# PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

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

**Name of PhD student:** CHRISTIAN GIOMMI

**Title of PhD research:** Protective role of Probiotics in reducing the harmful effects of environmental contaminations.

**Name of PhD supervisor:** Prof.ssa. OLIANA CARNEVALI

**Research lab name:** Laboratory of Development and Reproductive Biology

**Cycle:**
[X] XXXVI
[ ] XXXVII

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

**DISVA instrumentation labs/infrastructure eventually involved in the project:**
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[X] Wet Lab.

**ABSTRACT (1000 characters, including spaces):**
Endocrine Disruptors (EDCs) are known to cause brain, gut, spleen and gonadal alterations as well as embryo development and ossification impairment in zebrafish. Probiotics are able to improve organism welfare and health and to boost both embryo development and ossification. A possible role of probiotics against the toxic effect of EDCs in the different tissues could then be hypothesized. Histology, immunohistochemistry and TUNEL assay were used to investigate physiological alterations caused by EDCs, while qRT-PCR allowed the expression analysis of genes involved in immune system, embryo development and ossification process. Spleen respiratory burst assay and binding/killing test were used to investigate the alterations on immune system. These techniques permit to study the mitigation of SLAB51 on brain, gut and spleen toxicity of Bisphenol A and the effect of *Bacillus subtilis* against the larval development and ossification alterations induced by Perfluorooctanoic acid in zebrafish.

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

**- BACKGROUND**

The plasticizer Bisphenol A (BPA) and the flame retardant Perfluorooctanoic acid (PFOA) are two nowadays Contaminant of Emerging Concerns (CECs) and their capacity to interfere with hormonal axis was deeply investigated, leading to the classification of this chemicals as Endocrine Disruptors (EDCs). Affecting the endocrine system, BPA is able to interfere with numerous biological functions, impairing immune system [1,2] and brain health [3] among others. In addition, PFOA was observed to interfere with thyroid hormone signalling leading to development delay and alteration [4,5]. On the other hand, probiotic administration beneficial role has been demonstrated at different biological levels, comprising immune system [6], brain function and behaviors [7], development [8] and ossification [9,10]. In the first year of my Ph.D., evidence of toxic effects mitigation at reproductive level emerged in zebrafish exposed to BPA using SLAB51 probiotic formulation [11]. In addition, positive roles of SLAb51 formulation on metabolism of fish affected by BPA exposure was described.

**- SCIENTIFIC AIMS**

The leading hypothesis of this study is to use probiotics to mitigate Bisphenol A and PFOA toxicity at different biological levels, focusing on brain, gut and spleen health and immune system in adult zebrafish exposed to BPA for 4 weeks and on larval development and ossification process in zebrafish exposed to PFOA for 3 weeks.

**- WORKPLAN AND RESEARCH ACTIVITIES**

**WP 1. Objective.** Investigation of SLAB51 mitigation capacity against BPA effects on brain and gut health and immune system using adult *Danio rerio* as experimental model.

**Methods**. A total of 80 adult zebrafish (40 male and 40 female) (*D. rerio*, AB wild-type strain) were divided into eight 10-L aquaria (10 fish/tank) with oxygenated water under controlled conditions (28.0 ± 0.5 °C) on a 14/10 h light/dark cycle and fed twice a day commercial food (Vipagran; Sera, Loessnitz, Germany). The experiment was set up in duplicate as follows:

- C: control fish fed twice a day with commercial food
- BPA: fish fed commercial food and exposed to 10 µg/L BPA (98% analytical purity, Sigma-Aldrich, Milano, Italy)
- BPA+P: fish exposed to 10 µg/L BPA and fed commercial food supplemented with SLAB51 at a final concentration of $10^9$ CFU/g
- P: fish fed commercial food and receiving a dietary supplementation of SLAB51 at a final concentration of $10^9$ CFU/g

At the end of the trial, fish were euthanized with 500 mg/L MS-222 (3-aminobenzoic acid ethyl ester, Sigma Aldrich) buffered to pH 7.4. All groups were sampled after 4 weeks of treatment. Histological analyses were performed on 4 µm thick slices produced by 5 brain and gut for both female and male fish fixed in bouin and embedded in paraffin, for each experimental groups, and stained with Hematoxylin and Eosin. Alcian blue staining was also performed on gut samples in order to quantify the presence of goblet cells. Immunohistochemical analysis was performed on brain samples to quantify the presence of TAU aggregates and Glial fibrillary acidic protein (GFAP). Furthermore, on the same samples, TUNEL assay was performed to assess the level of apoptosis cell death. For each experimental group, total RNA was extracted from 5 female and 5 male guts, using RNAeasy® Minikit (Qiagen, Milano, Italy). qRT-PCRs were performed with SYBR green in a CFX thermal cycler (Bio-rad, Milano, Italy). Ribosomal protein 13 (rpl13) and ribosomal protein 0 (rplp0) mRNAs were used as internal standards in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Respiratory burst assay was performed on white blood cells isolated from zebrafish spleen in order to measure the reactive oxygen species (ROS) produced by neutrophiles before and after stimulation with phorbol 12-myristate 13-acetate (PMA; FLUKA Biochemika), which induced its production. In addition, a binding/killing test was performed on the same samples to quantify the number of macrophages out of 100 cells that was able to phagocytise *Saccharomyces cerevisiae* cells which

were incubated together with the sample. One-Way ANOVA followed by Dunnett's multiple comparison test was performed using statistical software package Prism5 (GraphPad Software, Inc. USA) with significance accepted at $P < 0.05$. The exact Fisher's Test was conducted in the R program to analyze ordinal data (histopathology of brain). P-value was corrected using Benjamini & Hochberg procedure and FDR < 0.05 indicates statistically significant differences among groups.

**Expected/Obtained Results**.

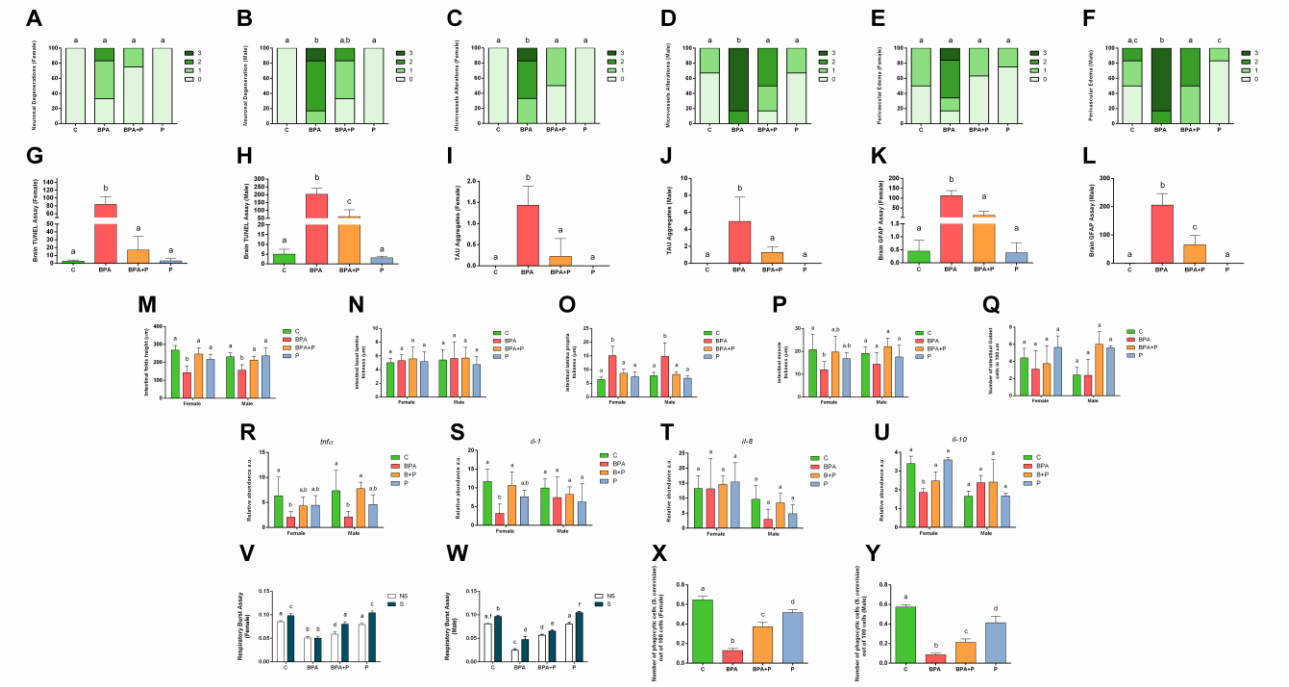

**Figure 1.** Histopathological evaluation of brain samples; A-B) neuronal degeneration, C-D) microvessels alterations and E-F) perivascular edema of female and male zebrafish brain respectively. 0 = no abnormality, 1 = mild abnormality, 2 = moderate abnormality and 3 = severe abnormality. Different letters indicate statistically significant differences (FDR < 0.05) among experimental groups (Exact Fisher's Test corrected using Benjamini & Hochberg procedure). Immunohistochemistry evaluation of brain samples; G-H) TUNEL assay, I-J) TAU aggregates and K-L) GFAP of female and male zebrafish brain respectively. Histological evaluation of gut samples; M) intestinal fold height (µm), N) intestinal basal lamina thickness (µm), O) intestinal lamina propria thickness (µm), P) intestinal muscle thickness (µm) and Q) number of intestinal goblet cells in 100 µm, in female and male fish respectively. Different letters indicate statistically significant differences (n = 5). P < 0.05) among groups (one-way ANOVA followed by Dunnett's multiple comparison test). Gene expression analysis**;** female and male gut mRNA expression values of R) *tnfα*, S) *il-1*, T) *il-8* and U) *il-10*, in the different experimental groups. Different letters indicate statistically significant change among groups (n = 5). P < 0.05 (two-way ANOVA followed by Tukey multiple comparison test) was considered statistically significant. Spleen immune system analysis; V-W) respiratory burst assay of female and male respectively and X-Y) binding/killing test of female and male respectively. Different letters indicate statistically significant differences. P < 0.05) among groups (one-way ANOVA followed by Dunnett's multiple comparison test).

At brain level, male fish seemed more prone to BPA toxicity due to the increase of neuronal degeneration, microvessel alteration and perivascular edema, while female reported only an increase of microvessel alteration. Regarding apoptosis, TUNEL assay indicates an increase of cell death in both male and female fish exposed to BPA. In these same fish, markers of neurodegeneration, TAU protein and GFAP, were also upregulated. In BPA+P fish, the counteracting effects of probiotics on BPA toxicity was clearly demonstrate, and the above-described biomarkers presented levels closer to C ones showing a complete recovery. Concerning gut, histological analysis revealed that BPA induced severe morphological alteration with a reduction of intestinal fold length and an increase of the lamina propria thickness which was not dependent on the sex. A reduction of intestinal muscle thickness was only observed in female fish. At molecular level, BPA induced a reduction of *tnfa* in both sexes and of *il-1β* and *il-10* only in female, suggesting that the contaminant is able to impair the immune system. Again, in BPA+P fish, probiotic reverted BPA effects both at morphological and molecular levels to C level. Considering spleen, respiratory burst assay revealed a reduction of ROS release in BPA group both in female and male, with female not responsive to stimulation with PMA compared to male. SLAB51 was able to mitigate this effect in BPA+P group by increasing this parameter to a level closer to C. Similar results were obtained in the binding/killing test, where BPA reduced the percentage of macrophages who were able to phagocytize *S. cerevisiae* in both sexes and BPA+P group showed an increased percentage compared to BPA ones. These results showed for the first time the presence of a counteracting effect of SLAB51 against BPA toxicity in the brain, gut and spleen, suggesting its possible use in the treatment of pathologies induced by a chronic exposure to environmental BPA levels and possibly other endocrine disruptors.

**WP 2. Objective.** To assess the mitigation ability of *Bacillus subtilis* var. *natto* against the PFOA toxicity at larval development and ossification process in *Danio rerio* larvae.

**Methods**. A total of 3000 zebrafish embryos (*D. rerio*, AB wild-type strain) were divided into twelve 500 mL beker (250 fish/beker), kept at the same condition of WP1 and fed twice a day with live rotifers. The experiment was set up in duplicate as follows:

- C: untreated control group
- P: treated with the probiotic *Bacillus subtilis* var. *natto* at a final concentration of $10^7$ CFU/ml
- 50 PFOA: exposed to a final concentration of 50 mg/L of PFOA
- 50 PFOA+P: exposed to a final concentration of 50 mg/L of PFOA and treated with probiotic at a final concentration of $10^7$ CFU/mL
- 100 PFOA: exposed to a final concentration of 100 mg/L of PFOA
- 100 PFOA+P: exposed to a final concentration of 100 mg/L of PFOA and treated with probiotic at a final concentration of $10^7$ CFU/mL

At the end of the trial, fish were euthanized following the same protocol of WP1. All groups were sampled at 7-, 14- and 21-days post fertilization (dpf). Larvae for morphometric and histological analysis were fixed in 4% Paraformaldehyde (PFA) and stored in 70% ethanol until processed. Morphometric analyses were performed under a stereomicroscope. For cartilages angle quantification, a double stain with Alcian Blue and Alizarin Red was performed overnight followed by a bleaching to remove larvae skin pigmentation and a clearing to remove the staining in excess. A stereomicroscope was used for images acquisition and morphometric and cartilage angles were analysed using ImageJ software. Five pools of 25 larvae per each time point were stored at -80°C for gene expression analysis performed following the same approach used inWP1.

**Expected/Obtained Results**.

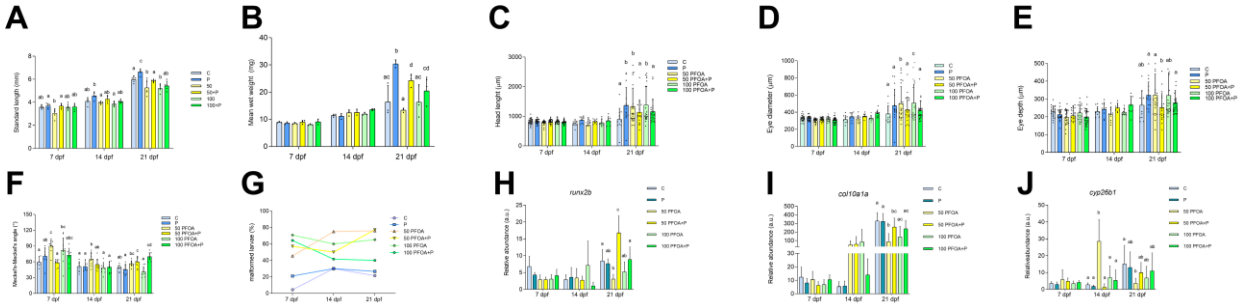


**Figure 2.** Morphometric analysis of zebrafish larvae at 7, 14 and 21 dpf of every group; A) standard length (mm), B) mean wet weight (mg), C) head length (µm), D) eye diameter (µm) and E) eye depth (µm). F) Meckel's-Meckel's (M-M) angle (°) of zebrafish larvae at 7, 14 and 21 dpf of every group after whole mount staining with Alcian Blue and Alizarin Red. G) Percentage of malformed larvae at every endpoint per every group. Gene expression analysis of H) *runx2b*, I) *col10a1a* and J) *cyp26b1*, in the different experimental groups at each endpoint. Different letters indicate statistically significant changes among groups. $P < 0.05$ (two-way ANOVA followed by Tukey multiple comparison test) was considered statistically significant.

Exposure to PFOA at both concentrations increased head length compared to C, and as a result also eye diameter was increased compared to C, while the body length was decreased. An overall alteration of head dimension was induced by PFOA, suggesting craniofacial malformations. The probiotic selected was able to restore these parameters to C level and to increase the body size uniformly. M-M angle was increased at each time point by the lowest dose of PFOA and restored to C level at 7 and 14 dpf, while at 21 dpf this malformation was present also in the co-treatment groups. Levels of total malformations were increased by both doses of PFOA and reverted only in the 100 PFOA+P group. The expression of three key genes involved in ossification were altered by the exposure to the lowest dose of PFOA, showing a non-monotonic dose response to the contaminant, which was able to reduce *runx2b*, involved in osteoblast differentiation, *col10a1a*, expressed by hypertrophic chondrocytes during endochondral ossification, and *cyp26b1*, involved in retinol metabolism and important for jaw morphogenesis, expression. Probiotic was capable to restore the C expression level regarding *runx2b* and *cyp26b1* and to partially reduce the alteration of *col10a1a* expression. For the best of our knowledge, these preliminary results represent the first evidence of probiotic mitigation against PFOA toxicity at larval development and ossification levels.

**- REFERENCES**


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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. Technology transfer and innovation, Prof. Donato Iacobucci. (24/02/2021-13/04/2021)
2. Designing research: European projects, Prof. Nicola Paone. (15/01/2021-29/01/2021)
3. Introduzione all'ambiente LaTeX per la redazione di documenti scientifici, Prof. Spinozzi Francesco. (14/05/2021-28/05/20219

4. Analisi di regressione mediante Microsoft Excel, Prof.ssa. Beolchini Francesca. (17/05/2021-21/05/2021)
5. Theory and application of complex networks, Prof.ssa. Ortore Maria Grazia. (4/10/2021)
6. Microbial-mediated processes in aquatic ecosystem: from basic to applied research toward solving environmental problems, Prof. Dell'Anno Antonio. (29/09/2021-30/09/2021)
7. Contaminant of emerging concerns (CECs): application of bioassays, biomarkers and precision cut tissue slices for studying their biological effects, Prof.ssa. Gorbi Stefania e Prof.ssa. Benedetti Maura. (14/10/2021)
8. Formazione specifica salute e sicurezza sul lavoro – rischio medio, UNIVPM.
9. Passato, presente e futuro dell'osservatore nazionale della pesca del tonno, gli studenti UNIVPM si raccontano (2/12/2020)
10. Progetti di innovazione nella regione Marche per lo sviluppo di una filiera della pesca e dell'acquacoltura più competitiva e sostenibile.
11. Future feeds in acquaculture: from experimental models to farmed fish species, Dr. Zarantoniello Matteo (14/04/2021).
12. Attendance to the seminar "Sfumature rosa in un mondo blue: testimonianze concrete dell'impegno delle donne nella protezione delle risorse marine, nella tutela dell'ambiente, nella valorizzazione della sostenibilità delle attività di pesca e nell'inclusione sociale", Prof.ssa. Giorgia Gioacchini (18/05/2022).

***List of periods spent abroad***

None.

***List of conferences/workshops attended and of contributions eventually presented***

1. 22/06/2021. GEI-SIBSC Symphosium (Virtual Meeting). Presenting a poster entitled: **Use of Probiotic to mitigate the reproductive disorders caused by BPA using Zebrafish as model**.

2. 22/09/2021-24/09/2021. The 3rd Fish Microbiota Workshop (Virtual Meeting).

3. 28/062022-01/07/2022. 14th International Congress on the Biology of Fish (Montpellier, France). Oral communication entitled: **Probiotic administration counteracts Bisphenol A reproductive toxicity in zebrafish.**

4. 04/09/2022-08/09/2022. 30th CECE & 9th ISFE Joint Conference of the European Society for Comparative Endocrinology and of the International Society for Fish Endocrinology (Faro, Portugal). Oral communication entitled: **A multidisciplinary approach to investigate probiotic mitigation against chronic Bisphenol A exposure effects at hepatic and gut levels in *Danio rerio.***

5. 20/09/2022-23/09/2022. 8th International Workshop on the Biology of Fish Gametes (Gdansk, Poland). Oral communication entitled: **Insights on reproductive gender specific toxicity induced by Glyphosate chronic exposure in *Danio rerio* adults.**

**Part 3. PhD student information on publications**

*If not yet published, please indicate the publication status (submitted, accepted, in preparation...)*

***List of publications on international journals***

J1. Giommi C, Habibi HR, Candelma M, Carnevali O and Maradonna F. Probiotic Administration Mitigates Bisphenol A Reproductive Toxicity in Zebrafish. Int J Mol Sci. 2021 Aug 27;22(17):9314. doi: 10.3390/ijms22179314.

J2. Giommi C, Ladisa C, Carnevali O, Maradonna F and Habibi HR. Metabolomic and Transcript Analysis Revealed a Sex-Specific Effect of Glyphosate in Zebrafish Liver. Int J Mol Sci. 2022 Mar 1;23(5):2724. doi: 10.3390/ijms23052724.

J3. Maradonna F, Fontana CM, Sella F, Giommi C, Facchinello N, Rampazzo C, Caichiolo M, Hoseinifar S, Dalla Valle L, Van Doan H and Carnevali O. A zebrafish HCT116 xenograft model to predict Anandamide Outcomes on Colorectal Cancer. (Under Review-minor revision, in Cell Death & Disease).

J4. Lombò Alonso M*, Giommi C*, Paolucci M, Notarstefano V, Montik N, Delli Carpini G, Ciavattini A, Ragusa A, Maradonna F, Giorgini E and Carnevali O. Preeclampsia disrupts the endocannabinoid system leading to macromolecular alterations in term placenta. * These Authors contributed equally. (Under review-minor revision, in International Journal of Molecular Sciences).

J5. Giommi C, Rossi G, Mangiaterra S, Ladisa C, Maradonna F, Habibi HR and Carnevali O. Evidence on the ability of probiotic to counteract Bisphenol A toxicity in female and male Danio rerio liver: a multidisciplinary study. (In preparation).

***List of publications on conference proceedings***

None.

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

B1. Endocrine-disrupting chemicals mediated health impairment in laboratory model organisms. **EDCs: Focus on metabolic alteration of mammalian and non-mammalian models.** Giommi C, Carnevali O and Habibi HR. (Book Chapter Submitted to Elsevier).

*13/10/2022*

Student signature

Supervisor signature