Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente, Ciclo XXXVII

Microplastics in aquaculture: accumulation and physiological effects in experimental models and species of commercial interest.

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INTRODUCTION

Microplastics (MPs; size < 5mm) contamination is a worldwide problem, and they reached all types of ecosystems. Their presence was detected in aquatic living organisms, cultured species included, posing serious concern for the aquaculture sector. The aim of this project was to study MPs assimilation and negative effects on both a model fish species and a species of commercial interest. Additionally, the project focused on identifying a mitigation strategy to counteract the adverse effects caused by MPs ingestion on fish and to reduce their absorption. A natural antioxidant, astaxanthin (**ASX**), was added to to the diets using microcapsules made of natural-based compounds (including starch, an effective MPs coagulant) able to stick to the fish feed. The project was divided in three distinct phases and included a collaboration with a national company, STM Aquatrade.

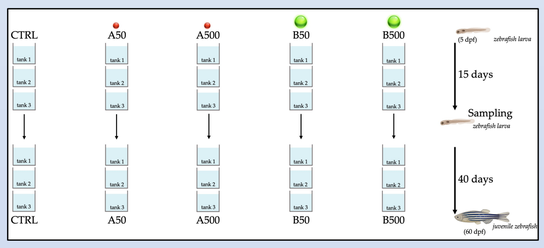
Phase I testing dietary MPs administration in zebrafish (*Danio rerio*) throughout their entire life cycle, with an additional final period of one month in which microencapsulated ASX was implemented in the test diets to verify the beneficial effects of both the antioxidant molecule and the coagulation compound against MPs ingestion. **Phase II** dietary MPs (only size 1-5 μm) administration to juvenile European seabass (*Dicentrarchus labrax*) starting from phase I results. The exposure lasted for two months, during which microencapsulated ASX was administrated either for the entire duration of the trial or for the last month to evaluate its potential beneficial activity. **Phase III** was performed *in vitro* to assess the potential efficacy of natural based microcapsules and their single components of MPs coagulation.

Diets formulation

Control: Zebrafish feed*
A50: Zebrafish feed + 1-5μm [50mg/kg]
A500: Zebrafish feed + 1-5μm [500mg/kg]
B50: Zebrafish feed + 40-47μm [50mg/kg]
B500: Zebrafish feed + 40-47μm [500mg/kg]

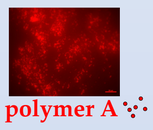
*Diets were prepared in the pilot feed mill facility available at the Department of Agriculture, Food, Environmental and Animal science of University of Udine (Italy). A control diet MPs-free (named Control) was formulated to resemble the proximate composition of a commercial standard diet available for zebrafish (Zebrafeed; Sparos LDA, OIhão, Portugal) Cospheric LLC (Goleta, CA, USA):
**https://www.cospheric.com/fluorescent_violet_tracer_microparticles_2um.htm
***https://www.cospheric.com/UVPMSBR_fluorescent_red_spheres_density0995.htm

Experimental design



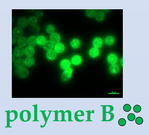
PREVIOUS RESULTS

Amino formaldehyde polymer
FMV-1.3**
size: 1-5μm
Peak emission of 636nm
when excited at 584nm



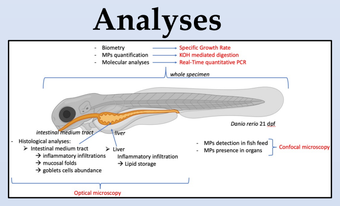
polymer A

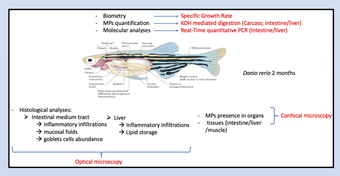
Polyethylene
UVPMS-BR-0.995***
size: 40-47μm
Peak emission of 607nm
when excited at 575nm



polymer B

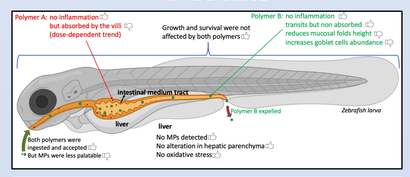
Analyses



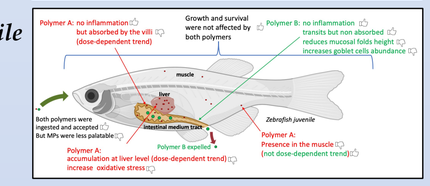


Results

zebrafish larvae



zebrafish juvenile



PHASE I

Methods. The same diets used in the previous study were administrated to zebrafish for a duration of 6 months. After this period, the experimental groups were divided into two distinct groups. For one month, one group continued with the same diets, while the other group was given diets supplemented with ASX (7g of microencapsulated ASX/kg of feed, corresponding to 175 mg of pure ASX). The analyses performed were the same as the previous study.

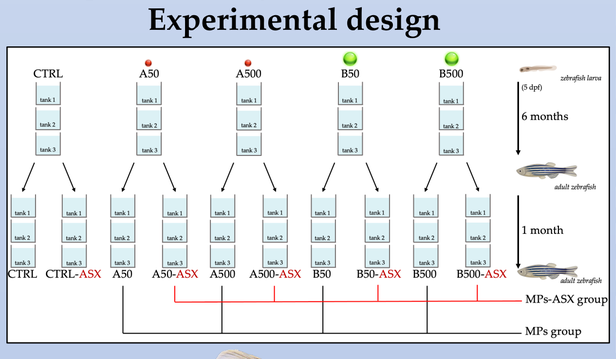
Diets formulation

Control
A50
A500
B50
B500
Control-ASX
A50-ASX
A500-ASX
B50-ASX
B500-ASX

Information about diets are represented in the previous section

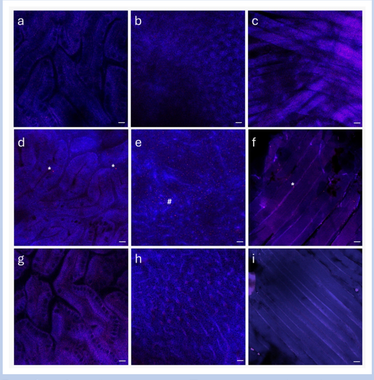
• = microencapsulated ASX

Experimental design



ZEBRAFISH

Results. In the groups fed exclusively with MPs diets, the results were similar to those observed in the previous study. However, once microencapsulated ASX was added to the feed, normal conditions were restored. ASX effectively reduced oxidative stress and restored normal mucosal folds height. Additionally, in the group fed A500-ASX diet, the amount of MPs detected in the organs was similar to that in the A50 and A500 groups, suggesting the formation of MPs aggregates too large to be absorbed at intestinal level.



Confocal microscopy. Representative images for zebrafish fed (**a-c**) CTRL-diets, (**d-f**) A-diets, (**g-h**) B-diets. (**a**,**d**,**g**) intestine; (**b**,**e**,**h**) hepatic parenchyma; (**c**,**f**,**i**) skeletal muscle. Scale bars = 20 μm. Asterisks indicate isolate MPs; # indicate groups of MPs.

Polymer A (1-5 μm)

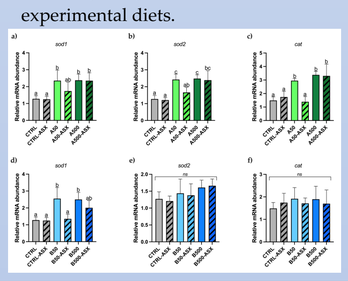
	CTRL	CTRL-ASX	A50	A50-ASX	A500	A500-ASX
Intestine	0	0	2.9 ± 0.3 ^{a}	2.6 ± 0.9 ^{a}	170.9 ± 20.6 ^{c}	20.5 ± 2.5 ^{b}
Liver	0	0	5.5 ± 1.7 ^{a}	5.5 ± 2.1 ^{a}	821.1 ± 95.5 ^{b}	12.2 ± 3.0 ^{a}
Muscle	0	0	2.0 ± 0.2 ^{a}	1.9 ± 0.9 ^{a}	48.0 ± 4.3 ^{b}	3.2 ± 1.8 ^{a}

Polymer B (40-47 μm)

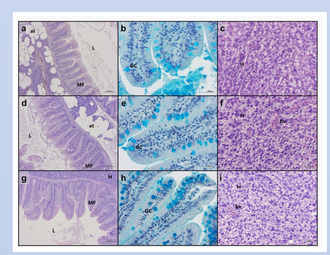
	CTRL	CTRL-ASX	B50	B50-ASX	B500	B500-ASX
Intestine	0	0	1.6 ± 0.3 ^{a}	1.5 ± 0.3 ^{a}	1.8 ± 0.3 ^{a}	1.6 ± 0.4 ^{a}
Liver	0	0	0	0	0	0
Muscle	0	0	0	0	0	0

Data are reported as mean ± SD (*n* = 15). a,b Within each line, different letters denote statistically significant differences ($p < 0.05$) among the experimental group.

MPs quantification after 10% KOH mediated digestion (number of microbeads/mg of tissue). Intestine, liver, and muscle of adult zebrafish fed experimental diets.



Real-time qPCR results. Relative mRNA abundance of genes involved in oxidative stress response (*sod1*, *sod2*, *cat*) analysed in the liver of adult zebrafish fed the experimental diets. Data are reported as mean ± SD (*n* = 5). $^{a-c}$ Different letters denote statistically significant differences among the experimental group. *ns*, no significant differences among the experimental groups ($p > 0.05$).



Histological evaluation of intestine and liver. Example of histomorphology of zebrafish fed (**a-c**) CTRL-diets, (**d-f**) A-diets, (**g-h**) B-diets. (**a**,**d**,**g**) intestine, scale bars = 100 μm; (**b**,**e**,**h**) details of Ab+ goblet cells, scale bars = 20 μm; (**c**,**f**,**i**) hepatic parenchyma, scale bars = 20 μm. Abbreviations: at, perivisceral adipose tissue; L, gut lumen; MF, intestinal mucosal folds; H, hepatocytes; GC, goblet cells; bv, blood vessels.

Polymer A (1-5 μm)

	CTRL	CTRL-ASX	A50	A50-ASX	A500	A500-ASX
Mucosal folds height (μm)	427.8 ± 12.9 ^{a}	430.4 ± 14.5 ^{a}	425.2 ± 11.2 ^{a}	426.3 ± 2.3 ^{a}	424.0 ± 14.6 ^{a}	427.5 ± 12.9 ^{a}
Ab+ goblet cells' relative abundance	+	+	++	++	++	++

Polymer B (40-47 μm)

	CTRL	CTRL-ASX	B50	B50-ASX	B500	B500-ASX
Mucosal folds height (μm)	427.8 ± 12.9 ^{a}	430.4 ± 14.5 ^{a}	391.2 ± 8.7 ^{b}	420.5 ± 14.1 ^{a}	390.9 ± 14.3 ^{b}	428.8 ± 21.4 ^{a}
Ab+ goblet cells' relative abundance	+	+	++	++	+++	++

Data of mucosal folds height are reported as mean ± SD (*n* = 15). a,b Different letters denote statistically significant differences ($p < 0.05$) among the experimental group. Ab+ goblet cells: + = scarce; ++ = diffused; + + + = highly abundant.

Histological indexes. Intestine of adult zebrafish fed the experimental diets.

PHASE II

Methods. Three experimental diets were formulated to cover the nutritional requirements of European seabass using the same amount of polymer A and microencapsulated ASX as in *phase I*. The feeding trial lasted two months. The experimental groups were subdivided as follows: (i) CTRL group: fish fed for 2 months CTRL diet; (ii) CTRL/CTRL-ASX group: fish fed for 1 month CTRL diet and for 1 month CTRL-ASX; (iii) CTRL-ASX group: fish fed for 2 months CTRL-ASX diet; (iv) A50 group: fish fed for 2 months A50 diet; (v) A50/A50-ASX group: fish fed for 1 month diet A50 and for 1 month A50-ASX; (vi) A50-ASX group: fish fed for 2 months A50-ASX diet. Analyses conducted were the same as previous studies with the addition of MPs detection in blood and adipose tissue.

Diets formulation

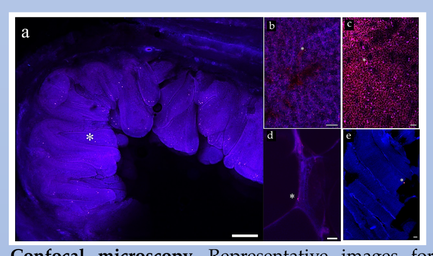
Control
Control-ASX
A50
A50-ASX

Information about MPs and microencapsulated ASX are reported in the previous sections

EUROPEAN SEABASS

Experimental design

Results. MPs were detected in all groups fed with polymer A diets. In the group fed the A50 diet, MPs quantity and oxidative stress levels were significantly higher compared to the CTRL and A50-ASX groups. Regarding histological index, the only notable difference was observed in the A50 group, which exhibited a significantly higher presence of supranuclear vacuoles compared to the other groups. When ASX was included in the diets from the beginning, the negative effects of MPs ingestion were counteracted, and the number of MPs absorbed was reduced. In the A50/A50-ASX group, where ASX was introduced midway through the feeding trial, normal conditions were partially restored, and the amount of assimilated MPs was somewhat similar to that in the A50-ASX group.

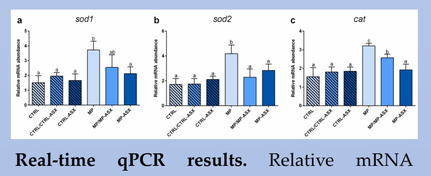


Confocal microscopy. Representative images for European seabass fed A50 diets (**a**) intestine, (**b**) liver, (**c**) blood, (**d**) adipose tissue, (**e**) muscle; Scale bars = 20 μm. Asterisks indicate isolate MPs; # indicate groups of MPs.

	CTRL	CTRL/CTRL-ASX	CTRL-ASX	A50	A50/A50-ASX	A50-ASX
Intestine	0	0	0	34.3 ± 4.7 ^{a}	12.0 ± 3.0 ^{b}	10.3 ± 1.5 ^{b}
Liver	0	0	0	72.3 ± 5.8 ^{a}	52.7 ± 11.4 ^{a}	26.3 ± 8.0 ^{b}
Muscle	0	0	0	3.0 ± 0.9 ^{a}	3.2 ± 0.7 ^{a}	0
Adipose tissue	0	0	0	7.1 ± 1.8 ^{a}	3.7 ± 0.9 ^{ab}	1.7 ± 0.6 ^{b}
Blood	0	0	0	17.6 ± 2.8 ^{a}	12.9 ± 1.5 ^{b}	7.7 ± 1.5 ^{c}

Data are reported as mean ± SD (n = 15). $^{a-c}$ Within each line, different letters denote statistically significant differences among the experimental groups.

MPs quantification after 10% KOH mediated digestion (number of microbeads/mg of tissue). Intestine, liver, muscle, adipose tissue and blood of juvenile European seabass fed experimental diets.

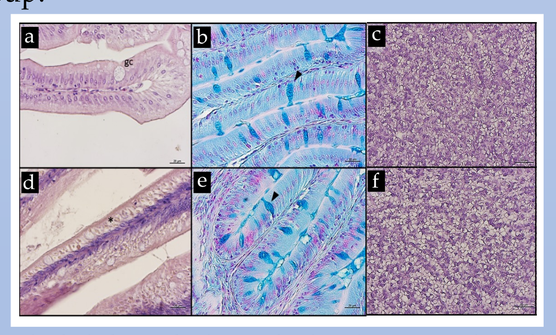


Real-time qPCR results. Relative mRNA abundance of genes involved in oxidative stress response (*sod1*, *sod2*, *cat*) analysed in the liver of juvenile European seabass fed the experimental diets. Data are reported as mean ± SD (*n* = 5). $^{a-c}$ Different letters denote statistically significant differences among the experimental group. *ns*, no significant differences among the experimental groups ($p > 0.05$).

	CTRL	CTRL/CTRL-ASX	CTRL-ASX	A50	A50/A50-ASX	A50-ASX
Mucosal fold height	566.1 ± 42.1	588.7 ± 35.5	569.1 ± 47.2	552.4 ± 30.4	583.6 ± 31.5	583.6 ± 44.3
Submucosa width	25.3 ± 2.1	25.9 ± 2.6	24.8 ± 2.3	26.7 ± 2.9	25.7 ± 2.6	26.3 ± 3.2
Mucosal fold fusion	+	+	+	+	+	+
Basal inflammatory influx	+	+	+	+	+	+
Supranuclear vacuoles	-	-	-	+++	+++	++
Ab+ goblet cells relative abundance	++	++	++	++	++	++

Values for mucosal fold height and submucosa width are shown as mean ± SD (n = 15).

Histological indexes. Intestine of juvenile European seabass fed the experimental diets. Data are reported as mean ± standard deviation (n = 15).

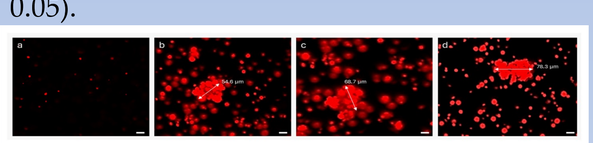


Histological evaluation of intestine. Example of histomorphology of distal intestine and liver from European seabass fed the experimental diets. (**a-d**) different details of mucosal and submucosal architecture from fish fed CTRL diet; (**e**) example of distal intestine sample from MP-ASX group; (**f**) focus on the basal portion of mucosal fold in fish from MP/MP-ASX group. (**g**,**h**) details of mucosal architecture from fish fed MP diet. Example of liver from **(i)** CTRL groups; **(l)** A50 groups. Abbreviations: sm, submucosa; gc, goblet cell; mf, mucosal fold. Symbols: arrowhead, Ab+ goblet cell; *, supranuclear vacuoles. Stainings: (**a-c**) and (**e-g**), Mayer's haematoxylin and eosin Y; (**d**) and (**h**), Alcian blue. Scale bars: (**a**) 100 μm; (**b-d**) 20 μm; (**e**) 50 μm; (**f-h**) 20 μm.

PHASE III

Methods. To verify the eventual coagulation activity of microcapsules on MPs, an *in vitro* experiment was conducted using two solutions of polymer A at 50mg/L and 500mg/L made with fresh water at which, empty microcapsules, microencapsulated ASX, ASX, and the two main compound of the microcapsules (starch and Arabic gum) were added separately. The components were added at 1x, 5x, and 10x to see the coagulation activity of these components after 6h. An *in vivo* test was conducted on zebrafish to which A50, A500, A50-ASX, and A500-ASX diets were administrated to quantify the faecal MPs amount. The pH was adjusted to be the same of the fish digestive tract.

Results. The *in vitro* test revelated that MPs coagulation occurred only in presence of starch. This coagulation was observed at 1x concentration of the compounds when the MPs solution was 500 mg/L, while the same result was achieved with 50 mg/L MPs solution at 10x concentration of the compounds. The *in vivo* experiment (data not shown) mirrored the *in vitro* findings; no significant difference was detected between A50 and A50-ASX groups. However, in A500-ASX group, the amount of MPs found in zebrafish faeces was significantly lower compared to A500 group ($p < 0.05$).



Coagulation in the *in vitro* experiment. (**a**) solution containing only MPs at 500 mg/L; (**b**) MPs solution (500 mg/L) added with microencapsulated ASX (1× concentration); (**c**) MPs solution (500 mg/L) added with empty microcapsules (1× concentration); (**d**) MPs solution (500 mg/L) added with starch (1× concentration). Scale bars: 20 μm.

MPs concentration	Concentration of added components	Solutions (1mL) MPs only	ASX	MPs + microencapsulated ASX	MPs + empty microcapsules	MPs + starch	MPs + Arabic gum
Solution 50	1×	-	-	-	-	-	-
50 mg/L of	5×	-	-	-	-	-	-
polymer A	10×	-	-	+	+	+	-
Solution 500	1×	-	-	+	+	+	-
500 mg/L of	5×	-	-	+	+	+	-
polymer A	10×	-	-	+	+	+	-

All the solutions were tested in triplicate. -: detection of coagulation events; +: absence of coagulation events. MPs = polymer A microbeads.

***In vitro* coagulation experiment** after 6 hours.

PERIOD ABROAD

This period was conducted at the Institute of Environmental Assessment and Water Research (Barcelona, Spain). The aim was to investigate different types of MP polymers and their respective plastic additives in digestive tract samples from fish collected in 2018 and 2022 at the Ebro river using liquid and gas chromatography. It was found that in 2018, the polymers present were polyethylene and polypropylene, while in 2022, they were polyethylene and polydimethylsiloxane. The presence of plastic additives was detected in all samples. Results are presently under analysis.