# PathoGelTrap

## New Blue Revolution through a pioneering pathogen-trapping technology based on bioselective hydrogel-forming proteins

H2020 – FET OPEN - Challenging Current Thinking

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## Deliverable No. 4.9

## Fish welfare assessment 3

PathoGelTrap™
Sustainable Prevention in Aquaculture

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# Executive Summary

Within the objectives of WP4 of the PathoGelTrap project, task 4.9 assesses the fish welfare during *in vivo* experiments. Due to restrictions in PGT liquid mass production, IZSVE revised the experimental set up in order to scale down the needs to perform animal experiments. IZSVe performed a citotoxicological test on GenScript chimera production. Moreover, the ecotoxicological impact of PGT liquid has been investigated through EC50 test on *Vibrio fisheri* and *Daphnia magna*.

Results from toxicological tests showed that PGT Liquid is safe at cellular level, as well as for Vibrio, while tests with Daphnia resulted in an EC50 of 36.41% of the working concentration.

IZSVE successfully downscaled the experimental set up for the *in vivo* trial, with the consequent reduction in the amount of PGT Liquid needed.

## List of acronyms/abbreviations

AWB= Animal Welfare Body
DL= Deliverable
EC= European Commission
EU= European Union
IHC= Immunohistochemistry
IZSVE= Istituto Zooprofilattico Sperimentale Delle Venezie
LLPS = Liquid-Liquid Phase Separation proteins
PGT= PathoGelTrap
Real Time PCR= Real-Time Polymerase Chain Reaction
SW: Smartwater Planet S.L.
VNNv= Viral Nervous Necrosis virus
VLP= Virus Like Particle
WP= Work-Package
YR= Yersinia ruckeri

## Glossary of terms

| Term | Explanation |
|---|---|
| *Betanodavirus* | Viral agent causative of Viral Nervous Necrosis, also known as viral encephalopathy and retinopathy, one of the target infective agents of the PGT project |
| *Yersinia ruckeri* | Bacterial agent causing Enteric Redmouth disease, one of the target infective agents of the PGT project |
| Pathogen | Organism able to cause disease |
| Antigen | Any substance that causes the immune system to produce antibodies against itself. In terms of pathogens, antigens are a protein (or part of it) exposed on its surface and capable of being recognized. |
| Liquid-liquid phase separation (LLPS) | Certain molecules (such as proteins) are rearranged into a dense phase that coexists with a dilute phase reminding liquid droplets. |
| Real Time PCR | A real-time polymerase chain reaction (real-time PCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR |
| Animal Welfare Body | The Animal Welfare Body serves as an interface with researchers, from whom it receives study projects involving the use of animals. The Animal Welfare Body liaises with the Ethics Committee of IZSVe to evaluate these projects and provide the opinions needed to apply for authorization from |

| | |
|---|---|
| | the Ministry of Health. The Body has the task of interfacing with the Ministry and then with the researchers in relation to project approval. |
| Chimera | In the context of this project, fusion protein construct formed by the candidate LCR and the affibody or the affibody-like molecule |
| EPC | Epithelioma Papulosum Cyprini cell line |
| E-11 | E11 is a clone of the cell line SSN-1 (Striped Snakehead Fry cell line) |
| VERO | African green monkey kidney cell line |
| RAW | Mouse monocyte macrophage cell line |
| MTT cell proliferation assay | The MTT assay protocol is based on the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product. Viable cells with active metabolism convert MTT into formazan. Dead cells lose this ability and therefore show no signal. |
| EC50 | Half maximal effective concentration (EC50) is a measure of the concentration of a drug, antibody or toxicant which induces a biological response halfway between the baseline and maximum after a specified exposure time. |
| Buffer B | The buffer for Chimera DPB1-P7.C3 and C4 suspension in filtered tap water containing 50 mM phosphate pH 7.4, 500 mM NaCl, 500mM Imidazole, 0.5M GdnHCl, + 50mM L-arginine and 50mM L- glutamic acid |
| Buffer C | The buffer for Chimera DPB1-P7.C3 and C4 suspension in filtered tap water containing 50mM L-arginine and 50mM L-glutamic |

# 1. Introduction

During the last decades, fish welfare has received increased attention from the public, and therefore in the world of research, industry and from governments. In general, stress could be defined as the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state (Barton 1997). In reference to fish species, "stress" means the condition in which the dynamic equilibrium of an organism, called homeostasis, is threatened or disturbed by the action of internal or external stimuli, commonly referred to as stressful (Wendelaar Bonga 1997). Measures of physiological stress response naturally feature prominently in studies of welfare. However, stress response is an adaptive function in the face of a perceived threat to homeostasis and, as suggested above, stress physiology does not necessarily equate to suffering and diminished welfare. In the short term, stress responses serve a very important function to preserve the individual. Welfare measures in aquaculture are, therefore, largely associated with tertiary effects of stress response that are generally indicative of prolonged, repeated or unavoidable stress (Huntingford et al. 2006; Iwama 2007). There is no single measure of welfare and although a wide range of physiological, biochemical and behavioural measures are used to assess welfare, none of these are considered reliable in isolation and multiple measures need to be taken. Indicators associated with chronic stress response provide a potential source of information concerning the welfare of the fish and are important because they allow the development of protocols that reduce stress. Behavioural and physiological measures are intrinsically linked and are dependent on one another for correct interpretation with regard to welfare. The most realistic assessment of welfare, however, is obtained through a series of information measures combined together through an appropriate statistical approach (Broom 1998).

Considering the potential fish welfare issues identified and the complexity of the measurement techniques, the objective of this deliverable is to assess the fish welfare during the in vivo trials to be performed in DL 2.6 in order to evaluate PathoGelTrap safety to fish.

# 2. Requirements for fish welfare assessment

## 2.1. Animal experiment authorization

IZSVE own an experimental aquarium authorized by the Italian Ministry of Health (Auth. Min. n. 19/2019-UT of 24/6/2019), with the permission to work with pathogens (BSL-2). Besides this authorization, the Italian Ministry requires that every experimental trial has to be authorized singularly before being performed (art. 31 of LD 2014/26). That means that for any in vivo trial, we need to complete several documents regarding the experimental set up, the measures implemented to cope with the 3R principles (Reduce, Replace and Refine) and the statistical analyses performed to support the results.

For the *in vivo* trials to be performed in PGT project (DL 2.6 and DL 3.5), IZSVE draft the Annex 6 (Art. 31 of LD 2014/26) in order to get the Authorization from the Italian Ministry of Health. On February 2022 the IMH approved the request (139/2022-PR).

Considering the results obtained by *in vitro* testing of the PGT liquid candidates (see D2.5) and filter (see D3.4), the Consortium decided to proceed initially with the *in vivo* trial only for one pathogen (Betanodavirus), one species (European sea bass) and one formulation (PGT liquid).

In order to reduce the amount of PGT liquid to be mass produced by GenScript (see D2.5), IZSVe revised the experimental set up of the *in vivo* trial, scaling down both the size and the number of the tanks and of the fish (see D2.6 for details).

## 2.2. Citotoxicity assay

Cytotoxicity results from the interference with structures or properties that are essential for cell survival, proliferation, or function. Cytotoxicity assays were among the first *in vitro* bioassay methods used to predict toxicity of substances to various tissues. *In vitro* cytotoxicity testing provides a crucial means for safety assessment and screening, and for ranking compounds.

Citotoxicity assays were performed to test 2 batches of PGT liquid from CSIC (Chimera DPB1-P7.C3 and Chimera DPB1-P7.C4) and their Buffer (Buffer B and Buffer C) and the 3 batches of PGT liquid produced by GenScript (Batch A, B and C; for details see D2.5).

Citotoxicity assays were performed on E-11 cell line at working concentration (1 µM).

Results showed that:

- Buffer B was extremely toxic for cell lines, showing a complete cell disruption
- Buffer C was not toxic for cell lines, and thus it was chosen as the definitive buffer also for mass production
- Chimeras from CSIC (in Buffer C) showed no toxicity on cell cultures, as well as the 3 batches produced by GenScript.

Based on these results, PGT Liquid produced by GenScript was considered suitable for the application in the *in vivo* trial, since there was no toxic effect on cell line, which are considered the first standard test to evaluate the toxicity of a solution.

## 2.3. *In vivo* toxicity

Despite the results from cell cultures, during the *in vivo* trial we observed deleterious effects in fish in the tank treated with PGT liquid (see D2.6 for experiment details).

At necropsy, compromised fish showed the typical clinical signs of death for anoxia, with wide open opercula and mouth. Checking the oxygen levels in the tank, the values were perfectly at physiological values (see D4.10). At optic microscope examination of fresh gills slide, gills appeared anaemic, clogged by protein aggregates (fig. 1). Immunohistochemistry (IHC) using antibody against histidine (HIS) highlighted that the protein aggregates in the gills filament where PGT liquid aggregates (fig. 2).

With these evidences, we can state that the death of the fish was caused by an acute hypoxia due to the impossibility for fish to breath correctly through the gills because of the clogging occurred by Chimera stuck to the filaments.

To solve this issue, IZSVE performed several tests using molecular sieves in order to remove PGT liquid from water (see D4.10 for details). A series of strategies are being implemented.

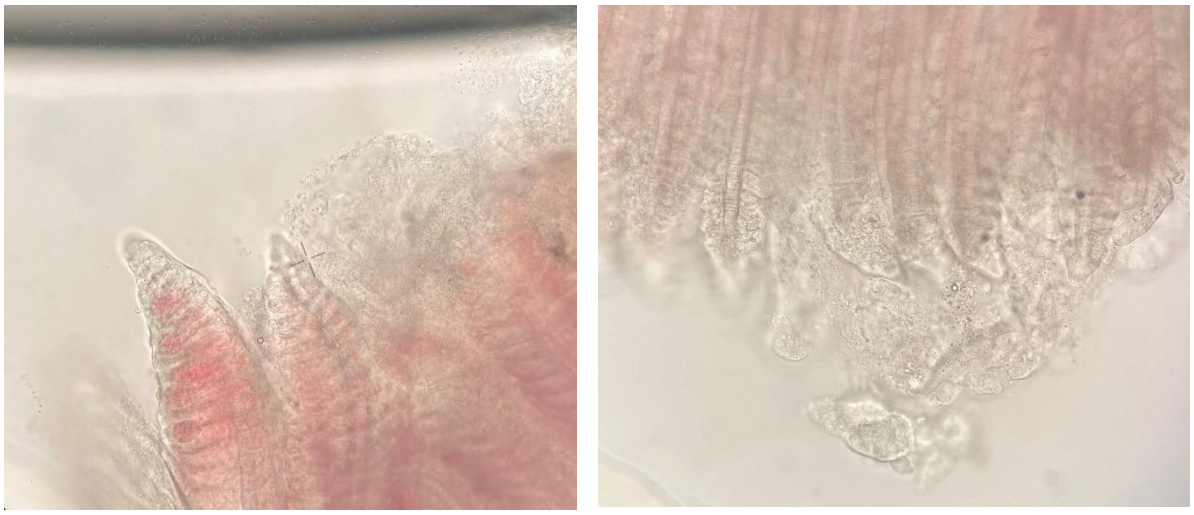

**Figure 1. Fresh slide from gills**. Gills lamellae appears anaemic and clogged by protein aggregates

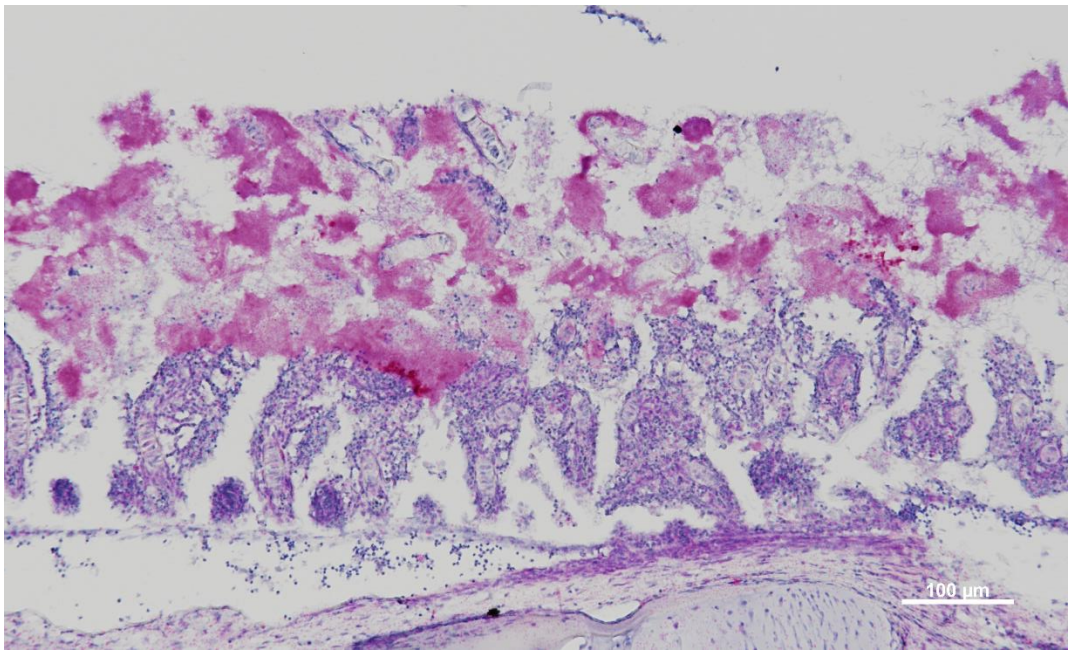


**Figure 2. Immunohistochemistry of gills with anti-HIS**. Chimera aggregates (in reddish color) are stuck onto gills lamellae

## 2.4. Welfare indicators

Due to the remodelling of the *in vivo* trial set up, the fish size needed was about 1 g (weight), making it impossible to carry out the selected tests for the evaluation of welfare parameters. Basing on that, the Consortium agreed to not perform biochemical markers assays.

The water quality parameters, used also as welfare indicators, are reported in D4.10.

**Table 1. Water parameters during in vivo trial.**

| Tank | pH | O2 mg/l | NH4+ mg/l | NO2- mg/l | NO3- mg/l | Salinity g/l | T °C |
|---|---|---|---|---|---|---|---|
| 1 | 8.5 | 8.20 | <0.1 | 0.3 | 3 | 25 | 25.5 |
| 2 | 8.4 | 8.43 | <0.1 | 0.2 | 5 | 25 | 25.2 |
| 3 | 8.5 | 8.54 | <0.1 | 0.2 | 3 | 25 | 24.8 |
| 4 | 8.5 | 8.23 | <0.1 | 0.1 | 2 | 25 | 25.6 |

## 2.5. Environmental toxicity

To investigate the environmental impact of the PGT liquid, IZSVE has entrusted the execution of ecotoxicity tests (EC50) on *Vibrio fisheri* and *Daphnia magna* to an accredited external laboratory (ARPAV).

Three samples were sent to ARPAV laboratory as follows (analysed following UNI EN ISO 11348-3:2019 and UNI EN ISO 6341:2013):

1) Chimera DBP1.P7-C3, concentrated at 1 µM (35 mg/l), for testing with *Vibrio fisheri*
2) Chimera mHP1α-R2 at 1 µM (33 mg/l), for testing with *Vibrio fisheri*
3) Chimera DBP1.P7-C3, concentrated at 1 µM (35 mg/l), for testing with *Daphnia magna*

Results from those assays showed that both Chimera DBP1.P7-C3 and Chimera mHP1α-R2 were not toxic for bacteria (EC50 > 90% and > 81.90%, respectively), but Chimera DBP1.P7-C3 was toxic for *Daphnia magna* at certain concentration (EC50 >36.41 %).

# 3. Overall monitoring and evaluation of results

- IZSVE scaled down the *in vivo* trial set up to meet the needs for mass production of PGT liquid. This meant also reducing the number of fish needed for the trial to combine the 3R regulation to the scope of the experiment.
- IZSVE performed cytotoxicity assays to determine the best buffer for PGT liquid production and tested the safety of the chimera mass production by GenScript, to be used in the *in vivo* trial.
- IZSVE performed ecotoxicological tests on PGT liquid by outsourcing. The results showed an acceptable safety of the solution when tested with Vibrio, but a certain toxic effect on Daphnia.
- Results from *in vivo* trials showed that PGT liquid in its current configuration, if it comes into contact with fish, may have a clogging effect on fish's gills, making breathing difficult.
- Molecular sieves and other strategies need to be further implemented to address this issue.

# 4. Conclusion

IZSVE got the authorization by the Italian Ministry of Health on February 2022 to perform animal experiments. Buffer C was evaluated as non toxic in cell culture and suggested as suitable buffer for PGT liquid mass production. PGT liquid from GenScript has been tested for safety on cell culture prior to the *in vivo* experiment.

To assess environmental risk, ecotoxicological tests were performed against *Vibrio fisheri* and *Daphnia magna*. The evidences from the *in vivo* trial supports the results that showed no toxicity of PGT liquid (cell culture and *V. fisheri* tests): the effect seen on fish and *Daphnia* is plausibly not due to a chemical toxicity but rather a bio-mechanical interference with gills, preventing breathing (also Daphnia has gills).Molecular sieves and other strategies are being implemented to address this issue.

# 5. References


- Barton B.A. (1997). Stress in sunfish: past, present and future – a historical perspective. In Fish Stress and Health in Aquaculture, Society for Experiment Biology, Seminar Series 62 (Iwama G.K., Pickering A.D., Sumper J.P., Schreck C.B., eds), pp. 1–31. Cambridge University Press.
- Broom D.M. (1998). Welfare, stress and the evolution of feelings. Advanc Study Behav 27, 371–403.
- Huntingford F.A., Adams C., Braithwaite V.A., Kadri S., Pottinger T.G., Sandøe P., Turnbull J.F. (2006). Current issues in fish welfare. J Fish Biol 68, 332-372.
- Iwama G.K. (2007). The welfare of fish. Dis Aquat Org 75, 155-158.
- Wendelaar Bonga S.E. (1997). The stress response in fish. Physiol Rev 77, 591-625.