PathoGelTrap

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

H2020 – FET OPEN - Challenging Current Thinking

Deliverable No. 4.2

WATER ASSESMENT

This project has received funding from the
European Union's Horizon 2020 Research and Innovation Programme
Under Grant Agreement no. 899616

Document Control

Deliverable	4.2
WP/Task Related	4
Delivery Date	06/05/21
Dissemination Level	PU
Lead Consortium member	SW
Contributors	ENRIQUE AMARÉ, ALEJANDRO GRANADO, GONZALO PÉREZ, CARLOS MAZORRA (SW)
Reviewers	ENDA CUMMINS (UCD)
Key Words	WATER QUALITY, SENSORS, SENSOR PLATFORM, MEDUSA

Revision History

Version	**Date**	**Notes**
V 1.0	06/05/2021	
V 1.1	28/05/2021	

Table of Contents

Executive Summary

Within the objectives of WP4 of the PathoGelTrap project, task 4.2 assesses the water and aquaculture management operational parameters in which the PGT products are going to work. SmartWater (**SW**) is developing a hardware device to measure over 85 water parameters such as pH, temperature, dissolved oxygen, conductivity, Oxidation Reduction Potential (ORP), turbidity and ions such as Na^+, K^+ y Cl^-, NO3- or NH4+. **SW** has constructed one device which will be used to control these parameters in IZSVE facilities during the *in vivo* experiments, providing relevant info to guarantee animal welfare, prevent diseases and avoid economic and production losses.

Relevant water quality conditions in fresh and marine waters aquaculture operations have been revised and presented to the scientific teams to inform the environmental water quality conditions of operation for the PGT products.

List of acronyms/abbreviations

AFB: Afibody
DL: Deliverable
IZSVE: Istituto Zooprofilattico Sperimentale Delle Venezie
LCR: Low complexity region
ORP: Oxidation Reduction Potential
PGT: PathoGelTrap project
RAS: recirculating aquaculture systems
SW: Smartwater Planet S.L.
UCD: University College Dublin
WP: Work package
Ppt: parts per thousand (used in salinity)

Glossary of terms

Term	Explanation
Pathogen	Organism able to cause disease
Water quality operation conditions	Water quality conditions typically found in aquaculture operations
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 Red-mouth disease, one of the target infective agents of the PGT project
Affibody	Small (6.5-kDa) engineered binding proteins based on a three-helix bundle motif of the Z domain derived from staphylococcal protein A used as a scaffold for sequence variation.
PGT products	The products that the project is designing to present the PGT technology, as a liquid form and as a filter form.

1. Introduction

PathoGelTrap envisioned technology proposes a new fish health management model within aquaculture that allows prevention and control of infectious diseases by selectively blocking the pathogens directly into the water, surpassing the current technological paradigm that focuses on disease prevention through the direct action over the fish by vaccines or broad-spectrum antibiotics, which alters the environmental microbiota. Besides, this technology opens the door for a disrupting way for future pathogenic disease control.

Water quality monitoring is essential to ensure, on the one hand, that the design of the PGT components (LCR_AFB) considers the working water quality environment that the PGT products will have to work in.

On the other hand, online, real-time water quality monitoring during *in vivo* experiments, will always ensure that fish welfare conditions are maintained and that water quality parameters during operation are recorded for analysis.

2. Water quality conditions

2.1. Target Diseases and water conditions

2.1.1. Betanodavirus

Betanodavirus is an RNA virus causing viral nervous necrosis (VNN) and viral encephalopathy and retinopathy (VER). The disease produces behavioural neurological signs (erratic spiral swimming, turns and changes in buoyancy), encephalitis and neuronal necrosis in the brain and eye. It especially affects larvae and young fish (acute conditions), with mortality being highly variable (from 0.5% to 100%). In older fish it can take chronic forms. Outbreaks in on-growing situations in sea bass can produce typical mortalities between 5 and 35%. Temperature is a very important factor in the occurrence of disease outbreaks and its dynamics (25°C seems to be a critical temperature). Subclinical infection with mild symptoms of anorexia and lethargy, which are very difficult to distinguish from other simple pathological or zootechnical problems, is also suspected.

In terms of its distribution, in 1987 the disease was described in barramundi in Australia and Malaysia. In 1988 it was detected in sea bass in Martinique. In 1991 it was detected in Norway in turbot, and in the same year it was also reported in sea bass in France. In 1995 it was described in the Mediterranean in sea bass in Greece and Italy. It is now distributed worldwide and cases have been detected and described on both coasts of the USA, northern Atlantic Europe and the Mediterranean, the Middle East, and Asia from India to Japan, and the south-east coast of Australia. It is also suspected on all other coasts, including the west coast of Africa, except for the extreme ends of the coast near the North and South Polar Circles.

The RNA virus appears in a number of different genetic variations (see Table 1). Isolates in Europe usually correspond to the RGNNV type and occasionally to the BFNNNV type. However, a publication from the University of Santiago de Compostela (2007) assigned 31 samples from the Iberian Peninsula to the SJNNV type in sole (8 samples), sea bream (18 samples) and sea bass (5 samples).

Table 1: Genetic forms of Betanodavirus RNA.

Genotipe	Serotipe	Main host	Optimum temperature (in vitro)
SJNNV	A	Stripped Jack, senegalese sole*, Sea-bass	20-25ºC
RGNNV	B	Red- spotted Grouper, Sea-bass	25-30ºC
BFNNV	C	Barfin Flounder, Halibut, Cod, Plaice or Japaneese flounder, 1 case in Sea-bass at low temperature	15-20ºC
TPNNV	C	Tiger Puffer	20ºC

It is also very likely that recombination between the different viruses occur and that specificity is not strict. It is also likely that the virus adapts to host maintenance conditions in certain environments. The disease has also been described in sea bass, yellowtail, turbot, sole (solea and senegalensis), common bream (puntazzo) and eel among others (up to more than 30 species).

Other species are considered to be vectors of infection, such as mullet, bream and snapper. It has also been detected in mussels (sometimes used in broodstock feed) and can survive in arthropods, including Artemia.

Although there are still many gaps in the epidemiology of the disease, horizontal transmission has been clearly demonstrated:

- Cohabitation with sick or infected fish (including wild fish), via water.

- Entry of new sick or carrier animals into the unit.

- Via containers of carrier or infected animals, including transport tanks or water changes.

- Via feeding (including cannibalism)

The relationship between the presence of the virus in the individual, the expression of the disease and its potential as a transmitter is complex and dependent on the individual's state of immunological competence. The incubation period found in larvae of different species is between 4 and 30 days, however temperature, immune system, genetics and even acquired immunity will influence the development of the disease. The virus can survive stable in seawater for more than a year.

In sea bass, horizontal transmission in a cage can evolve in about 35 days, with temperatures above 25°C favouring the outcome of the outbreak, and with mortality between 10% and 80%.

2.1.2. *Yersinia ruckeri*

Yersinia ruckeri is a Gram-negative, rod-shaped enterobacterium, and the agent causing Enteric Red-mouth disease (ERM) in trout and other salmonids. ERM is an acute or chronic bacterial infection in freshwater fish, but also in marine environments. The mortality rates of ERM are usually low in the initial phase of the disease and then increase rapidly, resulting in severe fish losses. This is especially true when the fish are exposed to stress, for example caused by poor water quality conditions. It causes haemorrhages on the body surface, reddening at the base on the fins, along the lateral line and on the head. Internally there can also be petechial haemorrhages on the liver, visceral fat and pyloric caeca. Other clinical signs include exophthalmia, darkening of the skin, splenomegaly and inflammation of the lower intestine with accumulation of thick yellow fluid.

ERM was first reported associated to losses in hatchery reared rainbow trout in the 1950´s in the USA. Since then, the appearance of *Yersinia* has been widely reported throughout the world, in Canada, Europe, South America, the Middle East, China, India and Australia. It has been also isolated from birds, reptiles and mammals, including humans.

Y. ruckeri can be transmitted by contact between infected and non-infected fish. The bacteria can then be released when the carrier fish become stressed, for example by high temperature or poor rearing conditions. The bacteria can survive at least 4 months outside the host, as expelled, for instance, by the faeces. Gills are an important route of entry of the bacteria.

There have been reports of survival of this bacteria in freshwater and full-strength seawater. ERM has also been described in seawater-adapted salmonids. Y. ruckeri has an optimal growth temperature of 28°C but outbreaks of disease occur at temperatures around 18°C

2.2. Water quality in operational conditions

PGT products will need to operate in the environmental conditions of normal rearing waters in aquaculture, both marine and freshwater. SmartWater has applied its experience within the aquaculture sector and surveyed the common operational conditions of water quality in diverse production environments, both in open water operations, flow-through tanks and in recirculating aquaculture systems (RAS), for the target species and microbes. Also it is important that the water quality parameters and the ranges considered are not only those conducive to pathology outbursts, but indeed those of normal, non-stressed conditions. This is consistent with the use of the technology as a preventive measure in normal situations.

2.2.1. Seabass rearing conditions

In the wild, adult European seabass (*Dicentrarchus labrax*) live in coastal waters down to about 100 m depth. They are euryhaline and eurythermal and are found in the littoral zone on various kinds of bottoms in estuaries, lagoons and occasionally rivers. This means that they can be exposed and be very tolerant to different conditions in terms of temperature and salinity. Salinity can vary from 30-32 ppt in spawning grounds, to 25-30 ppt along the coastline, and even brackish or fresh water in hunting grounds. Temperature wise, conditions can also cover a wide range, from 4-5 ºC to over 25 ºC.

Seabass is farmed mainly in what is called in the Industry the “Mediterranean area”, which in reality goes from Madeira in the West to Israel in the East, but also in the Black Sea and some RAS operations in, for instance, the UK or Germany.

Rearing conditions of the initial stages of hatcheries, from broodstock and eggs to nursery fry, are carried out in tanks inland, in flow-through or RAS units. In this situation, some control over the water quality parameters is possible, and these are adapted to as close as possible to the optimal conditions for the species and the developmental stage.

In general, full strength salinity (30-32 ppt) is used when possible, although in some cases farms are situated in estuaries that present changing salinity conditions. In any case salinity stays above 25 ppt most of the time. Temperatures are controlled and adjusted to the needs of the rearing phase. Broodstock fish are kept under regulated thermoperiods along the year to control egg production while maintaining adequate ingestion of food and fish welfare, from 5-6ºC to 20-21ºC simulating natural thermoperiods. Eggs and larvae are kept initially in temperatures around 15ºC and slowly brought up to temperatures around 20ºC when they become fry. Nursery temperatures can be kept around the twenties, but can rise as high as 25ºC or more in the summer. This can affect the risk of disease outbreaks.

On-growing of seabass can be carried out in RAS but is most commonly done in coastal waters within enclosed pens or cages. Here fish are exposed to natural temperature and salinity conditions that fluctuate naturally. High temperatures in the summer or low temperatures in the winter can cause different disease outbreaks, and Betanodavirus outbreaks are produced typically in high temperature situations during the summer.

2.2.2. Trout rearing conditions

Most wild trout species live in fresh water in North America, North of Asia and Europe, but some species have been introduced to Australia and New Zeeland. They live in different environments like lakes, rivers, streams and even the sea. They have low tolerance for pollution and they are indicative of clear, un-contaminated waters. Many species are anadromous, meaning that in the wild they migrate from the sea to fresh water bodies to reproduce and spawn.

Rainbow trout (*Oncorhynchus mykiss*) is by far the most common cultured species. This species can live in fresh, brackish and full salinity waters. It can survive in a wide range of temperatures (from 0 to 27°C), but normal spawning and growth takes place in a narrower range (from 9 to 14°C). Optimal temperatures are between 10 and 15 °C and temperatures higher tan 25 °C can be lethal.

Also pH is a sensitive parameter. According to developmental stage, trout can handle different pH ranges, which reflect the migrating behaviour between fresh and salty waters.

Rainbow trout is farmed throughout the world as an intensive monoculture. There is some grow-out carried out in cages in lakes or even in brackish or marine waters, and there are also some RAS operations. However, trout is reared to commercial size mainly in earth or concrete tanks, in flow through systems, with very little control over temperature and other water quality parameters (but dissolved oxygen content).

Water sources are normally river waters (or lakes) that are conducted to flow through the rearing units. Frequently, well-waters are available, with more stable quality conditions (temperature and pH), and are used in combination with river waters.

As with the seabass, conditions in commercial operations are partially controlled to try to provide the best productivity, in terms of survival and growth as much as of fish welfare.

Rearing conditions for eggs and larvae are best within a temperature of 8 to 11 °C and a pH between 6,5 and 8. As the fish grow they can tolerate wider ranges of pH (5,5 to 9,5) and temperature (7 to 18 °C), with optimal feeding occurring at temperatures between 13 and 15 °C. At pH below 5,0 trout´s capacity to regulate the concentration of CL^- and NA^+ in plasma is severely compromised.

2.2.3. PGT Operational conditions

Considering the water conditions described above, both for the farming practices of each target species and for the epidemiology of the diseases considered, a range of relevant water quality parameters in which the PGT products had to be operative had to be defined. Temperature, pH and Salinity have been considered the most relevant parameters.

Temperature conditions are of special importance for the development of infection and the dynamics of the infectious outbreak. Given that the water treatment with PGT aims at preventing the outbreak of disease, the operational range of temperatures of PGT should be ample enough to be effective at the regular rearing temperatures outside those considered optimal for the disease agents.

The suggested physiochemical characteristics of both salt- and freshwater to be used during PGT product design and experiments are showed in Table 2. This conditions are consistent with the ones found in most culture operations of both species.

Table 2: Water physiochemical conditions for operation of PGT.

	Temperature	pH	Salinity (Conductivity)
Fresh: Trout	10-20 °C	6-9	0.005 – 0.05 S/m
Marine: Seabass	15-28 °C	6-9	25-37‰

The definition of what is considered “fresh-water” in terms of Cl content has been assessed through the bibliography available to inform by request of the scientific teams of “normal” parameters (see Table 3).

Table 3: Comparison of river water and sea water composition collated from published literature.

Ions	Average River water (mM/l)	Average sea water (mM/l)	River water ratio to Cl	Sea water ratio to Cl
Hco_{3}^{-}	0.86	2.38	5.375	0.0044
So_{4}^{-}	0.069	28.2	0.43125	0.0517
Cl^{-}	0.16	545	1	1
Ca^{2+}	0.33	10.2	20.625	0.0187
Mg^{2+}	0.15	53.2	0.9375	0.09761
Na^{+}	0.23	468	14.375	0.8587
K^{+}	0.03	10.2	0.1875	0.0187

All these characteristics are also used to find appropriate LCR candidates in DL 2.1 and WP4 for experimental trials.

The project has collected three representative types of water samples:

- One fresh water sample from a commercial trout farm in Central Spain
- One seawater sample from a commercial seabass hatchery I Northern Spain
- One saltwater sample from the facilities of IZSE where the in vitro and in vivo tests will take place

These samples have been divided in aliquots at CSIC and used for the manipulation of PGT products.

2.2. Sensors Platform

Water quality monitoring is a sensitive part of the validation trials to be carried out during the project. This is relevant both from the point of view of maintaining design operational parameters and from the fish welfare perspective.

SmartWater has developed an advanced sensor Platform called Medusa, a rechargeable plug&play multifunctional IoT device continuously measuring water quality, both in marine and freshwater.

Medusa is a small (approx.30cm diameter) floating platform that can be deployed in a pond or tank, collecting real time water quality data, and sending them to the data serves for Data management. There are options for 85 different parameters. The data transmission from the sensors is done via 4G and thanks to Cloud technology it is stored and sent to the desired device. Through an app, an alarm system can be configured and any change in any of the measured parameters can be received on any mobile device. Figure 1 shows a schematic vision of how the Medusa sensors platform collects, manages and makes available the information on water quality parameters.

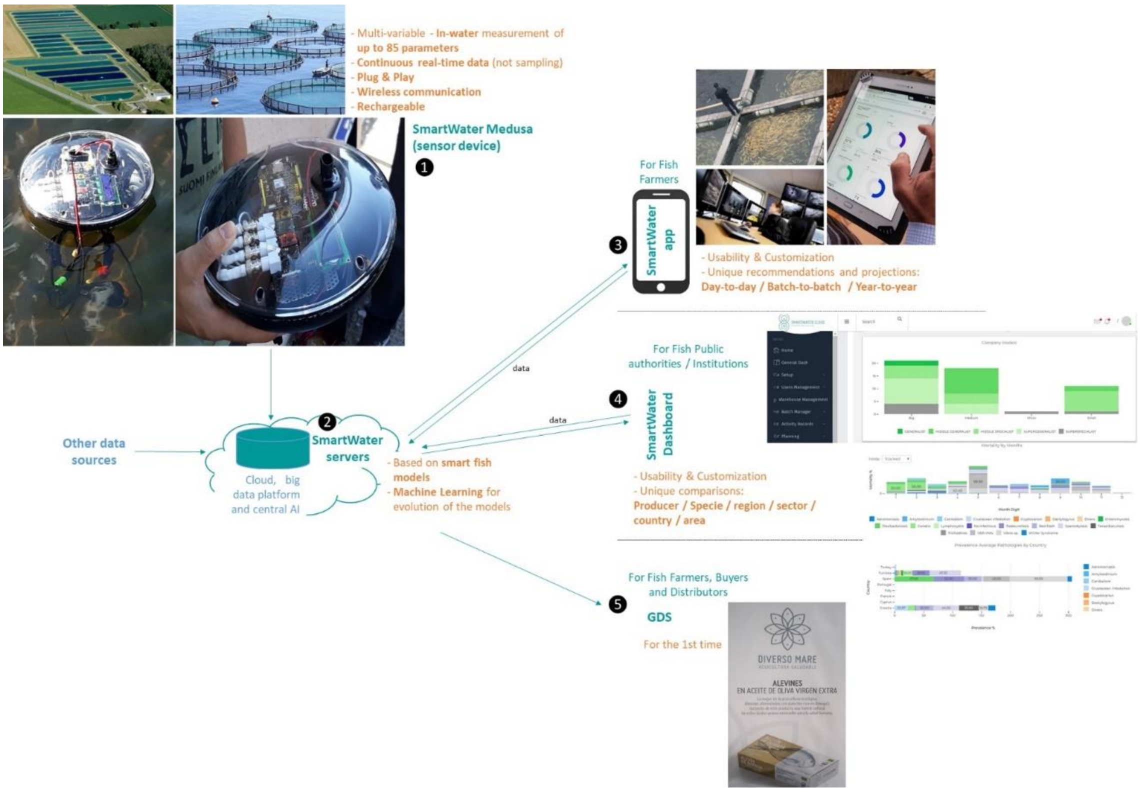

Figure 1: Medusa water quality parameters data collection architecture.

From the initial stages of the project, the Medusa has been adapted to the requirements of the PathoGelTrap project.

- Sensors configuration has been develop for O2, Temperature, pH and Conductivity, and electronics adapted accordingly. These are critical water quality parameters in aquaculture production operations, both in marine and freshwater.
- A suitable physical sensor for continuous measurements of NH4, which is also a critical parameter for fish welfare, is proving to be a difficult development. A possible virtual NH4 sensor is being adapted to the configuration of the PGT Medusa
- Communication requirements are being met, to be able to stablish data transmission and monitoring and adequate alarms.

The adapted model has been consolidated and tests have been carried out in more controlled environments and in relevant environments. To further develop the virtual ammonium sensor, tests are being carried out at the ZOO Aquarium in Madrid, in quarantine ponds provided by the zoo management. First, the parameters are being measured without the presence of fish and then measurements will be made in tanks occupied by fish and in which feed will be added to measure ammonium variations. Following this, medusa will be used to collect water quality measurements in aquaculture environments.

SW is making available to the project enough devices for the follow up of the experimental validation treatments.

3. Overall monitoring and evaluation of results

- Relevant water quality parameters have been collected regarding the target pathogens, their epidemiology and disease dynamics and the culture conditions of the model affected fish species.
- Initial, standard commercial water quality operational parameters for the design of the PGT products have been defined for the Project.
- Standard water samples from relevant sources are available and being used for the design and manipulation of PGT products
- Development of the sensors platform Medusa is ready for pH, dissolved oxygen, temperature and salinity (as conductivity) to be utilised in water quality monitoring for *in vivo* experiments.
- Suitable physical or virtual NH4 sensors are being pursued.
- Water quality monitoring in relevant aquaculture operations is being planned and will be reported in following editions of this Deliverable

4. Conclusion

Water quality characterisation and monitoring is essential for the definition of water quality working parameters of the PGT products and for ensuring these parameters are followed during in vivo experiments, as well as for guaranteeing fish welfare during these experiments.

Water quality working conditions are specified in Table 2.

A real-time water quality monitoring device (Medusa) is made available to the project by **SW**.

5. Bibliography

G.N. Frerichs, A. Tweedie, W.G. Starkey, R.H. Richards. Temperature, pH and electrolyte sensitivity, and heat, UV and disinfectant inactivation of sea bass Dicentrarchus labrax/ neuropathy nodavirus. Aquaculture 185 : 13–24. 2000.

Kumar, G., Menanteau-Ledouble, S., Saleh, M. et al. Yersinia ruckeri, the causative agent of enteric redmouth disease in fish. Vet Res 46, 103. 2015.

J. Mendez, D. Cascales, A.I. Garcia-Torrico, Jose A. Guijarro. Temperature-Dependent Gene Expression in Yersinia ruckeri: Tracking Specific Genes by Bioluminescence During in Vivo Colonization. Front Microbiol. 9: 1098. 2018

Panzarin et al. Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe. Infection, Genetics and Evolution 12: 63–70. 2012

Panzarin et al. In vitro study of the replication capacity of the RGNNV and the SJNNV betanodavirus genotypes and their natural reassortants in response to temperature. Veterinary Research 2014, 45:56

Romalde, J. L., J. L. Barja, B. Magarinos, and A. E.Toranzo. Starvation-survival processes of thebacterial fish pathogen Yersinia ruckeri. Systematic and Applied Microbiology 17:161–168. 1994.

Thorsen, B. K., Ø. Enger, S. Norland, and K. A. Hoff. Long-term starvation survival of Yersiniaruckeri at different salinities studied by microscop-ical and flow cytometric methods. Applied and En-vironmental Microbiology 58:1624–162. 1992.