UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Sorocaba

PROGRAMA DE PÓS-GRADUAÇÃO em
Ciências Ambientais

ANGÉLICA IRASEMA SIBAJA LUIS

NANOTECNOLOGIA APLICADA À AQUICULTURA: DESENVOLVIMENTO DE SISTEMAS PARA A FORMULAÇÃO DE DIETAS EM PEIXES BASEADOS EM NANOPARTÍCULAS DE QUITOSANA E PCL CONTENDO ÁCIDO ASCÓRBICO

Sorocaba

2021

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Dissertação apresentada como requisito para a obtenção do título de Doutor em Ciências Ambientais da Universidade Estadual Paulista "Júlio de Mesquita Filho" na Área de Concentração Diagnóstico, Tratamento e Recuperação Ambiental

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Sorocaba

2021

L953n

Luis, Angelica Irasema Sibaja

Nanotecnologia aplicada à aquicultura: desenvolvimento de sistemas para a formulação de dietas em peixes baseados em nanopartículas de quitosana e PCL contendo ácido ascórbico / Angelica Irasema Sibaja Luis. -- Sorocaba, 2021

98 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, Sorocaba

Orientadora: Prof. Dr. Leonardo Fernandes Fraceto

Coorientadora: Profa . Dra . Vera Lúcia Scherholz Salgado

1. nanopartículas poliméricas. 2. ácido ascorbico. 3. toxicidade. 4. aquicultura. 5. peixe-zebra. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Ciência e Tecnologia, Sorocaba.
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TÍTULO DA TESE: NANOTECNOLOGIA APLICADA À AQUICULTURA: DESENVOLVIMENTO DE SISTEMAS PARA A FORMULAÇÃO DE DIETAS EM PEIXES BASEADOS EM NANOPARTÍCULAS DE QUITOSANA E PCL CONTENDO ÁCIDO ASCÓRBICO

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Câmpus CNPEM / Centro Nacional de Pesquisa em Energia e Materiais, Laboratório Nacional de Nanotecnologia

Sorocaba, 16 de julho de 2021

AGRADECIMENTOS

Agradeço a Deus por todas as oportunidades que me deu, por iluminar minha mente e fortalecer meu coração, obrigada por todas as pessoas que foram colocadas ao longo deste caminho, têm sido minha companhia e apoio.

Ao meu filho Julio, por ser meu motivo, minha força, meu tudo, não tenho palavras para definir o amor que sinto por você. Obrigada por ser luz em meus dias sombrios

À minha família pelo apoio, principalmente à minha irmã Maria e ao meu cunhado Felipe, por todo o amor e apoio que me deram, por cuidar do meu filho de forma especial, serei eternamente grata a vocês.

Agradeço ao meu namorado por estar na minha vida, apesar da distância. Você sempre esteve ao meu lado nos momentos difíceis e nunca me deixou desistir. Estar ao seu lado é a melhor coisa que me aconteceu, espero que Deus nos conceda muitos mais anos juntos.

Ao meu orientador o Prof. Dr. Leonardo Fernandes Fraceto pelo apoio, instrução, colaboração e paciência, proporcionado nestes anos. Por me dar sua ajuda em tempos difíceis. Sempre serei grata por me aceitar em seu grupo de pesquisa. Obrigada!

A minha coorientadora a Prof. Vera e o Henrique da EMBRAPA, por todo o apoio que me deram durante a minha estadia no laboratório, pelos conhecimentos adquiridos, durante a realização dos testes de toxicidade.

Um agradecimento aos meus colegas Estefânia e Jhones pelo apoio incondicional nestes anos, pelo conhecimento e experiência adquiridos.

Aos meus colegas de laboratório, pela amizade e pela ajuda que me deram nesse período. Muito obrigada.

Ao programa de Pós-Graduação em ciências ambientais e a Universidade Estadual Paulista Júlio Mesquita Filho (UNESP), pelo espaço, oportunidade e por me proporcionarem e um conhecimento de qualidade.

Ao programa PROPAT-BRASIL-MÉXICO, pela oportunidade que me proporcionou de vislumbrar um grandioso horizonte.

A CONACYT pelo suporte financeiro outorgado para a realização desta pesquisa.

A fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e CNPq, pelo suporte e apoio concedido.

E a todos que direta ou indiretamente fizeram parte da minha formação, muito obrigada.

ABSTRACT

Nutrient loss in aquaculture is a common problem that this industry has faced in recent years many essential nutrients are not tapped or do not arrive in the necessary quantities and can increase the risk of disease. As is the case with ascorbic acid (AA), which is an essential macronutrient for fish growth and reproduction. This vitamin is unstable under the action of high temperatures, oxygen and heat. In this context, several methods are being developed to improve the stability of AA, as is the case with systems based on synthetic or biodegradable polymers. The present work describes the preparation and characterization of the polycaprolactone (PCL) and chitosan (CS) nanoparticles loaded with ascorbic acid (AA), as well as the toxicological effects in zebrafish (*Danio rerio*), evaluating the development, behavior and enzymatic activity. Both nanoparticle systems had an average size of 314 and 303 nm, polydispersity index of 0.36 and 0.28, high encapsulation efficiency and good physical chemical stability for 90 days. The two nanoparticle systems provided protection against the degradation of AA exposed to an oxidizing agent, compared to AA alone. The LC_{50} found were 330.7, 57.4 and 179.6 mg/L for plain AA, the CS nanoparticle formulation, and the PCL nanoparticle formulation, respectively. The CS and PCL nanoparticles loaded with AA at a concentration of 50 mg/L, showed less toxicity in the development of zebrafish compared to AA isolated at the same concentration. The use of these nanosystems in this study was promising in the protection of AA, and the PCL nanoparticles containing AA showed less toxicity in zebra fish and greater protection of AA compared to chitosan nanoparticles containing ascorbic acid. Nanotechnology offers a new alternative for the use of nutrients in aquaculture, protecting against degradation and reducing the loss of these nutrients, leading to greater weight gain and growth of fish, making this activity sustainable.

Keywords: Nanoparticles, ascorbic acid, toxicity, aquaculture

RESUMO

A perda de nutrientes na aquicultura é um problema comum que esta indústria tem enfrentado nos últimos anos. Muitos nutrientes essenciais não são aproveitados ou não chegam nas quantidades necessárias e podem aumentar os riscos de doenças. Como é o caso do ácido ascórbico (AA), que é um macronutriente essencial para o crescimento e reprodução dos peixes. Esta vitamina é instável sob a ação de altas temperaturas, oxigênio e calor. Neste contexto, diversos métodos estão sendo desenvolvidos para melhorar a estabilidade do AA, como é o caso dos sistemas a base de polímeros sintéticos ou biodegradáveis. O presente trabalho descreve o preparo e a caracterização das nanopartículas de Policaprolactona (PCL) e quitosana (CS) carregadas com ácido ascorbico (AA), bem como os efeitos toxicológicos em zebrafish (*Danio rerio*), avaliando o desenvolvimento, comportamento e atividade enzimática. Ambos sistemas de nanopartículas apresentaram um tamanho médio de 314 e 303 nm, índice de polidispersão de 0.36 e 0.28, eficiência de encapsulação alta e uma boa estabilidade físico química por 90 dias. Os dois sistemas de nanopartículas bridaram proteção contra a degradação do AA exposto a um agente oxidante, comparado com o AA isolado. As CL 50 encontrados foram 330,7, 57,4 e 179,6 mg/L, para AA isolado, nanopartículas de CS e nanopartículas de PCL, respectivamente. As nanopartículas de CS e PCL carregadas com AA na concentração 50 mg/L, apresentaram menor toxicidade no desenvolvimento do zebrafish em comparação com o AA isolado na mesma concentração. O uso destes nanosistemas neste estudo foi promissor na proteção de AA, sendo que as nanopartículas de PCL contendo AA apresentaram uma menor toxicidade no zebrafish e maior proteção do AA em comparação com as nanopartículas de quitosana contendo ácido ascorbico. A nanotecnologia oferece uma nova alternativa para o uso de nutrientes na aquicultura, protegendo contra degradação e reduzindo a perda destes nutrientes, levando a um maior ganho de peso e crescimento dos peixes, tornando esta atividade sustentável.

Palavras-chave: nanopartículas, ácido ascorbico, toxicidade, aquicultura

Sumário

INTRODUÇÃO GERAL

Em 2018, a produção de peixes no mundo foi de 179 milhões de toneladas, sendo que 156 milhões de toneladas foram destinas para o consumo humano, com uma estimativa de 20,5 kg per capita e 22 milhões de toneladas foram destinadas para a produção de óleo e farinha de peixes. A pesca indiscriminada tem levado a uma queda na produção de peixes. Mundialmente, a produção aquícola supera a pesca por captura e o número de espécies cultivadas e registradas pela FAO aumentou em 31,8%, passando de 472 em 2006 a 622 milhões de toneladas em 2018. As espécies mais cultivadas são peixes de aleta, moluscos, crustáceos entre outras espécies de animais (SOFIA, 2020).

O alto consumo de peixes se deve principalmente por representarem uma fonte importante de proteínas e ácidos graxos ômega-3 nas dietas humanas (PRADEEPKIRAN, 2019). Apesar do crescimento acelerado desta indústria, a aquicultura vem enfrentado vários problemas como, por exemplo, a influência das dietas na aparição de doenças (BLAZER, 1992).

Uma alimentação que não cumpre com os requisitos nutricionais pode afetar a eficiência alimentar e o crescimento do organismo e consequentemente o aumento e a susceptibilidade a doenças. Dietas desequilibradas também podem induzir a antagonismo entre os nutrientes e níveis altos de nutrientes poderiam induzir a uma aparição de sinais de toxicidade. Porém os dados de biodisponibilidade de nutrientes se limitam a algumas espécies (OLIVA-TELES, 2012). A fim de melhorar a resistência a doenças e o sistema imunológico nos peixes são usados nutrientes dietéticos como proteínas, minerais, lipídios e vitaminas. (LIM; WEBSTER, [s.d.]). A maior parte das vitaminas não estão disponíveis e tem que ser adicionadas nas dietas. Esse é o caso da vitamina C ou ácido ascórbico (AA). A vitamina C é uma das vitaminas mais estudadas, e é solúvel em água. Ela é produzida por microrganismos e plantas, embora alguns animais não tem a capacidade de sintetiza-la, devido à falta de L-gulonolactona oxidase, responsável pela biossíntese de AA (DAWOOD; KOSHIO, 2016), tendo então que ser fornecidas nas dietas (ENGELKING, 2015). O ácido L-ascórbico é estável quando seco e, em condições alcalinas ou neutras, é facilmente oxidado. A oxidação molecular pode ser devido à luz, calor, umidade ou outros oxidantes. O ácido ascórbico é oxidado e convertido em ácido desidroascórbico, depois é convertido em ácido 2,3-dicetogulônico. Esse processo de oxidação ocorre rapidamente na alimentação dos peixes, principalmente quando entra em contato com a água (CHEBROLU et al., 2012; O'KEEFE, 2001).

A vitamina C é micronutriente essencial para manter as funções fisiológicas e o crescimento de organismos aquáticos. Quando os peixes são expostos a dietas deficientes de ácido ascorbico podem apresentar algumas anomalias como hemorragia, alterações da cartilagem de suporte, hemorragias petequiais, hiperplasia de tecido, anorexia, perda de apetite, malformação, diminuição do crescimento, escoliose e lordose (O'KEEFE; TECHNOLOGIES, 2001).

As indústrias produtoras de rações para peixes vem enfrentando vários desafios como a perda de funcionalidade da vitamina C durante o processo de fabricação e armazenamento, já que nestes processos a vitamina pode estar exposta a oxigênio, e altas temperaturas (ALISHAHI et al., 2011a; GADIENT; FENSTER, 1994). A degradação da vitamina C nos alimentos também pode ser afetada por outros agentes, como a desidratação catalisada pelo ácido ascórbico e a destruição pela luz (SANDNES, 1991). O ácido ascórbico tem uma cinética de degradação rápida (primeira ordem). A reação de Maillard, que é causada pela luz e calor (RIPOLL; CLEMENT, 2016), é responsável por 75% dessas ocorrências. Muitos métodos tem sido testados e usados com o fim de reduzir a perda da vitamina C. Umas das alternativas é proteger tanto fisicamente como quimicamente o ativo por meio do recobrimento, encapsulamento e a microencapsulação aumentando a sua estabilidade e evitando que seja degradado rapidamente (DESAI; LIU; PARK, 2006a; KUMAR VEMULA et al., 2007; MORIBE et al., 2011; WANG et al., 2003). Por outra parte, a estabilidade desta vitamina poderia melhorar se fossem usados envoltórios biodegradáveis como é o caso das nanopartículas.

A nanotecnologia é um tema novo para a indústria alimentícia e poderia criar uma barreira capaz de inibir as interações químicas que desfavorecem os ingredientes ativos. A nanoencapsulação de vitaminas tem tido avanços consideráveis, assim como os nano-hidrogéis e as nano-fibras. Estes nanocarreadores, são produzidos a partir de componentes de alimentos, como é o caso da quitosana, ou componentes sintéticos, como é o caso do PCL (KATOUZIAN; JAFARI, 2016) .

Apesar dos benefícios que a nanotecnologia pode trazer, há uma preocupação quanto a sua biossegurança e sua aplicação no ambiente e na aquicultura. Porém, é importante entender e conhecer os efeitos que esta tecnologia pode causar aos organismos (ELSAESSER; HOWARD, 2012). No entanto, estudos de toxicidade tem que ser realizados para avaliar o risco das nanopartículas no ambiente e em organismos não-alvo. Nesse sentido, o zebrafish (*Danio*

rerio) tem sido utilizado nos testes de toxicidade já que proporciona a oportunidade de avaliar os efeitos dos compostos e de diminuir o risco de toxicidade nos peixes (MOURA et al., 2019).

Desta forma o objetivo principal deste trabalho foi preparar e caracterizar sistemas nanocarreadores a partir de matrizes naturais e sintéticas de quitosana e PCL, para carregar a vitamina C. Para que a aplicação destes sistemas nanocarreadores contendo a vitamina C seja realizada de uma forma segura, foram avaliados os possíveis efeitos tóxicos no desenvolvimento do zebrafish. Foram investigados parâmetros de desenvolvimento físico como malformação, taxa de sobrevivência, taxa de eclosão e parâmetros comportamentais, atividade da enzima AchE (acetilcolinesterase), assim como ganho de peso.

O primeiro capítulo da tese comenta sobre a importância da aquicultura para o sustento alimentar. Esta atividade apresentou um grande crescimento nos últimos anos, e, ao fornecer alimentos para humanos, é também geradora de empregos. Outro tema que é abordado neste capítulo, são os problemas que a aquicultura tem enfrentado em relação à nutrição, os suplementos que são necessários para o desempenho das atividades dos peixes, bem como as novas tecnologias que podem ser aplicadas para a preservação das características dos diferentes nutrientes que são adicionados às dietas dos peixes, como é o caso da nanotecnologia. A nanotecnologia pode resolver os principais problemas que a aquicultura enfrenta, como o tratamento de doenças, a reprodução, a produção e a nutrição. Também se discute sobre a importância de um regulamento ou legislação para que a utilização destes nanomateriais seja feita de forma segura, reduzindo os impactos ao ambiente e à biota aquática.

No segundo capítulo são apresentados o preparo e caracterização das nanopartículas (quitosana (CS) e policaprolactona (PCL), assim como ensaios de toxicidade usando zebrafish (*Danio rerio*) em testes de comportamento, desenvolvimento, ganho de peso e atividade enzimática. Os resultados mostram uma eficiência de encapsulação alta e uma estabilidade físico química por 90 dias. As nanopartículas de quitosana e PCL apresentaram tamanhos de 314 – 303 nm e um índice de polidispersão de 0.36 – 0.28, respectivamente. Ambos os sistemas forneceram proteção contra a degradação do AA exposto a um agente oxidante em comparação com o AA isolado. A CL50 encontrados foram, 330,7 mg/mL, 176,6 mg/mL e 57,4 mg/mL, obtida para AA isolado, nanopartículas de PCL carregadas com AA e nanopartículas de quitosana carregadas com AA respectivamente. Nos ensaios de toxicidade na concentração de 50 mg/L, as nanopartículas de quitosana e PCL carregadas com AA apresentaram menor toxicidade no desenvolvimento do zebrafish em comparação ao AA isolado na mesma concentração. Houve uma diminuição da atividade enzimática, mas esta não afetou o

comportamento natatório das larvas de zebrafish avaliados. Esta diminuição pode estar relacionada às alterações morfométricas observadas como a curvatura da cauda. Estes resultados abrem novas perspectivas para o uso da nanotecnologia em aquicultura quanto a rapidez no crescimento dos animais e diminuição de perdas de nutrientes mostrando assim a viabilidade de seu uso na alimentação dos peixes.

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CHAPTER I - INTELLIGENT MATERIALS APPLIED IN FISH NUTRITION

(Artigo não submetido)

ABSTRACT

Aquaculture has been affected by the bioavailability of nutrients in diets, if the diets do not meet the dietary requirements, could generate other problems such as limited growth, generating economic losses. It is important to provide the organism with all essential nutrients in adequate amounts, for the final product to be of high quality. Many essential nutrients, have to be added to diets because they are not found in quantities necessary to meet the organism needs. But some essential nutrients such as, for example vitamins E and C, may be unstable in their pure form and have to be added to diets. The instability of these nutrients can generate losses during processing and storage of rations. Therefore, new alternatives that can effectively protect nutrients have been sought. Nanotechnology applied to aquaculture could solve many problems related to nutrition, increasing stability, slow release, protection against degradation and increased efficiency of the active ingredients. These systems open new perspectives for use in aquaculture for nutritional supplementation of fish, thus contributing to a better development of fish.

Keywords: aquaculture, nutrition, fish, nanotechnology, food

1. INTRODUCTION

Poor management of natural resources has led to losses in marine and continental waters due to changes in watercourses, pollution and fishing resources (GRZYBOWSKI; GLIŃSKA-LEWCZUK, 2019). The exploitation of fishing resources occurred after the Second World War (BEARE et al., 2010). Fish consumption has become of extremely important, since it is an important source of carbohydrates, proteins and omega 3, at low cost(THILSTED et al., 2011). Great part of our global aquatic resources has suffered a significant deterioration and decrease, especially in schools of fish (VASILAKOPOULOS; MARAVELIAS; TSERPES, 2014). In the level worldwide, fisheries have suffered a sharp decline, driving the growth of aquaculture (OECD-FAO, 2020). For this reason, new strategies have been searching that contribute to the production of aquatic foods (JENNINGS et al., 2016).

In 2012 humanity on earth reached more than 7 billion people, for 2050 there is an estimate that the earth will reach 9 billion people, consequently to a greater need for nutrition (ZANNA, 2020). The population is constantly growing and aquaculture has a significant share in the production of aquatic foods, as it provides 47% of food resources, mainly in countries with high population density, as well as in developing countries (OECD-FAO, 2020). Small-scale fish farming is an activity practiced between 2000-1000 BC. In 473 BC., the first study on the cultivation of carp (*Cyprinus carpio*) was documented in China (FAO, 1988). Aquaculture started to make great contributions from the 20th century, providing food security to man and became an important sector (DUNHAM, R. A, 2000). In recent years, aquaculture has been one of the fastest growing industries in the world and finds himself is constantly expanding (STÉVANT; REBOURS; CHAPMAN, 2017).

Aquaculture can occur in several bodies of water (rivers, irrigation tanks, reservoirs and coastal areas) (BOSTOCK et al., 2010), taking into account several factors such as soil type, topography, supply, dynamics and water quality. Fish farming ranges from extensive, semi-intensive and extensive aquaculture (BOYD, 2003; NAYLOR et al., 2000).

However, this industry faces several problems such as the appearance of diseases. The disease outbreaks in the raise of fish are directly related to poor implementation of biosecurity measures, such as poor water quality (BERA et al., 2018). A limiting factor in fish farming is water quality as it can influence growth rate, health and feed efficiency. Fish diseases can be prevented by maintaining good water quality in cultivation, as well as good nutrition and

feeding (WANJA et al., 2020). The diets of aquatic organisms are composed of nutrients that are physiologically important in animal and cellular metabolism. They are also essential for health, reproduction and growth. To maintain a good diet in fish, more than 40 nutrients must be included, the requirements of each nutrient will depend on the weight and age of the animal. Quantitatively, these requirements may show some difference between species that can be influenced by the stage of development, gender, age, physiological factors and cultivation environment. It is important that the nutrients available in the diets adjust to the nutritional requirements of each species of fish, aiming that the higher part of the nutrients be absorbed and harnessed by organisms, thereby reducing excess nutrients in fecal excretion (LALL; DUMAS, 2015).

Among the nutrients are lipids, proteins, carbohydrates, minerals and vitamins (LALL; DUMAS, 2015). A part of these nutrients such as vitamin C and E is not produced by aquatic animals and must be added to the feed (LALL; DUMAS, 2015). Nutrients added to diets can be lost in the processing or storage of rations, due to exposure to high temperatures, light and oxygen (CRAIG et al., 2017; KRAMER, 1977). Many methods are used in order to protect and improve the stability of these compounds, as is the case of encapsulation, coatings and microencapsulation (AMARAL; ANDRADE; CONTO, 2019; TACON ALBERT G. J., 1988). In this sense, nanotechnology applied to aquaculture can solve many problems related to animal health and nutrition, as well as the development of new formulations capable of decreasing the rate of degradation, increasing efficiency and reducing the volatilization of compounds sensitive to high temperatures, oxygen and light (LUIS et al., 2019).

In this context, the objective of this chapter was to address the main problems that fish nutrition faces. It is presented the role of the main nutrients and the problems they show for their incorporation into the feed as degradation and solubility, among others, are discussed. Finally, the approach is highlighted on how nanotechnology has contributed to solving the problems that aquaculture faces.

2. AQUACULTURE AND ITS IMPORTANCE

Global fisheries suffered a great decline caused by indiscriminate fishing, thus driving the growth of aquaculture (COLE et al., 2009; DE SILVA, 2001). This decline is mainly due to the large deficit of marine and freshwater organisms captured (SOFIA, 2020). According to (FAO, 2018a), world aquaculture had an annual growth of 5.8% in the period from 2000 to 2019.

In 2018, China was one of the countries with the largest production of edible fish in the world, followed by Asia, the Americas, Europe, Africa and Oceania (SOFIA, 2020). This industry has a significant participation, since in 2016 it provided 47% of food resources, especially in countries with high population density, in addition to developing countries. An example of this is the American company (Sino Agro Food, inc), one of the largest in the world that operating in China. Currently, more than 598 species are cultivated; mainly fish, crustaceans, mollusks, frog (*Rana spp*), soft shell Chinese tortoise (*Trionyx sinensis*), Sea cucumber (*Apostichopus japonicus*) among others (SOFIA, 2020).

Aquaculture as a food producer, began to give contributions from the 20th century and became an important sector, mainly for providing food security to man (COLE et al., 2009; DE SILVA, 2001). Aquaculture is responsible for the supply of fatty acids, omega 3 and 6, essential amino acids and proteins, being responsible for approximately 17% of the animal protein consumed (COLE et al., 2009; DE SILVA, 2001). In addition to including food production, aquaculture plays an important role in the restoration species in danger of extinction, recovery of threatened wild species and restoration of habitats (FROEHLICH; GENTRY; HALPERN, 2017; HOLZWEISSIG, 1998). This activity is considered sustainable, as it generates jobs for women, men and youth (SOFIA, 2020). According to Sofia, (2020), in 2018, 20,533 thousand people were employed, being that most of them belong to developing countries where small-scale artisanal aquaculture is practiced and the types of jobs can vary from permanent to temporary with full-time or seasonal working hours.

Like all activity, there are advantages and consequently disadvantages, in the cultivation of fish in captivity and aquaculture can cause negative impacts on the environment (COLE et al., 2009). Among these effects, we can highlight the accumulation of nutrients and organic matter composed of fecal matter and remains of uneaten food (BOYD et al., 2020). Studies confirm the increase in organic matter in cropping systems in coastal areas and inland Waters (CHUA THIA ENG; PAW; GUARIN, 1989; SARÀ et al., 2004).

Several factors influence the accumulation of organic matter, including the cultivated species, depth and currents, type of management and the quality of the feed. The remains of feed and feces contain high levels of phosphorus (P), nitrogen (N) and carbon (C), that accumulate in the sediment and also lead to water eutrophication and organic contamination of the environment (COLE et al., 2009). In eutrophication, there is an increase in the production

of microalgae due to the accumulation of these elements, causing the death of fish due to oxygen depletion (SCHNEIDER et al., 2005).

To avoid eutrophication, farms regularly make a mass balance, where the nutrient load resulting from food aquaculture is based on the difference between nutrients in the feed and the nutrients withheld or to take advantage of by the shrimp or fish. However, quantitative information on how these nutrients is carried into the environment is still quite limited, even more that these nutrient discharges vary according to the production system and the specie (VERDEGEM, 2013). For example, in intensive systems, P and N, are the main contaminants, whereas in watertight crops, they can be used and integrated with other activities, to reduce the use of resources, waste discharges, leaching and degradation (SCHNEIDER et al., 2005).

Fish crops integrate with macroalgae crops. System integration and water reuse are steps that are seldom used in aquaculture due to the additional costs they can generate (VERDEGEM, 2013), however, despite this disadvantage they are show themselves efficient. An integrated system with macroalgae (*Lactuca linneaus*), was able to retain 30% nitrogen and 20% phosphorus in ration (VERDEGEM, 2013). The cultivation of organisms producing waste integrated with oysters improves water quality and reduces the risk of a negative environmental impact as they are natural water filters in *in situ* cultivation (TROELL et al., 2003).

Another challenge facing aquaculture is the non-efficient processing of nutrients within water purification and recirculation systems, because this influences in proportion and composition of nutrients that are discharged and accumulated in aquaculture systems (VERDEGEM, 2013).

The accelerated growth of the industry has led to several problems other than those reported, such as the emergence of diseases and new pathogens. These problems, that are directly related to water quality, disease resistance and nutrition, directly impact on the sustainable development of this industry (OLIVA-TELES, 2012). Diseases caused by bad nutrition are difficult to define, since these can be caused by deficiency (malnutrition), excess (over nutrition) or imbalance of the required nutrients (lipids, proteins, vitamins and minerals) (SHEFAT; KARIM, 2018a). Extreme weakness from lack of food can be caused by poorly balanced diets in relation to the proportion of nutrients. The problems caused by malnutrition such as slow growth rate, reduced fertility, decreased appetite, pathological lesions, susceptibility to diseases, can disappear when changing the feed (OLIVA-TELES, 2012;

SHEFAT; KARIM, 2018a). When fish are uncontrolled feed, the excess is converted in fat and deposited in organs and tissues that affect physiological functions (CRAIG et al., 2017).

Aquaculture plays an important role in the economy and sustainability as it improves income, increases food security and economic development in fishing and aquaculture communities. Aquaculture production in 2018 was 114.5 million tons of live weight, with a total sale of 263.6 billion USD. Of which 821 million tons were aquatic animals, 32.4 million tons were aquatic algae and 66 billion tons of pearls and sea shells (FAO, 2020). The failure or success of aquaculture companies depends on disease management and it depends on researchers and producers. Losses of fish or shrimp to disease can cause prejudices not only nationally, but can also generate impairments in commercial transactions. International economic losses are subject to the quarantine process, for disease control, and failure to comply with this rule can lead to a total ban on imports; on the other hand, other countries may be affected by the transmission of diseases by infected products from aquaculture causing losses in production and decreased profits (FAO, 2020). The application of good food management practices can avoid these problems. A quality diet, balanced, complete and good cultivation conditions contribute to the prevention of these diseases (SHEFAT; KARIM, 2018a).

3. NUTRITION AND NUTRITIONAL PROBLEMS

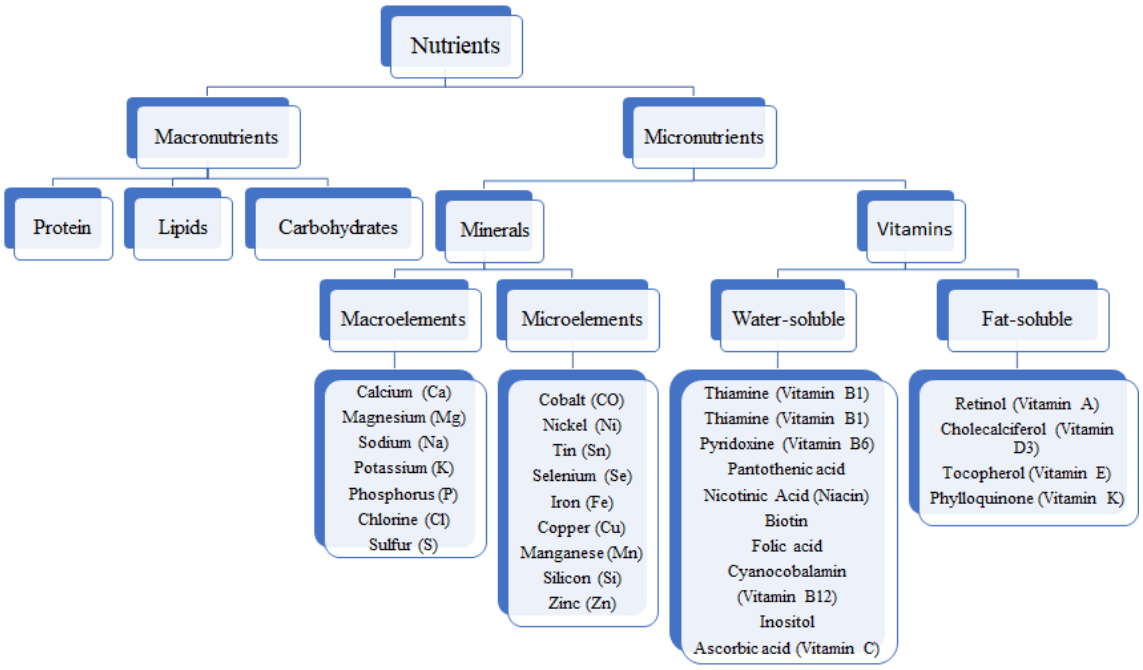
Currently aquaculture search a balance between sustainability and production of efficient food and, in this context, fish nutrition is very important as studies the main sources of energy and essential nutrients (GATLIN, 2003; OLIVA-TELES, 2012).

Aquaculture production can be affected by the bioavailability of nutrients in diets. It is important to maintain a balanced diet and proper nutrition. If the diet does not meet these requirements or is not administered correctly, will not be properly digested, generating other problems, as limited growth and stools with high nutrient content, that cause economic losses and environmental contamination (OLIVA-TELES, 2012). To maintain a good diet, it is necessary to take into account the various factors that can influence or affect nutritional needs, among which are: the quality of artificial foods, the availability of natural foods, the physicochemical quality of water, the temperature and size of the fish (JOSHUA; ADOGBEJI, 2017).

As described above, nutrition in aquaculture is of utmost importance, to maintain the good quality of crops and the final product. The purpose of feeding fish in captivity is to provide them with all essential nutrients, providing adequate amounts that maintain a balance between nutrients, thus promoting the development of defenses against infections and decreased response to stress (ELŻBIETA TERECH-MAJEWSKA, 2012; OLIVA-TELES, 2012).

Nutrients are fundamental for growth, reproduction, enzyme functioning, hormone synthesis, tissue renewal, among other physiological functions, of aquatic organisms and fish (HAMRE, 2011). Nutrients are divided into two groups, macronutrients (carbohydrates, lipids and proteins) and micronutrients (vitamins and minerals) (Figure 1) (ELŻBIETA TERECH-MAJEWSKA, 2012; OLIVA-TELES, 2012). These nutrients are added to artificial diets and can also be used to enrich live foods to improve nutrition.

Figure 1. Nutrients that are added to artificial diets and can also be used to enrich live foods to improve nutrition.



Fish must be fed with adequate amounts of dietary ingredients viewing that their fulfilled nutritional requirements are met. When these needs are not met, there can be serious consequences on the health of organisms, however, if these nutrients are managed in very high amounts can also affect the fish health, showing signs of toxicity. The most important aspect is

to administer the proper amounts, that can satisfy nutritional needs (JOSHUA; ADOGBEJI, 2017). In fish or shrimp farms where supplementation is not performed with live food, artificial food has to meet the dietary requirements of each species, taking into consideration the life cycle, physiological characteristics and development phase (HAMRE et al., 2013).

Among the essential nutrients are proteins, macromolecules formed by amino acids (essential and non-essential) joined by peptide bonds (OLIVA-TELES, 2012) and for this nutrient to be tapped, proteins must be hydrolyzed releasing free amino acids which will be absorbed through the wall of the digestive tract and distributed through the blood to the organs, being used for the synthesis of new proteins, destined for growth, tissue repair and reproduction (JAHAN-MIHAN et al., 2011).

There are several types of proteins distributed in the fish's body. Among them, are the fibrous proteins (elastin, keratin, collagen, etc.). Elastin is found in connective tissues, arteries, elastic cartilage and bladder. Keratin is found in small amounts and collagen is a component of the bone matrix, connective tissues, skin, fins, operculum and blood vessels (LALL; DUMAS, 2015).

The lipids or fats, are organic molecules, formed by carbon atoms, hydrogen and oxygen, found in the tissues of plants and animals, are insoluble in water and classified into five groups: fatty acids, waxes, phospholipids, steroids and sphingomyelins. In fish, the lipids, serve as a source of energy and act as regulators of metabolism (GOMES et al., 2019). The lipids act as absorption and transport pathways for fat-soluble vitamins, as A, E and K. They provide essential fatty acids such as omega-3 and omega-6 (WATANABE, 1982). Lipids are an important source of energy and the requirement for these can vary between lipid digestion and the eating habits of each species (GLENCROSS, 2009).

Carbohydrates are organic substances, soluble in water, solids at room temperature and are the main constituent of the energy reserves of plant and animal cells (GERSCHENSON; ROJAS; FISSORE, 2017). They provide energy at low cost but are considered non-essential, biologically speaking, but are incorporated into diets to reduce costs in addition to increase lipid retention and fish proteins, thus reducing nitrogen levels downloaded into farm effluents (KAMALAM; MEDALE; PANSERAT, 2017).

Minerals are important to maintain good growth and a proper fish metabolism. Minerals can be absorbed through the surface of the body or through the gills, However, the amount

absorbed on many occasions is not necessary to meet the fish's requirements, so they should be added to the diet (LALL; DUMAS, 2015). Eighty to ninety percent of phosphorus and calcium are present in bones, scales and of fish teeth. Approximately 60% of magnesium is stored in the skeleton of fish (FAO, 1978). Minerals regulate the exchange of water and solutes, maintain osmotic pressure, provide rigidity and strength to the skeleton, regulate the pH, provide body acid-base balance and participate as cofactors in metabolism and in catalysis (WITTEN et al., 2016).

Vitamins are micronutrients, a fundamental component for human health and for aquatic organisms. These micronutrients contribute to the development and growth of organisms, strengthen the immune system, helping to prevent disease (KATOUZIAN; JAFARI, 2016). Vitamins are needed primarily for the formation of coenzymes, which are organic or metal-organic molecules that bind enzymes to initiate or promote catalytic activities. Vitamins can be classified according to their solubility, water soluble vitamins (water soluble) and fat-soluble (lipid soluble) (LITWACK, 2018).

Fat-soluble vitamins are characterized as aliphatic and aromatic hydrocarbons. They are mainly related to immunological processes, antioxidant activities and maintenance of tissue structures; in this group are vitamin A (β-Carotene), D (1,25-Dihydroxyvitamin D3, Active Form), E (α-tocopherol) and vitamin K (K1, phytyl-menaquinone and K2, multiprenyl-menaquinone) (KOHLMEIER, 2015). Water-soluble vitamins, are involved as coenzymes in the processes linked to the metabolism of organic nutrients: lipids, proteins and carbohydrates. Within this classification are the group B vitamins, B1, aneurin (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxal phosphate, pyridoxine and pyridoxamine), B9 (folic acid), vitamin B12 (cobalamin), vitamin B7 (biotin/vitamin H) and ascorbic acid (vitamin C) (PINCHEN, 2018). Table 1 presents a summary of the main essential nutrients studied in fish diets, their benefits when included in adequate amounts into the diets, and also the problems caused due to its insufficiency.

Table 1. Main essential nutrients studied in fish diets, their benefits and problems due to their insufficiency.

Nutrients	Benefits	Problems caused by disability	References
Protein	- Repair worn out fabrics and damaged - The catabolized protein acts as an energy source - Formation of new tissues - Contributes at the formation of enzymes and hormones, as well as biological substances such as hemoglobin and antibodies	- Increased mortality rates - Dorsal and caudal wing erosion - Cataracts - Lordosis - Scoliosis - Renal calcinosis	(AGARWAL et al., 2019)
Lipids	- Source of metabolic energy - Biological vehicles in the absorption of vitamins A, E and K - Support for vital organs - Contribute to buoyancy - Source of steroids, omega-3 and omega-6	- Increase in mortality rates - Hepatosomatic liver enlargement, pale and swollen liver -Decrease in spawning - Limited growth	(IZQUIERDO et al., 2000)
Carbohydrates	- Provide energy - Retains lipids and proteins - Reduction the nitrogen level	- Low insulin production -Reduced glucose utilization by deficit of glucose transporting molecules	(HEMRE; MOMMSEN; KROGDAHL, 2002)
Minerals	- Essential constituents of teeth and bones - Tissue constituents - Participate in the body acid-base balance - Regulate blood pH - Cofactors in metabolism	- Limited growth - Bone Demineralization - Anorexia - High mortality rates - Erosion of skin and fins - Tail deformation - Cataracts	(GATLIN, 2003)

Vitamin B	-Coenzyme in the metabolism of carbohydrates, reductase and oxidase enzymes - And important in protein and energetic metabolism - Maintenance of nervous tissue	Changes in body coloration - Anorexia - Growth reduction - Edema	(AGARWAL et al., 2019; FAO, 1989; HANSEN; WAAGBØ; HEMRE, 2015)
Vitamin C	-Antioxidant -Promotes collagen biosynthesis -Important modulator of the REDOX system	-Limited growth -Lordosis -Scoliosis - Hemorrhages in fins and intestine - Twisted and deformed gill filaments - High mortality rate - Decrease in hatch rate - Anorexia - Hemorrhages external and internal - Tail fin wear- Exoftalmia - Reduction of stress resistance	(AGARWAL et al., 2019; AL-HARBI, 2001; O'KEEFE; TECHNOLOGIES, 2001; SHEFAT; KARIM, 2018a)
Vitamin A	- Maintenance of mucus-secreting epithelial tissue from the skin, reproductive tract, gastrointestinal tract and bones - Maintenance of normal vision	- Limited growth - Depigmentation - Renal hemorrhage - Eye opacity - Hemorrhagic lesions on the wings and skin - Loss of mucosa in the skin	(AGARWAL et al., 2019; SHEFAT; KARIM, 2018a)
Vitamin D	- It is important in the metabolism of phosphorus and calcium	- Anorexia	(SHEFAT; KARIM, 2018b)

		- Limited growth	
Vitamin E	Improves disease resistance, maintains immunity, collaborates in vitamin C biosynthesis - Stabilization of the biological membrane and inhibit the proliferation of some cells - Importance to DNA biosynthesis and cellular respiration	- Anemia - Muscular dystrophy - Exophthalmia - Ascites - Atrophy of pancreatic tissue - Exudative diathesis - Low hatching and growth rates	(FAO, 1989; HAMRE, 2011; HE et al., 2017; ROEM; KOHLER; STICKNEY, 1990)
Vitamin K	- Facilitation blood production - Release of various proteins - Participation in calcium transport	- Anemia - Bleeding of the skin	(GASCO et al., 2018; SHEFAT; KARIM, 2018b)

Essential nutrients such as minerals, proteins and vitamins are administered through artificially formulated or natural foods (CRAIG et al., 2017). Information on nutritional needs in aquaculture is still limited (ZHANG et al., 2020). Live food is mainly used in the larval stages of crustaceans, in all stages of mollusk development and in fish larvae and post-larvae (BARROS; VALENTI, 2003; DHERT; SORGELOOS, 1992). Planktonic organisms such as artemia, copepods, cladocerans, rotifers and protozoa are used to that end (FAO, 1996; GOGOI; SAFI; DAS, 2016; W.RASDI; QIN, 2014). In feeding with phytoplankton, a variety of microalgae can be used, including chlorophylls and diatoms (BROWN, 2002). The most used groups of zooplankton are copepods and rotifers (AJI, 2011; DAS et al., 2012).

Zooplankton is used as the first food for the first stages of life in several fish species. Microalgae are used in the first larval stages of crustaceans, bivalve mollusk and some fish species (AJI, 2011; DAS et al., 2012), and are also used to enrich zooplankton in feeding fish larvae and other organisms. In addition to providing energy, proteins and vitamins, they also contribute to steroids and pigments (DAS et al., 2012).

Microalgae are an important part of food supplements in the aquaculture industry as they contain high values of proteins, even among the different species of microalgae. These strains can be used in aquaculture, cannot produce any signs of toxicity, your cell wall must be digestible and must have high nutritional quality. Among the most used species we can find *Chaetoceros*, Chlorella, Isochrysis, *Tetraselmis*, *Pavlova*, *Phaeodactylum*, *Nannochloropsis*, Thalassiosira e *Skeletonema* (SPOLAORE et al., 2006).

The importance of microalgae in aquaculture is directly related to nutrition, because in addition to high protein values, they also provide essential vitamins such as A, B, C and E and are rich in chlorophyll. In the larval stage, microalgae are used as a single food for a short period of time, and as a supplement, they can be used fresh, in order to pigment the meat of the salmon or to promote other biological activities. However, the use of live microalgae in aquaculture is a challenge because of high costs, as well as production, storage and concentration difficulties (SPOLAORE et al., 2006).

The availability of live food can enhance production success, achieving high survival and growth rates for shellfish and fish offspring (CARNEIRO et al., 2003). However, to achieve this success, it is necessary to know the nutritional components of natural foods, nutritional status of live food can improve (GHOSH; DEY, 2015). In many occasions, artificial feeds do not meet the necessary nutritional requirements and these can be supplemented with live food to ensure the yield of shellfish and fish larvae (CARNEIRO et al., 2003).

4. CHALLENGES IN PRODUCING A QUALITY ARTIFICIAL DIET

The aim of aquaculture is to achieve good fish growth in a short time (GASCO et al., 2018). The foods used in modern aquaculture are a mixture of raw materials which provide essential nutritional requirements. Among these ingredients there are vitamins, minerals, pigments, oils, which when mixed can satisfy the demands of micro and macronutrients (HUA et al., 2019). Diets, in addition to satisfying the nutritional fish needs, must also be of high quality to improve environmental sustainability (GASCO et al., 2018).

According FAO Annual Report (2018), deficit of quality feeds, lack of feeding methodologies and techniques to meet the basic needs of farmed fish, are some of the challenges facing aquaculture currently. In aquaculture production costs are in the range of 50 to 70%, which includes ingredients and input costs. The basis of the most used ingredients for the

formulation of fish foods are rice, wheat, corn, soy and large amounts of fish oil (source of lipids) and fish meal (source of protein), used by farms and also in commercial aquaculture (HUA et al., 2019; RANA; SIRIWARDENA; HASAN, 2009).

Fish oil is a highly rich commercial source of polyunsaturated fatty acids. In 2006, aquaculture used about 85.5% of the total fish oil produced. Joint to the growth of aquaculture, in the future the use of fish oil in feed will not be economically sustainable and this could be switched to vegetable oils. However, fish oil is a source of eicosapentaenoic acid and docosahexaenoic acid, and these fatty acids are not present in vegetable oils (OLIVA-TELES, 2012). Considering the costs increase of oil and fish meal during last years, breeders are looking for new alternatives to obtain low-cost proteins, such as soy, microbial proteins, blood and bone meal (HUA et al., 2019; NAYLOR et al., 2000; RANA; SIRIWARDENA; HASAN, 2009). Animal or vegetable flours are also being used as a substitute for fish meal at a lower cost (AYOOLA, 2010).

Animal or vegetable by-products, such as grains, flour and fish waste, among others, are, therefore, used in the preparation of feeds in the cultivation of aquatic organisms (TACON; HASAN, 2009). These by products must be balanced specifically for each species and must provide the necessary nutrients in the required percentages of fats, vitamins, minerals, carbohydrates, etc. for each type of cultivation, species and developmental stage of the organism (ELANGOVAN; NATHAN; AHILAN, 2017).

Each fish species and development stage require, therefore, different diets. Along with that, it is also very important to identify the photoperiod, temperature and metabolism of the cultivated fish. Among the fishes' metabolic cycles, the hormonal cycle is the most interesting, where lipogenesis and protein accumulation are controlled. Deficiencies due to lack of essential nutrients at maturation are related to egg size, reduced fertility and egg hatchability. All these factors must be observed in order to produce high quality fingerlings, essential for the aquaculture industry (HARDY, 1999).

Aquaculture depends on knowledge of different areas as economic, technological, and biological engineering (KRAUSE; MIKKELSEN, 2017). Among these areas, food is an extremely important topic, since it allows to obtain a high survival rate and an excellent growth of the cultivated organisms, improving the quality of the products (FAO, 1989). To formulate

a product is needed basic knowledge related to dietary requirements for each species, quality and quantity of nutrients (CRAIG et al., 2017).

Artificial feeds are classified according to water content, as dry (less than 20% water), semi wet (20 to 50% water) and wet (more than 50% water). These artificial feeds are presented in an extruded or pelletized form (MISRA; SAHU; JAIN, 2002). Extrusion is one of the most used methods for processing fish feed, it is a traditional method, very flexible, with low energy consumption and can be manufactured in continuous processing mode, with the objective of producing products with different textures, shapes, flavors and colors, from basic ingredients (RIAZ; ASIF; ALI, 2009).

On the other hand, there is little information about ingredients stability during the extrusion process. It is known that, for example, the vitamin A, C, and D are more sensitive to oxidation, vitamin A, folic acid, and biotin are more sensitive to heat, and vitamin K may be the most affected by moisture. The vitamins most affected in this process are vitamin A, B1, C and E, as well as folic acid. During prolonged times at high temperatures, vitamins can lose about 65% of their original activity. However, it is not only the factors listed above that influence the stability of vitamins. Other important points that should also be taken into account are the quality of the raw material, cooking temperature, moisture, screw rotation speed and the flow of material, size of the matrix, power input, between others (RIAZ; ASIF; ALI, 2009).

The processing of feed ingredients can be done by cooking or thermal, where the raw material is submitted in a digester at high temperatures (FAO, 1986). This processing is economical and simple, but can be harmful to proteins, affecting the availability of amino acids and digestibility. The longer the raw material exposure time to high temperature, the lower the nutritional value and digestibility (HAMRE et al., 2013).

Another process used is chemical hydrolysis, which can be alkaline or acid. In this process, the raw material undergoes dramatic changes in pH, making it difficult to control. This process advantages are its low cost and flavor improvement, but there are serious disadvantages, such as denaturation of amino acids, especially tryptophan, essential to nutrition. Also, there is ash incorporation along with the components during the procedure (PETROVA; TOLSTOREBROV; EIKEVIK, 2018; WISUTHIPHAET; KONGRUANG; CHAMCHEUN, 2015).

The enzymatic hydrolysis, another process used for processing ingredients, uses low pressure and temperatures, not having loss of amino acids. In addition to improving the availability of amino acids, this technique also promotes increased protein digestibility (SIDDIK et al., 2020). Another advantage is that it uses less steam and energy in cooking the ingredients due to the breakdown of peptide bonds of amino acids that occurs by proteolytic enzymes, generating peptide chains with variable length depending on the protein source (RAO et al., 1998; SIDDIK et al., 2020).

Hydrolyzed proteins are more easily absorbed than whole proteins, that helps in the first animals' life stages because the absorption of the food is inefficient, instead of adult animals have no difficulty in absorption (HOU et al., 2017). There are still many questions to be elucidated regarding the application of hydrolyzed proteins since some studies have shown the benefits that this process has on the immune system, in growth, intestinal health and improved production yield (AKSNES et al., 2006; KOLKOVSKI; TANDLER, 2000; PLASCENCIA-JATOMEA et al., 2002; SUWAL et al., 2018).

Food additives are added in small amounts in fish feed, in order to preserve the characteristics and improve the dispersion of ingredients. Among them, are preservatives, binders, food colors and stimulating foods (ALEMAYEHU; GEREMEW; GETAHUN, 2018).

The aquaculture industry could achieve greater efficiency and production with the addition of supplements containing immunostimulants (BARMAN et al., 2013). An immunostimulant is a compound of natural origin that modulates the immune system increasing resistance of the organism against diseases. The immunostimulants most commonly used are the oligosaccharides as the glucans, which are added to the fish feed and are able to alter the intestinal flora and boost the immune system (RINGØ et al., 2011). Even though the research on supplementation with trace elements and minerals is important due to their fish live cycle essential processes, its progress is slow (PRABHU; SCHRAMA; KAUSHIK, 2014).

The minerals chelation with peptides or amino acids increases bioavailability for fish and other animals. Chelate is a mineral bonded to a non-metallic entity (DAYYANI; ABADI; FARHANI, 2013)[103]. The purpose of chelation is to protect the mineral against complexation to compounds and avoiding its insolubility in the digestive tract, ensuring the absorption of minerals through the membranes (MILES; HENRY, 2000). After the mineral is absorbed in the digestive tract and distributed in the fish's body.

MacroGard® is an immunostimulant, a commercial supplement of β-glucanas, used in animal and aquatic organisms' feed. Use of this product increases the growth of crustaceans, improves biological functions and modulates the immune response (MIEST et al., 2016).

Ascorbic acid phosphate derivatives (vitamin C) have anti-scurvy activity in some organisms, such as tilapia, marine shrimp, Japanese sole, catfish and yellowtail (WANG et al., 2003). Therefore, the phosphorus derivatives of the original compound are more stable, as is the case of ascorbyl polyphosphate. These components have also been demonstrated to have a bioavailability similar to the parent compound (BAE et al., 2012). Some products containing ascorbic acid derivatives are currently marketed as Aqua-Stab ™ and branded products Stay-C ™ (O'KEEFE; TECHNOLOGIES, 2001).

The company BIOSTADT launched on the market WOCKCEETM, a source of vitamin C based on polyphosphate, is digestible, stable and absorbed efficiently as an ingredient in fish and shrimp feeding (BIOSTADT, 2020). DSM is a global company that offers a range of products that improve animal health and supplements that help to rations lower costs and the environmental impact, as is the case of the ROVIMIX® Stay-C 35 which contains stable vitamin C, of ROVIMAX® NX that collaborates in a strengthening of the immunity in fish and the RONOZYME® P which is an enzyme that helps to rations costs decrease as well as contributing to reducing the environmental impact (DCM, 2020).

Other supplements, such as chitosan, also have been studied, not only as a supplement in the feeding as well as stabilizer. Marchetti et al., (1999) hey studied the stability of vitamins coated with grease during the ration manufacturing and storage process. The recovery of vitamins after the granulation process was higher mainly for ascorbic acid. The krill exoskeletons and crabs are used in carp and dorado diets, to get a brighter coloring. However, chitin supplementation in diets and its influence on growth have not been studied. Kono Michiko, (1987) studied the effects of supplemented diets with chitosan, chitin and cellulose, and verified the influence on the growth of the Japanese anguilla, jurel (*Trachurus trachurus*), dorado. Among the results found that the diets supplemented with chitin (10 %) improved the growth rate of japanese anguilla, jurel (*Trachurus trachurus*) and dorado.

Aquaculture, as an industry in Latin America, is affected by a lack of support for the development of research in this sector. The worldwide level Technavio announced the top five producers of fish feed ingredients. Among them, it can be finding the companies Alltech,

ADDCON, Cargill, Grupo Biomar e Cermaq. According to a global survey carried out by (ALLTECH, 2020), the total production of balanced feed for aquaculture was 41%, with China, Bangladesh Vietnam being the main producers. Europe showed a decline in feed production in Russia, due to increased imports. One of the problems that aquaculture has faced in the nutrition area is the loss of nutrients due to low hydro stability, which causes leaching and disintegration (MARTINEZ-PORCHAS; MARTINEZ-CORDOVA, 2012).

The nutrition has become a controversial topic among researchers and companies in the search for sustainability in aquaculture. Due to the lack of essential nutrients in many dietary supplements, the vast majority of commercialized foods do not contain the necessary amount of essential nutrients, as these can be lost during manufacture, transport or prolonged storage, in many cases these nutrients may be present in artificial feed but in very low percentages, insufficient to carry out the fish's activities generating other problems, such as significant economic losses (HALVER, 2003).

Companies specializing in nutritional products and new generation companies have joined forces in the search for new alternatives that improve the stability of nutrients, ensuring that they arrive in quantities that meet the nutritional needs of the fish. New technologies are opening aquaculture industry ways in search of food improvements, as is the case with nanotechnology.

5. APPLICATION OF NANOTECHNOLOGY IN AQUACULTURE

Nanotechnology is the science responsible for the study of the materials application, synthesis, characterization. Its size scale can vary from nanometer to a billionth of a meter (SAINI; SAINI; SHARMA, 2010). These materials can be designed for interaction at the molecular or subcellular level. The applications of these materials in aquaculture range from water treatment, disease control, nutrient controlled release and efficient drug delivery, labeling and barcoding (KHOSRAVI-KATULI et al., 2017; LUIS et al., 2019). Nanotechnology is a new field and there are few studies on its application in aquaculture. Although this new technology is in the first stages of development, it can bring many benefits for solving problems such as production, animal health, reproduction as well as the treatment of diseases. Nanotechnology in fish nutrition can face many challenges, such as efficiency in providing essential nutrients, transporting nutrients (enzymes, nutraceuticals, antimicrobials and food additives), hormones,

reducing waste, improving bioavailability, solubility and protecting nutrients from degradation (CAN et al., 2011; LUIS et al., 2019; SHAH; MRAZ, 2019; WISDOM et al., 2018). Encapsulation of these nutrients can be carried out by various natural or synthetic matrices, as shown in Table 2.

Table 2. Different nanosystems applied in aquaculture.

Nanoparticles	**Method**	**Characterization**	**Application**	**References**
Chitosan-Cyclodextrin-TPP **Vitamin C**	Ionic gelation	Zeta potential - 35 mV Size – 300 nm EE – 15 %	Study of cytotoxicity, tissue and cell uptake in rotifers and plant larvae	(JIMÉNEZ-FERNÁNDEZ et al., 2014)
Chitosan - TPP **Vitamin C**	Ionic gelation	Zeta potential – 49 mV Size – 185 nm	Chitosan nanoparticles, for the protection of ascorbic acid in the digestive tract of rainbow trout *(Oncorhynchus mykiss).*	(ALISHAHI et al., 2011b)
Chitosan - TPP	Ionic gelation	Size – 30-60 nm EE – 79.9 %	Chitosan nanoparticles for oral administration of the OMP gene in fish	(VIMAL et al., 2012)
Alginate -Span 80 **Corn oil**	Controlled Emulsification	-	Microencapsulation of inactive *A. hydrophila* cells. For oral immunization of fish.	(RODRIGUES et al., 2006)
Chitosan – DNA- TPP	Ionic gelation	Size – 200 – 284.4 218.9 - nm EE – 91.5 %	Preparation of chitosan-DNA microparticles against *V. parahaemolyticus* in bream (*Acanthopagrus schlegelii*)	(LI et al., 2013)
PLGA- PLA - DNA	Double emulsion solvent evaporation	Zeta potential – -31.3 - -26.5 mV Size – 98.6 – 223.5 nm	PLA/PLGA nanoparticles, for the encapsulation of Omp antigen the *A. hydrophila* as an	(RAUTA; NAYAK, 2015)

		EE – 59.33 - 44 %	antigenic carrier in Carp rohu (*Labeo rohita*)	
PLGA - DNA	Double emulsion solvent evaporation	Zeta potential – 3.24 – 2.85 mV Size – 320 nm – 3-4 um EE – 27 – 24 %	Nanoparticles and microparticles, loaded with DNA, for intramuscular injection in Atlantic salmon	(HØLVOLD et al., 2013)
PLGA-DNA	Emulsion and spray dried	EE: 63%	Encapsulation of PLGA nanoparticles containing DNA for the treatment of lymphocytic in Japanese platija (*Paralichthys olivaceus*)	(TIAN; YU, 2011)

It is well known that compounds used in fish diets are sensitive to temperature, light and oxygen. These bioactive, when incorporated in the diets, can be hydrolyzed in the intestinal tract of fish (ALISHAHI et al., 2011b; ALISHAHI; AÏDER, 2012a). Since they can also be destroyed by the acidity of the tract, the nanoencapsulation can improve their absorption and provide protection to them in gastrointestinal conditions (ARANAZ et al., 2009; LUO et al., 2011), as shown in figure 2.

Figure 2. Nanotechnology biodegradable molecules production can play an important role in incorporation of nutrients in feed for fish, as they can improve absorption, controlled release and stability. As well as providing protection in gastrointestinal conditions.

Encapsulation of these bioactives is a promising way to overcome the problems facing aquaculture industry. The encapsulation consists of using polymeric materials or particles of liquids, solids or small gas droplets. After encapsulation, the active compound can be released in a controlled manner (SILVA et al., 2014). Nowadays, materials such as minerals, vitamins, dyes, enzymes, sweeteners, are being encapsulated, which are used in the food industry (CANO-SARABIA; MASPOCH, 2012). Chitosan is considered a candidate as an encapsulating agent due to its muco-self-adhesive properties, non-toxicity and biocompatibility and biodegradability (KRAVANJA et al., 2019). The positive effects that the chitosan presents in aquaculture has already been documented to improve survival, growth of some species such as the rainbow trout (*Oncorhynchus mykiss*) (MESHKINI et al., 2012), madrigal carp (*Cirrhina mrigala*) (SHANTHI MARI et al., 2014) and tilapia (*Oreochromis niloticus*) (WANG; LI, 2011).

The use of chitosan as a nano-carrier of active compounds is at an initial stage, (ALISHAHI et al., 2011b; ALISHAHI; AÏDER, 2012b), study in the use of chitosan nanoparticles containing vitamin C in rainbow trout diets is important to improve asset stability. Among the results found in a search for this paper, chitosan nanoparticles containing vitamin C increased the useful life of the active compared to vitamin C alone. Also, it demonstrated that nanoparticles protect the active ingredient from the damage caused in the gastrointestinal tract of trout, leading to a controlled release for a period of time of 48 hours. On the other hand, Jiménez-Fernández et al, (2014) studied the potential of chitosan-cyclodextrin nanoparticles as a route of vitamin C supply. The nanoparticles were prepared by the ionic gelation method, and

it was found an encapsulation efficiency of 15%, sizes between 253-258 nm, zeta potential of 33 mV and polydispersity index of 0.34-0.38. The nanoparticles were exposed in sea water and obtained a 7% release of vitamin C for a period of 2 hours, indicating stability in sea water. Also, it had the capacity to penetrate the intestinal epithelium of *S. senegalensis* larvae. Rotifers fed with nanoparticles containing vitamin C showed an increase in the content of this vitamin. This study demonstrated that nanoparticles are a tool for the administration of water-soluble compounds. Chitosan nanoparticles were also used for the treatment of white spot syndrome virus in shrimp. Shrimp treated with the nanoparticles showed high survival rates for 30 days (JIMÉNEZ-FERNÁNDEZ et al., 2014).

Fernández-Díaz; Yúfera, (1995) reported the capacity of Dorada (*Sparus aurata*) to digest encapsulated diets, emphasizing that this depends mainly on the coating (stiffness and thickness) of the capsule. In this study, it was found that thin-walled gelatin microcapsules have a rapid degradation in comparison with thicker and resistant materials. Among other species of commercial value, the use of nanotechnology efficient delivery of food supplements has already been studied, Ashouri et al. (2015), demonstrating that the feeding supplementation of nano-Se (1 mg/kg) improved the growth of carp. There were also improvements in the antioxidant defense system. Asaikkutti et al. (2016) found that magnesium oxide nanoparticles added to shrimp diets (*Macrobrachium rosenbergii*) improved growth, survival, antioxidant activity, and final weight of the organism. Muralisankar et al, (2016), found that copper nanoparticles (20 mg/kg) added to diets positively influence growth and increased activity of freshwater langoustine digestive enzymes (*Macrobrachium rosenbergii*). Another study performed by (IZQUIERDO et al., 2017), showed that Se, Zn and Mn nanoparticles when supplementing improves mineralization and the growth in bream larvae (*Sparus aurata*). The use of nanoscale materials is growing rapidly, and with it also grows the concern in relation to its nanotoxicity. Some nanoparticles can cause lung damage, renal toxicity, hepatotoxicity and immunotoxicity in animals (LIU et al., 2016).

TiO_2 nanoparticles caused respiratory toxicity and blood oxidative stress in rainbow trout (*Oncorhynchus mykiss*) (FEDERICI; SHAW; HANDY, 2007; SCOWN et al., 2009). TiO_2 nanoparticles are also absorbable in the intestinal and gill epithelium of fish (SCOWN et al., 2009). Farkas et al, (2010) found that gold and silver nanoparticles showed effects on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. The toxicity of Ag nanoparticles in fish can occur in several ways, affecting oxidative stress and metal mobilization (GAGNÉ et al., 2012).

Nanoparticles of nickel, silver, chromium oxide and cobalt oxide affected growth, behavior, oxidative stress and tissue structure in *L. rohita*. These effects on the fish are dependent on the type of particle, as cobalt oxide nanoparticles were more toxic than silver nanoparticles (KANWAL et al., 2019).

The behavior of nanoparticles depends on their physicochemical properties (size, zeta potential, and shape) and their kinetic energy. The aggregation of the particles is directly related to these properties (SHAW; HANDY, 2011). The smaller a nanoparticle is, the greater the interaction between the volume and the surface, increasing the toxicity and reactivity of these. Small nanoparticles can penetrate animal and plant tissue. Particles smaller than 100 nm can be absorbed by cells by penetrating the cell membrane (SAJID et al., 2015). The nanoparticles have a very high reactivity in catalytic process the particles do not degrade, which can lead to a series of reactions in biological systems, this reactivity makes the particles highly toxic. Another factor that influences in reactivity is the surface charge (SAJID et al., 2015). Nanoparticles can be toxic to both single-celled organisms, daphnia and fish (SAJID et al., 2015). The nanoparticle uptake pathway in fish can be through the body walls or through the gills (NOWACK; BUCHELI, 2007).

The forms of entry of nanomaterials in the environment can be intentional or unintentional (product degradation) and through wastewater treatment plants sludge, effluents and ground water aquifers (BISWAS; WU, 2005). There is a difficulty in knowing the concentrations that are being released into the environment. The changes of these nanomaterials as sedimentation, agglomeration and surface area, can influence the range and route of release into the environment. These changes or transformations affect the transport, toxicity, and fate of nanoparticles in the environment, on which lies the importance of understanding and characterizing these changes or transformation (MAURER-JONES et al., 2013).

The transport of nanoparticles in the environment depends on their characteristics, size, aggregation and sedimentation in cells. The sedimentation in cells is of paramount importance for determining the absorption rate. The absorption of nanoparticles into mammalian cells depends on the size and surface, in some cells this uptake is produced by phagocytosis or endocytosis, and can be stored in the mitochondria of the cells, which can cause a toxic reaction (TAGHAVI et al., 2013).

In risk assessment, it is necessary to identify environmental routes, the resources and applications of nanoparticles (WARHEIT, 2018). The nanoparticles released into the environment interact with water, soil, and air. These interactions can change the nanoparticles' surface properties, aggregation, change in charge among other properties. These effects of nanoparticles have already been studied in soils and in aquatic biota (ecosystems) (BELAL; EL-RAMADY, 2016). However, more information is required about toxicity and behavior of nanoparticles being that the available data may be insufficient to assess concentrations and quantity expected nanoparticles in the environment. For more information about toxicity, it is necessary to know and evaluate the expected concentrations and amounts of the nanoparticles in the environment (WARHEIT, 2018). The challenges that nanotechnology presents are broad, it is necessary to develop a regulatory framework for the use of nanomaterials, as well as studies of the acute and chronic effects of nanoparticles (JEEVANANDAM et al., 2018; NOWACK; BUCHELI, 2007; RANA; KALAICHELVAN, 2013). It is important to develop a regulatory framework to be able to evaluate toxicity in the aquatic environment. For that, more studies are necessary with the aim of decreasing damage and toxicity (JAHAN et al., 2017). Toxicology studies are few and these indicate that nanoparticles can be toxic at low concentrations. So far there is not much information about absorption, transfer and mobility that determines the risk of nanoparticles for the environment and organisms (RANA; KALAICHELVAN, 2013).

Despite all the studies related to toxicology, the information may be insufficient, because these studies focus on in somehow many types of nanomaterials (silver, carbon nanotubes, metal nanoparticles, aluminum and titanium). Information such as degradation, deposition, transport models is not yet available, since it requires a very large investment to solve or understand the behavior of nanomaterials in the environment (GUPTA; XIE, 2018).

The regulatory protocols in the use of nanoproducts are adapted from the protocols used for chemical substances, and is subject to regulation in each country. However, the FDA has warned about the need for tests standardization and the development of protocols for testing biological effects. In 2007, the European union restricted the use of chemicals in REACH where it alerts that commercialization purposes products have to be environmental and toxicological tested. The EPA in the United States had encouraged the toxicological study of metallic and carbon nanoparticles. The USEPA is developing a new rule (SNUR) that can guarantee a review of nanoscale materials (SAJID et al., 2015). The use of this technology requires a political and

scientific debate to adequate environmental legislation about their application and evaluation well as techniques for analysis and environmental impacts.

6. CONCLUSIONS AND FUTURE PROSPECTS

Nanotechnology is among the most recent technological advances and is part of several production processes, which requires regulation or legislation. The properties that these nanomaterials exhibit have improved the performance of a wide variety of services and products, such as pharmaceuticals, food processing, and energy distribution (AMETA et al., 2020; KHAN; SAEED; KHAN, 2019). In many countries the use of nanotechnology is growing more and more and is being applied in various areas mainly in pharmaceuticals, biomedicine, advanced materials, agriculture and recently in aquaculture (HUANG et al., 2015). Every technological innovation brings changes to the environment, from development till the disposal. Nanotechnology, besides bringing innovation, can also benefit the environment. However, for this fact to happen, it is necessary that regulatory agencies assess their positive and negative impacts and research centers that study the interaction and the characteristics of these nanomaterials on how they can affect the environment and human beings.

Nanotechnology offers various benefits such as reduction in the use of hazardous materials, energy efficiency, remediation of degraded areas, greenhouse gas emissions, nanostructured conductors and nanoadditives (ELSAESSER; HOWARD, 2012; MAKSIMOVIĆ; OMANOVIĆ-MIKLIČANIN, 2017; TARAN et al., 2020). Thanks to all these technological advances that nanotechnology offers to the world, it is considered as one of the greatest revolutions. Nevertheless, the new properties of these materials can rise concerns in relation to promoting undesirable events. Every substance can be toxic depending on the frequency of exposure, dose and route of administration (ELSAESSER; HOWARD, 2012).

There are many benefits that nanotechnology can offer to aquaculture. Currently, different systems are used that can strengthen fishing and aquaculture. In the meantime, there is a concern on nanosystems toxicity. That is the reason why regulation is essential to the safe use of these systems. The application of nanotechnology in aquaculture can be in a slow, but promising way. It can solve fundamental problems as quality diet formulation and disease control.

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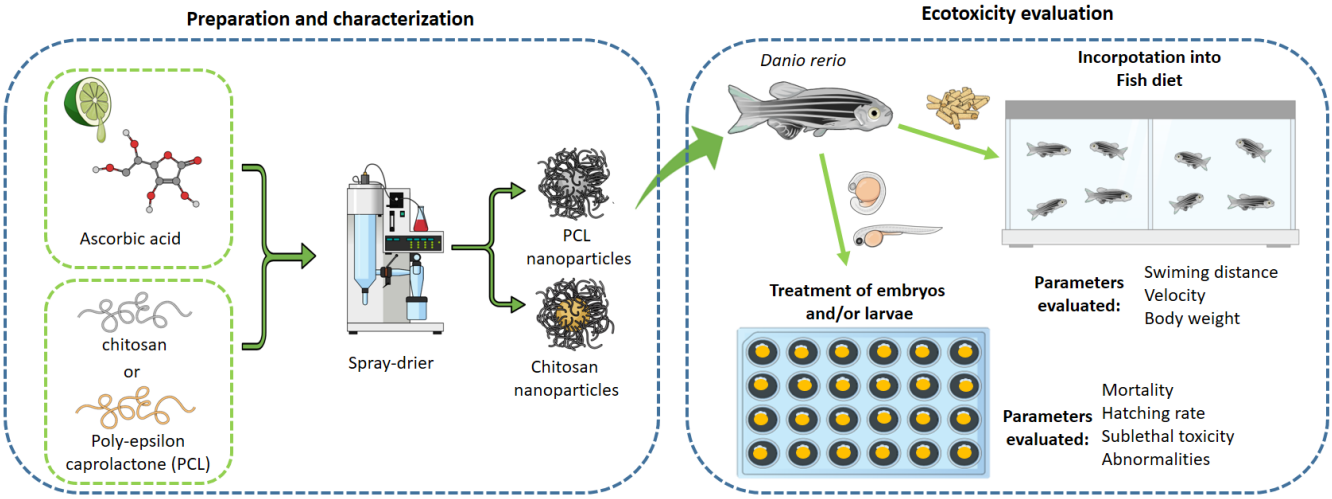
CHAPTER 2

ECOTOXICITY EVALUATION OF POLYMERIC NANOPARTICLES LOADED WITH ASCORBIC ACID FOR FISH NUTRITION IN AQUACULTURE



LUIS, A. I. S.; CAMPOS, E. V. R.; OLIVEIRA, J. L.; VALLIM, J. H.; PROENÇA, P. L. F.; CASTANHA, R. F.; DE CASTRO, V. L. S. S.; FRACETO, L. F. Ecotoxicity evaluation of polymeric nanoparticles loaded with ascorbic acid for fish nutrition in aquaculture. **Journal of Nanobiotechnology**, [s. l.], v. 19, n. 1, p. 163, 2021.

ABSTRACT

Ascorbic acid (AA) is a micronutrient essential for the mechanisms of reproduction, growth, and defense in fish. However, the biosynthesis of this micronutrient does not occur in fish, so it must be supplied with food. A difficulty is that plain AA is unstable, due to the effects of light, high temperature, and oxygen, among others. The use of nanoencapsulation may provide protection and preserve the physicochemical characteristics of AA for extended periods of time, decreasing losses due to environmental factors. This study evaluated the protective effect of nanoencapsulation in polymeric nanoparticles (chitosan and polycaprolactone) against AA degradation. Evaluation was made of the physicochemical stability of the nanoformulations over time, as well as the toxicological effects in zebrafish (*Danio rerio*), considering behavior, development, and enzymatic activity. For the statistical tests, ANOVA (two-way, significance of $p < 0.05$). Both nanoparticle formulations showed high encapsulation efficiency and good physicochemical stability during 90 days. Chitosan (CS) and polycaprolactone (PCL) nanoparticles loaded with AA had mean diameters of 314 and 303 nm and polydispersity indexes of 0.36 and 0.28, respectively. Both nanosystems provided protection against degradation of AA exposed to an oxidizing agent, compared to plain AA. Total degradation of AA was observed after 7, 20, and 480 min for plain AA, the CS nanoparticle formulation, and the PCL nanoparticle formulation, respectively. For zebrafish larvae, the LC_{50} values were 330.7, 57.4, and 179.6 mg/L for plain AA, the CS nanoparticle formulation, and the PCL nanoparticle formulation, respectively. In toxicity assays using AA at a concentration of 50 mg/L, both types of nanoparticles loaded with AA showed lower toxicity towards the development of the zebrafish, compared to plain AA at the same concentration. Although decreased activity of the enzyme acetylcholinesterase (AChE) did not affect the swimming behavior of zebrafish larvae in the groups evaluated, it may have been associated with the observed morphometric changes, such as curvature of the tail. This study showed that the use of nanosystems is promising for fish nutritional supplementation in aquaculture. In particular, PCL nanoparticles loaded with AA seemed to be most promising, due to higher protection against AA degradation, as well as lower toxicity to zebrafish, compared to the chitosan nanoparticles. The use of nanotechnology opens new perspectives for aquaculture, enabling the reduction of feed nutrient losses, leading to faster fish growth and improved sustainability of this activity.

Keywords: Ascorbic acid, polymeric nanoparticles, toxicity, zebrafish, aquaculture.

1. INTRODUCTION

With the global population predicted to increase by approximately 30% to 9.7 billion in 2050, one of the greatest challenges currently facing the world is food security (FISHER et al., 2017). Aquaculture can actively contribute to ensuring an adequate food supply, providing high quality protein to feed the growing world population, especially in developing countries (EL BASUINI et al., 2020). However, in efforts to maximize production, the aquaculture sector faces challenges regarding the emergence of diseases, which are very common in intensive production systems (DOAN et al., 2020). Therefore, it is necessary to develop suitable strategies that can ensure the sustainable development of aquaculture (SHAH; MRAZ, 2019). The Blue Growth initiative aims at the sustainable global development of aquaculture, enabling the sector to contribute to the Sustainable Development Goals (SDGs) of the United Nations, especially Goals 2 and 14. Goal 2 concerns the achievement of zero hunger, food security, and improved nutrition, while Goal 14 aims at the conservation and sustainable development of the oceans, seas, and marine resources (FAO, 2015, 2018b).

Among the strategies for the development of sustainable aquaculture, the utilization of natural sources of vitamins to improve the growth and development of aquatic species has been studied (AMIR et al., 2019; DAWOOD; KOSHIO, 2018; EL BASUINI et al., 2020). Vitamins act mainly as cofactors of enzymes, so their inadequate supplementation can be detrimental to organism growth and survival, with increased vulnerability to diseases and infections (GAO et al., 2014; SHAHKAR et al., 2015). Due to their coenzyme activities, vitamins contribute to normal metabolic functioning and the maintenance of health (DAWOOD; KOSHIO, 2018). The hydrophilic vitamin L-ascorbic acid (AA) is an essential micronutrient required by aquatic animals, since they do not have the ability to synthesize it (AMIR et al., 2019; DAWOOD; KOSHIO, 2018).

It has been reported that dietary supplementation with AA can positively affect growth performance, immunological responses, and survival rate, as well as increase the resistance to stressors in different fish species (DAWOOD; KOSHIO, 2018). The amount of AA required in the diet varies according to species, size, age, breeding condition, and the form of AA administered (DOAN et al., 2020). In its pure form, ascorbic acid is unstable in alkaline

environments, where its oxidation is accelerated, leading to its loss at rates influenced by factors including temperature, oxygen, light, and pH, among others (SHERAZ et al., 2015). Therefore, it is important to develop new technologies capable of protecting this molecule against premature degradation, increasing its stability and bioavailability

Nanoencapsulation offers a way to improve the stability of AA (DESAI; PARK, 2005; FANGYUAN DONG, 2016). The materials used for nanoencapsulation include a variety of matrices that may have natural or synthetic origins (LUIZ DE OLIVEIRA; RAMOS CAMPOS; FRACETO, 2018). One such material, chitosan, is derived from chitin, obtained mainly from crustaceans such as crabs, although this polymer is also found in the cell walls of fungi. After being separated, chitin is submitted to an alkalinization or acidification process. Chitosan easily penetrates cell and transcellular walls, is positively charged, forms complexes by hydrophobic interactions or hydrogen bonds, and is soluble in acidic media (MOHAMMED et al., 2017). In fish, chitosan can adhere to the gills and skin, due to the interaction between chitosan nanoparticles and the layer of mucopolysaccharides and mucoproteins that covers the organism (COSTA et al., 2016). Other polymer widely used to nanoencapsulation is poly (ε-caprolactone), which is an aliphatic polyester, biodegradable and biocompatible. It is a FDA (US Food and Drug Administration) approved polymer with high permeability to many compounds, non-toxicity and reasonably low cost that renders this polymer attractiveness for aquaculture applications (WITT; SCHEPER; WALTER, 2019).

Despite the potential benefits of nanocarrier systems, concerns have been raised about the biosafety of their use in aquaculture. Therefore, it is necessary to evaluate and characterize the possible ecotoxicological risks resulting from their use, in order to make decisions about the implementation of these nanotechnologies in agricultural practices (KAH et al., 2018). Hence, it is essential to understand the potential effects of nanocarrier systems towards both target and nontarget organisms. For this purpose, the zebrafish (*Danio rerio*) has been used to assess the effects of different compounds on the fish community (MOURA et al., 2019). Monitoring of the development of *Danio rerio* provides a rapid way to evaluate the environmental risks of nanomaterials (SHAW et al., 2016).

Decrease the premature degradation of AA, the aim of this work was to prepare and characterize polymeric nanoparticles (chitosan and polycaprolactone) used to encapsulate AA,

followed by evaluation of their effects on the development of zebrafish embryos and larvae. In addition, nanoparticulate systems with or without AA were evaluated for possible toxic effects on the development of these organisms. Different parameters of physical development were evaluated, including malformations, hatching and survival rates, and behavioral indicators. The AchE activity was also determined, since its alteration can lead to impaired motor function (BERTUZZI; AMPATZIS, 2018). This study opens perspectives for the development of systems for fish nutrition in aquaculture, aiming at more sustainable production that ensures the quality of life of the organisms.

2. MATERIALS AND METHODS

2.1. MATERIALS

L-ascorbic acid (99%), trehalose dihydrate, poly-ε-caprolactone, and chitosan (50,000–190,000 Da) were obtained from Sigma-Aldrich and were used without further purification. Acetonitrile and methanol (HPLC grade) were obtained from J.T. Baker. Low molecular weight chitosan and the chemicals used in the oxidation assays (Oxone® and EDTA) were obtained from Sigma-Aldrich. The AchE assays employed acetylthiocholine iodide and DTNB (5,5′-dithiobis (2-nitrobenzoic acid) acquired from Sigma-Aldrich.

2.2. NANOPARTICLES SYNTHESIS

2.2.1. CHITOSAN NANOPARTICLES

Nanoparticles composed of chitosan and sodium tripolyphosphate (TPP) were prepared by the ionic gelation technique at room temperature (25ºC), followed by spray drying, according to (DESAI; LIU; PARK, 2006b). A 250 mL volume of an aqueous solution of acetic acid (0.5% v/v) was prepared containing low molecular weight chitosan (1% w/v). Separately, 10 mL of an aqueous solution was prepared containing 2.5 g of AA. The AA solution was added to the chitosan solution and the resulting mixture was homogenized for 10 min, under magnetic stirring at 800 rpm. This solution was then dripped into 10 mL of TPP solution (1% w/v) under magnetic stirring at 800 rpm for 10 min. The final concentration of AA was 0.01 mg/mL. Control nanoparticles were prepared using the same method described above, without addition of AA. In order to guarantee the nanoparticles dispersibility and their regular spherical shape, trehalose dihydrate (1% w/v) was used as additional excipient (WONG; JOHN, 2016). The

nanoparticles were dried using a laboratory spray dryer with a 1 mm diameter nozzle (Model MSD 1.0, Labmaq, Brazil). The operating conditions were inlet temperature at 110.4 °C, outlet temperature at 72 °C, feed rate of 0.3 L h^{-1}, atomization flow of 40 L h^{-1}, and drying flow of 1.50 m^3 min^{-1}. The powder obtained was stored at room temperature until use.

2.2.2. POLYCAPROLACTONE NANOPARTICLES

Polycaprolactone nanoparticles were prepared by the double emulsion-evaporation method at room temperature (25ºC), with slight modifications (PEREIRA et al., 2014). For the organic phase, 400 mg of PCL were dissolved in 20 mL of dichloromethane. The aqueous phase was PVA (3 mg mL^{-1}) dissolved in ultrapure water. AA was dissolved in ethanol and mixed with the aqueous phase containing PVA, followed by ultraturrax homogenization for 1 min at 14,000 rpm. The resulting mixture was added to the organic phase and homogenized for 8 min by ultraturrax at 14,000 rpm. The organic solvent was evaporated using a rotary evaporator and the formulation was concentrated to a volume of 10 mL. The final concentration of AA was 5 mg mL^{-1}. The nanoparticles were dried using a laboratory spray dryer with a 1 mm diameter nozzle (MSD 1.0, Labmaq, Brazil). In order to guarantee the nanoparticles re-dispersibility and their regular spherical shape, trehalose dihydrate (1%) was used as additional excipient (WONG; JOHN, 2016). The operating conditions were inlet temperature at 110.4 °C, outlet temperature at 65.4 °C, feed rate of 0.3 L h^{-1}, atomization flow of 40 L h^{-1}, and drying flow of 1.50 m^3 min^{-1}. The powder obtained was stored at room temperature until use.

2.1. NANOPARTICLES CHARACTERIZATION

Evaluation was made of the physicochemical characteristics of both nanoparticle formulations, including the mean diameter, polydispersity index, zeta potential, pH, and encapsulation efficiency. For these analyses, the dried nanoparticles (10 mg) were resuspended in 1 mL of ultrapure water. The samples were stored in amber flasks at room temperature, protected from light.

2.1.1. MEAN DIAMETER AND POLYDISPERSITY INDEX

The mean diameter and polydispersity index were determined by the photon correlation spectroscopy technique, using a ZetaSizer Nano ZS 90 instrument (Malvern Instruments) at an

angle of 90°. The nanoparticle solutions were diluted 1,500 times with deionized water. The measurements were performed in triplicate (n = 3).

2.1.2. ZETA POTENTIAL AND pH

The surface charges of the nanoparticles were determined by zeta potential measurements using the microelectrophoresis technique, employing a ZetaSizer Nano ZS 90 analyzer (Malvern Instruments). The samples were diluted 1,500 times and analyzed in triplicate. The pH values of the different formulations were obtained using a potentiometer (Tecnal) previously calibrated with pH 7 and pH 4 standards.

2.1.3. ASCORBIC ACID (AA) ENCAPSULATION EFFICIENCY

The AA encapsulation efficiency was determined by the ultrafiltration-centrifugation method. The nanoparticles were centrifuged using a 10 kDa cellulose filter (Millipore) that allowed the passage of non-encapsulated AA. The encapsulation efficiency was determined as the difference between the amounts of AA added to the formulation (100%) and present in the filtrate (non-encapsulated AA). The quantification of AA was performed by HPLC, with UV detection at 250 nm. The separation was performed using a HyperClone C18 column (250 × 4.6 mm, 5 μm), with a mobile phase consisting of a 95:5 (v/v) mixture of methanol and 1% (v/v) acetic acid. Quantification employed an analytical curve ($y = 1.99x - 5.83$) with correlation coefficient of 0.99903, limit of detection (0.277 μg mL^{-1}) and quantification (0.926 μg mL^{-1}).

2.1.4. AA RELEASE KINETICS AND MATHEMATICAL MODELING

The profiles of AA release from the nanoparticles were evaluated using a two-compartment system composed of a donor compartment (2 mL) and an acceptor compartment (50 mL), separated by a cellulose membrane (1000 Da exclusion pore size) under slight stirring and room temperature (25 ºC). Aliquots (1 mL) were periodically collected from the acceptor compartment, with the volume being replaced with 1 mL of water to maintain a constant volume. The aliquots removed were analyzed by HPLC and the results were converted to percentages of released AA. The assays were performed in triplicate. Zero order, first order, Higuchi and Korsmeyer-Peppas mathematical models were used to elucidate the release mechanism of AA from all formulations (HAIDAR, 2018).

2.1.5. AA OXIDATION KINETICS

The oxidation profiles of AA, alone and encapsulated in the CS and PCL nanoparticles, were evaluated using the method described previously (SALKIĆ; SELIMOVIĆ, 2015). A stock solution of AA was prepared by dissolving 50 mg of AA in 250 mL of stabilizing solution (EDTA). The absorbance of this solution was measured at 265 nm, with the stabilizing solution used as the background. Separately, a solution of Oxone (potassium peroxymonosulfate, Sigma-Aldrich) was prepared in 50 mL of EDTA solution. An aliquot of AA solution was mixed with 3 mL of Oxone solution and completed to 25 mL with the stabilizing solution, resulting in a solution with 10 μg mL^{-1} of AA. The same procedure was performed for the CS and PCL nanoparticles loaded with AA. The absorbance value of the solution containing only AA, minus the absorbance value of the solution containing AA and Oxone, was proportional to the concentration of AA present in the sample. For oxidation studies under aquarium conditions, 9 aquaria were used (3 per treatment), each containing a final volume of 3 L and fitted with an aeration pump. The parameters set for the experiment were 28 °C, pH 7 ± 0.5, and 25 μg mL^{-1} of AA (the concentration used in the in vivo assays). At predetermined times, 1 mL aliquots were collected, and the amount of AA was determined using HPLC. The results were presented as % viable ascorbic acid. The assays were performed in triplicate ($n = 3$).

2.2. IN VIVO STUDIES

2.2.1. MAINTENANCE AND REPRODUCTION OF THE ANIMALS

The breeding fish (*Danio rerio*, wild-type strain) were kept in a Rack Hydrus system (Model ZEB-40, Alesco), under controlled conditions of 28 ± 0.2 ºC, conductivity of 400 ± 0.2 μS, and pH 7.0 ± 0.2. The pH was controlled using solutions of 30 g L^{-1} sodium bicarbonate (Synth) and 1 mL L^{-1} HCl (37%, ACS, Scharlau). The conductivity was adjusted with 30 g/L saline solution (Red Sea salt or Instant Ocean). The embryos were kept in reconstituted water prepared according to the protocol of the USEPA (2002). The experiments carried out in this work were approved by the Animal Use Ethics Committee (CEUA) of Embrapa Environment (protocol nº 12/2018).

2.2.2. TOXICITY EVALUATION USING ZEBRAFISH

The embryos and larvae were divided into control groups (embryo medium only) and treatment groups with addition of ascorbic acid (AA), empty chitosan nanoparticles (NPs_CS), chitosan nanoparticles loaded with AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL

nanoparticles loaded with AA (NPs_PCL_AA). Preliminary tests were performed to determine the lethal concentration (LC_{50}, mg/L). For this experiment, 24-well polystyrene plates were used, with the embryos being individually exposed to 2 mL of the test solution (n = 24 per group). The concentrations used for each exposure were 3.12, 6.25, 12.5, 25, 50, 100, 200, and 400 mg/L. The same dilutions were used for the control nanoparticles without the presence of AA. The positive control was 4 mg L^{-1} of 3,5-dichloroaniline (≥ 98%, Sigma-Aldrich). The plates were examined every 24 h to determine the mortality of the embryos and larvae, using a stereomicroscope (Model SMZ 2 LED, Optika) and Optika View v. 7.1.1.5 software. After 96 h of exposure, the LC_{50} was determined for each group, using probit analysis performed with Statgraphics Centurion XVII v. 1.17.04 software.

2.2.3. EVALUATION OF FISH EMBRYO ACUTE TOXICITY (FED)

The embryos were exposed to different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}) of the treatments (n = 24 per group). The exposure was carried out for 96 h under the test conditions described in OECD protocol 236 (OECD, 2013). The embryos were kept individually in 24-well polystyrene plates (n = 24 organisms per group), with addition of 2 mL of test solution to each well. The temperature was maintained at 26 ± 0.2 °C and a light/dark photoperiod of 14/10 h was provided. Determinations were made of mortality and morphological abnormalities including changes in heartbeat, presence of coagulation, undetected tail, change in head formation, disintegration of larvae, pericardial edema, yolk sac edema and opacity, and defective development of the somite.

The total length of the larvae (mm) was measured from the mouth to the terminal portion of the tail. The embryos and larvae were evaluated every 24 h, using a stereomicroscope to determine the occurrence of malformations and/or mortality. After exposure for 96 h, the live larvae were photographed and their total lengths were measured using Optika View v. 7.1.1.5 software previously calibrated with a millimeter scale.

Mortality was defined as the presence of coagulation, lack of heartbeat, failure of somite development, and undeveloped tail. The hatching rate was calculated considering the successful hatching of embryos in relation to the total number of embryos in each replicate. The observed malformations included pericardial edema, tail deformity, yolk sac edema, and spinal curvature. Pericardial edema was identified as swelling due to an increase in the volume of fluid in the

pericardium, which is the portion of the coelomic cavity that separates the heart from the body wall (WU et al., 2019). The presence or absence of an inflated swimming bladder was determined for the embryos 120 h post-fertilization (hpf), using a microscope.

The malformation rate was determined considering the percentage of malformed larvae in relation to the total number of larvae that hatched during the test. At the end of the exposures (96 h), the larvae (n = 10 per group) were photographed and the total length was measured using the Optika View v. 7.1.1.5 software, at 2 × magnification. The equipment was previously calibrated using a millimeter scale.

2.2.4. EVALUATION OF ZEBRAFISH MOTOR ACTIVITY

For evaluation of the behavioral alterations caused by the different treatments, the embryos were divided into the following groups: control (embryo medium only), ascorbic acid (AA), chitosan nanoparticles (NPs_CS), chitosan nanoparticles loaded with AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles loaded with AA (NPs_PCL_AA). Zebrafish embryos (n = 36 per group) were exposed (96 h) under sublethal and acute toxicity conditions, using the different treatments at concentrations of LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}. After 96 h, the larvae were distributed individually in 96-well plates, with each well containing 100 µL of embryo medium and 100 µL of test solution (total volume = 200 µL). The behaviors of the larvae were recorded individually for 10 min (at 28 °C) and were analyzed using EthovisionXT software (Noldus). The parameters considered were speed (mm/s) and distance covered.

2.2.5. EVALUATION OF ACETYLCHOLINESTERASE (AChE) ACTIVITY

AChE activity was determined according to the method described previously [28]. The embryos (2 hpf) were exposed for 96 h to different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}) of the treatments: control (embryo medium only), ascorbic acid (AA), chitosan nanoparticles (NPs_CS), chitosan nanoparticles loaded with AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles loaded with AA (NPs_PCL_AA). The exposures were performed in sterile polystyrene Petri dishes. Live larvae were collected 96 h after the start of exposure, for the determination of AChE activity. The larvae were washed (3 times) with cold (4 °C) phosphate buffer (0.5 mol/L) at pH 7.0, followed by storage in Eppendorf microtubes containing 0.5 mL of phosphate buffer (n = 5 pools of 10 larvae per group). The readings were performed in triplicate. The samples in phosphate buffer were homogenized with an ultraturrax,

followed by centrifugation for 10 min at 10,000 g and 4 °C. The supernatant was used for the analysis of AChE. The protein concentrations in the samples were determined as described previously by Bradford, (1976). Five pools of 10 larvae were analyzed for each group, with the readings performed in triplicate. A 96-well microplate spectrophotometer (Sunrise, Tecan) was used for the analysis. The AChE activity was determined as described previously by Ellman et al., (1961), modified for microplates Da Silva De Assis, (1998). For this, 15 µg of the homogenate was added to 15 µL of 9 mmol/L acetylcholine (acetylthiocholine iodide, Sigma-Aldrich) and 70 µL of 0.75 mmol/L DTNB (5.5′-dithiobis (2-nitrobenzoic acid), Sigma-Aldrich). The absorbance at 412 nm was monitored for 5 min, at 30 °C (11 cycles, with 30 s between readings).

2.2.6. EVALUATION OF ZEBRAFISH BODY WEIGHT

Seventy zebrafish larvae with initial weight 1.8–1.9 mg and length 7.748–8.398 mm were distributed in 7 aquaria with volumes of 3 L (n = 10 organisms per group) and were exposed to the different treatments for 61 days. The control group was fed with commercial food (TetraMIN), while the other groups were fed with TetraMIN plus different concentrations (25, 50, and 100 mg/kg) of chitosan nanoparticles loaded with AA (NPs_CS_AA) or PCL nanoparticles loaded with AA (NPs_PCL_AA). The fish were fed to satiety, three times a day. At the end of the experimental period, the fish were fasted for 24 h. After this period, individual measurements were made of the weights and total lengths of the fish from each treatment. The results were presented as the means of the final weights and lengths of 10 organisms.

3. RESULTS AND DISCUSSION

3.1.CHARACTERIZATION OF THE POLYMERIC NANOPARTICLES

Chitosan nanoparticles cross-linked with sodium tripolyphosphate were prepared by the ionic gelation method, while polycaprolactone nanoparticles were synthesized using the emulsification/evaporation method. In both cases, the synthesis was followed by spray drying. The physicochemical properties of the nanoparticles were determined before and after the spray drying process.

For the chitosan nanoparticles (NPs_CS) and the chitosan nanoparticles containing AA (NPs_CS_AA) in aqueous suspension, the average diameters were 316 ± 5 nm and 337 ± 3 nm,

respectively. The polydispersity indexes for these nanoparticles ranged from 0.39 to 0.40, while the zeta potentials were between 47 and 76 mV (Table 1).

Table 1. Physicochemical characterization of the nanoparticles loaded with AA. The parameters determined were the mean particle diameter (MD) obtained by the dynamic light scattering method, polydispersity index (PDI), zeta potential (ZP), and encapsulation efficiency (EE). The values are the means of three determinations.

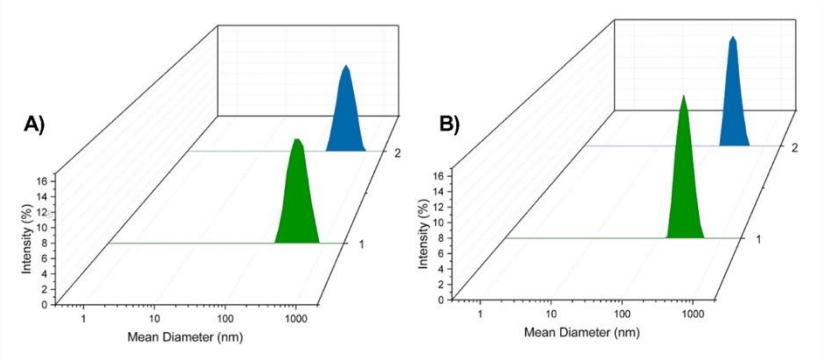
Samples	MD (nm)	PDI	ZP (mV)	EE (%)
Nanoparticles before spray drying				
NPs_CS	316 ± 5	0.39 ± 0.13	47 ± 0.7	-
NPs_CS_AA	337 ± 3	0.40 ± 0.01	76 ± 0.5	98 ± 0.1
NPs_PCL	329 ± 1	0.23 ± 0.10	-19 ± 0.2	-
NPs_PCL_AA	350 ± 5	0.34 ± 0.10	-20 ± 0.1	95 ± 0.4
Nanoparticles after spray drying				
NPs_CS_SD	294 ± 1	0.33 ± 0.02	41 ± 0.5	-
NPs_CS_AA_SD	314 ± 3	0.36 ± 0.01	60 ± 1.7	96 ± 0.5
NPs_PCL_SD	281 ± 1	0.21 ± 0.05	-15 ± 0.2	-
NPs_PCL_AA_SD	303 ± 1	0.28 ± 0.06	-18 ± 0.4	91 ± 0.2

For the PCL nanoparticles (NPs_PCL) and the PCL nanoparticles containing AA (NPs_PCL_AA) in aqueous suspension, the average diameters were 329 ± 1 nm and 350 ± 5 nm, respectively. The polydispersity indexes were between 0.23 and 0.34, while the zeta potentials for these nanoparticles were negative, with values of -19 ± 0.2 mV for NPs_PCL and -20 ± 0.1 mV for NPs_PCL_AA (Table 1). Both nanoparticulate systems showed high encapsulation efficiencies, with values of $98 \pm 0.1\%$ for NPs_CS_AA and $95 \pm 0.4\%$ for NPs_PCL_AA. However, there were significant decreases in the encapsulation efficiency during storage (data not shown), which could be attributed to the high aqueous solubility of AA (330 g/L). Furthermore, it is known that AA presents rapid degradation in aqueous solution. For these reasons, together with the fact that the objective of this work was to prepare nanoparticles to be incorporated into fish diets, it was decided to submit these nanoparticles to a spray drying process, in order to extend the stability of the AA and improve its biological activity.

After drying the nanoparticles, they were resuspended in distilled water and their physicochemical characteristics were determined, in order to evaluate the effect of the drying

process. The average diameters of the chitosan nanoparticles (NPs_CS_SD) and the chitosan nanoparticles containing AA (NPs_CS_AA_SD) presented reductions of 22 and 23 nm, respectively. Decreases of the polydispersity index were also observed for both formulations, although the chitosan nanoparticles loaded with AA showed the same size distribution pattern before and after the spray drying process (Fig. 1A). Chitosan nanoparticles are electrostatically stabilized, so the strong cationic charge of the nanoparticles produced could be an indication of good stability of the nanoformulation. Another important parameter that did not undergo significant alteration was the efficiency of encapsulation of AA in the chitosan nanoparticles, with a value of $96 \pm 0.5\%$ obtained.

Figure 1. Size distributions obtained using the DLS technique. **A**) Chitosan nanoparticles loaded with AA, before the spray drying process (NPS_CS_AA) (1) and after spray drying (NPs_CS_AA_SD) (2). **B**) PCL nanoparticles containing AA, before spray drying (NPs_PCL_AA) (1) and after spray drying (NPs_PCL_AA_SD) (2).



Similar to the behavior observed for the chitosan nanoparticles, the dried PCL nanoparticles showed reductions of 48 and 47 nm for NPs_PCL_SD and NPs_PCL_AA_SD, respectively (Table 1). Decreases of the polydispersity index also occurred after the drying processes, but the size distribution was not modified, as shown in Fig. 1B. The zeta potentials showed small increases, compared to the nanoparticles before the drying process, but remained negative, which is a characteristic of PCL. The zeta potential values for these nanoparticles were below the values recommended in the literature for a stable colloidal system. It is well known that the zeta potential is responsible for the colloidal stabilization of the nanoparticles in suspension, preventing their aggregation, due to the electrostatic repulsion of the particles, thus, the higher the value of the zeta potential (in module), the greater the electrostatic repulsion and the lower the tendency to form aggregates. However, in the case of the PCL formulations,

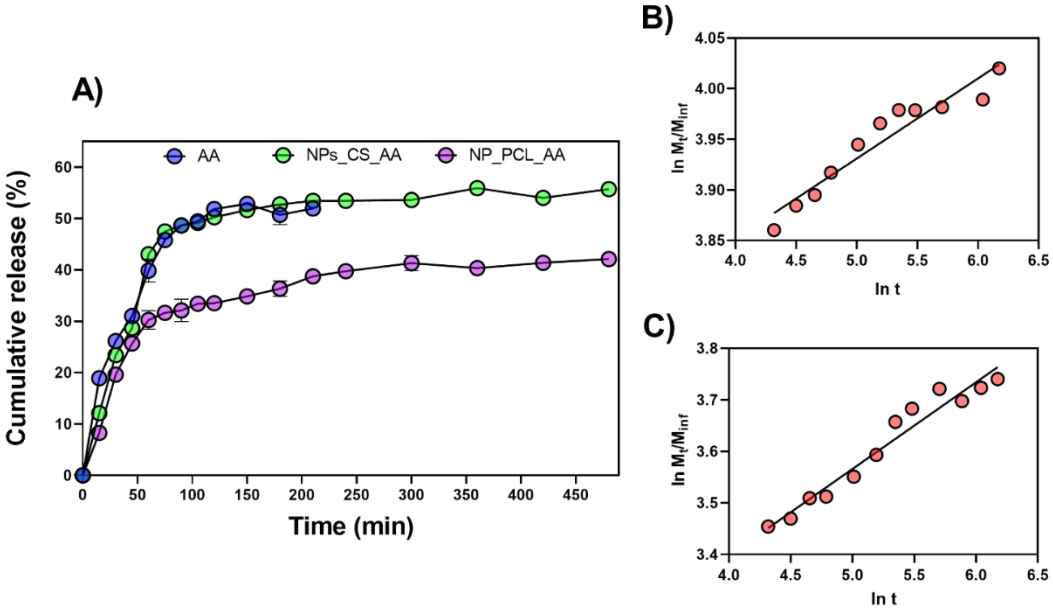
PVA was used as a stabilizing agent, with the main stabilization mechanism of this surfactant involving steric hindrance, rather than electrostatic charge effects.

A high AA encapsulation efficiency ($91 \pm 0.2\%$) was observed, indicative of strong affinity of the compound for the nanoparticles. The physicochemical characterization data demonstrated that the drying process did not cause changes capable of destabilizing the system, indicating that this process could be applied to both types of nanoparticle, in order to improve the stability of AA. All the results presented below were performed using nanoparticles that were spray dried and subsequently resuspended before each experiment.

3.2.KINETICS OF AA REALESE FROM THE NANOPARTICLES

The profiles of release of AA from the chitosan nanoparticles (NPs_CS_AA) and the PCL nanoparticles (NPs_PCL_AA) are shown in Fig. 2A. For non-encapsulated ascorbic acid, the concentration of this compound in the acceptor compartment was $39 \pm 0.2\%$ after 60 min, reaching $51 \pm 0.9\%$ after 210 min, followed by a decrease of the concentration, due to degradation of the compound in the aqueous medium, which precluded further concentration measurements for this system.

Figure 2. A) In vitro release kinetics profiles for ascorbic acid (AA), chitosan nanoparticles containing ascorbic acid (NPs_CS_AA), and PCL nanoparticles containing AA (NPs_PCL_AA), for analyses performed in triplicate, with quantification by HPLC. Fitting of the Korsmeyer-Peppas mathematical model for the release of AA from **B)** NPs_CS_AA and **C)** NPs_PCL_AA.



The release patterns observed for NPs_CS_AA and NPs_PCL_AA were similar, with faster release of AA in the first hour of the experiment, followed by a more constant release (Fig. 2A). After 60 min, releases of $43 \pm 0.1\%$ and $30 \pm 0.1\%$ were observed for NPs_CS_AA and NPs_PCL_AA, respectively. In comparison with the non-encapsulated AA, the chitosan nanoparticles were not able to modify the AA release profile, with maximum release of $55.7 \pm 0.03\%$ after 480 min. In contrast, the association of AA with the PCL nanoparticles significantly delayed the release of AA, with release of $42 \pm 0.03\%$ of the encapsulated AA after 480 min. A previous study evaluated the release of AA from chitosan nanoparticles in seawater (pH 8), with only $6.57 \pm 0.52\%$ of the AA released after 120 min, which was 7.6 and 5 times less than observed here for the chitosan and PCL nanoparticles, during the same period of time (JIMÉNEZ-FERNÁNDEZ et al., 2014). The lower release of AA observed by these authors can be explained by the difference of the nanoparticulate system. In this system, there is the presence of cyclodextrin, thus, AA is complexed with cyclodextrin, which was further involved by CS. In this way, the AA needs to diffuse from the CD and later, from the CS to be released to the medium,thus, the $6.57 \pm 0.52\%$ release refers to the AA that is mainly dispersed close to nanoparticles surface.

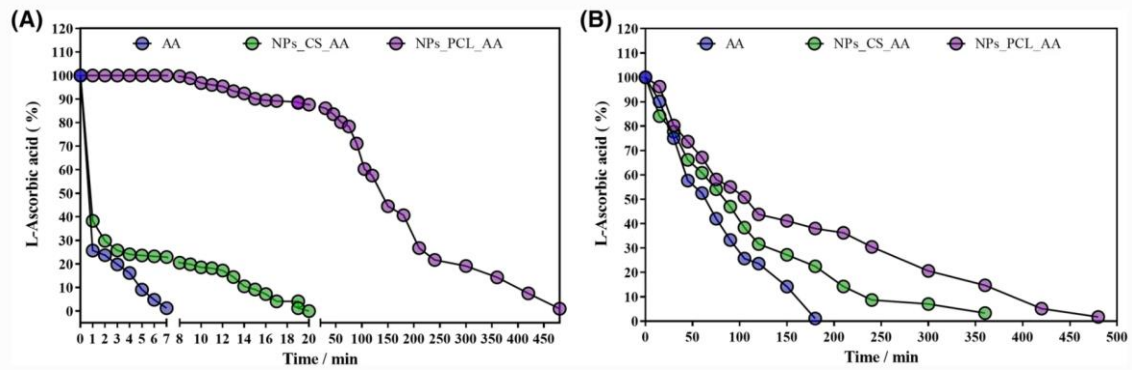
The mechanisms of release of AA from the chitosan and PCL nanoparticles were investigated by application of the zero order, Higuchi, and Korsmeyer-Peppas mathematical models. The Korsmeyer-Peppas model provided the best fit to the release data for both the CS

and PCL nanoparticles, with r^2 values of 0.9189 and 0.9433 for NPs_CS_AA and NPs_PCL_AA, respectively (Fig. 2B, C). For both systems, the release exponent (n) value was less than 0.5 (n = 0.28 for the chitosan nanoparticles and n = 0.38 for the PCL nanoparticles), indicating that the release mainly occurred by diffusion of AA through the swollen polymeric matrix and through the hydrophilic pores (Fickian diffusion). The release constants (k) for the chitosan and PCL nanoparticles were 0.1899 and 0.07912 min^{-1}, respectively, indicating that faster release of AA occurred from the CS nanoparticles, compared to release from the PCL nanoparticles. Brito & colleagues evaluated the release of hydro soluble vitamins from chitosan nanoparticles. The release constant (k) for vitamin C released from chitosan nanoparticles at pH 7.4 and pH 3 were 1.0 and 1.4 min^{-1} respectively (DE BRITTO et al., 2014). In other hand, when the release rate of vitamin C was evaluated from polycaprolactone-polyethylene glycol microparticles in three different pH (2.8, 7.4 and 9.6), the obtained values were between 0.0127 to 0.0493 min^{-1} (OZSAGIROGLU; GUVENILIR, 2016). In this way, the results found here corroborate with previous published results in the literature, where the release rate of AA is faster when encapsulated into chitosan nanoparticles in comparison to PCL nanoparticles.

3.3.AA OXIDATION KINETICS

The oxidation of AA can occur according to both anaerobic and aerobic mechanisms, depending on the availability of oxygen. In the non-oxidative pathway, AA is hydrolyzed to produce an acyclic structure. In the oxidative pathway, oxidation occurs due to the presence of oxygen, producing dehydroascorbic acid (BLAUG; HAJRATWALA, 1972). The oxidation of ascorbic acid was induced by the presence of peroxymonosulfate, resulting in a two-stage reaction in which an ascorbate free radical is firstly produced, followed by the donation of a second electron to produce dehydroascorbic acid, in a reversible process. Dehydroascorbic acid undergoes a ring opening reaction, resulting in 2,3-diketogulonic acid and causing loss of vitamin activity (SALKIĆ; SELIMOVIĆ, 2015). Figure 3 shows the oxidation profiles of non-encapsulated AA and AA encapsulated in the chitosan or PCL nanoparticles.

Figure 3. Oxidation of non-encapsulated ascorbic acid (AA) and ascorbic acid encapsulated in chitosan nanoparticles (NPs_CS_AA) and PCL nanoparticles (NPs_PCL_AA). The oxidation of ascorbic acid was **A** induced using peroxymonosulfate and **B** occurred under aquarium conditions simulating the natural environment of fish



For the non-encapsulated AA, addition of the oxidizing agent to the solution resulted in a fourfold decrease (2.5 ± 0.01 µg/mL) of the AA concentration, relative to the initial value, in the first minute of the experiment (Fig. 3A). Total degradation of the AA present in the solution was observed after 7 min. The AA present in the chitosan NPs also showed rapid (2.6-fold) degradation during the first minute of the experiment, in the presence of the oxidizing agent. Nonetheless, the encapsulation of AA in the chitosan NPs was able to decrease the degradation rate, which stabilized between 2 and 8 min, followed by progressive degradation until complete removal of the AA after 20 min. Encapsulation of AA in the PCL NPs resulted in much slower degradation of the compound, with total degradation only reached after 8 h.

In addition, the degradation of AA, free or encapsulated in the CS and PCL nanoparticles, was evaluated in aquarium simulations representing the normal conditions of the fish environment (Fig. 3B). This experiment with a constant aeration system simulated the natural degradation of AA in aquaculture systems, where there is continuous movement of the water. It has been reported that greater movement of water (solvent) accelerates the degradation of AA (LAING; SCHLUETER; LABUZA, 1978).

As shown in Fig. 3B, faster degradation was observed for the non-encapsulated AA. In the first 60 min of the experiment, almost half of the AA present in solution had already been degraded, while no AA remained in the solution after 120 min, showing that the AA was totally degraded. For the NPs_CS_AA and NPs_PCL_AA, the AA degradation rates were slower, with $60.88 \pm 0.05\%$ and $67.15 \pm 0.52\%$ of the AA remaining after 60 min, and total degradation of AA after 360 and 480 min, respectively. These results demonstrated the effectiveness of the nanosystems in protecting AA against degradation and corroborated the data obtained using the peroxymonosulfate oxidizing agent. In addition, these results showed that under aquarium

conditions, the use of the nanoparticles could promote an increase in the amount of AA in solution, compared to non-encapsulated AA. The results obtained in these tests corroborated the release data, with the greater degradation observed for NPs_CS_AA being due to the faster release of the compound, compared to the release from NPs_PCL_AA, with the latter promoting a more sustained release of the AA protected within the nanoparticles.

AA solutions are highly susceptible to rapid degradation and oxidation by oxygen dissolved in alkaline solutions (FATIBELLO-FILHO; VIEIRA, 2000). Stevanović et al. (2007) prepared poly(D,L-lactide-co-glycolide) nanospheres for the encapsulation of ascorbic acid and evaluated the in vitro degradation of the particles in the presence and absence of ascorbic acid. In both cases, degradation and complete release of the ascorbic acid occurred after 8 weeks. Only 10% of the ascorbic acid was released in the first 24 days, after which agglomeration of the particles resulted in the formation of a porous film. The encapsulation of AA in the nanoparticles provided protection against reactions, temperature, humidity, and oxygen, consequently improving both stability and bioavailability.

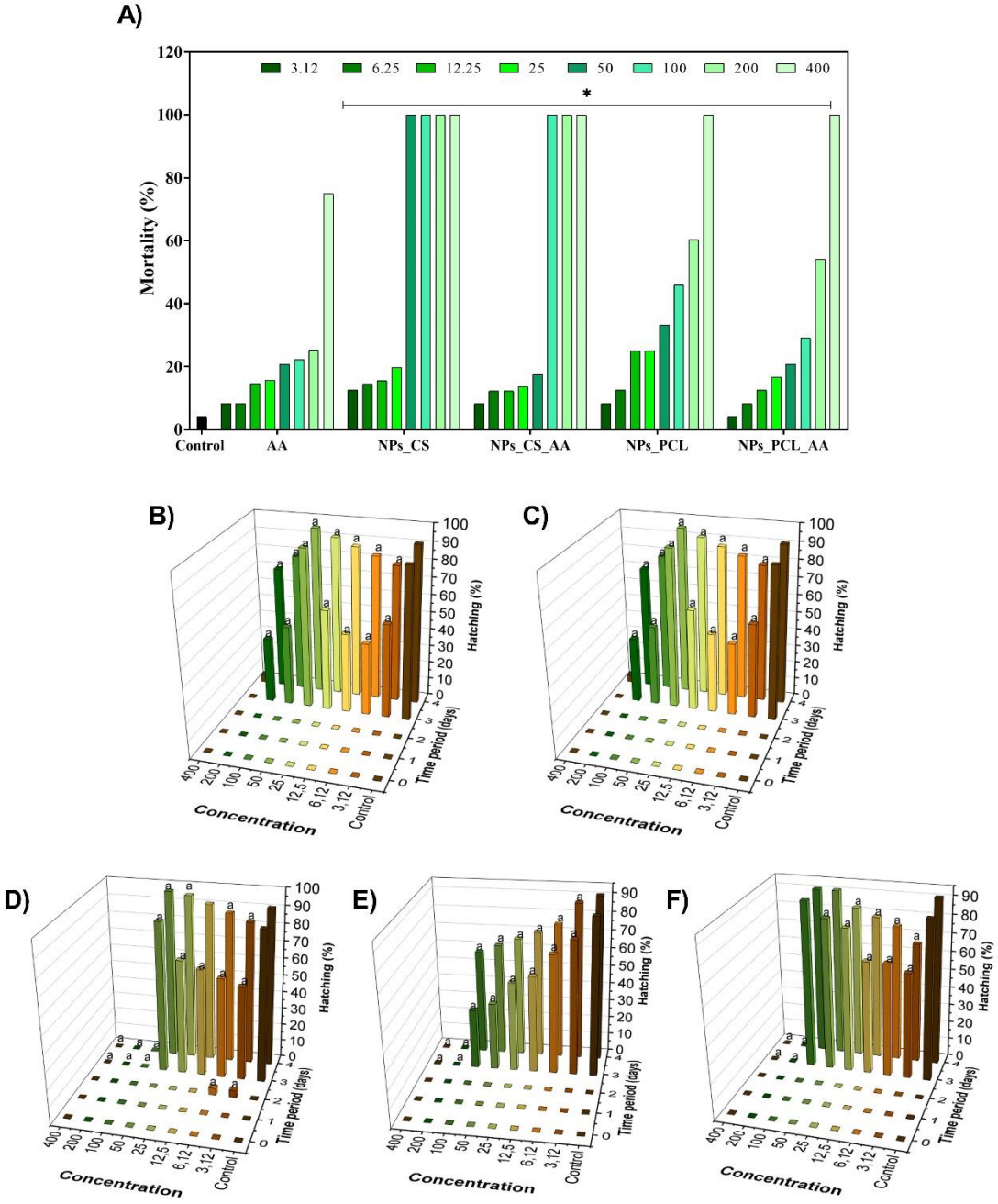
The degradation of AA is influenced by factors including oxygen, pH, temperature, metal ions, enzymes, light, water activity, and oxidizing agents (nitrite), among others (SMUDA; GLOMB, 2013). Studies have mainly focused on the degradation of ascorbic acid in fruits and other foods, which generally follows first order kinetics (KADAKAL; DUMAN; EKINCI, 2017). When oxygen is present, the rate of degradation of AA is faster (DENNISON; KIRK, 1978). This work is the first study of the oxidation of AA (99% purity) encapsulated in polymeric nanoparticles, comparing the results obtained with induction using peroxymonosulfate and under aquarium conditions, which opens up new opportunities for future research.

3.4.TOXICITY EVALUATION USING ZEBRAFISH

The use of nanomaterials has grown exponentially, accompanied by increasing concerns about their toxicities due to occasional or occupational exposures. Several factors can influence the toxicity of the materials, both intrinsic to them (size and shape, surface charge, composition, and state of agglomeration, among others) and extrinsic (such as pH, ionic strength, and medium composition). In this work, the LC_{50} values were determined for non-encapsulated AA, chitosan nanoparticles, chitosan nanoparticles containing AA, PCL nanoparticles, and PLC

nanoparticles containing AA. Zebrafish embryos were used as a model organism, following the OECD 236 protocol (OECD, 2013). The concentrations used were 3.12, 6.25, 12.5, 25, 50, 100, 200, and 400 mg L^{-1}. The accumulated mortality percentage was measured after 96 h (Fig. 4A).

Figure 4. A Mortality rates of embryos and larvae exposed to the different treatments (control, AA, NPs_CS, NPs_CS_AA, NPs_PCL, and NPs_PCL_AA) at different concentrations (3.12, 6.25, 12.25, 25, 50, 100, 200, and 400 mg/L). **B–F** Hatching rates of embryos exposed to ascorbic acid (AA), chitosan nanoparticles (NPs_CS), chitosan nanoparticles containing AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA), respectively. For the statistical tests, ANOVA (two-way, significance of $p < 0.05$) was used to observe the statistical differences between groups with the same concentration, where a indicates a statistically significant difference, compared to the control group



The groups treated with non-encapsulated AA showed mortality rates below 30% for all the concentrations tested, with the exception of the highest concentration (400 mg L^{-1}), where mortality was 75% (Fig. 4A), which could have been due to the pro-oxidant effect of AA at high concentrations, causing oxidative damage to DNA (GARCÍA-RODRÍGUEZ; ALTAMIRANO-LOZANO, 2017).

When AA was encapsulated in the chitosan NPs (NPs_CS_AA), mortality of less than 20% was observed up to a concentration of 50 mg L^{-1}, while higher concentrations caused 100% mortality of the organisms. Chitosan nanoparticles without encapsulated AA (NPs_CS) were also tested at the same dilutions as the NPs containing AA (Fig. 4A). These formulations were more toxic to the zebrafish, with a concentration of 50 mg L^{-1} causing 100% mortality of the organisms. The zebrafish treated with PCL nanoparticles (NPs_PCL) and PCL nanoparticles containing AA (NPs_PCL_AA) showed concentration-dependent mortality. In both cases, mortality of less than 50% was observed up to a concentration of 100 mg L^{-1}. However, the highest concentration (400 mg/L) caused 100% mortality.

The mortality data indicated that the chitosan nanoparticles were more toxic to the zebrafish embryos and larvae, compared to the PCL nanoparticles. Probit analysis, performed with Statgraphics Centurion XVII v. 1.17.04 software, enabled calculation of the $LC_{50,}$ LC_{40}, LC_{30}, LC_{20}, and LC_{10} values for the different formulations tested (Table 2).

Table 2. Average lethal concentrations (mg/L) for zebrafish embryos and larvae exposed to different treatments: ascorbic acid (AA), chitosan nanoparticles (NPs_CS), chitosan nanoparticles containing ascorbic acid (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA)

LC – 96 h (mg/L)	**AA**	**NPs_CS**	**NPs_CS_AA**	**NPs_PCL**	**NPs_PCL_AA**
LC_{50}	330.7	28.9	57.4	168.9	179.6
LC_{40}	275.8	24.8	48.1	137.5	146.1
LC_{30}	217.1	20.3	38.1	103.8	110.2
LC_{20}	148.3	15.2	26.4	64.5	68.2
LC_{10}	53.0	8.0	10.2	9.9	10.0

The LC_{50} for non-encapsulated AA was 330.7 mg L^{-1}, while reductions of 11.4-fold and 5.7-fold, respectively, were observed for the LC_{50} values of the chitosan nanoparticles (28.9 mg L^{-1}) and chitosan nanoparticles containing AA (57.4 mg/L), compared to the non-encapsulated AA. The LC_{50} values for the NPs_PCL and NPs_PCL_AA formulations were 168.9 and 179.6 mg L^{-1}, respectively, around twofold lower than the LC_{50} for the non-encapsulated AA.

The LC_{50} was significantly higher after the encapsulation of AA in the chitosan nanoparticles (NPs_CS_AA), compared to the nanoparticles alone (NPs_CS). This increase could have been related to the ability of ascorbic acid to modulate or decrease genotoxicity, regenerating other oxidants and removing free radicals.

Similar results were obtained elsewhere for the LC_{50} of chitosan nanoparticles for zebrafish embryos ($LC_{ß} = 23.26$ mg L^{-1}) (YUAN et al., 2016). In this case, the encapsulated ascorbic acid acted to reduce the toxic effects of the chitosan particles (GARCÍA-RODRÍGUEZ; ALTAMIRANO-LOZANO, 2017).

The incorporation of AA into the NPs_PCL system also decreased the mortality rate, which could be attributed to the effect of AA in reducing toxicity. This effect was reported by Stevanović et al. (2002), who found that the incorporation of ascorbic acid significantly reduced genotoxicity in *Oreochromis mossambicus* exposed to ethyl methanesulfonate. Sarma et al. (2009) found that the incorporation of ascorbic acid in the diet reduced the toxicity of the acaricide endosulfan, tested using *Channa punctatus*. The antioxidant activity of AA can act to decrease the toxicity of certain compounds. However, the observed behavior may have been more related to the release of AA from the NPs.

The toxicity of PCL nanoparticles has been evaluated in other in vitro and in vivo studies. Quercetin-loaded polycaprolactone microspheres were evaluated for their in vitro cytotoxicity in synovial cells (NATARAJAN et al., 2011). The treatments were carried out for 48 h, in the presence and absence of the active substance (quercetin). The results showed that the PCL microspheres caused cytotoxicity in the cells, suggesting that the system could be used in future treatment methodologies. In other work, Youssouf et al. (2019) tested the effects of oligo-carrageenan micelles grafted with polycaprolactone, containing curcumin. It was found that the micelles were not cytotoxic in zebrafish and that they also decreased the anti-inflammatory effects of curcumin and improved cell absorption. It was concluded that the micelles were safe nanocarriers.

In addition to mortality, evaluation was made of the influence of the formulations on the hatching of zebrafish. The embryos were exposed to non-encapsulated AA, chitosan NPs (NPs_CS), chitosan NPs containing AA (NPs_CS_AA), PCL NPs (NPs_PCL), and PCL

nanoparticles containing AA (NPs_PCL_AA), at concentrations of up to 200, 25, 50, and 100 mg L^{-1}, respectively. The concentrations were defined according to the mortality tests and concentrations higher than the LC_{50} of each formulation were not used.

The hatching rates of the embryos were lower after 48 h of exposure to AA, compared to the control group (Fig. 4B). However, after 96 h, the hatching rate of the embryos exposed to a concentration of 50 mg/L was similar to that of the control group. For concentrations of 100 and 200 mg L^{-1}, more pronounced decreases in hatching were observed after 96 h of treatment. Lower hatching rates were also observed for the embryos exposed to the chitosan NPs (Fig. 4C). However, for these groups, the hatching decreased in a dose-dependent manner, according to the increase in the concentration of nanoparticles. Interestingly, when the embryos were exposed to nanoparticles loaded with AA, the hatching rate was also lower, compared to the control group, but hatching increased with increase of the concentration of nanoparticles, after 96 h of exposure (Fig. 4D). For the embryos treated with the PCL NPs, the hatching rate was inversely proportional to the concentration of nanoparticles present in the medium, with a higher concentration of NPs resulting in a lower hatching rate (Fig. 4E). All the concentrations tested caused a significant reduction in the hatching rate after 96 h of exposure, with the exception of the lowest concentration tested (3.12 mg L^{-1}). For the embryos exposed to the PCL NPs containing AA, as well as those exposed to the chitosan nanoparticles, the hatching rate increased with increase of the concentration of NPs in the medium (Fig. 4F).

Similar results were reported by Hu et al. [47], who evaluated the toxicity of chitosan nanoparticles and chitosan nanoparticles containing Tween 80, with average sizes of 247 ± 20 and 251 ± 15 nm, respectively, using zebrafish as a model organism. Dose-dependent decrease of the hatching rate and increase of mortality were observed for both formulations, with an embryo mortality rate of around 100% at a concentration of 50 mg/L. The LC_{50} values for the chitosan nanoparticles and the chitosan nanoparticles containing Tween were 23.26 and 25.06 mg L^{-1}, respectively, which were very similar to those found in this study (Table 2).

Hu et al. (2011) found that chitosan nanoparticles with sizes of 200 and 340 nm caused significant mortality of zebrafish embryos, compared to the positive control (ZnO nanoparticles), at concentrations of 40 and 30 mg L^{-1}, respectively. For both types of

nanoparticle, the toxicity to the embryos was dose- and time-dependent, with the smaller nanoparticles having greater toxic effects.

In contrast, Wang et al. (2016) tested the toxicity of chitosan nanoparticles (84.86 nm) towards zebrafish embryos, using different concentrations (150 to 400 mg L^{-1}), obtaining an LC_{50} approximately tenfold higher (280 mg L^{-1}) than found in the present study. In another zebrafish toxicity study, Abou-Saleh et al. [49] found that chitosan nanoparticles in the size range 100–150 nm presented no toxic effects until the highest concentration tested (200 mg L^{-1}). These differences between the studies suggest the need for further research investigating the effects of different preparation methods, since it is possible that specific components of the nanoparticles could have induced toxic effects in the zebrafish embryos.

Evaluation was also made of morphological changes in the zebrafish embryos and larvae (Additional file 1: Figure S1). For all groups exposed to low concentrations, no abnormalities were observed in the embryos and larvae. However, high concentrations caused toxic effects including edema and opacity of the yolk sac, tail malformation, clotted egg, change in head formation, and disintegration of larvae. The non-encapsulated AA caused effects at concentrations of 25 and 50 mg L^{-1}, with the organisms showing higher numbers of malformations, compared to the organisms exposed to AA encapsulated in the NPs_CS_AA, at the same concentrations. After exposure to 100 mg L^{-1} of NPs_ CS_ AA, the larvae disintegrated after hatching. The embryos showed changes in the formation of the head and were coagulated when exposed to NPs_CS concentrations of 50 mg L^{-1} or higher (Additional file 1: Figure S1).

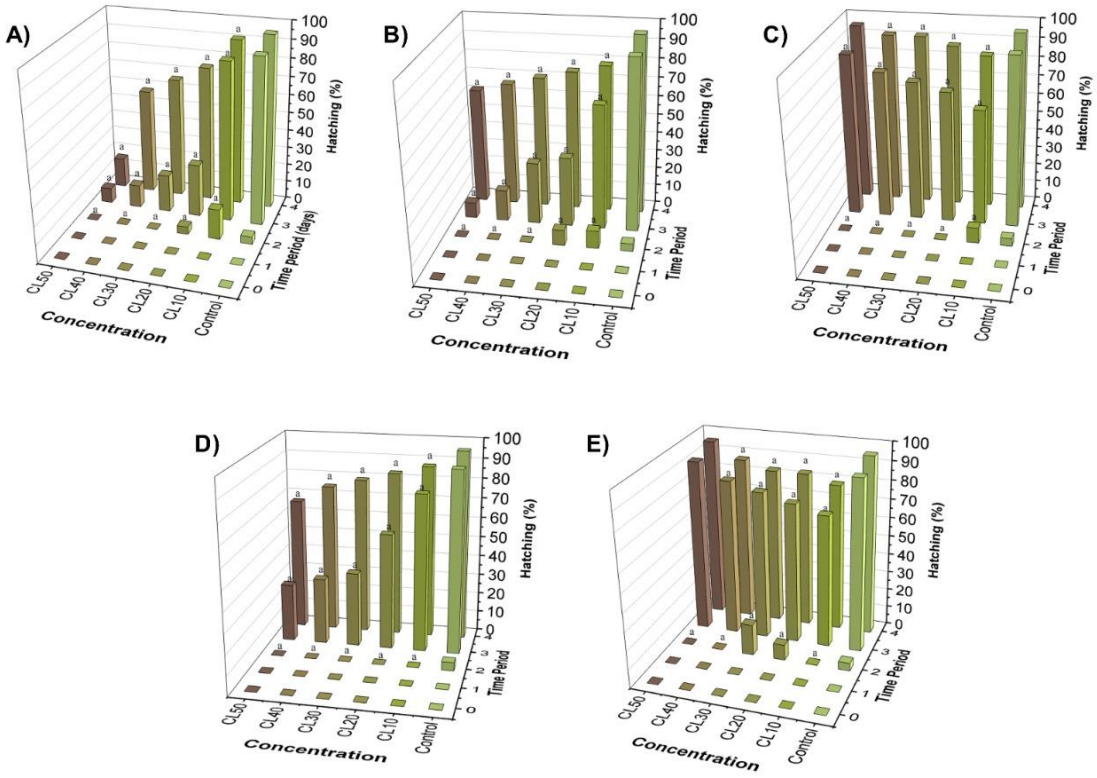
Malformations were also observed in the animals treated with PCL nanoparticles and PCL nanoparticles containing AA. However, at the same concentration (100 mg L^{-1}), fewer types of malformations were observed in the animals exposed to NP_PCL_AA, compared to non-encapsulated AA (Additional file 1: Figure S1). It should be noted that ascorbic acid (AA) is a vitamin and has limited toxicity, although high concentrations (above 200 mg L^{-1}) can cause some toxic effects. The results showed that the chitosan nanoparticles had toxic effects in the embryos, but were less toxic when combined with ascorbic acid, which led to fewer morphological abnormalities and a higher hatching rate, due to the controlled release of AA from the NPs.

The results indicated that concentrations of up to 50 and 100 mg L^{-1} of AA encapsulated in NPs_CS_AA and NPs_PCL_AA, respectively, would be promising for the incorporation of these systems as a vehicle for this vitamin in fish feed. These doses are higher than those necessary for the satisfactory development of fish (MERCHIE; LAVENS; SORGELOOS, 1997; OMONIYI; IYIOLA, 2018). For the incorporation of AA in the feed, advantages of the systems proposed here are that they can reduce oxidation of the compound and prolong its shelf life, due to the high encapsulation efficiency (above 90%, for both nanoparticulate systems), providing stability during 90 days of storage (data not shown).

3.5.EVALUATION OF FISH EMBRYO ACUTE TOXICITY (FED)

The morphological changes (Additional file 1: Figure S2) of the embryos and larvae were evaluated for 96 h exposure to different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}) of all the formulations. After fertilization, the embryos were individually exposed in 24-well polystyrene plates kept in an incubator at 26 ± 1 °C. The lethal effect on the embryos was indicated by the absence of somites, egg coagulation, and loss of independent movement. Hatching was used to assess toxicity, since it is influenced by several environmental and endogenous factors. Figure 5 shows the daily rate of embryo hatching. For all treatments, at the lowest concentrations tested (LC_{10} and LC_{20}), hatching started after 48 h of exposure, while at higher concentrations, hatching started after 72 h of exposure. For embryos treated with non-encapsulated AA, hatching decreased in a dose-dependent manner, with only 16.6% of the embryos hatching after 96 h of exposure at the highest concentration tested (LC_{50}) (Fig. 5A). Although ascorbic acid has an antioxidant effect, at high concentrations it can act as a pro-oxidant, causing oxidative damage to DNA (GARCÍA-RODRÍGUEZ; ALTAMIRANO-LOZANO, 2017).

Figure 5. Hatching rates of embryos and larvae exposed to the treatments at different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}): **A** ascorbic acid (AA), **B** chitosan nanoparticles (NPs_CS), **C** chitosan nanoparticles containing AA (NPs_CS_AA), **D** PCL nanoparticles (NPs_PCL), and **E** PCL nanoparticles containing AA (NPs_PCL_AA). For the statistical tests, ANOVA (two-way, significance of $p < 0.05$) was used to identify differences between the groups, where a indicates a statistically significant difference, compared to the control group



The embryos treated with PCL nanoparticles at the LC_{50} concentration showed a hatching rate of 66.7% after exposure for 96 h, which was 1.4-fold lower than observed for the control group after the same exposure time. In contrast, the hatching rate values for the animals treated with NPs_PCL_AA were higher than 79% for all the concentrations tested. In the case of NPs_CS_AA, the hatching rate increased with increase of the concentration of AA present in the medium. For the animals exposed to the LC_{50} concentration, the hatching rate after 96 h was 95.8%, which was the same as the value observed for the control after 96 h. These results indicated that the sustained release of AA acted to reduce the effects of the nanoparticles on the hatching of embryos, since in the presence of AA there was no significant difference for this parameter, when compared to the control group.

Embryo malformation was assessed using images obtained with a stereomicroscope (Model SMZ 2 LED, Optika), at 2 × magnification. Additional file 1: Figure S2 shows the malformations of embryos and larvae observed for each treatment at the LC_{50} dose, after 96 h of exposure. Regardless of the treatment, the embryos presented malformation of the yolk sac. However, the embryos exposed to non-encapsulated AA showed greater severity and number

of abnormalities such as edema and opacity of the yolk sac, edema of the pericardium, malformation of the tail, and bent spine.

All these teratogenic alterations, with the exception of tail malformation, were also observed for the embryos treated with chitosan nanoparticles. Embryos exposed to the PCL nanoparticles showed malformation, edema, and opacity of the yolk sac, as well as pericardial edema. These same malformations, except pericardial edema, were observed for the embryos treated with PCL nanoparticles containing AA.

Hu et al. (2011b) also observed dose-dependent malformations including bent spine and opaque yolk, following exposure of the organisms to chitosan nanoparticles (mean diameter of 200 nm). However, no significant malformations were observed in embryos exposed to chitosan nanoparticles with mean diameter of 340 nm. Yuan et al. (2016) observed non-inflated swim bladder and bent spine in embryos exposed to both chitosan and chitosan/Tween 80 nanoparticles.

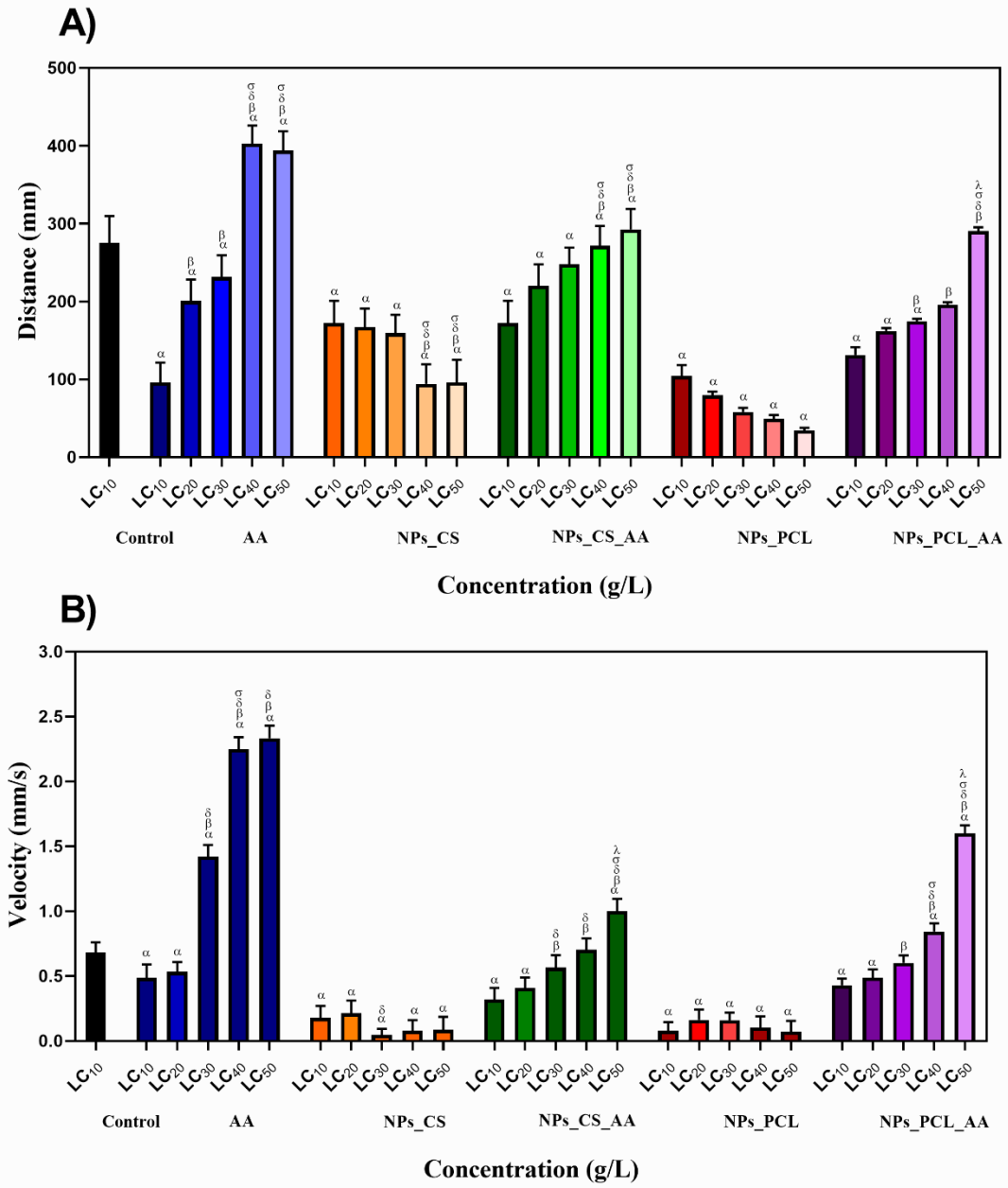
The embryos treated with nanoparticles containing AA showed only edema and opacity of the yolk sac. Encapsulation of the AA led to decreased toxicity, evidenced by fewer malformations, compared to the non-encapsulated compound and the control nanoparticles. These results suggested that the interaction between ascorbic acid and nanoparticles could decrease the toxic effect of the carrier system, while providing efficient release of the active compound.

3.6.EVALUATION OF ZEBRAFISH MOTOR ACTIVITY

The use of zebrafish as a model for behavioral studies opens up new possibilities for assessing the toxicity of nanoparticles. In order to identify the effects of the treatments on motor development, evaluation was made of the speed of the zebrafish larvae and the distance traveled. Prolonged exposure to stressors can generate an adaptation response, consequently affecting the health of the body. When exposed to harmful compounds, fish may show stress responses such as slowing down, in order to save energy, or adoption of avoidance behavior, in order to increase the chance of survival (KALUEFF et al., 2013).

Analysis was made of the behavioral biomarkers (speed and distance covered) for larvae exposed to different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}) of the formulations (non-encapsulated AA, chitosan nanoparticles (NPs_CS), chitosan nanoparticles containing AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA). The embryos were exposed for 96 h, with subsequent evaluation of distance and speed using EthovisionXT software (Fig. 6).

Figure 6. Evaluation of the motor development biomarkers **A** distance traveled and **B** speed for zebrafish (*Danio rerio*) larvae exposed to the different treatments: control, ascorbic acid (AA), CS nanoparticles (NPs_CS), CS nanoparticles containing ascorbic acid (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing ascorbic acid (NPs_PCL_AA). The exposures were performed for 96 h at different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}). For the statistical tests, ANOVA (two-way, significance of $p < 0.05$) was used to evaluate differences among the groups, with α, β, δ, σ, and λ indicating statistically significant differences from the control, LC_{10}, LC_{20}, LC_{30}, and LC_{40} groups, respectively ($n = 9$)



Both the larvae exposed to non-encapsulated AA and the larvae exposed to chitosan nanoparticles containing AA showed a dose-dependent increase in the distance traveled, with higher concentrations resulting in greater distances traveled (Fig. 6A). Compared to the control group, the distance traveled was shorter, up to the LC_{30} concentration, for the animals exposed to AA and those exposed to NPs_CS_AA. However, for non-encapsulated AA at the LC_{50}, there was a significant ($p < 0.05$) increase in the distance traveled, compared to the control group, whereas use of the encapsulated ascorbic acid (in both types of nanoparticle) at the LC_{50} did not result in a significant difference in the distance traveled, compared to the control. The larvae

exposed to the chitosan and PCL nanoparticles (without ascorbic acid) presented dose-dependent reductions in the distance traveled, compared to the control group.

The swimming speed behavior was similar to that for the distance covered. Larvae treated with non-encapsulated AA and AA encapsulated in chitosan and PCL nanoparticles showed dose-dependent increases in speed, while larvae treated with chitosan and PCL nanoparticles (without ascorbic acid) presented decreased swimming speed with increased concentration of nanoparticles in the medium (Fig. 6B). There is a known relationship between speed and distance traveled, where the increased swimming activity may be driven by the escape instinct or the response to stress (AHMAD et al., 2012).

The external integument or skin is a form of protection for fish, with the mucosa and scales playing important roles in protecting against pathogens, in addition to providing osmoregulatory and respiratory functions (ÁNGELES ESTEBAN, 2012; XU et al., 2016). The motor behavior inhibition caused by the chitosan and PCL NPs could have been due to the mucoadhesiveness of the nanoformulations, since fish epithelial cells are coated with a mucus layer rich in mucopolysaccharides and mucoproteins, enabling nanoparticles to adhere to the gills and skin (COSTA et al., 2016). The absence of the antioxidant effect of AA may have influenced the results, since for both types of nanoparticle containing AA, the locomotion activity gradually increased with increase of the AA concentration. Larvae exposed to the LC_{40} and LC_{50} concentrations of both systems (NPs_CS_AA and NPs_PCL_AA) were more active, compared to the control group.

However, different to the present findings, previous studies found that the treatment of embryos with chitosan nanoparticles caused an abnormal increase in locomotion activity (hyperactivity), compared to the control group (ABOU-SALEH et al., 2019; YUAN et al., 2016). It was suggested that the chitosan nanoparticles were able to negatively affect the embryo nervous system, but did not affect muscular development. As noted by Wong et al. 2010), anomalies observed in locomotion can be caused by changes in neuromuscular function and problems with neuronal innervations or muscles.

The increases in activity of the AA, NPs_CS_AA, and NPs_PCL_AA groups could be related to the electrical signals of primary motor neurons, on which tail movement and increased

swimming depend (BUSS; DRAPEAU, 2001). Primary motor neurons can periodically or spontaneously depolarize (KNOGLER et al., 2014), with the frequency of this depolarization being related to the behavior of the fish, whereby increases in the electrical signals and muscle contraction lead to increases of speed and the distance traveled. For the organisms exposed to NPs_CS and NPs_PCL, the locomotion activity was lower in comparison with all the other groups tested, with no significant differences between the treatments. It is likely that the larvae were in a process of habituation, defined as a decrease in the conduct response, due to sensory or motor fatigue (RANKIN et al., 2009).

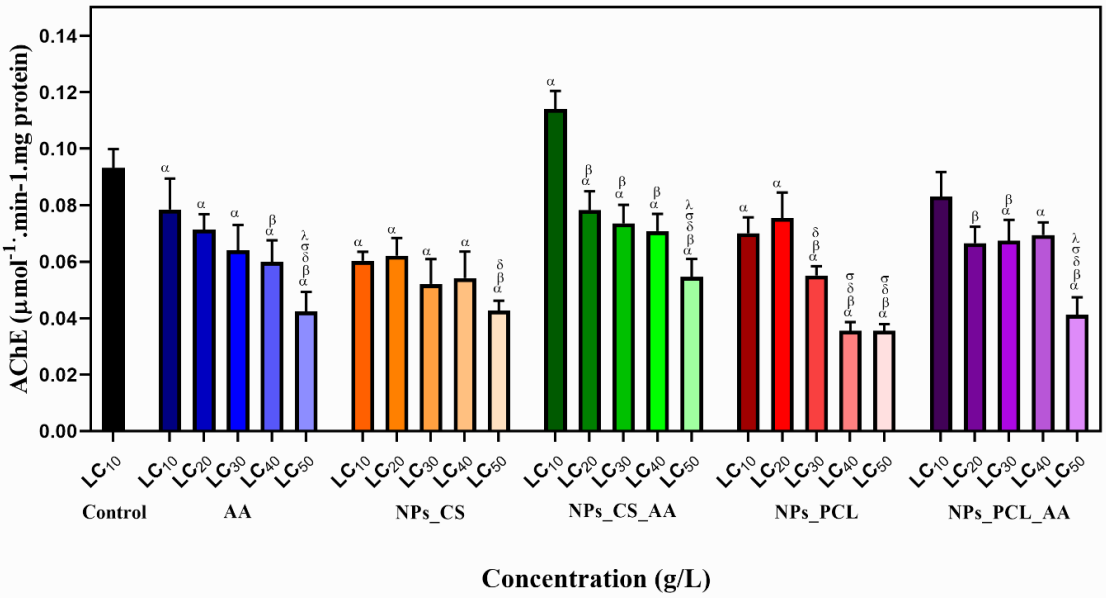
For both types of nanoparticle containing AA, the locomotion activity gradually increased with increase of the AA concentration, with the LC_{50} concentration causing the larvae to become more active than the organisms in the control group. In addition to these factors, the distance traveled by the zebrafish larvae was also related to the energy reserve provided by the yolk sac or yolk vesicle, which stores a nutrient substance (calf) responsible for providing the embryo with nutrients from the beginning of development until the start of exogenous feeding by the larva (MOMMSEN; WALSH, 1988). The loss of nutrients or failures in the absorption of nutrients from the yolk sac can result in decreased embryonic growth, due to decreased blood flow and cardiac changes (MAJEWSKI et al., 2018; PARK et al., 2020; QIANG et al., 2016). Other changes observed include pericardial edema, yolk sac edema, opaque yolk, tail malformation, and bent spine.

3.7.EVALUATION OF ACETYLCHOLINESTERASE (AChE) ACTIVITY

In the areas of neuropharmacology and neurotoxicology, zebrafish provides a very useful model for study of the effects of different compounds, enabling investigation of mechanisms of action as well as functional effects, such as behavioral changes. Swimming behavior can be used as a sensitive endpoint for detection of the sublethal effects of pollutants in fish (EISSA et al., 2010).

The specific AChE activity was determined for the larvae exposed to the different treatments (non-encapsulated AA, chitosan nanoparticles (NPs_CS), chitosan nanoparticles containing AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA)) at different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}). Statistical analysis employed the Kruskal–Wallis test ($p = 0.05$) followed by the Dunn test (Fig. 7).

Figure 7. Acetylcholinesterase (AChE) activities of zebrafish larvae after exposure for 96 h to different concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}) of ascorbic acid (AA), CS nanoparticles (NPs_CS), CS nanoparticles containing AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA), compared to the control. For the statistical tests, Kruskal-Wallis test ($p < 0.05$) followed by Dun test was used to evaluate differences among the groups, with α, β, δ, σ, and λ indicating statistically significant differences from the control, LC_{10}, LC_{20}, LC_{30}, and LC_{40} groups, respectively (n = 9)



Most of the treatments caused a significant dose-dependent decrease in AChE activity, compared to the negative control. An exception was the chitosan nanoparticles containing encapsulated AA, at the lowest concentration tested (LC_{10}), where the activity of this enzyme was significantly higher, compared to the control group. Wang et al. (2009) showed that the inhibition of the AChE enzyme by nanoparticles can be caused by its adsorption or interaction with the particles. It was observed that following adsorption, the nanoparticles inhibited AChE differently, depending on their chemical composition.

ACh plays a crucial role in the central and neuromuscular synapses of the cholinergic system. When ACh is released into the synaptic cleft, it is rapidly degraded by AchE (BEHRA et al., 2002). Since AChE is the enzyme responsible for ending cholinergic activity in the synaptic cleft, AChE inhibition leads to accumulation of the neurotransmitter acetylcholine

(ACh), resulting in increased stimulation of cholinergic neurons. Consequently, inhibition of the AChE enzyme can affect the balance and locomotion of the exposed animals. Excessive hydrolysis of ACh by AChE may lead to muscle hypoactivity and decreased behavioral responses (TIERNEY, 2011). In severe cases, it can also cause collapse of the central nervous system, resulting in loss of muscle control or even death (ČOLOVIĆ et al., 2013; GOLOMBIESKI et al., 2008). Also, the cholinergic system may be involved in morphometric alterations such as curvature of the fish tail, due to abnormal muscle development caused by inhibition of acetylcholinesterase activity and the lack of hydrolysis of AChE (PAMANJI et al., 2015; ROY; ZAMBRZYCKA; SANTANGELO, 2017). Hence, it is important to establish the relationship between locomotion behavior and biochemical responses such as AChE activity (BERTUZZI; AMPATZIS, 2018).

Alteration of this enzyme activity can lead to motor function impairment in zebrafish, due to the change in ACh levels at the neuromuscular junction (BERTUZZI; AMPATZIS, 2018; ENGENHEIRO et al., 2005). Behavioral impairment of silver catfish has been attributed to inhibition of cerebral AChE activity and oxidative damage (SERAFINI et al., 2019). Similarly, Baldissera et al. (2021) suggested that exposure to aquaculture-relevant concentrations (11 mg L^{-1}) of trichlorfon, an organophosphate pesticide, could elicit behavioral abnormalities associated with impairment of locomotion activity, a condition that can be caused by cerebral oxidative damage, depression of the antioxidant defense system, and inhibition of AChE activity (RODRIGUES et al., 2020). Studies have suggested the existence of a threshold relationship between AChE inhibition and swimming performance. Sandahl et al. (2005) observed significant decreases in swimming activity at an exposure concentration of chlorpyrifos as low as 0.6 µg L^{-1}, corresponding to a relative AChE activity of 77.2%, compared to the control.

Tierney et al. (2007) suggested that the AChE inhibition threshold was determined by the type of muscle used for any given swimming modality (swimming powered by aerobic muscle, or swimming powered anaerobically, depending on the difference in the amounts of red and white tissues). Critical swimming capacity may remain available, even when AChE inhibition reduces spontaneous swimming activity. This suggests that any anticholinesterase exposure impairing spontaneous swimming, a swimming mode that enables foraging, may still leave fish with maximum swimming performance, leading to a proportional increase in the use

of white muscle, which may differentially impair swimming behaviors such as predator avoidance.

However, for these observed effects, it can be difficult to distinguish between cholinergic (central and peripheral) mechanisms and other systems that participate in the processes of locomotion and swimming. It is interesting that two organophosphate pesticides (chlorpyrifos and malathion) were found to cause opposing behaviors in zebrafish larvae, related to swim speed (hyperactivity *vs.* hypoactivity) and rest. The swim speed and rest behaviors appeared to be correlated to AChE activity in larvae treated with chlorpyrifos, but not in larvae treated with malathion (RICHENDRFER; CRETON, 2015). The findings suggest that there may be other neural parameters that are affected and that influence larval behavioral changes. Gene modulation may be involved in cholinergic neurotransmission (CLEMENTE et al., 2019), while pharmacological and optogenetic manipulations of cholinergic neurotransmission in the hippocampal and amygdala regions suggest a specific role of cholinergic receptors in modulating the conditioned fear and extinction responses (RODRIGUES et al., 2020). Furthermore, in rapid acceleration movements, red muscle not only produces power, but also has an important role in tuning the effective body stiffness. The ongoing activity of red muscle may be a consequence of the motor neuron recruitment pattern for red and white muscle, with motor neurons for red muscle being recruited earlier, due to their low resistance, and remaining activated even when motor neurons for white muscle are recruited (SCHWALBE et al., 2019).

It is also interesting to note that ascorbic acid plays a role in neuronal differentiation, maturation, myelin formation, and modulation of neurotransmission, as in cholinergic systems (TRAVICA et al., 2017). Based on these results, it is plausible that AA interferes with the cholinergic system, depending on the dose and possibly the fish stage of development. Although, in the present study, a decrease in AchE levels was observed, Narra et al. (2015) showed that the acetylcholinesterase activity of rockfish was significantly increased by dietary AA supplementation, mitigating chlorpyrifos toxicity. In addition, dietary AA supplementation in the rockfish induced an increase in AChE activity (KIM; KANG, 2016). Hence, it is not yet clear whether chitosan can affect the role of AA in development of the cholinergic neurotransmitter system, since chitosan nanoparticles can remain adhered to the mucosal surfaces of fish (COSTA et al., 2016). Another possibility is that the AA kinetics induced by

nanoparticles, at the larval development stage selected here, might influence the enzyme activity at maturity, resulting in a non-synergistic effect.

Since locomotion behavior in organisms is important for avoiding predators, seeking food, and reproduction, it is a parameter of considerable ecological relevance (CORRIERE et al., 2020; SCHWALBE et al., 2019). In the present work, the nanoformulations did not appear to negatively affect swimming performance, despite the AChE decrease. On the contrary, the velocity and distance covered by the zebrafish larvae increased, in a response evoked to assist the fish in escaping from predators. Future studies will be needed to further elucidate these effects in zebrafish larvae.

3.8.EVALUATION OF ZEBRAFISH BODY WEIGHT

The control of fish nutrition is essential in the aquaculture industry, in order to maintain good health, strong immune systems, and resistance to disease (ALISHAHI et al., 2011a). The use of natural vitamins in aquaculture has been shown to provide good growth, satisfactory development, and normal cell function in aquatic organisms (YOSSA et al., 2015).

Ascorbic acid encapsulated in the CS and PCL nanoparticles was incorporated at different concentrations (25, 50, and 100 mg/kg) in a commercial fish feed (TetraMIN). Figure 8A shows the fish sizes obtained for the control group (TetraMIN alone) and the nanoparticle groups. The fish in the control group showed an average size of 20 mm, while the fish that received the commercial feed plus PCL nanoparticles (NPs_PCL_AA) at different concentrations showed a larger size of 23 ± 1.1 mm, representing an increase of 15%, compared to the control group. Similar results were obtained for the fish in the groups that received the commercial feed plus CS nanoparticles (NPs_CS_AA) at different concentrations, with an average size of 24 ± 0.8 mm, representing an increase of 20%, compared to the control group. The results showed that the higher the concentration of AA added to the food, the greater the size of the fish. Both groups treated with 100 mg/kg showed a significant difference in relation to the control group and the other concentrations, whereas there was no significant difference in fish size between the NPs_CS_AA and NPs_PCL_AA groups. These results indicated that neither colloidal system affected growth of the fish.

Figure 8. Evaluation of body length **A** and weight **B** of zebrafish fed different diets. The control group was fed commercial food (TetraMIN), while the other groups were fed TetraMIN supplemented with different concentrations (25, 50, and 100 mg/kg) of NPs_CS_AA and NPs_PCL_AA. Statistical tests were performed using ANOVA (two-way, significance of $p < 0.05$) to evaluate differences among the groups, where α, β, and δ indicate statistically significant differences from the control, 25 mg/kg, and 50 mg/kg groups, respectively ($n = 10$)

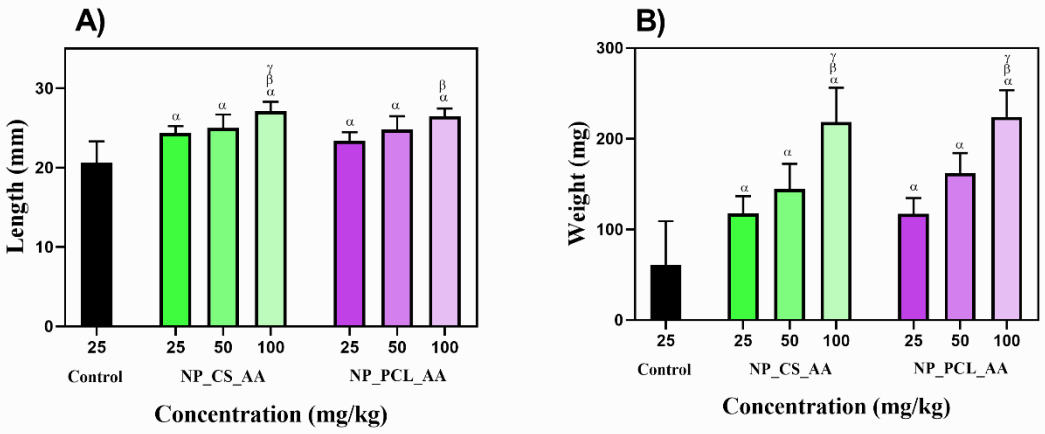


Figure 8B shows the weights of the fish after exposure to the different treatments. An average final weight of 60.97 mg was obtained for the control group fed the commercial food (TetraMIN). The groups fed the commercial food with the addition of NPs_PCL_AA and NPs_CS_AA showed higher weights, compared to the control, with the weight increasing as the AA concentration was increased, similar to the effect observed for fish length. The final mean weights for the groups fed 25, 50, and 100 mg kg^{-1} of NPs_CS_AA were 117.5 ± 19.10, 144.5 ± 27.71, and 218.7 ± 48.3 mg, respectively. The corresponding values for the NPs_PCL_AA group were 117.4 ± 17.1, 162.32 ± 21.8, and 223.9 ± 29.55 mg, respectively. These results showed that the AA stimulated the development of the fish, with no apparent harm caused by the nanocarrier systems.

The effects of AA supplementation on fish growth were studied by Nsonga et al. (2010), who quantitatively determined the dietary requirement of AA for the tilapia species, as well as the effect of this vitamin on fish growth. Different concentrations of AA (20, 40, 60, and 80 mg kg^{-1}) were tested and it was found that supplementation with AA at 60 mg kg^{-1} in the feed increased weight gain and maintained good fish health. Supplementation at 80 mg kg^{-1} did

not significantly increase fish development, compared to the 60 mg kg^{-1} dosage. In the present work, a supplementation dosage of 100 mg/kg had a greater effect on fish development, compared to the lower dosages tested (25 and 50 mg kg^{-1}). In the work of Nsonga et al. (2010), it is possible that AA degradation may have occurred, since the vitamin was not protected, which contributed to the absence of any difference between the effects of the dosages of 60 and 80 mg/kg. In the present case, the AA was protected, enabling it to be more effectively directed at the fish, consequently resulting in better fish development when increasing amounts of AA were added to the feed. In other work, supplementation of the fish diet with chitosan nanoparticles had positive effects on body weight, weight gain, and specific growth rate of grey mullet (*Liza ramada*) (DAWOOD et al., 2020). Similar results were observed for Nile tilapia (ABD EL-NABY et al., 2019; ALISHAHI et al., 2011a; CORRIERE et al., 2020; NAIEL et al., 2020; YOSSA et al., 2015), rainbow trout (KHANI OUSHANI et al., 2020), silver carp (YOUNUS et al., 2020), and loach (CHEN; CHEN, 2019) fed diets enriched with chitosan and/or chitosan nanoparticles. These positive effects of chitosan could be attributed to its ability to activate digestive enzymes, as well as to inhibit pathogenic bacteria, while activating beneficial ones (QIN et al., 2014).

Although dietary supplementation with micro-scale chitosan has shown positive results in the growth of some fish species, another study found that supplementation of the diet with micro-scale chitosan (at 5 g kg^{-1}) did not lead to any significant difference in silver carp growth parameters, compared to control groups (YOUNUS et al., 2020). On the other hand, the authors showed that the animals supplied with a diet supplemented with chitosan nanoparticles (5 g kg^{-1}) showed 29% greater weight gain, higher SGR, and better FCR, compared to the control and micro-scale chitosan groups (YOUNUS et al., 2020). These results could be explained by the higher absorption and bioavailability of chitosan nanoparticles in the fish gastrointestinal tract (RAJESH KUMAR et al., 2008), as well as increased residence time of the nanoparticles, which increased the contact with the absorptive epithelium, consequently directly influencing fish growth (ABDEL-TAWWAB; RAZEK; ABDEL-RAHMAN, 2019; YOUNUS et al., 2020). Greater growth of fish supplemented with chitosan nanoparticles, compared to those supplemented with micro-scale chitosan, has been observed for species including *Oreochromis nicoticus* (ABDEL-TAWWAB; RAZEK; ABDEL-RAHMAN, 2019; WANG; LI, 2011) and *Clarias gariepinus* (UDO; ANWANA, 2018). However, it should be noted that for the same diet formulation, there were differences in the weight gains of the

different species tested, which could have been related to the fish growth rate (PHATARPEKAR et al., 2000).

4. CONCLUSIONS

The results of this work demonstrated that the chitosan and PCL nanocarrier systems could be effectively loaded with ascorbic acid. High encapsulation efficiencies were obtained for both systems, with values of $96 \pm 0.5\%$ for NPs_CS_AA and $91 \pm 0.2\%$ for NPs_PCL_AA, enabling protection of the vitamin against degradation. The formulations presented average nanoparticle diameters of 314 ± 3 and 303 ± 1 nm, respectively, and were stable during storage. In vitro studies showed that both systems provided sustained release of AA, while oxidation tests showed that the PCL nanoparticles provided longer protection. In toxicity tests, the NPs_PCL_AA formulation showed a lower mortality rate, compared to the chitosan nanoparticles formulation, with LC_{50} values of 57.4 and 179.6 mg mL^{-1} for NPS_CS_AA and NPs_PCL_AA, respectively. Encapsulation of the AA resulted in decreased malformations in the zebrafish embryos and larvae, compared to the non-encapsulated compound. The larvae exposed to AA encapsulated in the chitosan and PCL nanoparticles showed dose-dependent increases of speed and distance traveled. In contrast, most of the groups showed significant dose-dependent decreases of AChE activity, which did not seem sufficient to affect behavior, but could have led to morphological changes influenced by the cholinergic system, such as curvature of the fish tail. The findings showed that the PCL nanoparticles system had greater potential to protect ascorbic acid and also presented lower toxicity, compared to the chitosan nanoparticles system, possibly due to the slower release of the active compound from the PCL nanoparticles. The results demonstrated that the use of nanotechnology can open up new perspectives in aquaculture, helping to reduce feed nutrient losses, promoting faster growth of fish, and potentially reducing production costs. The use of nanotechnology in aquaculture can make an important contribution to the sustainable provision of healthy products in the food industry. The findings also demonstrated the suitability of zebrafish developmental parameters for use in environmental risk assessments of compounds and nanomaterials.

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CHAPTER 3 - CONCLUSÕES GERAIS E PERSPECTIVAS

(Artigo não submetido)

A produção da aquicultura pode ser afetada pela biodisponibilidade de nutrientes nas dietas e, por isso, é importante manter uma dieta adequada. Se a dieta não atender aos requisitos ou não for utilizada corretamente, não será digerida adequadamente, gerando outros problemas, como crescimento limitado, os quais acabam causando perdas econômicas e contaminando o ambiente. O ácido ascórbico (AA) é um antioxidante essencial para manter o processo fisiológico de diferentes animais e um complemento alimentar benéfico para melhorar o crescimento, sobrevivência, desenvolvimento esquelético e resistência ao estresse em alevinos. Contudo, o AA é instável na sua forma pura o que leva a sua degradação e perda influenciada por vários fatores (altas temperaturas luz, umidade, etc.). A associação da nanotecnologia com a nutrição em peixes poderia melhorar a estabilidade deste composto.

Nesse contexto, o presente trabalho apresentou o desenvolvimento de dois sistemas carreadores para ácido ascórbico. Tal estudo visa resolver um problema de nutrição em peixes, fornecendo proteção contra degradação prematura do ativo. Também foram realizados estudos de toxicidade para que a aplicação destes sistemas nanocarreadores seja feita de uma maneira segura, visando minimizar os impactos no ambiente.

As formulações apresentaram um tamanho médio de 314 e 303 nm, índice de polidispersão de 0.36 e 0.28, eficiência de encapsulação alta e uma boa estabilidade físico química por 90 dias. Os dois sistemas de nanopartículas proporcionaram proteção contra a degradação do AA exposto a um agente oxidante, comparado com o AA isolado. As CL 50 obtidas foram 330,7, 57,4 e 179,6 mg/L, para AA isolado, nanopartículas de CS e nanopartículas de PCL, respectivamente. As nanopartículas de CS e PCL carregadas com AA na concentração 50 mg/L, apresentaram menor toxicidade no desenvolvimento do zebrafish em comparação com o AA isolado na mesma concentração. O uso destes nanosistemas neste estudo foi promissor na proteção do AA, sendo que as nanopartículas de PCL contendo AA apresentaram uma menor toxicidade no zebrafish e maior proteção do AA em comparação com as nanopartículas de quitosana contendo ácido ascorbico.

Até o momento, são poucos os trabalhos relacionados à nanotecnologia aplicada à nutrição de peixes. Assim, este trabalho abre novas diretrizes para o uso desses sistemas de forma segura. Os resultados encontrados neste trabalho são promissores para a aplicação desses

sistemas na aquicultura. Entretanto a comercialização de um produto a base desses sistemas tem um caminho longo, já que requer mais estudos para avaliar a sua viabilidade. Desta forma a nanotecnologia integrada com a aquicultura, pode melhorar a eficiência e eficácia de compostos instáveis como o ácido ascorbico, já que ambos os sistemas foram eficazes para o carreamento desta vitamina, apresentaram baixa toxicidade, além de aumentar o ganho de peso em peixes.

SUPPLEMENTARY MATERIAL

Figure 1S. Morphological abnormalities (pericardial edema, skin lesions, undeveloped tail, tail alterations, and bent spine) observed in the zebrafish embryos and larvae exposed to the different treatments at concentrations of 25, 50, and 100 mg/mL: non-encapsulated ascorbic acid (AA), chitosan nanoparticles (NPs_CS), chitosan nanoparticles containing AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA). The images were acquired using a stereomicroscope (Model SMZ 2 LED, Optika), at 2x magnification.

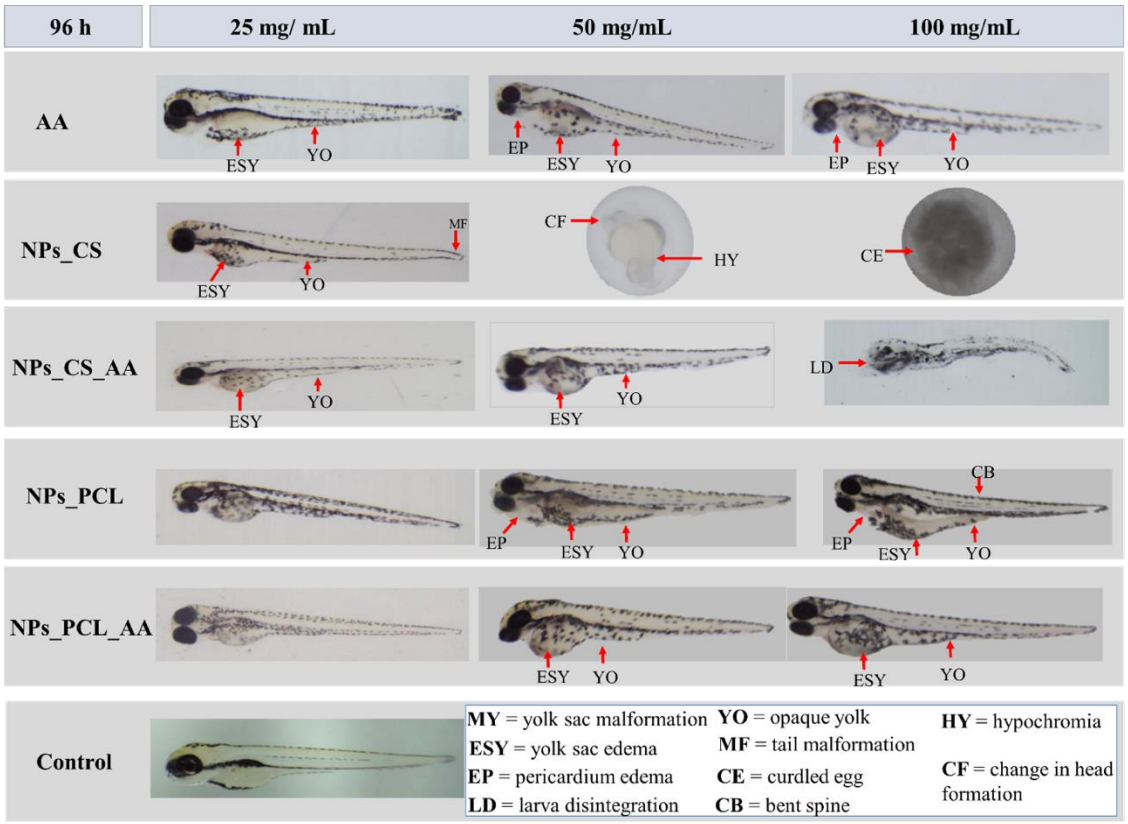
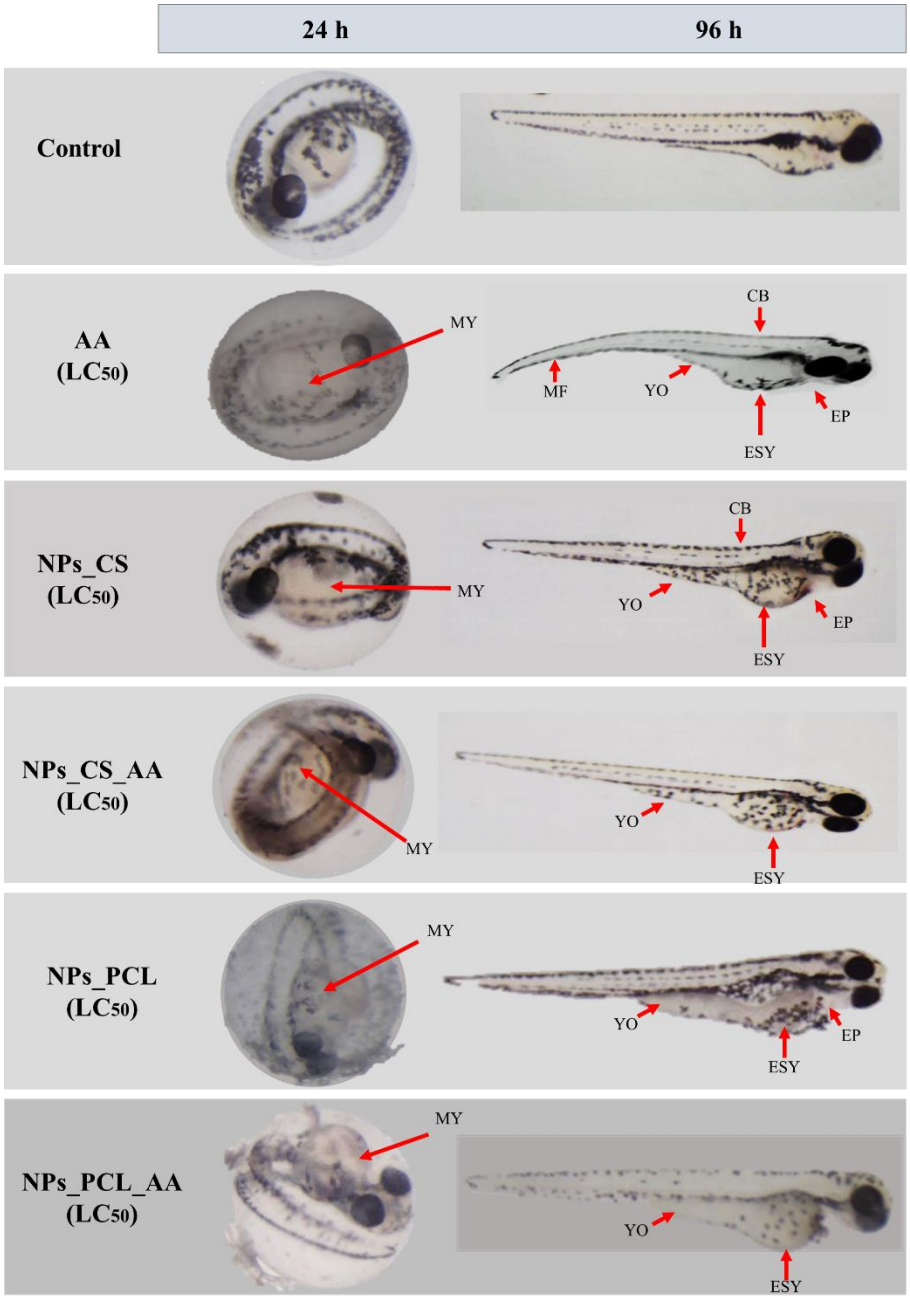


Figure 2S. Morphological abnormalities (yolk sac malformation and edema, pericardial edema, opaque yolk, tail malformation, and bent spine) observed in the zebrafish embryos and larvae exposed to the different treatments: non-encapsulated ascorbic acid (AA), chitosan nanoparticles (NPs_CS), chitosan nanoparticles containing AA (NPs_CS_AA), PCL nanoparticles (NPs_PCL), and PCL nanoparticles containing AA (NPs_PCL_AA). The images were acquired using a stereomicroscope (Model SMZ 2 LED, Optika), at 2x magnification.



Embrapa

Meio Ambiente

M.CEUA.CNPMA Nº 2

Jaguariúna, 13 de fevereiro de 2018.

Vera Lucia Scherholz Salgado de Castro
Embrapa Meio Ambiente

AUTORIZAÇÃO PARA A REALIZAÇÃO DE ATIVIDADES DE ENSINO OU DE PESQUISA CIENTÍFICA

Certificamos que a proposta intitulada "Nanotecnologia aplicada à aquicultura: