UNIVERSITÀ POLITECNICA DELLE MARCHE

Dipartimento di Scienze della Vita e dell'Ambiente
DISVA

PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: Cattaneo Nico

Title of PhD research: Microplastics in aquaculture: accumulation and physiological effects on experimental models and species of commercial interest.

Name of PhD supervisor: Prof. Olivotto Ike
Research lab name: Reproductive and Developmental Biology Lab, DiSVA

Cycle:
[X] XXXVII
[ ] XXXVIII

PhD Curriculum:
[X] Marine biology and ecology
[ ] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[X] Aquarium
[ ] MassSpec lab
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[ ] Simulation/informatics lab
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ABSTRACT (1000 characters, including spaces):
Microplastics (MPs) are globally distributed and also the aquaculture sector is affected by this contamination. This study aims to comprehensively investigate the effects of MPs on fish and proposes potential mitigation strategies. The research comprises four phases. Phase one used the model organism zebrafish (*Danio rerio*), exposing them to MPs-contaminated diets from larvae to juveniles with different polymer sizes and concentrations to better understand the possible different biological responses by different fish life-cycle stages. In the second phase, the study explored astaxanthin (ASX), a potent natural antioxidant, as a dietary supplement to counter the MPs adverse effects in fish. The third phase focused on European sea bass (*Dicentrarchus labrax*) on a trial using diets formulated after the previous. Additionally, a last phase was to test the possible cellular MPs uptakes using an *in vitro* organoid model based on rainbow trout (*Oncorhynchus mykiss*) intestinal cells.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Microplastics (MPs; plastics size < 5 mm) are globally diffused (Shahul Hamid et al., 2018; Tang et al., 2023) and have been detected in most living organisms, including aquatic ones (Ma et al., 2020; Naidu et al., 2017; Oliveira et al., 2020). Also the aquaculture sector is affected by this emerging pollutant (Chen et al., 2021; Miao et al., 2023) since MPs can easily reach the farmed fish (Dehm et al., 2022; Wu et al., 2023) through the water or the feed (Castelvetro et al., 2021; Chen et al., 2018; Hanachi et al., 2019; Wu et al., 2020). Several studies have highlighted that size, shape, concentration and chemical features are key characteristics in determining MPs toxicity in fish which may also differently react in relation to their life-cycle stage and the species (Bobori et al., 2022; Pannetier et al., 2020; Pirsaheb et al., 2020; Wang et al., 2020; Zhang et al., 2022). In addition, fish hardly recognise MPs once they are implemented in aquafeed (Rainieri et al., 2018; Xiong et al., 2019), leading to a transit in the gastrointestinal tract and, depending on their size (Bhagat et al., 2020; Cattaneo et al., 2023), to a consequent absorption at the intestinal level (De Sales-Ribeiro et al., 2020; Lu et al., 2016). After being absorbed, studies have demonstrated that MPs can be translocated to other organs or tissues such as the liver (Abbasi et al., 2018; Zitouni et al., 2021)or the muscle (Di Giacinto et al., 2023; Makhdoumi et al., 2021; Zeytin et al., 2020), raising concerns also for human health. On this regard, a number of studies have demonstrated that MPs tend to be accumulated in fish liver, preventing a consequent translocation to other tissues (Jovanović et al., 2018; Ye et al., 2021; Zeytin et al., 2020) but leading to systemic stress and increased oxidative stress response at hepatic level (Capó et al., 2021; Iheanacho and Odo, 2020; Xiao et al., 2023). Despite several methods have been tested to reduce the MPs use (Dong et al., 2023; Selvasudha et al., 2023) and release into the environment (Lange, 2021; Siddique et al., 2008), as well as to remove them from the water (Gao et al., 2022; Lengar et al., 2021; Poerio et al., 2019; Shen et al., 2020), a short-term complete disappearance of MPs pollution is presently utopic. Since most of the studies related to fish MPs exposure highlight that one of the most common detected disorders is oxidative stress (Espinosa et al., 2019; Iheanacho et al., 2023; Miao et al., 2023; Xia et al., 2020; Yedier et al., 2023), a possible solution could be represented by antioxidant molecules that have demonstrated to have a beneficial effect on fish welfare. The carotenoid astaxanthin (ASX) is considered one of the stronger natural antioxidant (Zhao et al., 2019) and investigate the possible beneficial effects of dietary ASX on fish growth and welfare to counteract the MPs-ingestion side effects could be of great interest for the aquaculture sector.

In this context, studies assessing the adverse effects of long-term MPs ingestion by fish and how to mitigate them are of fundamental importance. Zebrafish (*Danio rerio*) is a model species widely used in nutrition (Chemello et al., 2022; Zarantoniello et al., 2021) and toxicology studies conducted on MPs (Bhagat et al., 2020; Qiao et al., 2019) because, compared to other finfish, it has a short life-cycle, high reproduction rate, and its whole genome is sequenced (Ribas and Piferrer, 2014; Ulloa et al., 2014). For these reasons, zebrafish is well suited for studies on long-term MPs exposure over different life-cycle stages and to investigate the possible implementation of ASX in fish feed to mitigate the negative effects caused by MPs ingestion. Finally, the results of these studies could be applied for other studies conducted on species of commercial interest.

In addition, several uptake mechanisms have been proposed for MPs uptake such as absorption, including endocytosis, transcytosis, and paracellular diffusion (Khan and Jia, 2023; Leslie et al., 2022). However, the knowledge of this phenomenon *in vivo* is still fragmentary and largely unknown, so that most pathways are only predicted. In this perspective, cell-based organotypic models represent a valuable tool to explore the molecular mechanisms at play. Therefore, a recently developed rainbow trout (*Oncorhynchus mykiss*) *in vitro* intestinal platform, consisting of epithelial and connective cells (Verdile et al., 2023) could possibly elucidate this phenomenon.

- SCIENTIFIC AIMS

In the present study, diets including microplastics of different sizes and at concentrations were prepared. The aim was to test the effects of such diets through a multidisciplinary approach: (i) on zebrafish exposed to a long-term MPs contamination over different life-cycle stages; (ii) make a comparison of the biological and physiological responses between zebrafish larvae and juveniles; (iii) investigate the possible beneficial effects of astaxanthin implemented in fish feed after a long-term MPs-exposure on zebrafish adults; (iv) use the previous results in order to perform a study on a species of commercial interest, the European sea bass (*Dicentrarchus labrax*), with MPs-contaminated diets and ASX-implementation; (v) test the possible MPs uptake pathways *in vitro* using a rainbow trout organotypic intestinal model.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

The objective is to assess the effects of a feed-induced MPs contamination during zebrafish development from larva to juvenile and to compare the possible different biological responses of the two fish life-cycle stages, giving particular emphasis to growth and welfare, by monitoring the gut and liver health status, the stress, oxidative stress and immune response, and to check the MPs fate to other target organs in relation to their size.

Methods.

A control diet containing fish meal as major protein source was prepared according to a commercially available standard diet for zebrafish (Zebrafeed, Sparos ltd, Portugal). Four experimental diets were prepared by adding to the basal diets two different sized fluorescent MPs (Polymer A: amino formaldehyde polymer, 1-5 µm; Polymer B: Polyethylene, 40-47 µm), both from Cospherics (Santa Barbara, USA) at two different concentrations (50 mg/kg and 500 mg/kg). Zebrafish were fed the experimental diets (daily dose corresponding to the 3% of the body weight) from 5 to 21 days post fertilization for larvae and until 2 months post fertilization for juveniles.

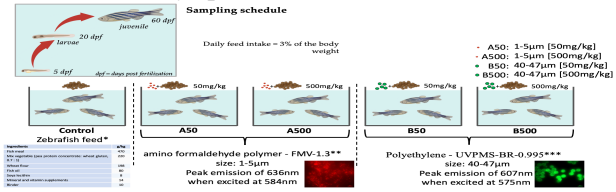

Figure 1. Experimental design

Obtained Results.

No significant differences in survival and specific growth rates were detected among the experimental groups for both zebrafish larvae and juveniles. The ingestion of the two polymers used in this study was confirmed both by confocal microscopy and MPs quantification in the zebrafish larvae and juveniles. However, only the polymer A (size 1-5 µm) was absorbed at intestinal level in both larvae and juveniles. The presence of polymer A microbeads was detected in liver and muscle samples only in juveniles suggesting a time-related translocation from the gastrointestinal tract. Furthermore, the MPs quantification in both whole larvae and in intestine and liver samples of juveniles highlighted a dose dependent accumulation of polymer A. Regarding polymer B (size 40-47 µm), no absorption was detected, but the transit through the intestinal tract caused a reduction of mucosal folds height and an increase in goblet cells relative abundance in B50 and B500 groups, suggesting a higher intestine lubrication. The absorption or the simple transit of both MPs in groups A and B did not cause inflammatory events at intestinal level nor alteration in the expression of immune markers in both larvae and juveniles. However, the accumulation of polymer A microbeads in liver samples of juveniles caused the upregulation of the oxidative stress markers.

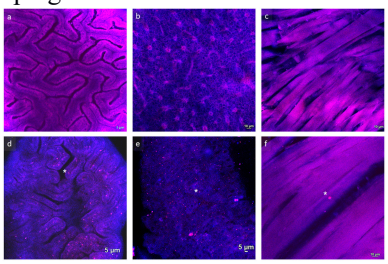

Figure 2. Representative images of (**a**,**d**) intestine, (**b**,**e**) liver, and (**c**,**f**) muscle samples of zebrafish juveniles fed Control (**a**-**c**) and A500 (**d**-**f**) diets. Asterisks indicate polymer A microbeads.

Table 1. MPs quantification (number of microbeads/mg) in whole zebrafish larvae and in the intestine, liver, and muscle of zebrafish juveniles fed the experimental diets.

		Control	A50	A500	B50	B500
Larvae	whole specimen	0	0.5 ± 0.2^{a}	3.5 ± 0.8^{b}	0	0
Juveniles	intestine	0	1.15 ± 0.45^{a}	61.93 ± 14.30^{b}	0.14 ± 0.01^{a}	0.64 ± 0.15^{a}
	liver	0	5.4 ± 1.6^{a}	231.1 ± 47.1^{b}	0	0
	muscle	0	0.3 ± 0.1^{a}	4.7 ± 1.2^{b}	0	0

Table 2. Histological indexes measured in the intestine of larvae and juveniles fed the experimental diets.

		Control	A50	A500	B50	B500
Larvae	Mucosal fold height	102.9 ± 15.0^{a}	86.7 ± 8.4^{ab}	88.0 ± 5.8^{ab}	73.2 ± 4.6^{bc}	65.7 ± 6.0^{c}
	Ab+ goblet cells' relative abundance	+	+	+	++	++
Juveniles	Mucosal fold height	94.9 ± 5.7^{a}	96.4 ± 8.8^{a}	88.2 ± 9.4^{a}	69.7 ± 7.9^{b}	70.1 ± 5.4^{b}
	Ab+ goblet cells' relative abundance	++	++	++	+++	+++

Ab+ goblet cells: + = 0 to 3 per villus; ++ = 3 to 6 per villus; +++ = more than 6 per villus.

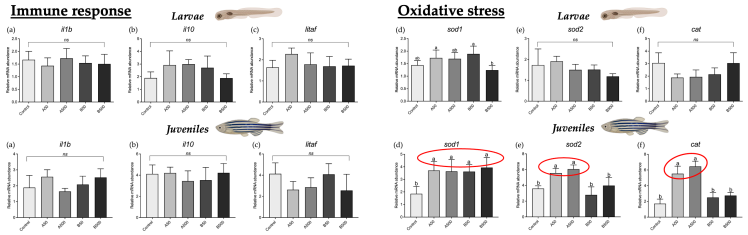

Figure 3. Relative mRNA abundance of genes involved in immune response (*il1b*, *il10*, and *litaf*) in intestine and oxidative stress (*sod1*, *sod2*, and *cat*) in liver analysed in zebrafish larvae and juveniles.

WP 2. Objective.

The objective is to mitigate the effects of a long-term feed-induced MPs contamination in zebrafish adult using ASX implemented it diets at 7g/kg of feed.

Methods.

Same methods used in WP1. After 6 months, zebrafish were divided in two sub-groups which were fed for one month the following diets: i) group MPs diets: same diets as WP1; ii) group ASX-MPs diets: same diets as WP1 implemented with 7g/kg of ASX.

Obtained Results.

Fish growth and survival rate were not affected by both MPs and MPs-ASX diets. As regards the fish fed the MPs-diets without the implementation of ASX the results were comparable to WP1. As regards the MPs-ASX diets the presence of both polymers inside fish was detected. ASX had a beneficial effect on the histomorphology of the groups fed de B-diets since they showed similar mucosal folds height and the amount of goblet cells in the group B500-ASX was analogue to

the other experimental groups. Considering the relative expression of genes involved in the immune response (*il1b*, *il10*, *litaf*), no significant differences were evident among the experimental groups fed the MPs-ASX diets and those fed the Control-diets. As expected, ASX had a positive impact in reducing the oxidative stress, since in group A50-ASX the expression of the genes analysed correlated to oxidative stress showed similar mRNA abundance compared to the CTRL groups. Nevertheless, in the group A500-ASX, which was administrated a diet containing MPs at a concentration 10 times than the natural one, the antioxidant activity of ASX appeared insufficient to reduce oxidative stress in adult zebrafish. ASX appears to have another notable benefit: a significant reduction in MPs accumulation within fish organs and tissues. When comparing the fish fed the A500-ASX diet to those fed the A500 diet, there was a significant decrease of approximately 10 times less MPs in the zebrafish intestine and muscle, and even 70 times less MPs in the liver. However, since in this trial ASX was microencapsulated (+Pop technology), the present study was unable to determine whether the reduction in MPs accumulation was attributed to the effects of ASX or the capsule that are retaining it, which may have the capacity to entrap the small MPs once ASX was released.

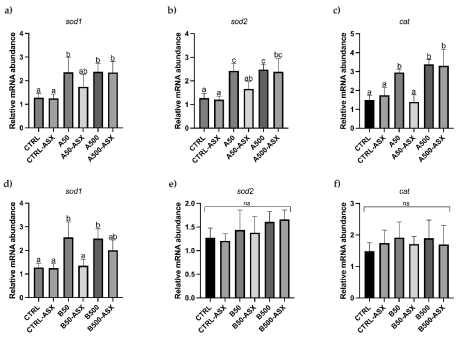

Figure 4. Relative mRNA abundance of genes involved in oxidative stress response (*sod1*, *sod2*, and *cat*) analysed in the intestine of adult zebrafish fed the experimental diets.

Table 3. MPs quantification (number of microbeads/mg) in the intestine, liver, and muscle of adult zebrafish fed experimental diets.

Polymer A (1-5 µm)						
	CTRL	CTRL-ASX	A50	A50-ASX	A500	A500-ASX
Intestine	0	0	0.9 ± 0.3 ^{a}	2.6 ± 1.9 ^{a}	170.9 ± 20.6 ^{b}	20.5 ± 12.0 ^{a}
Liver	0	0	5.5 ± 1.7 ^{a}	5.5 ± 5.1 ^{a}	821.1 ± 295.5 ^{b}	12.2 ± 5.0 ^{a}
Muscle	0	0	2.0 ± 0.2 ^{a}	1.9 ± 0.9 ^{a}	48.0 ± 4.3 ^{b}	5.2 ± 2.8 ^{a}
Polymer B (40-47 µm)						
	CTRL	CTRL-ASX	B50	B50-ASX	B500	B500-ASX
Intestine	0	0	0.6 ± 0.3 ^{a}	0.4 ± 0.2 ^{a}	0.8 ± 0.2 ^{a}	0.6 ± 0.4 ^{a}
Liver	0	0	0	0	0	0
Muscle	0	0	0	0	0	0

Table 4. Histological indexes measured in the intestine of adult zebrafish fed the experimental diets.

Polymer A (1-5 µm)						
	CTRL	CTRL-ASX	A50	A50-ASX	A500	A500-ASX
Mucosal fold height (µm)	127.8 ± 2.9 ^{a}	130.4 ± 4.5 ^{a}	125.2 ± 1.2 ^{a}	126.3 ± 2.3 ^{a}	124.0 ± 4.6 ^{a}	127.5 ± 2.9 ^{a}
Ab+ goblet cells' relative abundance	+	+	++	++	++	++
Polymer B (40-47 µm)						
	CTRL	CTRL-ASX	B50	B50-ASX	B500	B500-ASX
Mucosal fold height (µm)	127.8 ± 2.9 ^{a}	130.4 ± 4.5 ^{a}	91.2 ± 8.7 ^{b}	120.5 ± 24.1 ^{a}	90.9 ± 14.3 ^{b}	128.8 ± 31.4 ^{a}
Ab+ goblet cells' relative abundance	+	+	++	++	+++	++

WP 3. Objective.

The objective is to use the results obtained in WP1 and WP2 in order to set a study performed on European sea bass (*D. labrax*), a species of commercial interest. *Methods and expected results:* same methods as WP2, analyses are under development.

WP 4. Objective.

Project in collaboration with the University of Milan (Italy). Test a rainbow trout (RT) *in vitro* organotypic intestinal platform to evaluate the MPs uptake using polymer A (1-5 µm).

Methods.

Some preliminary tests have been performed on RT proximal and RT distal intestine epithelial cells cultured directly onto plastic surface to: i) exclude any aspecific toxic effect; ii) verify MPs uptake; iii) identify the most suitable MPs concentration (12.5 mg/L^{-1}, 25 mg/ L^{-1}, 50 mg/ L^{-1}). The organotypic intestinal platform was made of RT prossimal and RT distal cells seeded on the upper surface of an Alvetex™ (AV) insert, a highly porous synthetic scaffolding, previously populated with fibroblasts. Platforms were exposed to MPs for 2, 4 and 6 hours, after an effective epithelial barrier was established, as indicated by the transepithelial electrical resistance (TEER) value reaching its plateau. MPs uptake and distribution within the scaffolding were evaluated through histological and confocal (Nikon A1R confocal microscope) analysis.

Obtained Results.

After 24 hours exposure, neither pathological changes nor alteration of cell viability were observed in both cell lines, regardless of the tested concentrations. MPs co-localization with F-actin around the nucleus, demonstrated that MPs internalization followed a dose-response pattern reaching the highest values at 50 mg/L^{-1} concentration. Since this corresponds to an environmental-relevant concentration it has been selected as the exposure dose in the AV platform. After 2 hours of exposure, MPs crossed the epithelial barrier; after 4 and 6 hours, they progressively reached the deeper regions of the scaffolding, being absorbed in both epithelial and stromal cells. Absorption appeared to be more effective in RT proximal intestine cell line than in the distal, suggesting that the latter discourages MPs absorption. This pattern reflects the behaviour of the intestine *in vivo* whose proximal tract ensures 70% of nutrient absorption. The project is still ongoing.

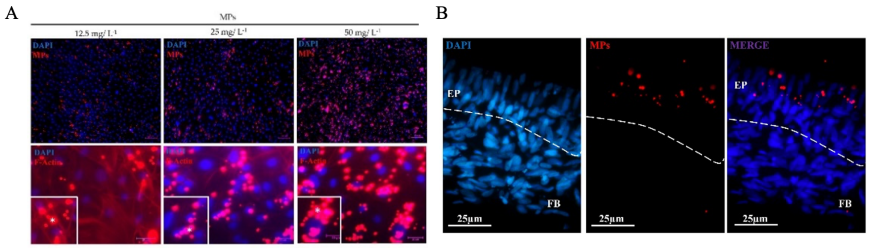

Figure 5. (**A**) F-actin staining showing MPs (red dots) absorption following a dose-dependent pattern in RT epithelial cells. (**B**) representative images of the AV platform showing that MPs cross the epithelial barrier *in vitro* and are absorbed by epithelial (EP, above the dotted line) and connective cells (FB, below the dotted line). Nuclei are counterstained with DAPI-blue signal.

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Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Design of Research (European Projects) – Prof. Paone Nicola

2. Technology Transfer and Innovation – Prof. Iacobucci Donato

3. Lyfe Cycle Assessment (LCA) – PhD Amato Alessia

4. "The resolution revolution in Cryo-electron-microscopy, in Structural Biology and in Life Sciences", Martino Bolognesi, Department of Biosciences, University of Milan - 7.06.22: 11h00, Aula Azzurra

5. Final examination (PhD students cycle XXXIV) - 8.06.22: 10h00, room s2 (BEM)

6. Inshore: second conference Project PSR (InShore) - 23.06.22: 10h-12h00, Aula Magna Agraria

7. Seminary: “Circular economy” (2022)

8. Seminary: “Donne nella pesca” (2022)

9. Shot of Science, speaker: “Marta Lombo” - 30.05.23

10. In vitro models mimicking human tissues and their cross-talk", Monica Mattioli Belmonte Cima, Dipartimento di Scienze Cliniche e Molecolari, Università Politecnica delle Marche - 13.06.23: 11h30-12h30

11. Aula Azzurra: “Facilities at DISVA” - 16.06.23: 11h-13h

12. Final examination (PhD students cycle XXXV) -15.06.23: 9h30-13h30

13. Aula Azzurra: "Mechanism of nano and microplastics capture by jellyfish mucin and its potential as a sustainable water treatment technology", Isam Sabbah, Prof. Ephraim Katzir, Department of Biotechnology Engineering, BRAUDE College of Engineering, Karmiel, Israel - 16.06.23: 10h-11h

14. Latex – Prof. Francesco Spinozzi

15. FTIR and Raman – Prof.sa Elisabetta Giorgini and Prof.ssa Giorgia Gioacchini

List of periods spent abroad

None.

List of conferences/workshops attended and of contributions eventually presented

1. Aquaculture Europe 2022 (EAS – European Aquaculture Society) – 27-30.09.22, Rimini (Italy). *Oral presentation*: Cattaneo, N., Zarantoniello, M., Conti, F., Randazzo, B., Frontini, A., Cardinaletti, G., Gioacchini, G., Olivotto, I. “The fate of dietary microplastics: a multidisciplinary laboratory approach to evaluate localization and physiological responses of zebrafish (*Danio rerio*) larvae”

2. Sealogy 2022 – 16-18.11.22, Ferrara (Italy). *Oral presentation*: Cattaneo, N. “Le problematiche delle microplastiche in acquacoltura e possibili soluzioni”

3. Aquaculture Europe 2023 (EAS – European Aquaculture Society) – 18-21.09.23, Wien (Austria). *Oral presentation*: Cattaneo N., Zarantoniello M., Conti F., Frontini A., Cardinaletti G., Gioacchini G., Olivotto I. “Dietary microplastics exoposure in different life-cycle stages: a study on zebrafish (*Danio rerio*) physiological responses and welfare from larvae to juveniles”

4. Aquaculture Europe 2023 (EAS – European Aquaculture Society) – 18-21.09.23, Wien (Austria). *Poster*: Verdile N., Cattaneo N., Camin F., Zarantoniello M., Conti F., Brevini Tiziana A.L., Olivotto I., Gandolfi F. “Microplastics uptake observed in a cell-based organotypic Rainbow trout (*Oncorhynchus mykiss*) intestinal platform”

Part 3. PhD student information on publications

List of publications on international journals

J1. Cattaneo, N.; Zarantoniello, M.; Conti, F.; Frontini, A.; Chemello, G.; Dimichino, B.; Marongiu, F.; Cardinaletti, G.; Gioacchini, G.; Olivotto, I. “Dietary microplastic administration during zebrafish (*Danio rerio*) development: a comprehensive and comparative study between larval and juvenile stages”. Animals, 13, 2256 (2023) DOI: 10.3390/ANI13142256

J2. In preparation.

List of publications on conference proceedings

List of other publications (books, book chapters, patents)

[11.10.23]

Student signature

Supervisor signature

Prof. Olivotto Ike