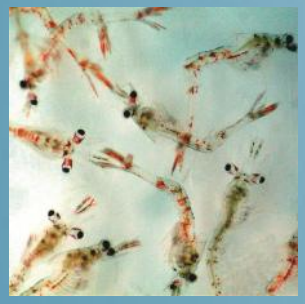
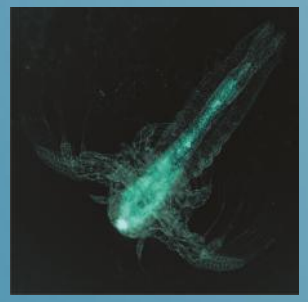
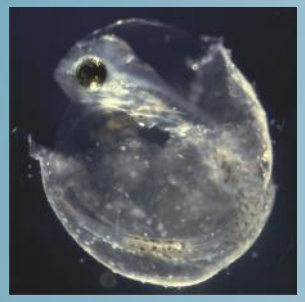
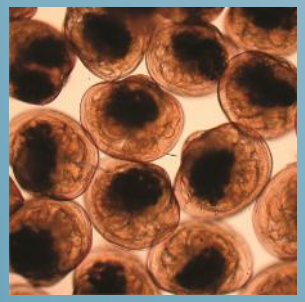




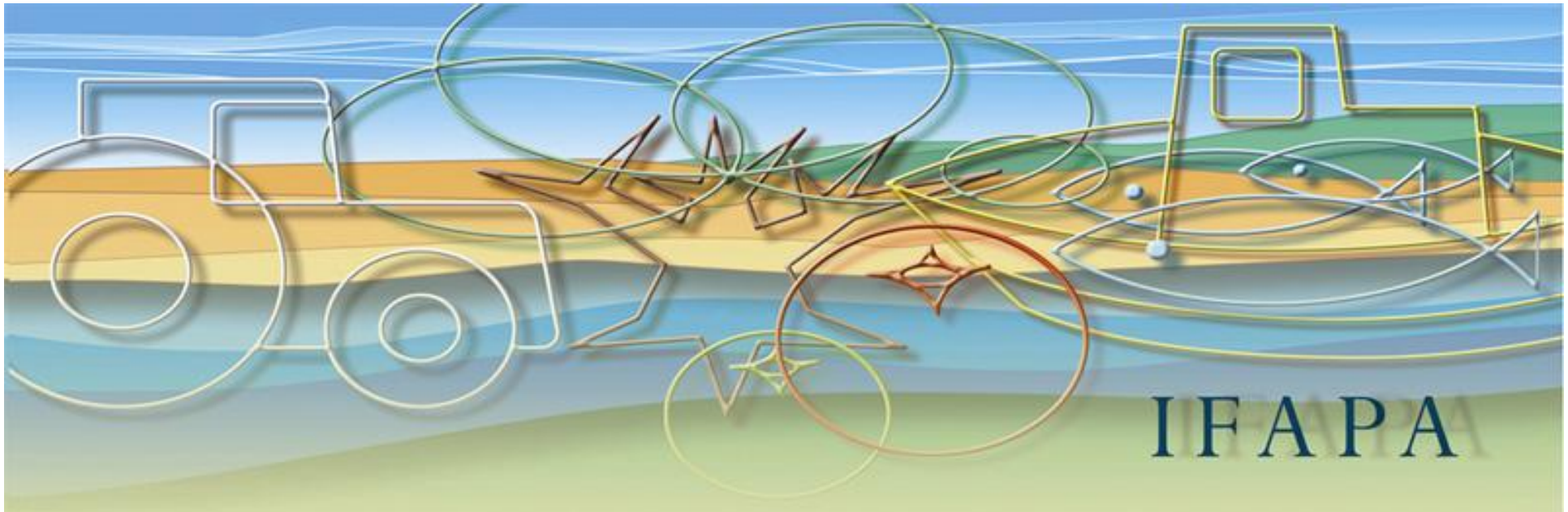
larvi 2013

6th fish & shellfish larviculture symposium

Nanoparticles as a novel delivery system for vitamin C administration in aquaculture

Eduardo Jiménez Fernández

ghent university, belgium, 2-5 september 2013



NANOPARTICLES AS A NOVEL DELIVERY SYSTEM FOR VITAMIN C ADMINISTRATION IN AQUACULTURE

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Introduction

Nanotechnology:

- Application of materials at the nanoscale to produce new products or processes.

Nanotechnology in aquaculture:

- New tools for fish biotechnology, nutrition, genetics, reproduction and aquatic health.

Feeding problems:

- Instability of ingredients in aquafeeds - poor delivery of micronutrients.

Outbreak of disease:

- One of the major stumbling blocks in the development and sustainability of aquaculture.

Polymer matrix nanoparticles:

- Attractive alternative in an attempt to solve these important problems in the aquaculture field.

Applications of Nanoparticles (NPs) in Aquaculture

- Food packaging
- Texture
- Physical properties of fish food
- **Micronutrient delivery systems**

Why nanoparticles as micronutrients delivering systems?

- High stability
- Biodegradable system
- Easy to store
- Large-scale applications
- High mucoadhesivity
- Absorption increases

The state of the art

Little information is available about the bioavailability, uptake and distribution of polymeric nanoparticles in aquatic organisms and their effects.

- Ascorbic acid (AA) loaded **nanoparticles increased the permanence time of AA** in rainbow trout digestive tract (Alishahi et al, 2011).
- No references to the use of polysaccharides complex to transport vitamin c.
- Polysaccharides complex can provide **better protection than CS-TPP NPs.**
- **No known references** of the use and evaluation of this type of particles in zebrafish, sole or rotifers.

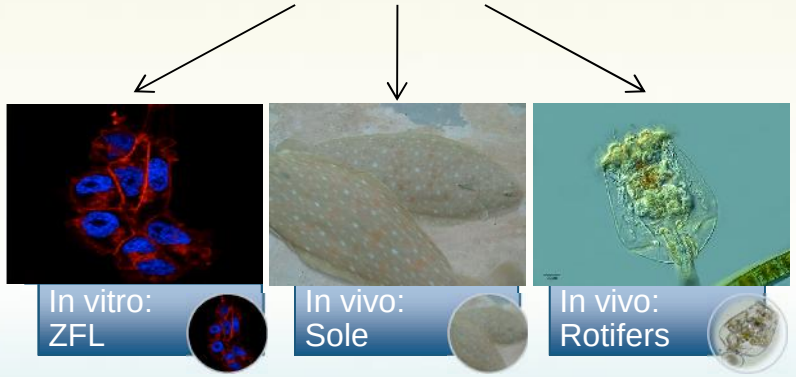
Targets:

General Objective

- To achieve an effective way to deliver vitamin C into the body of marine fish larvae in culture

Specific Objective

- Prepare chitosan-cyclodextrin nanoparticles (NPs) loaded with vitamin C in order to determine their properties, cytotoxicity and uptake in cells and tissues of different aquatic organisms.



Contributions to achieve

- To investigate the use of NPs in aquaculture and contribute towards the understanding of nutritional requirements of marine and suspension-feeders, including larvae of many fish species.

Methods and Results

Nanoparticle preparation methodology

- Delivering system tested:
  - Ionotropic gelation (CS-CD-TPP)
- Ascorbic acid was used as a model of hydrosoluble micronutrients (5/1; CS/AA w/w).
  - *The AA content was analyzed on a Finnigan Surveyor Plus HPLC system.*
- Fluorescein isothiocyanate (FITC)-labeled BSA (BSA-FITC) was used as a fluorescein model protein to evaluate the properties of nanoparticles and to perform uptake studies.

NPs made by Ionotropic gelation (CS-CD-TPP)

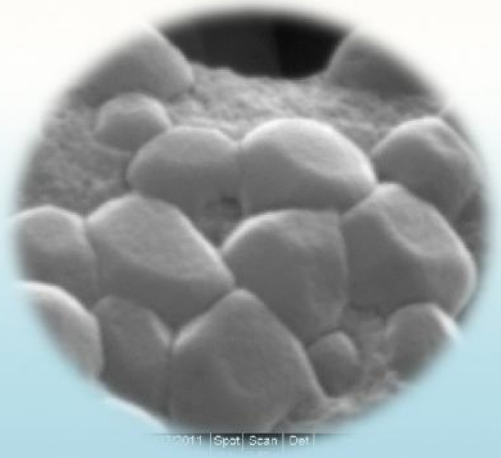
Chitosan-CD-TPP (CS-CD-TPP) nanoparticles were prepared by ionotropic gelation as described Teijeiro-Osorio et al, (2009)

Compounds were added to a CD-TPP solution under magnetic stirring at room temperature

The CD-TPP solution was added by peristaltic pump under CS

Particles were isolated by centrifugation at 4ºC for 60 min at 18000 rpm

Nanoparticles were then freeze-dried for use



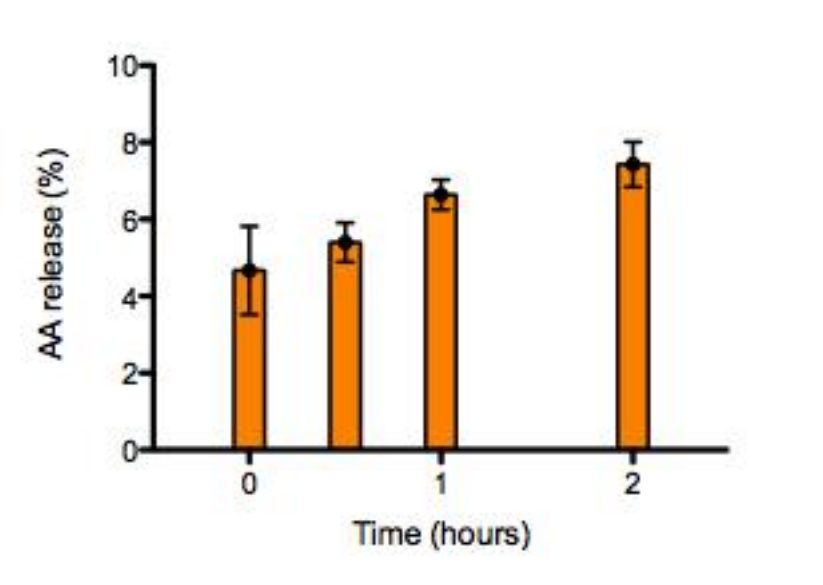
Physico-chemical properties of NPs

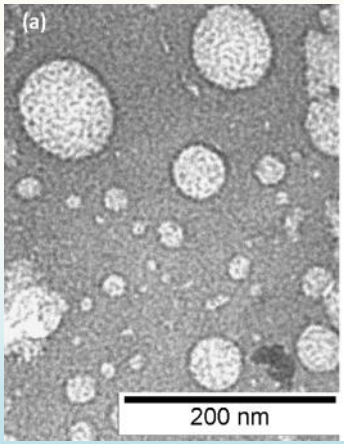
Table 1. Physico-chemical properties of non-loaded NPs, AA-NPs and FITC-NPs.

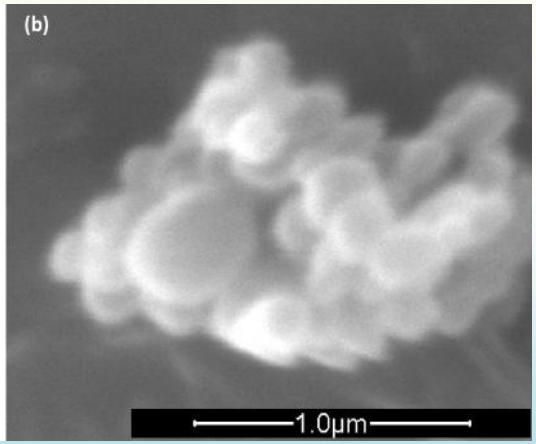
Nanoparticles	Size (nm)	Polydispersity index	Zeta potential (mV)	Yield (%)	EE (%)	LC (w/w)	AA release in 2 h (%)
Non-loaded NPs	253,1±32,1	0,37±0,06	35,5±1,13	23,4±2,6	-	-	-
AA-NPs	255,3±22,9	0,34±0,04	34,1±1,6	32,8±4,3	15,7±1,2	4,47±1,5%	6,57±0,52
FITC-NPs	258,5±16,9	0,38±0,03	37,7±1,5	27,6±3,0	-	-	-

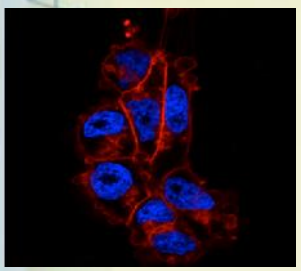
EE = encapsulation efficiency.

LC =AA loading capacity



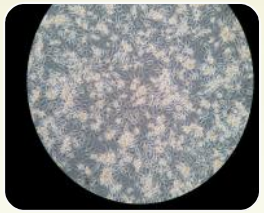




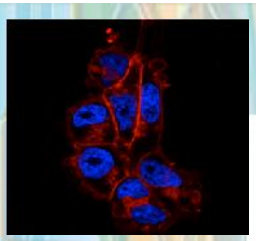


NPs in vitro evaluation

ZFL cell culture and cell toxicity studies

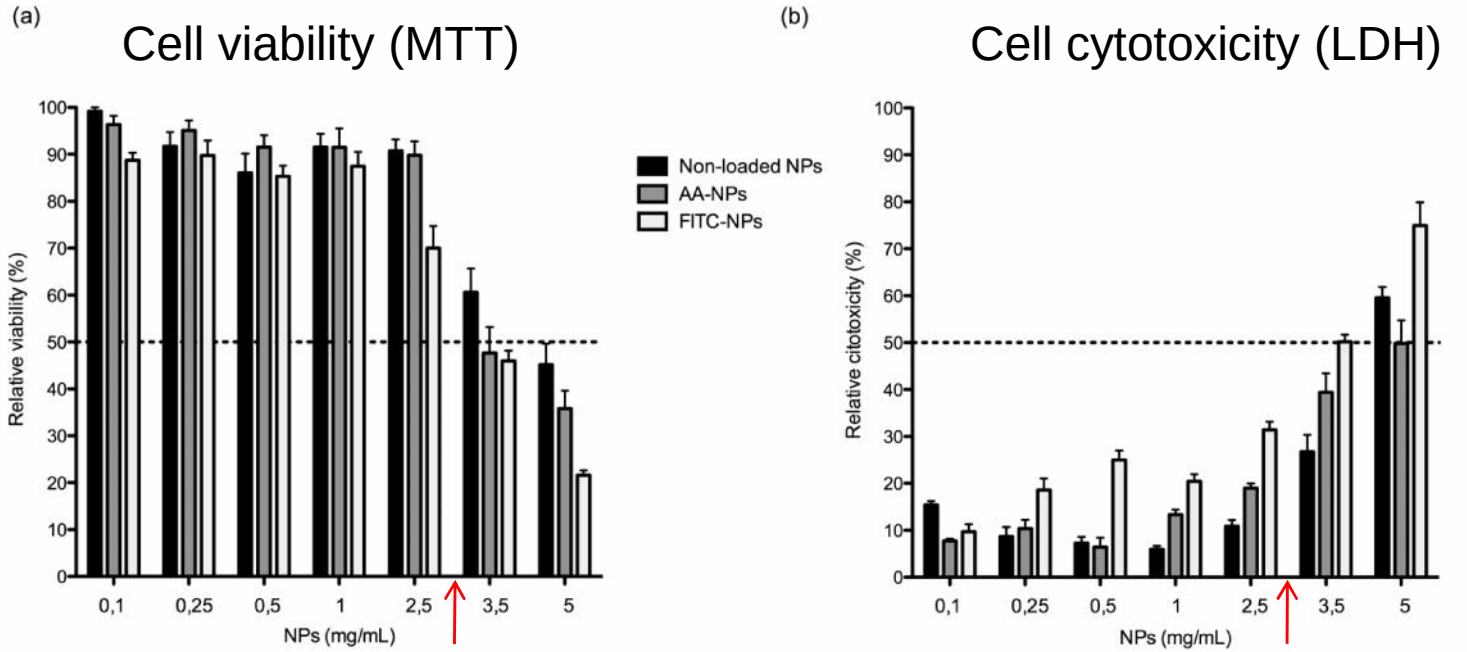


- Using zebrafish hepatocytes (ZFL).
- MTT/LDH assays.
- ZFL cells were seeded in 24-well plates 24 hours before toxicity studies.
- Assay doses: 0, 0.1, 0.25, 0.5, 1, 2.5, 3.5 and 5.0 mg/ml of NPs for 24 h.



Cell toxicity studies

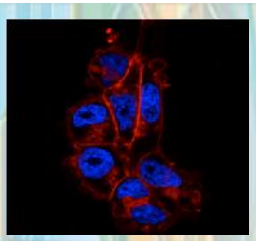
Methods and results



Sensitivity of ZFL cells toward various doses of different nanoparticle formulations. a) Cell viability by MTT assay; b) Cell cytotoxicity by LDH assay. (Mean ± SE). Statistical significance was accepted for $p<0.05$.

EC50(mg/ml)	MTT	LDH
FITC-NPs	3,0±0,38	3,48±0,65
Non loaded	4,41±0,84	3,9±0,3
AA-NPs	3,98±0,31	4,88±0,42

- **Cell viability did not decrease up to a higher NP concentration of 2.5mg/ml.**
- **To avoid potential toxic side-effects, these NPs should be used at doses below 2.5mg/ml.**



ZFL cell uptake studies

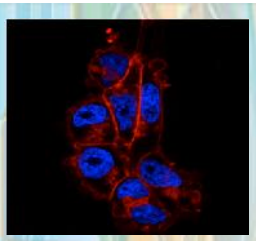
- Using Bovine serum albumin (BSA) labeled with fluorescein isothiocyanate (BSA-FITC) loaded in NPs to evaluate the cell uptake.

- Time-course and dose response of endocytosis:
  - Time course: 1, 3, 6, 12, 16 and 24 h post-treatment with 1mg/ml of FITC-NPs.
  - Dose response: 6 h using different FITC-NPs doses (0.25, 0.5, 1 and 2.5 mg/ml).

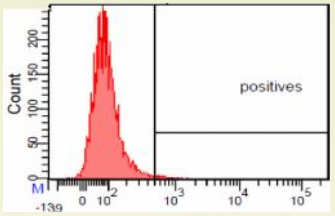
- Quantify the uptake of NPs by flow cytometry.

- Confocal microscopy (at 3h and 16h).

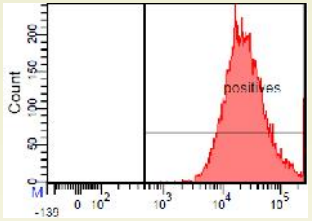
Time course and Dose response



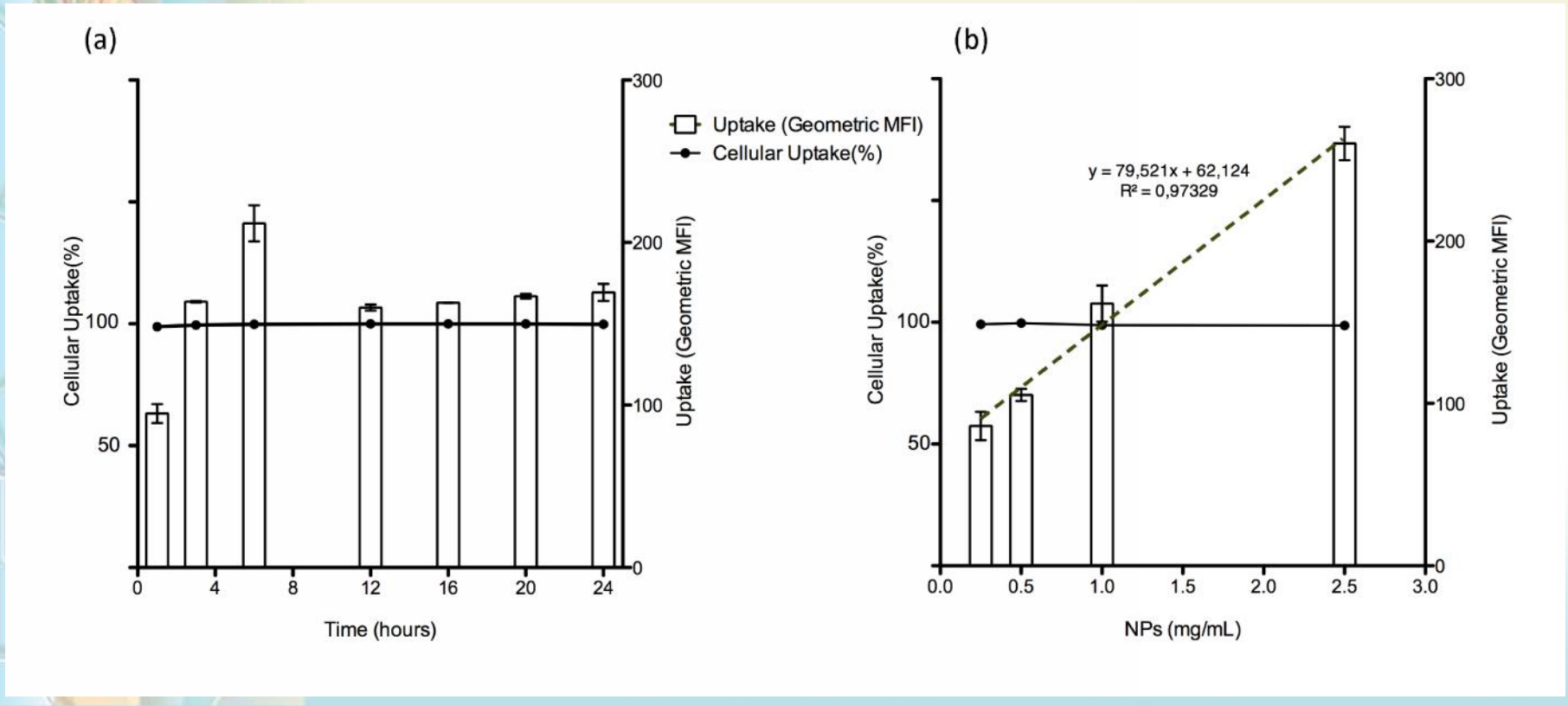
Flow cytometry



Control



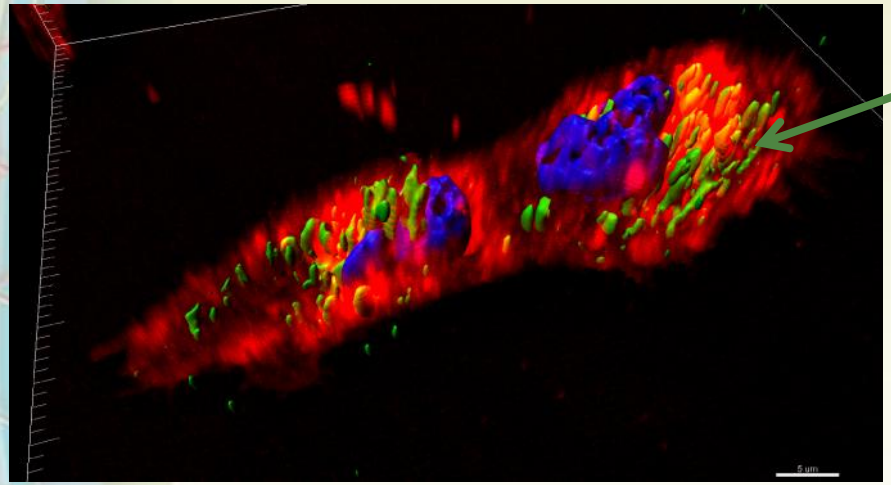
FITC-NPs

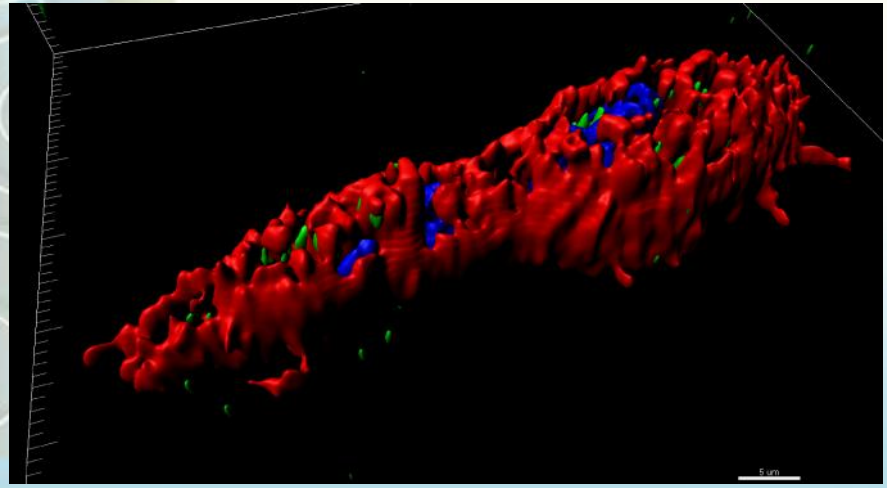


Time course (a) and dose-response (b) of ZFL cells treated with FITC-NPs analysed by cytometry

In vitro uptake of BSA-FITC delivered by nanoparticles after 3h

3h





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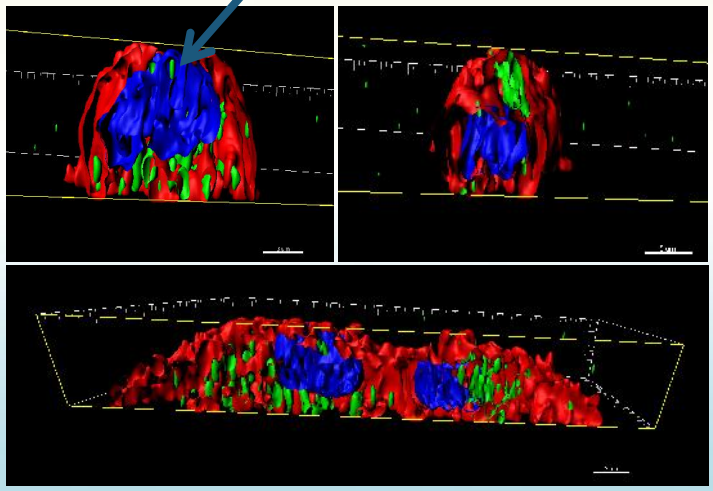
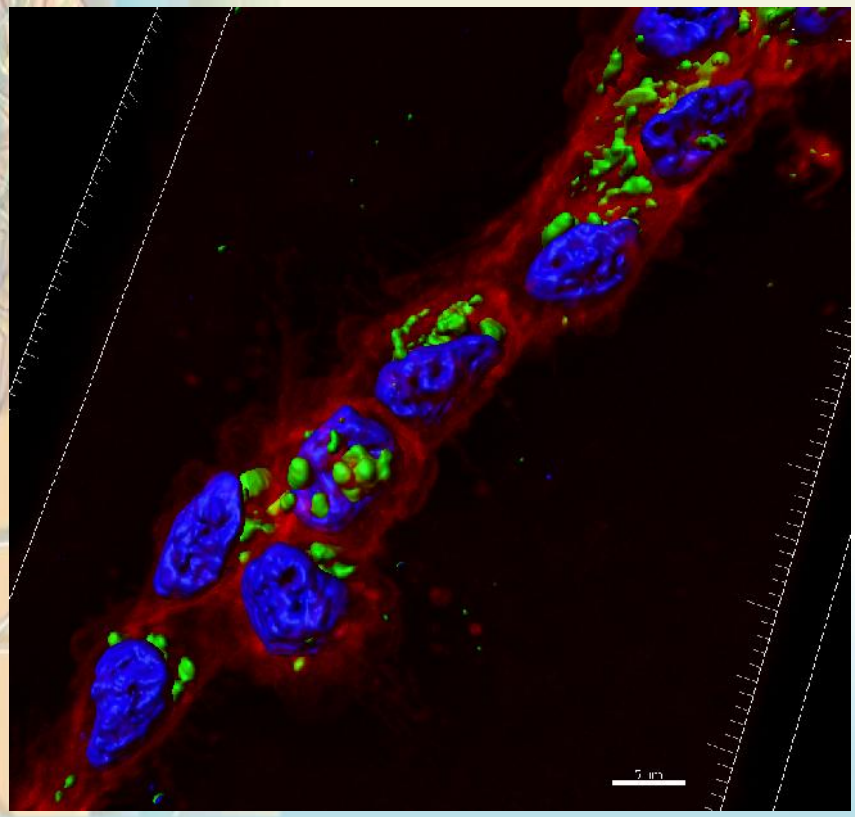
Cell nuclei



(masking + clipping)

In vitro uptake of BSA-FITC delivered by nanoparticles after 16h

16h



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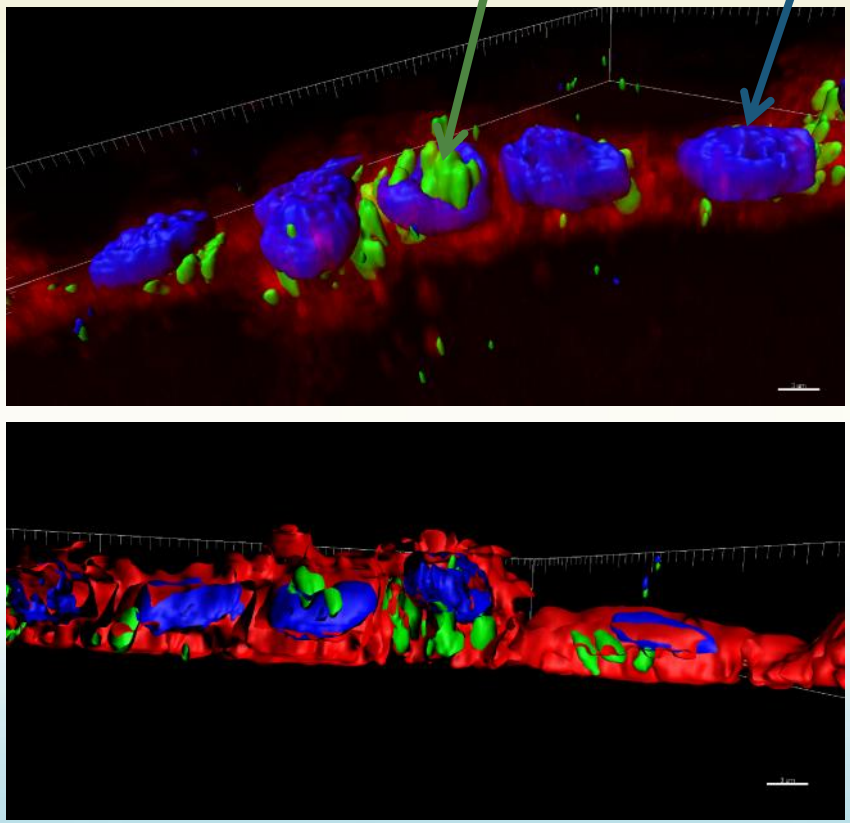

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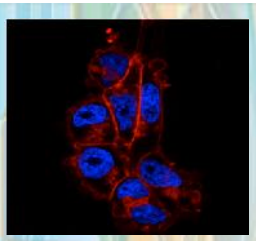
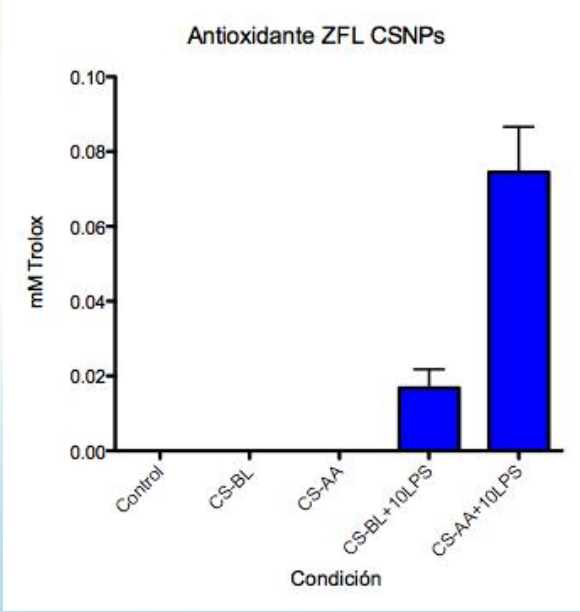


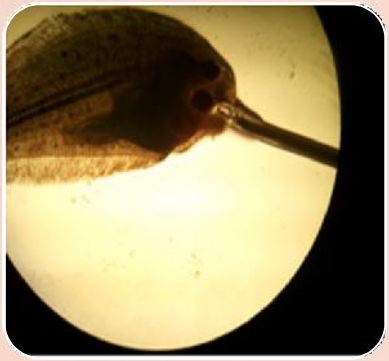
Effect of NPs on total cell antioxidant capacity

- 2,5mg/ml of AA-NPs for 6h.
- LPS to induce cell oxidative stress.
- Antioxidant assay Kit.
- Trolox equivalents.



NPs in vivo evaluation

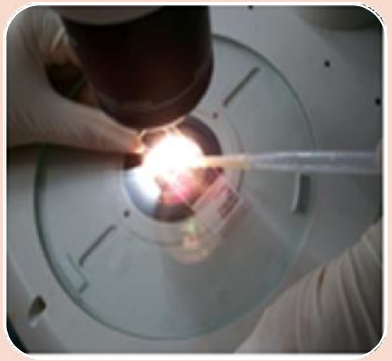
Solea senegalensis post-metamorphic larvae



BSA/BSA-FITC loaded nanoparticles were dispersed in PBS. Nanoparticles were administered to sole fish larvae by gavage.

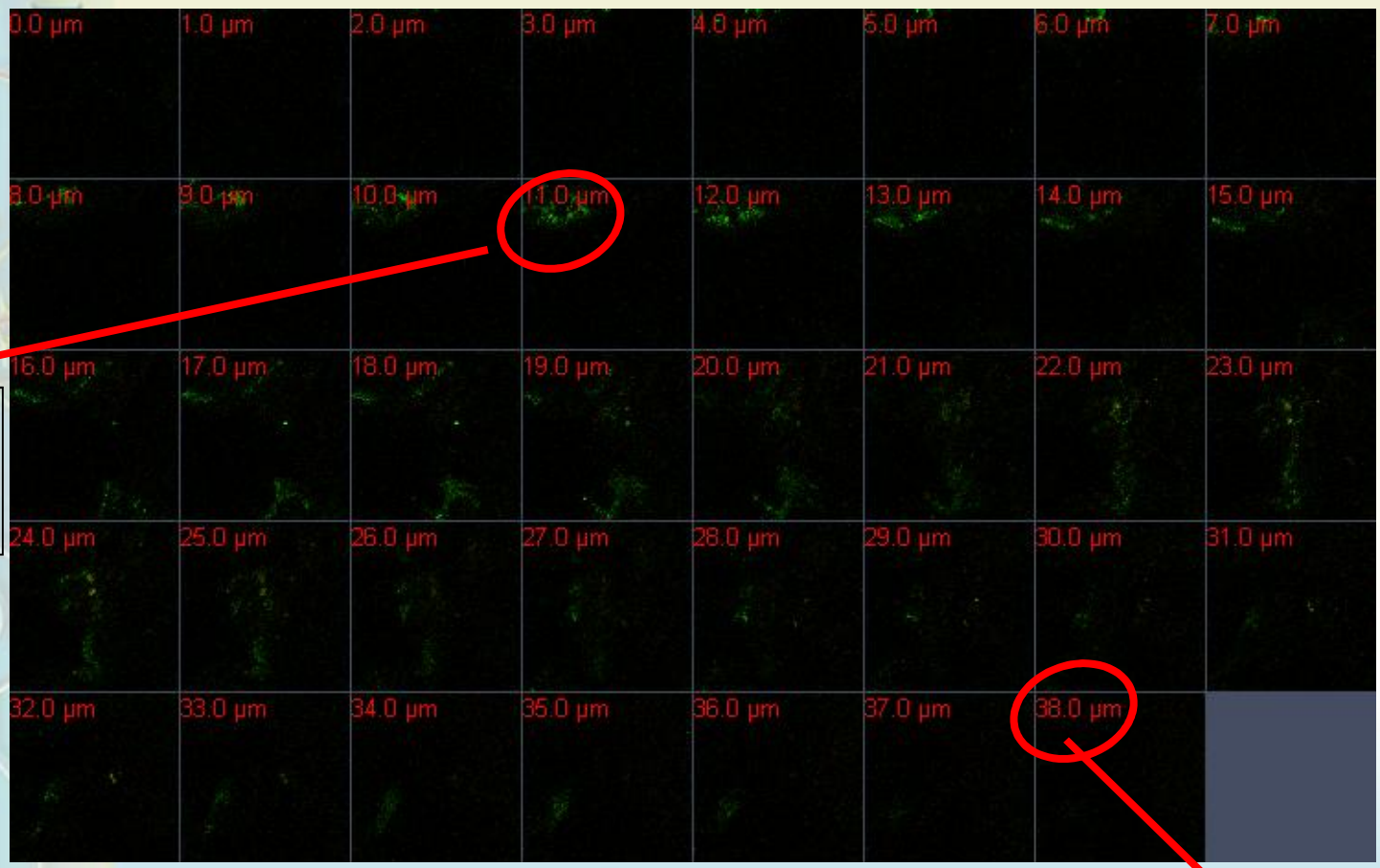
After 2h, the whole intestine was removed and flushed with PBS.

Each intestine was fixed with paraformaldehyde, permeabilized by immersion in Triton X-100 and stained with a solution of BODIPY® 665/676) in methanol.



Intestines were mounted on glass slides and analyzed using a confocal laser scanning microscope.

Penetration into the intestinal tissue…

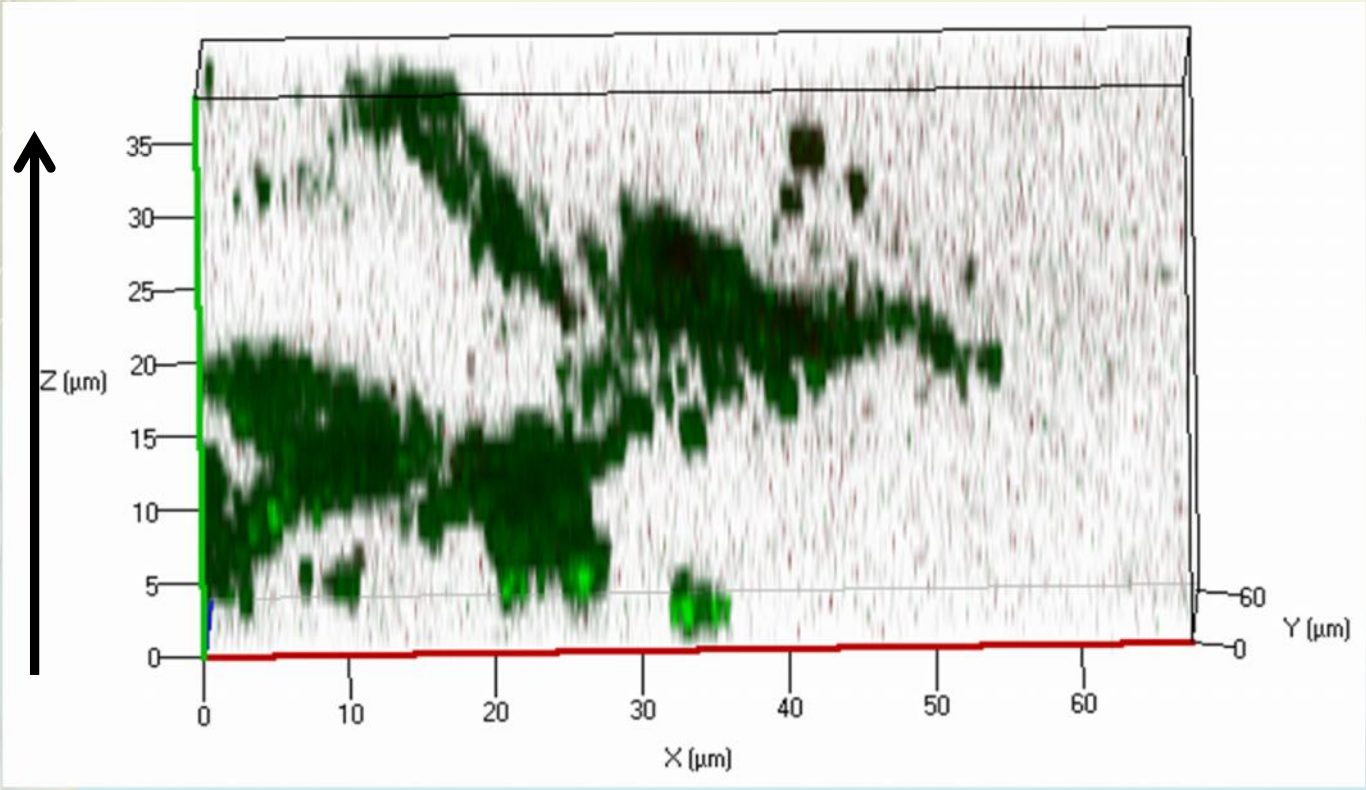


Max. Fluorescence intensity

z-stack (µm)

Focusing in BSA-FITC nanoparticles

Penetration into the intestinal tissue…

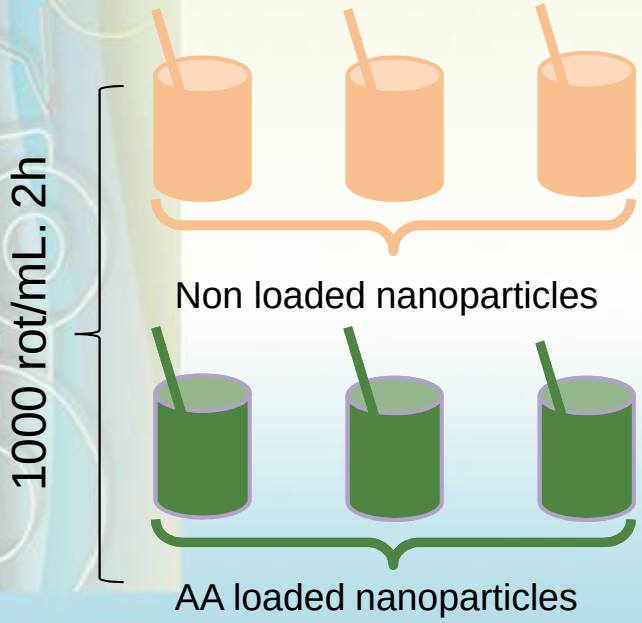


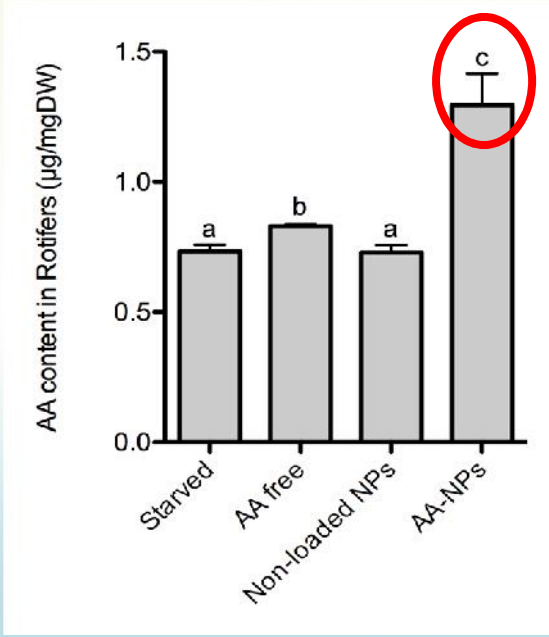
Rotifers *(Brachionus plicatilis)* enrichment with NPs

AA is an essential micronutrient for marine fish that plays a key role in a range of physiological processes.

Since AA is very unstable, we are using chitosan nanoparticles as AA carriers.

In vivo assay: Rotifers were placed in small containers (150ml) after 24 hours of starvation, then experimental nanoparticles were supplied for 2 hours.

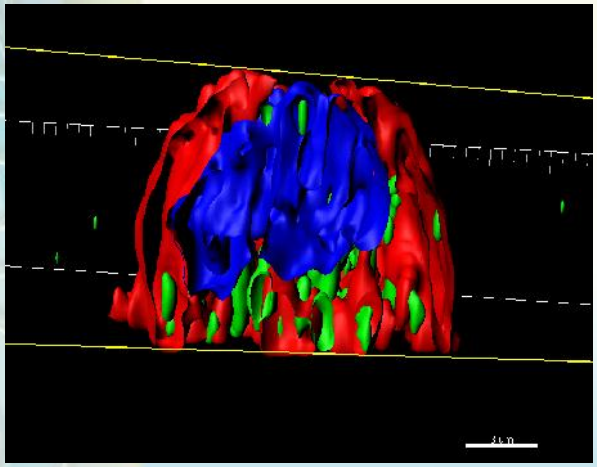




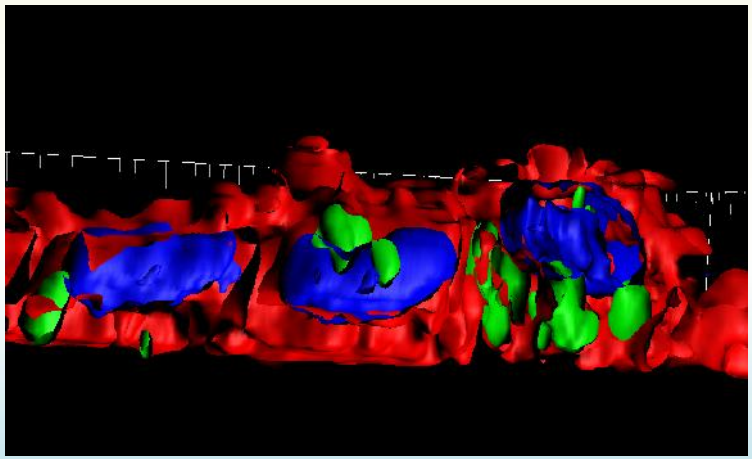
Key conclusions

BSA-FITC delivered by **CS-CD-TPP** nanoparticles is completely internalized in ZFL cell both at 3 and 16 hours. Fast delivery as also seen by FACS (max. uptake at 6 hours and 100% cells endocyting FITC-NPs)

3h

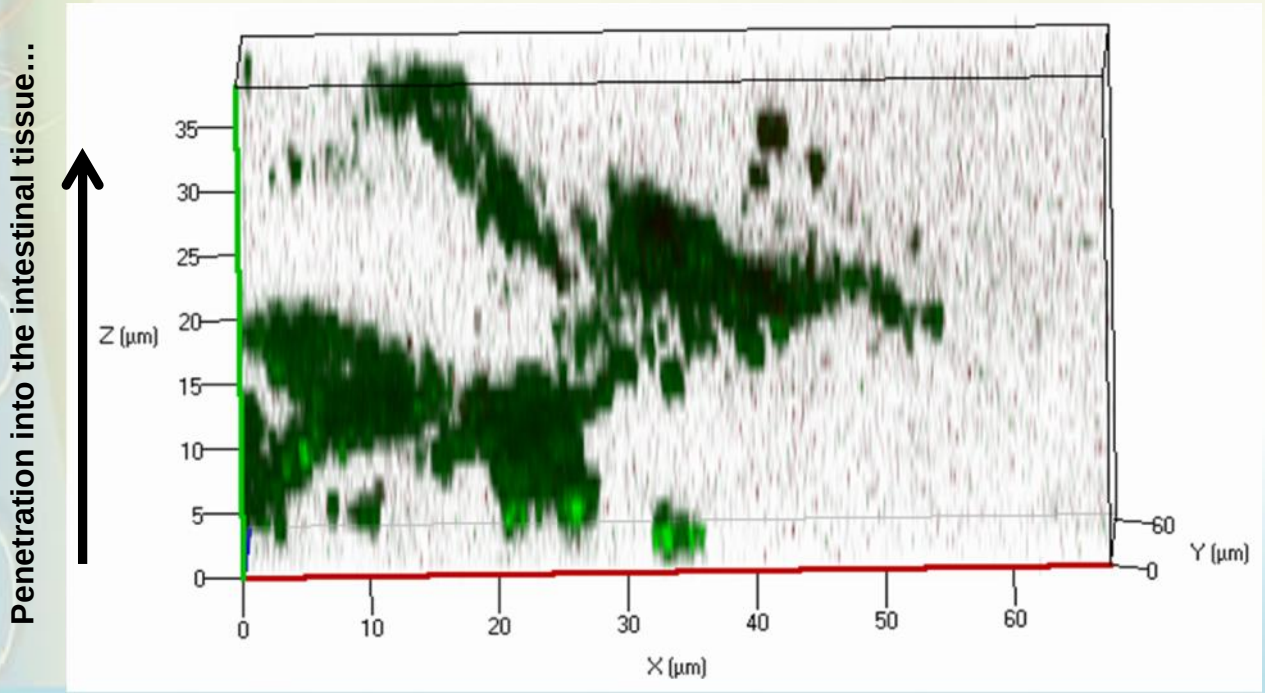


16h



Key conclusions

The NPs prepared for the present work were able to penetrate the intestinal epithelium of *Solea senegalensis* post-metamorphic larvae.



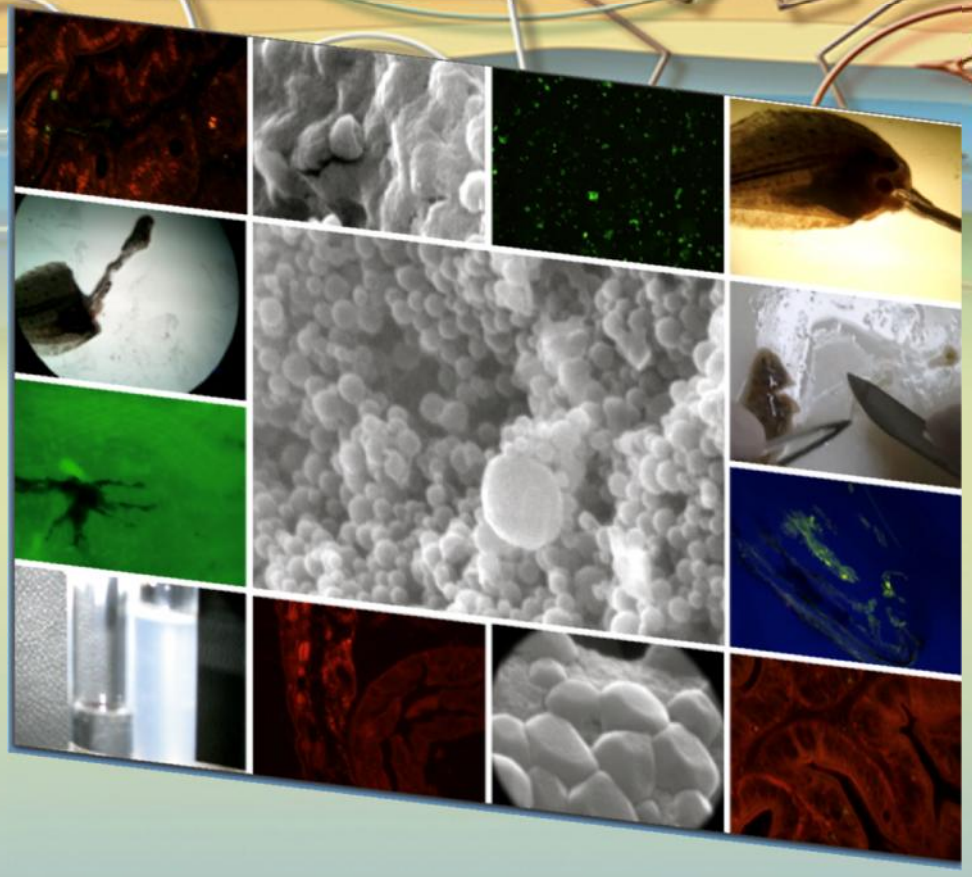
To Summarise:

The overall goal of this work was to assess the potential of nanoparticles (NPs) based on **biodegradable and non-toxic materials** as a vehicle for the **delivery of vitamin C** to aquatic organisms and to know their response to this type of particles

We have obtained **nanoparticles** with **small size, positive charge and they are able to be internalised** by zebrafish hepatocytes increasing the total antioxidant capacity. In addition, this delivery system is **able to penetrate through the intestinal epithelium** in *Solea senegalensis* larvae

Nanoparticles can function like **carriers of essential micronutrients** for marine fish or larvae prey, such as ascorbic acid.

The NPs developed in the present work are functional and might represent an **interesting tool for oral administration of active compounds**, opening new possibilities in nutrition studies and other fields in aquaculture.



IFAPA

Thank you for your attention¡

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