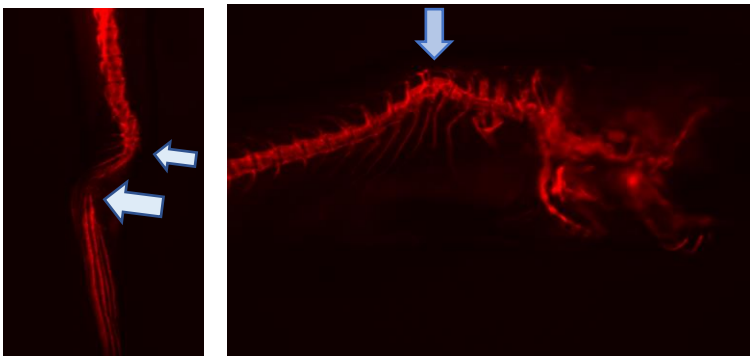


Figure 36 - A. CF (co-feeding) individual with severe scoliosis in caudal fin vertebrae and caudal fin. B. CF (co-feeding) individual with severe abdominal kyphosis.

Ossification in zebrafish occurs bidirectionally (Bird and Mabee, 2003). The location of these major deformities possibly indicates the areas undergoing development at 8 dpf when rotifers were replaced with diet. Until this time, zebrafish were provided nutrients in a live feed form. The transition from live feed to diet is a stressful event on fish development (Martins et al., 2019) and could have had a significant effect on early bone development of the fish. Martins *et al.,* (2019) found improved growth and reduced skeletal deformities in zebrafish transitioned to microdiets at 8 dpf, however this study was done using A*rtemia* nauplii. By 5.5 mm all centra are visible, by 9 mm all major skeletal structures are formed (Bird and Mabee, 2003). In trial 3, at 15 dpf all treatment groups fed the mixed microalgae enrichment had an average length greater than 9 mm. Further research is needed into the proper time to wean zebrafish from live feed to micro-diet however, 15 dpf may be a good time to undergo this transition to minimize skeletal deformities (Bird and Mabee, 2003). Further research must be done to fully understand the nutritional effects on skeletal development of zebrafish larvae however, by waiting until 15 dpf to begin feeding with commercial diets when major skeletal structures have developed, severe deformities such as scoliosis and kyphosis could be minimized.

6. Conclusions

The understanding of zebrafish nutritional requirements is vital to the continued use of this model organism. A microalgal blend for the enrichment of rotifers ensures zebrafish are provided with a balanced nutritional profile which will minimize differences in growth and skeletal development to allow greater confidence in experimental results.

In trial 1 it was determined that a microalgae paste was better suited for rotifer enrichment and zebrafish growth. Those zebrafish fed rotifers enriched with *Nannochloropsis* sp. paste outperformed all fish groups fed on other microalgal species, paste and powder, including those given *Nannochloropsis* sp. powder. Microalgal pastes are also cheaper to produce and dissolve better when added to enrichment bottles which is beneficial to both the company responsible for production and rotifer enrichment.

In order to determine possible microalgae to include in a blended product, trial 2 investigated the effects of single microalga enrichments on zebrafish growth and skeletal development. Again, zebrafish given rotifers enriched using *Nannochloropsis* sp. had better results than all other groups, but some microalgae species exhibited qualities that if incorporated into a microalgae blend could improve growth and development. For this reason, five microalgae, *Nannochloropsis* sp., *Isochrysis* sp., *Tetraselmis* sp., *Spirulina* sp. and *Skeletonema* sp., were selected based on their nutritional profile and effects on zebrafish growth and skeletal development.

The results from trial 3 showed less variation amongst treatment groups than either of the previous two trials. All rotifer fed treatments performed well in terms of length, weight, survival and skeletal deformities and with less variation within treatments. Although blended microalgae groups were not significantly larger than those enriched using *Nannochloropsis* sp., they were slightly larger and with a lower standard deviation. These fish were also larger at 15 dpf than those used in other feeding trials (Kaushik et al., 2011; Martins et al., 2018) however, they were smaller than those reared by Best *et al.* (2010) using saltwater rotifers at 30 dpf.

By minimizing the standard deviation in growth and survival, researchers using zebrafish for growth studies can be more confident in the experimental results. As a model organism for bone development, minimizing severe deformities will provide a better understanding of what causes deformities and how to prevent them.

An enrichment blend could likely have an *n-3:n-6* PUFA ratio between 0.42 and 0.81, have a well-balanced fatty acid profile high in all EFA. A protein content between 34% and 59%, although the free amino acid content is most likely the most important aspect and have a low Ca:P ratio as well as high levels of Sr, above 88 mg/kg.

More research should be done to optimize a blended microalgae formula for rotifer enrichment and obtain a better understanding of larval zebrafish nutritional requirements. Amino acid profiles of the enrichment blends and enriched rotifers, as well as vitamin profiles should be determined. Blends using other microalgae could be tested, as well as altering the profile of microalgae used in this study.

Although much research is still needed, our results show that a blended rotifer enrichment formula has many advantages over single microalga enrichment and will be beneficial to the research community by optimizing zebrafish larval growth and skeletal development.

7. Annex: 1

Table A: Main fatty acid composition of microalgae trial 1 (n = 3).

Fatty Acid %	NPA	NPO	IPA	IPO	TPA	TPO
C6:0						
C8:0						
C10:0						
C11:0						
C12:0	0.18 ± 0.12	0.39 ± 0.02				
C13:0						
C14:0			13.26 ± 0.07	14.34 ± 0.11		
C15:0		0.35 ± 0.01	1.75 ± 0.02	1.54 ± 0.02		
C16:0	29.23 ± 0.71	24.56 ± 0.22	21.10 ± 0.25	20.40 ± 0.22	71.54 ± 2.93	42.20 ± 0.74
C17:0			0.26 ± 0.00			
C18:0	0.29 ± 0.10	0.49 ± 0.22	0.64 ± 0.10			
C20:0						
C21:0						
C22:0						
C23:0						
C24:0						
Σ SFA	29.70	25.79	37.01	36.28	71.54	42.20
C14:1	6.17 ± 0.16	5.54 ± 0.05				
C15:1	0.62 ± 0.03			0.93 ±0.27		
C16:1	28.02 ± 0.31	31.00 ± 0.94	10.57 ± 0.21	12.57 ± 0.07		7.16 ± 0.17
C17:1		0.57 ± 0.13		0.74 ± 0.09		
C18:1c	4.09 ± 0.10	2.70 ± 2.54	14.19 ± 0.07	14.30 ± 0.13	3.34 ± 0.10	33.86 ± 0.81
C18:1t			2.33 ± 0.13	2.24 ± 0.02		4.63 ± 0.18
C20:1						
C22:1						
C24:1						
Σ MUFA	38.89	39.81	27.09	30.77	3.34	45.64
C18:3n-3						
C18:3n-6	0.26 ± 0.06					
C18:2n-6c	2.54 ± 0.00	4.68 ± 0.15	7.27 ± 0.02	1.28 ± 0.07	12.87 ± 1.05	6.17 ± 0.11
C18:2n-6t						
C20:4n-6	4.48 ± 3.98		0.82 ± 0.05	1.03 ± 0.00		
C20:5n-3	23.82 ± 2.30	29.14 ± 1.25	1.08 ± 0.02	1.17 ± 0.00	12.24 ± 3.88	5.99 ± 0.17
C20:3n-3	0.30 ± 0.08	0.54 ± 0.01				
C20:3n-6						
C20:2n-6						
C22:6n-3			26.46 ± 0.17	29.48 ± 0.13		
C22:2						
Σ PUFA	31.40	34.35	35.64	32.95	25.11	12.16
TOTAL (µg/mg)	42.88	49.11	20.84	23.56	9.48	19.70
Σ n-3	24.12	29.68	27.54	30.65	12.24	5.99
Σ n-6	7.28	4.68	8.10	2.31	12.87	6.17
Σn-3 : Σn-6	3.31	6.34	3.40	13.29	0.95	0.97
PUFA / SFA	1.06	1.33	0.96	0.91	0.35	0.29

ZF- Zebrafeed®; NPA – *Nannochloropsis* sp. Paste; NPA 10 ppt – *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO – *Nannochloropsis* sp. Powder; IPA - *Isochrysis* sp. Paste; IPO – *Isochrysis* sp. Powder; TPA – *Tetraselmis* sp. Paste; TPO – *Tetraselmis* sp. Powder.

Table B - Main fatty acid composition of Zebrafeed® and enriched rotifers in trial 1 (*n* = 3).

Fatty Acid %	ZF	NPA ROTS	10 ppt ROTS	NPO ROTS	IPA ROTS	IPO ROTS	TPA ROTS	TPO ROTS
C6:0								
C8:0								
C10:0								
C11:0								
C12:0	1.41 ± 0.22	1.61 ± 0.06						
C13:0								
C14:0	0.97 ± 0.05	3.13 ± 0.24	3.10 ± 0.34	2.99 ± 1.18	3.76 ± 0.20	3.30 ± 1.54	1.75 ± 0.13	1.39 ± 0.04
C15:0		0.81 ± 0.02	0.57 ± 0.18	1.34 ± 0.04	1.38 ± 0.20	2.20 ± 0.44	0.55 ± 0.01	0.82 ± 0.29
C16:0	28.12 ± 0.71	33.31 ± 1.04	33.09 ± 0.02	35.81 ± 2.02	33.77 ± 0.19	37.11 ± 0.81	34.61 ± 1.66	32.46 ± 0.46
C17:0		0.06 ± 0.01	0.63 ± 0.03		1.02 ± 0.00	1.27 ± 1.03		
C18:0	7.67 ± 0.10	3.61 ± 0.05	4.28 ± 0.73	6.21 ± 0.29	4.37 ± 0.13	4.71 ± 0.78	5.81 ± 0.42	4.46 ± 0.04
C20:0								
C21:0								
C22:0								
C23:0								
C24:0								
Σ SFA	38.17	42.53	41.67	46.36	44.30	48.58	42.72	39.14
C14:1								
C15:1								
C16:1	3.06 ± 0.22	24.04 ± 0.55	23.23 ± 0.47	18.78 ± 1.10	13.89 ± 0.02	20.10 ± 3.23	14.15 ± 0.12	13.50 ± 0.05
C17:1								
C18:1c	10.52 ± 0.12	9.26 ± 0.15	8.88 ± 0.23	13.31 ± 0.05	16.65 ± 0.32	10.46 ± 0.40	17.29 ± 1.31	20.40 ± 0.13
C18:1t	2.29 ± 0.13	4.94 ± 0.01	4.61 ± 0.05	6.10 ± 0.90	6.17 ± 0.00	6.03 ± 0.80	4.13 ± 0.30	5.05 ± 0.12
C20:1	1.03 ± 0.03	1.82 ± 0.18	1.85 ± 0.14	2.27 ± 0.64	3.70 ± 0.15	1.77 ± 0.65	3.04 ± 0.22	3.27 ± 0.17
C22:1	2.54 ± 0.17	0.65 ± 0.10	0.59 ± 0.03	0.57 ± 0.28	1.05 ± 0.00		0.80 ± 0.12	1.15 ± 0.02
C24:1	0.40 ± 0.03	0.23 ± 0.08	0.20 ± 0.13	0.88 ± 0.49	0.63 ± 0.07	2.16 ± 0.07	1.06 ± 0.70	0.44 ± 0.43
Σ MUFA	19.84	40.95	39.37	41.90	42.09	40.51	40.47	43.81
C18:3n-3								
C18:3n-6					1.75 ± 0.03		1.31 ± 0.14	0.03 ± 0.02
C18:2n-6c	32.54 ± 0.12	5.81 ± 0.05	7.43 ± 0.11	8.97 ± 1.93	0.51 ± 0.06	6.79 ± 0.73	10.64 ± 0.43	9.56 ± 0.08
C18:2n-6t								
C20:4n-6	0.12 ± 0.02	4.15 ± 0.09	4.55 ± 0.43	0.85 ± 0.72	4.81 ± 0.00	2.23 ± 0.97	2.00 ± 0.01	2.57 ± 0.10
C20:5n-3	3.15 ± 0.08	6.16 ± 0.28	6.52 ± 0.77	1.91 ± 1.16	6.09 ± 0.05	1.90 ± 1.33	2.87 ± 0.03	4.88 ± 0.19
C20:3n-3		0.42 ± 0.02	0.43 ± 0.10					
C20:3n-6								
C20:2n-6			0.02 ± 0.01					
C22:6n-3	6.18 ± 0.10				0.96 ± 0.27			
C22:2								
Σ PUFA	41.99	16.54	18.95	11.73	14.12	10.91	16.81	17.03
TOTAL (µg/mg)	20.78	16.10	16.59	8.34	11.95	6.61	12.11	15.98
Σ n-3	9.33	6.59	6.96	1.91	7.04	1.90	2.87	4.88
Σ n-6	32.66	9.96	12.00	9.82	7.08	9.01	13.95	12.15
Σn-3 : Σn-6	0.29	0.66	0.58	0.19	1.00	0.21	0.21	0.40
PUFA / SFA	1.10	0.39	0.46	0.25	0.32	0.22	0.39	0.44

ZF- Zebrafeed®; NPA – Rotifers enriched with *Nannochloropsis* sp. Paste; NPA 10 ppt – Rotifers enriched with *Nannochloropsis* sp. treatment with rotifers reared at 10 parts per thousand (ppt); NPO – Rotifers enriched with *Nannochloropsis* sp. Powder; IPA - Rotifers enriched with *Isochrysis* sp. Paste; IPO – Rotifers enriched with *Isochrysis* sp. Powder; TPA – Rotifers enriched with *Tetraselmis* sp. Paste; TPO – Rotifers enriched with *Tetraselmis* sp. Powder.

Table C - Main fatty acid composition of microalgae in trial 2 (n = 3).

Fatty Acid %	IE2	TE2	SPIRULI	SKEL	PHAEO	CHC
C6:0						
C8:0						
C10:0						
C11:0						
C12:0						
C13:0				2.37 ± 0.11		
C14:0	12.80 ± 0.27	0.54 ± 0.19		26.05 ± 0.40	5.75 ± 0.05	6.97 ± 0.02
C15:0	1.92 ± 0.03			0.78 ± 0.03	0.44 ± 0.04	1.71 ± 0.03
C16:0	19.71 ± 0.19	58.22 ± 0.41	56.60 ± 0.60	21.38 ± 0.74	19.97 ± 0.01	34.84 ± 0.81
C17:0						
C18:0	1.08 ± 1.07	3.06 ± 0.33	0.43 ± 0.06	1.84 ± 1.25		1.47 ± 0.82
C20:0						
C21:0						
C22:0						
C23:0						
C24:0						
Σ SFA	35.52	61.82	57.03	52.42	26.16	44.99
C14:1						
C15:1						
C16:1	12.18 ± 0.05	5.68 ± 0.20	6.08 ± 0.28	36.61 ± 0.43	34.63 ± 0.26	36.34 ± 1.34
C17:1	0.68 ± 0.48					
C18:1c	13.28 ± 0.00	2.63 ± 0.83	1.19 ± 1.07	4.29 ± 0.14	0.71 ± 0.08	1.96 ± 0.17
C18:1t	3.31 ± 0.13				0.47 ± 0.14	4.93 ± 0.00
C20:1		0.74 ± 0.26				
C22:1						
C24:1					0.13 ± 0.00	
Σ MUFA	29.45	9.06	7.27	40.90	35.94	43.23
C18:3n-3						
C18:3n-6		7.90 ± 0.14	15.60 ± 0.19	3.75 ± 1.12	0.10 ± 0.03	1.03 ± 0.53
C18:2n-6c	13.18 ± 0.21	7.00 ± 0.13	20.10 ± 0.06	2.15 ± 0.04	2.74 ± 0.02	
C18:2n-6t						
C20:4n-6	1.55 ± 0.04	1.12 ± 0.10			0.66 ± 0.01	
C20:5n-3	1.87 ± 0.03	13.10 ± 0.26		0.78 ± 0.07	33.19 ± 0.32	12.42 ± 0.30
C20:3n-3						

C20:3n-6						
C20:2n-6						
C22:6n-3	18.44 ± 1.56				1.22 ± 0.35	0.75 ± 0.67
C22:2						
Σ PUFA	35.03	29.12	35.70	6.68	37.90	14.20
TOTAL (µg/mg)	19.07	8.04	16.66	7.54	25.81	14.46
Σ n-3	20.31	13.10	0.00	0.78	34.41	13.17
Σ n-6	14.73	16.02	35.70	5.90	3.49	1.03
Σn-3 : Σn-6	1.38	0.82	0.00	0.13	9.86	12.79

Table D - Main fatty acid composition of Zebrafeed® and enriched rotifers in trial 2 (n = 3).

Fatty Acid %	IE2 ROTS	TE2 ROTS	SPIRULI ROTS	SKEL ROTS	PHAEO ROTS	CHC ROTS
C6:0						
C8:0						
C10:0						
C11:0						
C12:0						4.83 ± 0.32
C13:0						
C14:0	4.36 ± 0.05	1.20 ± 0.13	2.10 ± 0.74	2.50 ± 0.06	1.63 ± 0.09	2.29 ± 0.08
C15:0	3.40 ± 0.16			0.91 ± 0.73	1.44 ± 0.03	
C16:0	20.91 ± 1.28	43.21 ± 3.11	34.05 ± 1.59	35.69 ± 0.44	26.70 ± 0.06	30.40 ± 0.92
C17:0	1.68 ± 0.09		1.40 ± 0.69			0.79 ± 0.34
C18:0	8.97 ± 0.13	8.20 ± 0.66	13.63 ± 9.72	4.83 ± 0.54	4.90 ± 0.11	6.07 ± 0.01
C20:0						
C21:0						
C22:0						
C23:0						
C24:0						
Σ SFA	39.32	52.61	51.17	43.94	34.67	44.39
C14:1						
C15:1						
C16:1	5.72 ± 0.25	7.77 ± 0.10	8.87 ± 1.77	20.12 ± 0.28	17.51 ± 0.26	16.42 ± 0.30
C17:1	3.05 ± 0.24					
C18:1c	10.05 ± 0.13	12.69 ± 0.44	5.43 ± 0.32	8.86 ± 0.54	6.78 ± 0.10	9.25 ± 0.22
C18:1t	4.69 ± 0.09	5.73 ± 0.54	2.08 ± 0.03	4.76 ± 0.13	4.28 ± 0.01	3.26 ± 0.25
C20:1	4.23 ± 0.05	2.84 ± 0.08	1.11 ± 0.04	1.57 ± 0.04	2.11 ± 0.11	2.15 ± 0.02
C22:1	3.84 ± 0.24	0.41 ± 0.08			0.39 ± 0.02	0.14 ± 0.01
C24:1	4.44 ± 0.30	1.54 ± 1.27		2.44 ± 0.16	0.44 ± 0.02	0.49 ± 0.05
Σ MUFA	36.02	30.99	17.49	37.76	31.51	31.71
C18:3n-3						
C18:3n-6	3.15 ± 0.32	3.29 ± 0.03	2.71 ± 0.35			
C18:2n-6c	6.20 ± 0.35	8.99 ± 0.02	18.85 ± 4.07	11.45 ± 0.46	12.40 ± 0.08	16.09 ± 0.19
C18:2n-6t						
C20:4n-6	4.17 ± 0.08	1.38 ± 0.33	2.97 ± 0.40	1.88 ± 0.01	3.08 ± 0.07	2.72 ± 0.16
C20:5n-3	2.92 ± 0.02	2.75 ± 0.31	2.20 ± 0.18	5.49 ± 0.06	14.40 ± 0.12	4.59 ± 0.45

C20:3n-3	2.46 ± 0.14		2.57 ± 0.36	0.08 ± 0.00	0.58 ± 0.11	
C20:3n-6						
C20:2n-6	3.42 ± 0.25		2.03 ± 0.67		1.59 ± 0.15	
C22:6n-3	2.36 ± 0.16					
C22:2						
Σ PUFA	24.66	16.40	31.34	18.90	32.05	23.39
TOTAL (µg/mg)	8.69	6.79	10.03	6.36	10.06	6.61
Σ n-3	7.73	2.75	4.78	5.57	14.98	4.59
Σ n-6	16.94	13.65	26.56	13.33	17.07	18.80
Σn-3 : Σn-6	0.46	0.20	0.18	0.42	0.88	0.24
PUFA / SFA	0.63	0.31	0.61	0.43	0.92	0.53

ZF- Zebrafeed®; NPA ROTS – Rotifers enriched with *Nannochloropsis* sp.; IE2 ROTS - Rotifers enriched with *Isochrysis* sp. Experiment 2; TE2 ROTS - Rotifers enriched with *Tetraselmis* sp. Experiment 2; SPIRULI ROTS – Rotifers enriched with *Spirulina* sp.; SKEL ROTS – Rotifers enriched with *Skeletonema* sp.; PHAEO ROTS – Rotifers enriched with *Phaeodactylum* sp.; CHC ROTS – Rotifers enriched with *Chaetoceros* sp.

Table E: Main fatty acid composition of microalgae, Zebrafeed® and enriched rotifers for trial 3 (n = 3).

Fatty Acid %	Blend A	A ROTS	Blend B	B ROTS	Blend C	C ROTS
C6:0						
C8:0						
C10:0						
C11:0						
C12:0					2.75 ± 0.12	2.48 ± 0.10
C13:0						
C14:0	1.82 ± 0.04	2.86 ± 0.02	2.63 ± 0.03	2.64 ± 0.15	4.38 ± 0.05	2.83 ± 0.01
C15:0	0.86 ± 0.21	1.73 ± 0.00		1.80 ± 0.17	1.74 ± 0.06	1.75 ± 0.03
C16:0	36.55 ± 0.12	26.60 ± 0.38	39.26 ± 0.18	25.03 ± 0.27	27.42 ± 0.41	25.55 ± 0.55
C17:0		0.85 ± 0.00		0.97 ± 0.08		0.86 ± 0.01
C18:0	9.80 ± 0.16	5.69 ± 0.14	3.62 ± 0.21	5.83 ± 0.30	2.93 ± 0.21	5.73 ± 0.00
C20:0						
C21:0						
C22:0						
C23:0						
C24:0						
Σ SFA	49.03	37.73	45.51	36.27	39.23	39.20
C14:1						
C15:1						
C16:1	10.19 ± 0.24	17.06 ± 0.21	12.32 ± 0.37	14.44 ± 0.35	20.86 ± 0.20	15.84 ± 0.60
C17:1	0.69 ± 0.02	1.49 ± 0.01		1.52 ± 0.11		1.36 ± 0.09
C18:1c	8.33 ± 0.05	6.98 ± 0.03	5.30 ± 0.17	6.28 ± 0.23	6.77 ± 0.19	6.88 ± 0.05
C18:1t	0.96 ± 0.02	5.02 ± 0.00	1.78 ± 0.17	4.81 ± 0.09	2.01 ± 0.08	4.86 ± 0.07
C20:1		2.12 ± 0.01		2.40 ± 0.28		2.27 ± 0.09
C22:1		1.71 ± 0.01		1.73 ± 0.13		1.75 ± 0.03

C24:1		1.76 ± 0.02		2.08 ± 0.08		1.86 ± 0.02
Σ MUFA	20.17	36.13	19.40	33.27	29.64	34.81
C18:3n-3						
C18:3n-6	1.26 ± 0.01	1.57 ± 0.00	9.08 ± 0.01	2.58 ± 0.13	2.07 ± 0.07	1.62 ± 0.04
C18:2n-6c	15.71 ± 0.03	5.39 ± 0.04	11.88 ± 0.06	7.38 ± 0.03	4.27 ± 0.19	5.41 ± 0.01
C18:2n-6t						
C20:4n-6	4.72 ± 0.13	7.49 ± 0.11	4.92 ± 0.05	6.68 ± 0.22	9.06 ± 0.06	7.27 ± 0.19
C20:5n-3	7.57 ± 0.18	8.81 ± 0.13	6.82 ± 0.11	7.61 ± 0.57	14.38 ± 0.16	8.36 ± 0.33
C20:3n-3	0.61 ± 0.01	1.65 ± 0.00	1.23 ± 0.08	1.91 ± 0.07	1.19 ± 0.06	1.72 ± 0.01
C20:3n-6						
C20:2n-6				1.71 ± 0.17		1.48 ± 0.18
C22:6n-3	0.59 ± 0.02	1.22 ± 0.01	1.17 ± 0.10	1.33 ± 0.10	1.60 ± 0.02	1.33 ± 0.01
C22:2						
Σ PUFA	30.46	26.14	35.09	29.20	32.57	27.18
TOTAL (µg/mg)	21.60	24.14	18.63	20.86	20.96	22.72
Σ n-3	8.77	11.68	9.21	10.85	17.16	11.40
Σ n-6	12.13	14.46	25.88	18.35	15.40	15.78
Σn-3 : Σn-6	0.72	0.81	0.36	0.59	1.11	0.72

NPA – *Nannochloropsis* sp. microalgae; NPA ROTS –rotifers enriched with *Nannochloropsis* sp.; ZF - Zebrafeed®; Blend A – Microalgae blend A; Blend A ROTS – rotifers enriched with microalgae blend A; Blend B – Microalgae blend B; B ROTS – rotifer enriched with microalgae blend B; Blend C – microalgae blend C; C ROTS – rotifers enriched with microalgae blend C.

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