Ref. Ares(2025)2960412 - 16/05/2025

AquaBioSens

On-site biological sensing for aquatic pollutants and biohazards

Grant Agreement: 101135432

D3.1 Molecular assays

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Project description

Acronym	**AquaBioSens**
Title	**On-site biological sensing for aquatic pollutants and biohazards**
Coordinator	Foundation for Research and Technology - Hellas (FORTH)
Reference	101135432
Type	Research and Innovation Actions (RIA)
Programme	**Horizon Europe (HORIZON)**
Topic	HORIZON-CL6-2023-ZEROPOLLUTION-01-6
Start	01.01.2024
Duration	36 months
Website	www.aquabiosens.eu
Consortium	**Foundation for Research and Technology - Hellas** (FORTH), Greece, Coordinator **Dublin City University** (DCU), Ireland **University of Southern Denmark** (SDU), Denmark **BIOPIX DNA TECHNOLOGY P.C.** (BIOPIX-T), Greece **ETT S.p.A**. (ETT), Italy **INTERSPREAD GmbH** (INSP), Austria **University of Southampton** (UOS), United Kingdom **National Oceanography Centre** (NOC), United Kingdom

Deliverable

Deliverable number	D3.1
Deliverable title	Molecular assays
Deliverable version	1.0
Lead beneficiary	FORTH
Work package number	WP2
Work package title	Environmental RNA sensing for harmful microbes and biotic pollution
Due date of delivery	30.04.2025
Actual date of delivery	16.05.2025
Dissemination level	Public
Type	**Report**
Rights	AquaBioSens Consortium
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Document history

Version	Date	Beneficiary	Description
1.0	16.05.2025	FORTH	Deliverable 3.1 Molecular Assays

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1 Executive summary

This Deliverable report presents the progress made under Tasks 3.1 and 3.2 of WP 3 in the AquaBioSens project, focused on the development of rapid, on-site molecular assays for the detection and quantification of harmful aquatic microbes. We have developed new assays using two fast and robust isothermal RNA amplification methods—specifically quantitative fluorescence Recombinase Polymerase Amplification (qfRPA) and quantitative fluorescence Loop-Mediated Isothermal Amplification (qfLAMP)—to enable real-time detection of target species using a portable fluorescence analyzer that is being developed under WP1.

Our target organisms include:

- *Alexandrium* spp., marine dinoflagellates producing paralytic shellfish toxins;
- *Microcystis aeruginosa*, a freshwater cyanobacterium producing liver-damaging microcystins.
- *Escherichia coli*, an indicator of fecal contamination in recreational and drinking waters.
- An additional target, *Pseudo-nitzschia* spp., a marine diatom producing domoic acid, is under reconsideration due to genetic variability and current test site limitations.

Each assay was designed to detect active cells via RNA targets, with a focus on genes involved in toxin biosynthesis or stress response. The assays were evaluated for specificity, inclusivity, and sensitivity using synthetic standards and native nucleic acids. The RPA approach proved suitable for *Alexandrium* and *Microcystis*, while LAMP was used for *E. coli* due to lower risk of false positives.

Key outcomes include:

- High sensitivity for the *Alexandrium* assay (detecting as few as 13 cells).
- Very high sensitivity for the *Microcystis* assay, allowing us to significantly reduce the required sample volume for analysis, while still meeting the statutory thresholds.
- Moderate sensitivity for *E. coli*, with minor improvements planned to meet statutory detection thresholds.

Challenges encountered included delays in obtaining necessary strains for exclusivity testing due to regulatory barriers and sourcing constraints. These gaps are being addressed through European collaborations and ongoing sequencing confirmation. The next steps involve assay testing on simulated and natural samples (Task 3.4), in preparation for field validation in WP6 starting in 2026.

These advancements mark a significant step toward near-instant, decentralized water quality monitoring, promising to replace labour-intensive, centralized methods with portable, accurate diagnostic tools that can better support early warning systems and environmental safety.

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2 Figures and Tables

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3 Acronyms & Abbreviations

Term	Description
ASP	Amnesic Shellfish Poisoning
ELISA	Enzyme Linked Immunosorbent Assay
HABs	Harmful Algal Blooms
HPLC	High-Performance Liquid Chromatography
MCYST-LR	Microcystin-LR
PSP	Paralytic Shellfish Poisoning
qfLAMP	Quantitative Fluorescence Loop Mediated Isothermal Amplification
qfRPA	Quantitative Fluorescence Recombinase Polymerase Amplification
RPA	Recombinase Polymerase Amplification

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4 Introduction

The scope of this deliverable is to provide an update on progress made by FORTH and NOC in developing molecular assays for a selection of harmful microbes under Task 3.1. Isothermal real-time amplification of RNA with fluorescence detection is being developed for key target species. The aim of these assays is to run them on the portable multichannel fluorescence analyzer, to enable fast, on-site quantification of hazardous microbes.

The selected target species include organisms that are responsible for globally distributed marine and freshwater Harmful Algal Blooms (HABs). These species produce toxins that have adverse effects on animals, including humans, as well as depleting oxygen from the water due to their high densities. As these blooms are increasing in frequency in some regions due to global warming, they have significant environmental and financial impacts. At present, most monitoring programmes rely on enumeration of cells by microscopy or quantification of toxins, both requiring centralized facilities and a minimum analytical time of 1-4 days, but often exceed a week. Additionally, species misidentification with a non-toxic relative is a risk, and cell toxicity varies with environmental conditions. Bloom dynamics are fast and microscopy approaches are prone to errors and don't allow for early warning of HABs.

We also focus on fecal coliform bacteria *Escherichia coli*, an indicator of sewage or other animal waste contamination, which are used globally as indicators of water quality for bathing and drinking waters. Again, enumeration methods rely on lab-based testing which delays the availability of the results by several days.

The on-site molecular tests we are developing will enable rapid quantification of harmful cells in less than 1 hour, providing near-instant results of water safety. Our assays target RNA instead of DNA, they quantify active cells. Using genes that are involved in the biosynthesis of the toxin will also indicate toxin production levels where applicable.

This deliverable will:

- Outline the progress made towards the assay design for 3 different organisms.
- Provide information on the sensitivity and specificity to the target organism for each assay.
- Outline the next steps towards the completion of the WP tasks.

Please note that this is the public deliverable pertaining to WP3 - Tasks 3.1 and 3.2 More information will be provided in the periodic report.

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5 Overview of isothermal RNA amplification approaches

Isothermal amplification of nucleic acids is preferable over PCR for portable devices because operating at a single temperature requires less electrical power. We have chosen the two most robust methods which are also tolerant to impurities in the nucleic acid sample, thus accommodating simpler nucleic extraction methods. Each method has specific pros and cons that make it suitable for a particular type of target gene or organism.

5.1 Quantitative fluorescence Recombinase Polymerase Amplification (qfRPA)

The recombinase assisted amplification reaction (Recombinase Polymerase Amplification, RPA) utilises recombination enzymes instead of temperature cycling to achieve strand invasion. This characteristic of RPA along with a suitable DNA polymerase allows for amplification at reduced temperatures ranging from 37-42 °C. Amplification is achieved using proprietary reagents manufactured by TwistDx Ltd. The reaction is amenable to the addition of reverse transcriptase (RT) resulting in a one-pot RT and amplification reaction for RNA amplification. The assay utilizes 2 primers and one probe with a fluorescent dye for real-time tracking of the amplification reaction. The primers can accommodate more sequence diversity than LAMP, making it better suited for assays that target genes with diverse sequences, or a need to capture a gene sequence across several species.

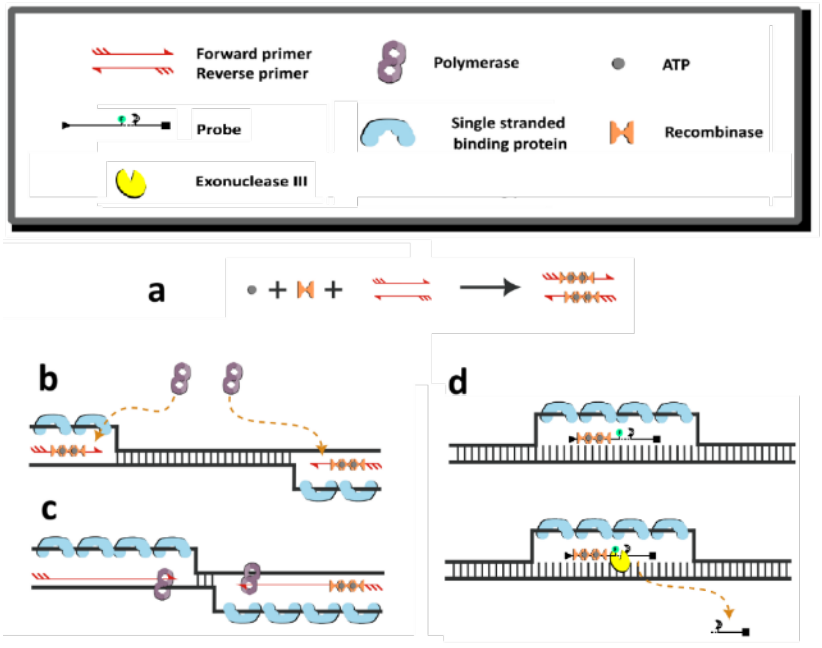

Figure 1. Overview of qfRPA. 1a) Primers interact with a Recombinase/ATP complex to form nucleoprotein filaments. 1b) Primer nucleoprotein filaments invade the double stranded DNA forming a displacement loop. The displacement loop is stabilised by single stranded binding proteins. 1c) The polymerase extends the primer while displacing other molecules. 1d) The probe carries a fluorophore and a quencher separated by a target abasic site. After hybridisation, the target abasic site is cleaved by an exonuclease to release the quencher from the fluorophore and generate fluorescence.

5.2 Quantitative fluorescence Loop mediated isothermal amplification (qfLAMP)

LAMP is carried out by a specific set of six primers, the design of which, although challenging, presents high specificity for a targeted genomic sequence. The different primers can selectively bind to eight regions on the gene and those include a pair of internal primers (FIP and BIP), a pair of external primers (F3 and B3) and a pair of loop primers (LF and LB). The amplification results in the formation of amplicons in dumbbell structures produced in larger quantities than other amplification approaches. These can be rapidly detected in real time by a fluorescent dye. Furthermore, LAMP already contains reverse transcriptase meaning that it can amplify RNA without the addition of extra RT enzyme. LAMP is a strong diagnostic tool due to the high sensitivity and specificity of the technique. However, the large number of primers makes it suitable only for very conserved gene sequences, usually within a single species with low diversity.

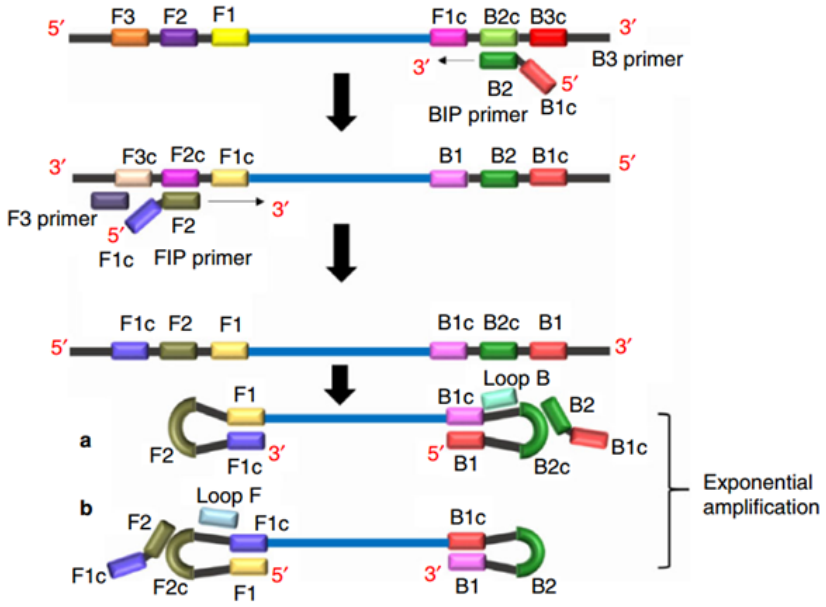

Figure 2. The amplification of DNA begins with the hybridization of the F2 region (FIP primer) with the complementary F2c region of the template strand. The FIP primer also, contains a F1c region in the 5' end that is important for the loop structure. The polymerase adds the complementary nucleotides in direction 5'--> 3'. Alongside the FIP primer, the F3 primer binds to the F3c region of the template strand. Upon the addition of the polymerase, F3 and FIP are elongated, and a loop-structure is formed. On the other template strand, the BIP primer contains a B2 region complementary to the B2c of the template and similarly, the B3 primer is complementary to the B3c region. The addition of nucleotides from the polymerase, in direction 5'--> 3' leads to the formation of another loop-structure. Ultimately, the result is the formation of dumbbell-shaped DNA, which the forward and backward loop primers hybridize in this particular step, contributing to the rapidity of the amplification.

6 Molecular assays for the quantification of active harmful microbes

6.1 Target species and assay design rationale

Initially 4 target species were selected for assays designs, based on their world-wide prevalence and need for monitoring. Assays have been developed for 3 of these targets. The rationale for our approach is explained for each species.

1. The toxic marine eukaryotic microalgae of the genus *Alexandrium* are well known for their large-scale blooms, called red tides due to the red colour of the cells. They produce saxitoxins, a group of potent neurotoxins that lead to paralysis in humans if they consume contaminated shellfish (PSP: paralytic shellfish poisoning). Some species such as *Alexandrium minutum* are expanding into polar regions and cause unprecedented blooms even in remote, but populated, locations. Shellfish farms in affected areas require frequent monitoring to close before any contaminated food is harvested, and require additional analysis to confirm the demise of the bloom before they can reopen. Current monitoring protocols use microscopy to enumerate cells in water samples and High-Performance Liquid Chromatography (HPLC) to measure toxin levels in seafood tissue samples. While these methods are effective, they require time, expertise and dedicated laboratory facilities. An assay targeting a gene involved in toxin production can rapidly quantify the toxin potential of water samples and thus provide a highly specific early warning system.
2. The toxic freshwater cyanobacteria *Microcystis aeruginosa* forms blooms in lakes, rivers and reservoirs globally, turning the water thick and green in colour. *M. aeruginosa* produces the cyanotoxin Microcystin, a toxin that damages the liver in exposed mammals including humans. Fish suffer inflammatory and endocrine damage when exposed, affecting fish farms that are situated in inland water bodies. Current monitoring strategies utilize toxin testing by ELISA, which take several days to complete. Cyanobacterial blooms in freshwater environments are usually monitored by microscopy to precisely quantify cyanobacterial cells. ELISA and HPLC are commonly used for detecting cyanotoxins—particularly Microcystin-LR (MCYST-LR), the most toxic and extensively studied toxin variant produced by *Microcystis aeruginosa*. These methods offer a comprehensive framework for assessing and tracking harmful algal blooms, however, they require complex lab-based equipment and a time-consuming analytical procedure.
3. The fecal coliform bacteria *Escherichia coli* are an indicator of sewage contamination in both fresh and marine coastal waters, that may result in illness. There is strict guidance regarding the limits of *E. coli* allowed in drinking and bathing waters, with mandatory closure of beaches, lakes and rivers in case of a rise in number. *E. coli* are normally enumerated by culture based methods in a laboratory. Although the enumeration method itself can be completed in a day, transport of samples to centralized facilities causes delays and, as the test requires live cells, sample transport over long distances may cause false results. We aim to create a highly sensitive molecular assay of live *E. coli* cells, by targeting heat-activated genes that will increase their expression with a short sample preheat step before the analysis.

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4. The toxic marine diatom *Pseudo-nitzchia* spp. forms large scale, persistent blooms spanning the coast of a whole continent in some areas, and producing the potent neurotoxin domoid acid. This toxin is responsible for Amnesic Shellfish Poisoning (ASP), causing amnesia if ingested and other neurological symptoms during exposure. Monitoring relies on cell counts by microscopy, however the genus includes both toxic and non-toxic species that often look alike. At the time of writing the proposal, the sequence information available for the genes involved in the domoic acid biosynthetic pathway was limited to only a few species. New genome information published in 2024 showed that the genes are too diverse to make a universal assay for all *Pseudo-nitzchia* species. We are considering whether to focus on a subgroup that is highly toxic, however, this group does not occur in our currently planned testing sites. We are looking for partners who could collaborate from locations where these blooms are found, before making a decision on whether or not to proceed with this fourth assay for the AquaBioSens project.

6.2 Assay characteristics

The selection of target genes for the assays has been described in detail in the report for Milestone 4. Their names and functions are also shown in Table 1.

Both LAMP and RPA have been used for assay development. RPA is prone to false positives for *E. coli* assays due to the manufacturing procedure of the reagents, so LAMP was chosen for this target. RPA was more suitable for other species which have significant intraspecific diversity in their gene sequences, as the RPA primers have more relaxed criteria in their design. The characteristics of the designed assays are summarized in Table 1.

The assays limits of quantification have been determined using both synthetic DNA standards, total genomic DNA and total genomic RNA. Analysis with synthetic RNA standards has been completed for the *Alexandrium* sxtA4 assay and pending for the *M. aeruginosa* mcyB and *E. coli* rpoH assays once the molecules are synthesized by the manufacturer.

To determine whether our assays have reached the required sensitivity according to the statutory requirements, or well-accepted limits, we assume that the sample collection would be 1 L of water and that the RNA will be concentrated into a 20 µL sample. A 5 µL subsample will be used in each assay reaction. This entails a 4-fold dilution of the sample, therefore, requires that the assay is 4x more sensitive to detect the required cell equivalents.

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Table 1. Overview of the designed assay specifications

Species	***Alexandrium* genus**	***Microcystis aeruginosa***	***E. coli***
Gene ID	*sxt*A4	*mcy*B	*rpoH*
Gene function	Saxitoxin biosynthesis	Microcystin biosynthesis	sigma - regulation of heat shock genes
Required sensitivity	In most European countries[1] For increased monitoring: 100 cells L^{-1} For action: 1000 cells L^{-1}	In European recreational waters[2] 20 million cells L^{-1}	EU-Bathing Water Directive[3] For excellent quality <5000 cfu L^{-1} Freshwater <2500 cfu L^{-1} Seawater
Assay type	RPA	RPA	LAMP
Assay run time	20 min	12 min	13 min
Limit of quantification (DNA)	Not applicable	DNA equivalent to 3900 cells (0.0015 ng) per reaction	DNA equivalent to 1000 cells (0.02ng) per reaction.
Limit of quantification (RNA)	RNA equivalent to 13 cells (0.1 ng) per reaction for *Alexandrium minutum*	RNA equivalent to 213,000 cells (1 ng) per reaction.	RNA equivalent to 4000 cells (0.04 ng) per reaction.
Improvement requirement	None.	None. Given the high sensitivity achieved, only 10-15 mL of water sample will be needed to achieve the required assay sensitivity.	Need to increase sensitivity at least 6.5 times. Optimization planned with chemical additives in the reaction.
Inclusivity	*A. minutum* *A. tamarense* *A. ostenfeldii* *A. pacificum*	*M. aeruginosa* (3 toxic strains)	*E. coli* (11 strains)
Exclusivity	*Anabaena cylindrica* (SXT producing cyanobacteria) *Planktothrix aghardii* (SXT producing cyanobacteria) *B. prasinos* (green algae) *P. trichornutum* (diatom) *T. pseudonana* (diatom) *E. huxleyi* (coccolithophore) Pending analyses on: *Pyrodinium bahamense* (sxt producing dinoflagellate) *Gymnodinium catenatum* (sxt producing dinoflagellate) *Alexandrium tamutum* (non toxic) *Alexandrium Andersonii* (non toxic)	*M. aeruginosa* (non-toxic) *Anabaena cylindrica* (MCY producing cyanobacteria) *Planktothrix aghardii* (MCY producing cyanobacteria)	Species IDs are subject to confirmation by sequencing. *Cobetia marina* *Escherichia albertii* *Escherichia blattae* *Escherichia hermanii* *Escherichia vulneris* *Klebsiella pneumonii* *Marinobacter hydrocarbonoclasticus* *Pantoea agglomerans* *Pseudoalteromonas tunicata* *Salmonella bongori* *Salmonella enterica* *Salmonella enterica (Nottingham)*

			Salmonella typhimurium

6.3 Next steps

6.3.1.1 Assay development initially progressed within the anticipated timeline. However, there have been some delays resulting in some gaps still remaining in the exclusivity testing. A key reason for this is that it has proven difficult to obtain certain cultured strains from culture collections. Some countries have recently banned export of cells, or even extracted nucleic acids, when the originating species is toxic or pathogenic. Therefore, we have obtained material from other labs around Europe that have been willing to collaborate, and we are still searching for more strains for a comprehensive validation of our assays. Some of the strains obtained require confirmation of their identity by sequencing, which is currently ongoing. Furthermore, an improvement is required for the sensitivity of the *E. coli* assay. The scale of the improvement required is small and we expect to achieve that with minor modifications to the LAMP protocol. This will close Task 3.2, which is covered in this Deliverable.

6.3.1.2 Following that, we will proceed to Task 3.4 which is to test the assays with simulated samples, or even natural field samples where possible. These are the key steps to be completed before the beginning of WP6 that will focus on field validation from the beginning of 2026.

7 Conclusions

Isothermal RNA amplification assays have been developed for 3 key organisms that are either toxic or indicate biotic pollution. After undertaking a series of optimization steps, the assays have achieved, or are close to achieving, the required sensitivity for statutory monitoring programs. They have been tested on a variety of related and unrelated microorganisms to ensure that they are amplifying RNA only from the required target group. This analysis is currently being expanded with cultured strains isolated from various locations around Europe. This will enable us to proceed with Task 3.4, which is to use the assays with simulated, or preferable real, samples for validation against the current statutory method of monitoring.

8 References

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