# AdriAquaNet

Enhancing Innovation and Sustainability in Adriatic Aquaculture

## Deliverable WP 4 - Task 4.3 WELFARE MONITORING

## Technical-scientific manual

## Manual for use on field of a “bivalent monitoring tool”

**Legnaro (PD), 30.06.2022**

# 1) INTRODUCTION

## Project

In the AdriAquaNet (AAN) project, WP4 aims to link expertise and do research and development to improve health and sustainability in aquaculture.
In particular, Task 4.3 is concerned with looking for best practices for welfare monitoring.

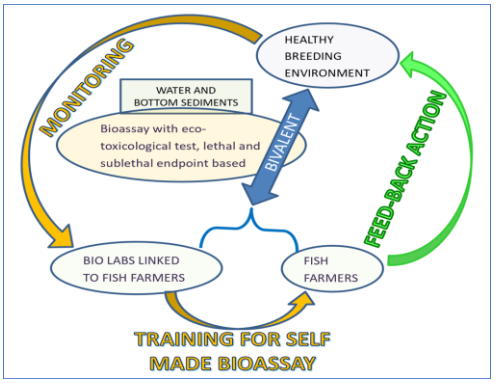


## Manual

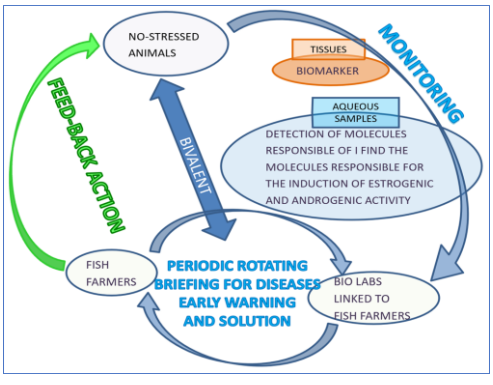
This Manual reports an overview of the results obtained from the monitoring work carried out to verify different approaches for biological investigation (bioassay) on an aquaculture plant, taken as an experimental site. The complete report with methodologies and comments for each type of bioassay analysis is in the scientific report submitted to the PP4 Istituto Zooprofilattico Sperimentale delle Venezie (assignment for the supply of the ecotoxicological investigation service in the AdriAquaNet project - CIG 8194562E79; CUP G26C18000600007).

## Issues and objectives

The intention of this Task was to obtain a bivalent monitoring tool, meaning that first of all it would assess the environmental matrices of the fish farm to establish the healthiness of the farm and identify the presence of possible stressogenic elements interfering with fish growth. Then, as a second aim of the tool, that it could be transferred as a method, to the fish farmers, or to a local laboratory committed to them; all for autonomous management of the farm's monitoring.
In order to understand the critical issues to be addressed and to understand the scope of the work and the purpose of this manual, here are 2 conceptual maps (the graphs reproduced here are in small size while the large ones are collected at the beginning of the annex to this manual, where the graphical materials are collected).




Graph 1




Graph 2



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The bivalence consists of the relationship between the matrix analysed with selected tests (bottom sediment, plant water alias pelagic environment, fish tissue) and the autonomy of the fish farmer to understand the data and implement feedback actions, either completely on his own or in partnership with a laboratory already committed to him. Bivalence gives information as a result of monitoring and at the same time stimulates feed-back actions on the fish farm, in order to have unstressed fish. The fish farmer will be able to learn how to manage all or part of the bioassay tests, thanks to specific training, or he will collaborate with a laboratory (collection of samples, management of survey sessions), and will discuss about the data obtained in a series of periodic briefings. In either case, the response will be to implement those actions that limit the stress on the fish and improve the healthy environment in the farm, favouring the fish's constant need to feed and to form biomass.



**Target**

Therefore, the main target audience of this Manual is fish farmers and any local laboratories connected to them. The need to which we respond is to broaden the panorama of methods and to select those that are sensitive to the variations we monitor and at the same time are not complicated in their execution.

**EPO indicators**

Therefore, with the work done and the results obtained, the following EPO indicators are considered to have been achieved:

- identification of new protocols, especially for assays with sub-lethal endpoints and for enzyme immunoassays;
- selection useful for the possible transfer of knowledge to fish farms (not possible until now due to the pandemic);
- first evidence for analytical protocols to be included in international guidelines for offshore fish farms.

## 2) MAIN PART

**Context**

The fish farms in which the survey scheme was to be applied were 3. For reasons related to the pandemic it was decided to select, as experimental project site, the plant of Friškina d.o.o., Movar Bay, k.o. Sevid , Rogoznica, ribogojilišno polje 1 Šibensko-kninska, legal seat in Split. This plant was comparable to many other smaller plants in the Adriatic Sea: with floating tanks, placed in a bay, without the direct impact of urbanised areas.



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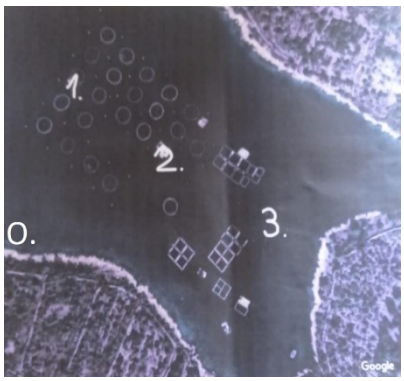
**Sampling**




The matrices taken into examination were the sediments lying under the cages, from which an elutriate (eluate) with standard water was obtained.
The sampling stations were identified according to a broad-coast gradient: station number 3 was the one closest to the shore, inside the bay, at a depth of between 5 and 8 metres; station 2 was the one central to the plant, at a depth of around 20 metres; station 1 was the one external to the plant facing out to sea, at a depth of 25 metres and deeper, with a muddy bottom; station O was the blank, taken at the head of the promontory south of the bay.
The codes and sampling periods were as follows and covered the time between summer, winter and early spring:

- 0-O-7-21 (B blank), 1-O-7-21 (OUT), 2-O-7-21 (MED), 3-O-7-21 (TOX) in mid-July 2021;
- 1-A-8-21, 2-A-8-21, 3-A-8-21 in the second half of August 2021;
- 1-B-11-21, 2-B-11-21, 3-B-11-21 in mid-November 2021;
- 1-C-03-22, 2-C-03-22, 3-C-03-22, in early March 2022.

**Methodology**

In order to have a battery of tests with different types of end-points and different types of parameters, the following assays were selected:

- - acute bioassays with bioluminescent bacteria on the solid phase (in contact with sediment)
- - acute bioassays with bioluminescent bacteria on the liquid phase (sediment elutriate)
- - chronic bioassays with unicellular planktonic algae on the sediment elutriate
- - acute bioassays with rotifers on the sediment elutriate
- - chronic bioassays with a planktonic copepod crustacean on the sediment elutriate
- - acute (double end-point) bioassays with barnacle larvae on the sediment elutriate
- - acute bioassays (double end-point) with juvenile jellyfish (ephire) on the sediment elutriate
- - enzyme immunoassays with yeasts for the detection of oestrogens or molecules with similar stimulation (oestrogenic activity)
- - enzyme immunoassays with yeasts for the detection of androgens or molecules with similar stimulation (androgenic activity)

The samples were all filtered at 0.45Micron in order to remove as much of the suspended particulate as possible. The fine fraction was a mixed detritus of organic origin, because there were many fragments of sea-grass, residual food and faecal pellets on the bottom; this generated a nutrient-rich silt.
Each test followed an internationally standardised protocol (ISO EN, ASTM, UNI ISO); in each replicate there were controls at 24 and 48 hours that validated each assay.

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In order to assess whether the differences from a normal situation were significant, sediment was also sampled away from the fish farm tanks and analysed (designated 'O'). This blank showed no signs of toxicity or the effect was always very low, zero at 24h and low significance at 48h.
It can therefore be stated that all the methods applied were correct and the tests valid.

**Results**



All the data obtained are collected in the 9 tables in the annex to this Manual (Graphical Annexes). Instead, below we summarise all the results by expressing them with judgements that summarise the significant aspect they highlighted (acute or chronic sensitivity, limits and interferences, toxicity, eutrophication, seasonality, broad-coast gradient).

| Type of test ---------- Sampling period | Bacteria on solid (SPT) | Bacteria on elutriate | Algae on elutriate | Rotifer larvae on elutriate | Eggs and copepod larvae on elutriate | Barnacle larvae on elutriate | | immature jellyfish (efire) on elutriate | | Testing for hormone-like molecules | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lethality | Immobility (sub-lethal endpoint) | Lethality | Immobility (sub-lethal endpoint) | androgens | oestrogen |
| 0 = negative reference sample (blank) | Zero acute toxicity signal | Zero acute toxicity signal | Medium eutrophication | Zero acute toxicity signal | Zero chronic toxicity signal | Zero acute toxicity signal | Acute toxicity signal at 48h (no at 24h) | Zero acute toxicity signal | Invalid | n.c. | n.c. |
| O = July'21 | Zero acute toxicity signal | Eutrophication, high inside the bay | Medium eutrophication offshore and in centre of the bay | Zero acute toxicity signal | n.c. | Zero acute toxicity signal | Acute toxicity signal at 48h (no at 24h) | Acute toxicity signal at all 3 sampling sites (at 24h only inside the bay) | Invalid | Probable presence | Measured presence |
| A = August'21 | Zero acute toxicity signal | Low eutrophication, acute toxicity signal inside the bay | Medium eutrophication offshore and in centre of the bay | Zero acute toxicity signal | Chronic toxicity signal inside the bay | Zero acute toxicity signal | Acute toxicity signal at 48h (no at 24h) | Acute toxicity signal at all 3 sampling sites (at 24h only inside the bay) | Invalid | Probable presence | Measured presence |
| B = November'21 | Toxicity signal offshore | Low eutrophication, acute toxicity signal inside the bay | Medium eutrophication offshore and in centre of the bay | Zero acute toxicity signal | Chronic toxicity signal inside the bay | Zero acute toxicity signal | Acute toxicity signal at 48h (no at 24h) | Acute toxicity signal at all 3 sampling sites (at 24h only inside the bay) | Invalid | Probable presence | Measured presence |
| C = March'22 | Slight acute toxicity signal in centre and inside the bay | Acute toxicity signal at all 3 sampling sites | Medium eutrophication offshore and in centre of the bay | Zero acute toxicity signal | Chronic toxicity signal at all 3 sampling sites | Acute toxicity signal at all 3 sampling sites | Acute toxicity signal at 48h (no at 24h) | Acute toxicity signal at all 3 sampling sites (also at 24h) | Invalid | Probable presence | Measured presence |

**TAB 10 interpretative overview of the results of all assays in the different periods**

Analysing the results as a whole, all the assays show us that the test responses are **different** in the period between **summer and autumn** (samples named 'O', 'A' and 'B') than in March (sample named 'C'). Thus, in **spring**, the tendency is to have worse responses, toxic results all along the gradient: this is shown by bacteria, *Acartia*, barnacle and juvenile jellyfish (efira).

There is also a marked **difference between the samples inside the bay and those offshore**: here is a tendency for the **in-bay samples to be more significant** in terms of toxicity, especially between **summer and autumn**; in **spring** the results show **uniform signs of toxicity**.



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The presence of a **widespread eutrophication** of the bottom sediment, which can be expected in a fish farm, is confirmed. Bioluminescent bacteria on the liquid phase and algae are stimulated in growing populations.

Observing this reaction by eutrophy, it can be deduced that the **acute toxicity signals are weak and that their detection is covered** by the nutrient load in the bottom. Only in March did the bacteria report toxicity in all three samples. That **acute toxicity is low** is also shown by the assays with rotifers, which do not show any signs of effect; in fact, rotifers respond only to massive contamination.
However, that **toxicity is of a chronic nature** is demonstrated by *Acartia* itself.



The **sub-lethal end-points** for acute toxicity **did not work well**, as they were very sensitive and suffered from a **'mechanical'**, non-toxic effect. Despite **filtering the samples at 0.45Micron**, there is a possibility that very small **fragments passed** through the filter and formed a silt, which then created an agglomerate that trapped the organisms and prevented them from moving and breathing. It is therefore determined that they are not applicable.

The most complex part of the methodological investigation was the application of tests to determine the **presence of molecules with oestrogenic and androgenic activity**, such as pharmaceutical residues, pesticides, packaging polymers, synthetic tank polymers and pipes. This was done to assess **growth interference** in a fish farm where stimulation for reproduction **limits feeding** instincts and alters the food conversion index. Our sample was always sediment **elutriate**, a novelty for the application of this test (already tested for: ultrapure, drinking and mineral water, surface water, wastewater, groundwater and well water, aqueous extracts). For the measurement of **'oestrogen-like'** the concentration of E2 lies in the range between the lower and upper limit of quantification, so molecules that induce oestrogen-like stimulation are present in the samples and can be **detected quantitatively and also quantified**. In the case of **'androgen-like'** DHT concentrations lie in the range between the detection limit and the lower limit of quantification, so samples can be **detected qualitatively but not quantified**.
We can thereby assert that **there are molecules that induce stimulation similar to oestrogens and androgens in the sediment**, as well as in the dissolved particulate matter coming from the anthropized area along the coastline and/or originating from the breeding plant and residing on site.



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## 3) CONCLUSION AND RECOMMENDATIONS



First of all, considering the stability of the sample in the centre of the fish farm, **monitoring with a reduced number of stations** is also considered functional. We recommend only **one station inside the bay** (the 3 in our case) and **one offshore** (the 1 in our case), as well as having a **blank** sampled at the promontory cape (the 0 in our case) each season.

Regarding the preparation of samples for testing, it is recommended to proceed with a **second filtration**, after decanting, with 0.22Micron; this is to avoid the formation of this fine silt with unpredictable mechanical interference.

With regard to the type of tests and the best period for their application, assuming a sort of control plan, we recommend the following **strategy: separate the application of the tests at two different points in different periods**.

**A)** The following three tests should be carried out **throughout the summer, late summer and early autumn on ay samples inside the by**:

- - chronic bioassays with unicellular planktonic **algae** on the sediment elutriate;
- - acute bioassays with **rotifers** on the sediment elutriate;
- - acute (lethality) bioassays with **barnacle** larvae on the sediment elutriate.

For acute assays, however, the **mortality endpoint** should be considered and **not the sub-lethal endpoint**. In addition, **more consideration must be given to the 24-hour results** than to the 48-hour results.
These assays allow us to **monitor the level of eutrophication** accumulated on the sea bottom and to detect any **occasional acute contamination**.
In addition, all these tests have the possibility of being **trained to fish farm staff**, implementing that **Bivalency** represented in **Graph 1** for feedback actions. This practical part could **not be implemented due to the pandemic**. In our specific case study, however, the staff's ability was evident, in order to learn methods, manage sediment sampling and water conservation.
Finally, let us remember that algae, rotifers, barnacles are **easily available in nature around the fish farm**; they are also most certainly manageable by the fish farmers, as they are generally **used in fish breeding**, for fry or for raising juvenile fishes, being the basis of every food chain.



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Acute bioassays with **bioluminescent bacteria** on the solid or liquid phase, due to the need for a specific instrument (luminometer) can be **commissioned to an external laboratory** in case of unclear situations or risk of contamination.

Bioassays with **juvenile jellyfish (ephire)** on the sediment elutriate, are **not properly applicab**le, due to the objective difficulty of obtaining larvae and their high sensitivity to environmental matrices.



**B)** The following three tests should be carried out **during winter on offshore samples**:

- - chronic bioassays with a **planktonic copepod** crustacean on the sediment elutriate
- **- enzyme immunoassays** with yeasts for the detection of oestrogens or molecules with similar stimulation (**oestrogenic activity**)
- - **enzyme immunoassays** with yeasts for the detection of androgens or molecules with similar stimulation (**androgenic activity**)
- It would be useful in this situation to also have a **chronic test, in contact** with the sediment.

Chronic assays and the detection of molecules inducing androgen-like and oestrogen-like activity, are best applied in winter and especially on offshore samples in order to **observe that there is no diffuse contamination** coming in. In winter, then, trace toxicity is **not covered by the fish farm's eutrophic load**.

In this second strategic hypothesis, it is necessary to identify a **local laboratory link to the fish farm**, that establishes an adequate and preventive monitoring plan. So operational briefing would be periodically conducted between the laboratory technicians and the fish farm staff, in order to understand the **possible risk of trouble or distress at fish wellness**, so to be able to identify possible solutions together.



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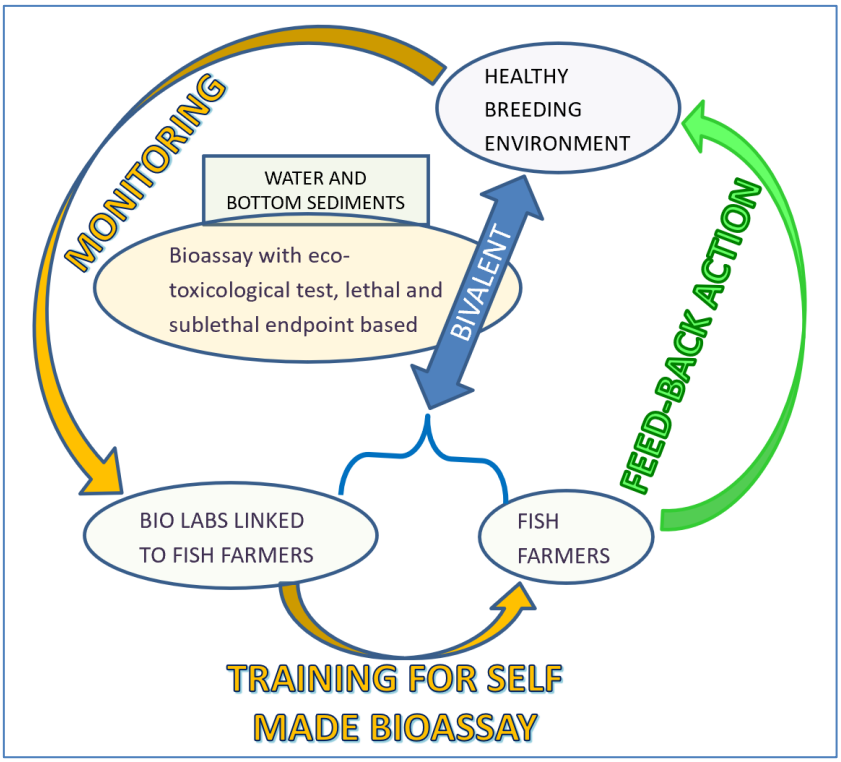
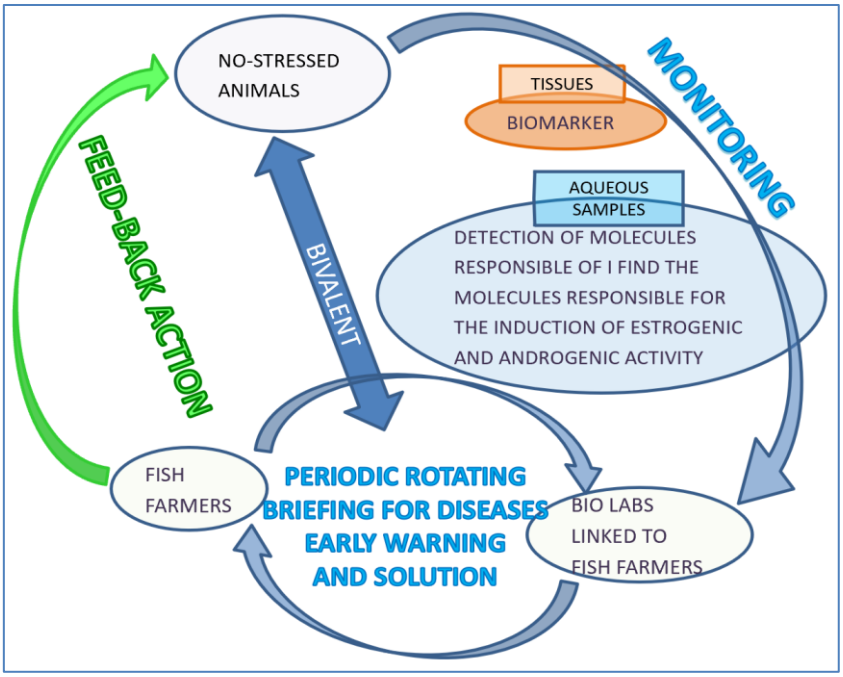
## GRAPHIC ANNEXES




**GRAPH 1 - Conceptual diagram of the first aspect of the bivalent tool**




**GRAPH 2 - Conceptual diagram of the second aspect of the bivalent tool**



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| reference * | Sample | Salinity | pH | Dissolved oxygen | Sand <1 mm | Pelite | Natural Tox Threshold | Measured Tox | Range at 95% confidence level of measured tox | Range at 95% confidence level of measured tox | $R^2$ | Toxicity Index S.T.I. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (psu) | | mg/L | (%) | (%) | (TU) | (TU50 p.s.) | (TU50 p.s.) | (TU50 p.s.) | | |
| * | 0-O-7-21 (ex B blank) | 33 | *** | *** | 97,34 | 2,66 | 33,70 | 0,00 | 0,00 | 0,00 | 1,00 | 0,00 |
| * | 1-O-7-21 (ex OUT) | 33 | *** | *** | 89,59 | 10,41 | 57,94 | 0,00 | 0,00 | 2,95 | 1,00 | 0,00 |
| * | 2-O-7-21 (ex MED) | 33 | *** | *** | 98,38 | 1,62 | 30,43 | 2,64 | 2,64 | 5,27 | 1,00 | 0,09 |
| * | 3-O-7-21 (ex TOX) | 33 | *** | *** | 96,85 | 3,15 | 35,22 | 0,00 | 0,00 | 3,17 | 1,00 | 0,00 |
| * | 1-A-8-21 | 33 | *** | *** | 93,10 | 6,90 | 46,95 | 9,59 | 3,20 | 22,37 | 0,92 | 0,20 |
| * | 2-A-8-21 | 33 | *** | *** | 94,42 | 5,58 | 42,84 | 12,40 | 6,20 | 18,60 | 0,97 | 0,29 |
| * | 3-A-8-21 | 33 | *** | *** | 92,58 | 7,42 | 48,58 | 23,20 | 20,88 | 25,52 | 1,00 | 0,48 |
| * | 1-B-11-21 | 33 | *** | *** | 94,39 | 5,61 | 42,92 | 90,13 | 82,29 | 101,88 | 0,99 | 2,10 |
| * | 2-B-11-21 | 33 | *** | *** | 93,23 | 6,77 | 46,54 | 31,35 | 28,22 | 37,62 | 0,99 | 0,67 |
| * | 3-B-11-21 | 33 | *** | *** | 90,63 | 9,37 | 54,68 | 19,66 | 15,73 | 23,59 | 0,99 | 0,36 |
| * | 1-C-03-22 | 33 | *** | *** | 93,14 | 6,86 | 46,84 | 9,66 | 6,44 | 9,66 | 1,00 | 0,21 |
| * | 2-C-03-22 | 33 | *** | *** | 95,92 | 4,08 | 38,14 | 38,22 | 27,80 | 48,65 | 0,98 | 1,00 |
| * | 3-C-03-22 | 33 | *** | *** | 93,88 | 6,12 | 44,52 | 43,16 | 38,84 | 47,47 | 1,00 | 0,97 |

*** parameters not measurable on wet sediment due to low volume; coincides with elutriate values

| SOLID TOXIC INDEX (S.T.I.) | CLASSE | TOXICITY LEVEL |
|---|---|---|
| S.T.I. ≤3 | A | NO TOXICITY |
| 3< S.T.I. ≤6 | B | MEDIUM TOXICITY |
| 6< S.T.I. ≤12 | C | HIGH TOXICITY |
| S.T.I. > 12 | D | VERY HIGH TOXICITY |

**TAB 1 acute bioassays with bioluminescent bacteria on the solid phase (in contact with sediment)**

| EC20, EC50 | inhibition effect at 100% sample concentration | TOXICITY LEVEL |
|---|---|---|
| EC20≥90% | effect<50% | NO TOXICITY |
| EC20<90% e EC50≥100% | 20%≤effect<50% | MEDIUM TOXICITY |
| 20%≤EC50<100% | effect≥50% | HIGH TOXICITY |
| EC50<20% | | VERY HIGH TOXICITY |

**TAB 2 acute bioassays with bioluminescent bacteria on the liquid phase (sediment elutriate)**

* EC50 = 3,632 mg L-1 (da 1,693 a 7,791 mg L-1) (3,5-diclorofenolo)

| reference * | Sample | Salinity | pH | Dissolved oxygen | inhibition effect at 100% sample concentration | EC50 (30 min.) | Toxicity level or eutrophication |
|---|---|---|---|---|---|---|---|
| | | (psu) | | (mg/L) | (%) | (%) | |
| * | 0-O-7-21 (ex B blank) | 35 | 7,66 | > 6 | 4,23 | n.c. | No toxicity |
| * | 1-O-7-21 (ex OUT) | 34 | 7,48 | > 6 | -21,85 | n.c. | possible eutrophication |
| * | 2-O-7-21 (ex MED) | 34 | 7,52 | > 6 | -19,04 | n.c. | possible eutrophication |
| * | 3-O-7-21 (ex TOX) | 35 | 7,92 | > 6 | -69,71 | n.c. | high eutrophication |
| * | 1-A-8-21 | 35 | 7,44 | > 6 | -2,09 | n.c. | No toxicity |
| * | 2-A-8-21 | 34 | 7,97 | > 6 | -24,84 | n.c. | possible eutrophication |
| * | 3-A-8-21 | 34 | 7,56 | > 6 | 7,87 | n.c. | No toxicity |
| * | 1-B-11-21 | 33 | 7,68 | > 6 | -4,50 | n.c. | No toxicity |
| * | 2-B-11-21 | 34 | 7,84 | > 6 | -21,62 | n.c. | possible eutrophication |
| * | 3-B-11-21 | 35 | 7,93 | > 6 | 8,24 | >100 (>1.000) | No toxicity |
| * | 1-C-03-22 | 34 | 7,42 | > 6 | 32,07 | >100 (>1.000) | medium toxicity |
| * | 2-C-03-22 | 34 | 7,78 | > 6 | 28,15 | >100 (>1.000) | medium toxicity |
| * | 3-C-03-22 | 34 | 7,98 | > 6 | 26,78 | n.c. | medium toxicity |





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| Sample | inhibition or stimulation effect at 100% sample concentration (+Δ% or -Δ%) ** % | Growing percentageat 100% sample concentration (72h) *** % | 72h EC20 (forcing extrapolation) % | 72h EC50 (forcing extrapolation) % | Toxicity level or eutrophication |
|---|---|---|---|---|---|
| 0-O-7-21 (ex B BLANK) | -15,38 | 115,38 | 1034,32 | 1164,51 | possible eutrophication |
| 1-O-7-21 (ex OUT) | -17,73 | 117,73 | 488,97 | 531,79 | possible eutrophication |
| 2-O-7-21 (ex MED) | -10,90 | 110,90 | 1356,15 | 1509,15 | possible eutrophication |
| 3-O-7-21 (ex TOX) | -11,79 | 111,79 | 1465,43 | 1669,29 | possible eutrophication |
| 1-A-8-21 | -15,67 | 118,98 | 189,96 | 156,24 | possible eutrophication |
| 2-A-8-21 | -17,18 | 117,18 | 194,27 | 199,15 | possible eutrophication |
| 3-A-8-21 | -14,05 | 114,05 | 181,94 | 112,03 | possible eutrophication |
| 1-B-11-21 | -12,32 | 121,76 | 176,33 | 148,87 | possible eutrophication |
| 2-B-11-21 | -9,22 | 109,22 | 1286,29 | 1467,45 | possible eutrophication |
| 3-B-11-21 | -8,55 | 110,14 | 1353,36 | 1547,74 | possible eutrophication |
| 1-C-03-22 | -10,89 | 110,89 | 159,80 | 163,64 | possible eutrophication |
| 2-C-03-22 | -12,58 | 123,67 | 267,31 | 249,33 | possible eutrophication |
| 3-C-03-22 | -6,45 | 105,12 | 1066,45 | 1232,79 | possible eutrophication |

** % growth (G%) = 100 x μi / μc

*** $I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100$

| EC20, EC50 | CLASS | inhibition effect at 100% sample concentration (+Δ%) | stimulation effect at 100% sample concentration (-Δ%) | TOXICITY LEVEL |
|---|---|---|---|---|
| EC20≥90% | A | <20Δ% | 20<-Δ%≤100 | NO TOXICITY |
| EC20<90% e EC50≥100% | B | 20≤Δ%<50 | - Δ%>100 | MEDIUM TOXICITY |
| 40%≤EC50<100 | C | Δ%≥50 | - | HIGH TOXICITY |
| EC50<40% | D | - | - | VERY HIGH TOXICITY |

**TAB 3 chronic bioassays with unicellular planktonic algae on the sediment elutriate**

| Sample | Lethality at 100% sample concentration (48h) | EC50 (%) | Toxicity level |
|---|---|---|---|
| Reference test (K2Cr2O7) EC50 = 213 mg L-1 (181-245 mg L-1) | | | |
| 0-O-7-21 (ex B blank) | 0,00 | n.c. | No toxicity |
| 1-O-7-21 (ex OUT) | 3,33 | n.c. | No toxicity |
| 2-O-7-21 (ex MED) | 6,66 | n.c. | No toxicity |
| 3-O-7-21 (ex TOX) | 10,00 | n.c. | No toxicity |
| 1-A-8-21 | 0,00 | n.c. | No toxicity |
| 2-A-8-21 | 6,66 | n.c. | No toxicity |
| 3-A-8-21 | 0,00 | n.c. | No toxicity |
| 1-B-11-21 | 6,66 | n.c. | No toxicity |
| 2-B-11-21 | 6,66 | n.c. | No toxicity |
| 3-B-11-21 | 3,33 | n.c. | No toxicity |
| 1-C-03-22 | 0,00 | n.c. | No toxicity |
| 2-C-03-22 | 0,00 | n.c. | No toxicity |
| 3-C-03-22 | 6,66 | n.c. | No toxicity |

| EC20, EC50 | inhibition effect at 100% sample concentration | TOXICITY LEVEL |
|---|---|---|
| EC20≥90% | effect<50% | NO TOXICITY |
| EC20<90% e EC50≥100% | 20%≤effect<50% | MEDIUM TOXICITY |
| 40%≤EC50<100% | effect≥50% | HIGH TOXICITY |
| EC50<40% | | VERY HIGH TOXICITY |

**TAB 4 acute bioassays with rotifers on the sediment elutriate**



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| Sample | sample concentration (%) | Lethality 48h at 100% sample concentration (%) | Lethality with Abbott correction (%) | naupli survival (%) | standard deviation | EC20 (%) | EC50 (%) | Toxicity level |
|---|---|---|---|---|---|---|---|---|
| **CRTL** | 0 | 3,33 | | 96,67 | 0,58 | | | |
| **0-O-7-21 (ex B blank)** | 100 | 23,33 | 20,69 | 76,67 | 1,15 | >90 (107,72) | >100 (525,93) | non tossico |
| **1-C-03-22** | 100 | 30,00 | 27,59 | 70,00 | 1,00 | <90 (80,42) | >100 (250,81) | tossicità medio-bassa |
| **2-C-03-22** | 100 | 20,00 | 17,24 | 80,00 | 0,00 | <90 (89,20) | >100 (340,23) | tossicità medio-bassa |
| **3-C-03-22** | 100 | 26,67 | 24,14 | 73,33 | 1,53 | <90 (89,62) | >100 (333,92) | tossicità medio-bassa |
| **3-A-8-21** | 100 | 16,67 | 13,79 | 83,33 | 1,15 | <90 (81,94) | >100 (565,87) | tossicità medio-bassa |
| **3-B-11-21** | 100 | 23,33 | 20,69 | 76,67 | 1,15 | <90 (86,23) | >100 (788,46) | tossicità medio-bassa |



**TAB 5 chronic bioassays with a planktonic copepod crustacean on the sediment elutriate**

| EC20, EC50 | inhibition effect at 100% sample concentration | TOXICITY LEVEL |
|---|---|---|
| EC20≥90% | effect<50% | NO TOXICITY |
| EC20<90% e EC50≥100% | 20%≤effect<50% | MEDIUM TOXICITY |
| 20%≤EC50<100% | effect≥50% | HIGH TOXICITY |
| EC50<20% | | VERY HIGH TOXICITY |

| Sample | Lethality at 100% sample concentration | | Immobility at 100% sample concentration | |
|---|---|---|---|---|
| | *24h* | *48h* | *24h* | *48h* |
| CTRL | 0 | 0 | | |
| 0-O-7-21 (ex B blank) | 1,4 | 10,1 | 12,9 | 100 |
| 1-O-7-21 (ex OUT) | 2,6 | 11,9 | 16,2 | 100 |
| 2-O-7-21 (ex MED) | 4,3 | 10,2 | 14,6 | 100 |
| 3-O-7-21 (ex TOX) | 8,7 | 14,7 | 10,5 | 98,1 |
| 1-A-8-21 | 0 | 1,3 | 8,3 | 100 |
| 2-A-8-21 | 1,3 | 3,7 | 6,8 | 100 |
| 3-A-8-21 | 3,8 | 7,2 | 7,2 | 95,4 |
| 1-B-11-21 | 1,4 | 3,8 | 6,7 | 100 |
| 2-B-11-21 | 2,5 | 5,2 | 6,1 | 100 |
| 3-B-11-21 | 4,2 | 6,0 | 4,8 | 100 |
| 1-C-03-22 | 1,0 | 25,2 | 9,8 | 100 |
| 2-C-03-22 | 2,3 | 25,3 | 8,5 | 100 |
| 3-C-03-22 | 0 | 22,4 | 8,6 | 100 |

| Lethality or Immobility at 100% sample concentration | TOXICITY LEVEL |
|---|---|
| effect<50% | NO TOXICITY |
| 20%≤effect<50% | MEDIUM TOXICITY |
| effect≥50% | HIGH TOXICITY |

**TAB 6 acute (double end-point) bioassays with baleen larvae on the sediment elutriate**

| Sample | Lethality at 100% sample concentration | | Pulse Frequency (PF) Inhibition at 100% sample concentration | |
|---|---|---|---|---|
| | *24h* | *48h* | *24h* | *48h* |
| CTRL | 0 | 0 | | |
| 0-O-7-21 (ex B blank) | 0 | 42 | 46 | 71 |
| 1-O-7-21 (ex OUT) | 35 | 75 | 96 | 100 |
| 2-O-7-21 (ex MED) | 44 | 80 | 98 | 100 |
| 3-O-7-21 (ex TOX) | 100 | 100 | 100 | 100 |
| 1-A-8-21 | 25 | 76 | 98 | 100 |
| 2-A-8-21 | 28 | 86 | 98 | 100 |
| 3-A-8-21 | 75 | 100 | 99 | 100 |
| 1-B-11-21 | 26 | 78 | 97 | 100 |
| 2-B-11-21 | 36 | 85 | 96 | 100 |
| 3-B-11-21 | 83 | 100 | 100 | 100 |
| 1-C-03-22 | 65 | 98 | 100 | 100 |
| 2-C-03-22 | 53 | 98 | 99 | 100 |
| 3-C-03-22 | 67 | 100 | 98 | 100 |

| Lethality or Pulse Frequency (PF) Inhibition at 100% sample concentration | TOXICITY LEVEL |
|---|---|
| effect<50% | NO TOXICITY |
| 20%≤effect<50% | MEDIUM TOXICITY |
| effect≥50% | HIGH TOXICITY |

**TAB 7 acute bioassays (double end-point) with juvenile jellyfish (ephire) on the sediment elutriate**



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| Sample | Sample e Dilution | Samples | | OD Phytase | | E2 Equivalents (EEQ) in ng/L | Measurement Uncertainty for Undiluted and Unspiked Sample |
|---|---|---|---|---|---|---|---|
| | | Qualitatively Detected | Quantifiable | Mean Value | Relative S. D. | | |
| 1-A-8-21 | undiluted | Yes | Yes | 0.848 | 3.6 % | 9.5 | 5.8 % |
| 1-B-11-21 | undiluted | Yes | Yes | 0.649 | 2.6 % | 7.6 | 5.3 % |
| 1-C-03-22 | undiluted | Yes | Yes | 0.534 | 2.0 % | 6.4 | 5.5 % |
| 3-A-8-21 | undiluted | Yes | Yes | 0.470 | 4.8 % | 5.8 | 5.8 % |
| 3-B-11-21 | undiluted | Yes | Yes | 0.463 | 2.8 % | 5.7 | 5.9 % |
| 3-C-03-22 | undiluted | Yes | Yes | 0.449 | 6.1 % | 5.5 | 6.0 % |
| 3-O-7-21 (ex TOX) | undiluted | Yes | Yes | 0.439 | 6.5 % | 5.4 | 6.1 % |
| 2-O-7-21 (ex MED) | undiluted | Yes | Yes | 0.428 | 3.2 % | 5.3 | 6.3 % |
| 1-O-7-21 (ex OUT) | undiluted | Yes | Yes | 0.491 | 4.3 % | 6.0 | 5.7 % |

**TAB 8 enzyme immunoassays with yeasts for the detection of oestrogens or molecules with similar stimulation (oestrogenic activity)**

| Sample | Sample e Dilution | Samples | | OD Phytase | | E2 Equivalents (EEQ) in ng/L | Measurement Uncertainty for Undiluted and Unspiked Sample |
|---|---|---|---|---|---|---|---|
| | | Qualitatively Detected | Quantifiable | Mean Value | Relative S. D. | | |
| 1-A-8-21 | undiluted | Yes | No | 0.519 | 3.3 % | 59.6 | 31.3 % |
| 1-B-11-21 | undiluted | Yes | No | 0.558 | 2.0 % | 80.1 | 24.5 % |
| 1-C-03-22 | undiluted | Yes | No | 0.561 | 4.2 % | 81.6 | 24.1 % |
| 2-A-8-21 | undiluted | Yes | No | 0.520 | 2.2 % | 60.1 | 31.1 % |
| 2-B-11-21 | undiluted | Yes | No | 0.545 | 4.0 % | 73.5 | 26.3 % |
| 2-C-03-22 | undiluted | Yes | No | 0.517 | 3.5 % | 58.4 | 31.8 % |
| 3-A-8-21 | undiluted | Yes | No | 0.530 | 3.8 % | 65.5 | 28.9 % |
| 3-B-11-21 | undiluted | Yes | No | 0.517 | 7.3 % | 58.3 | 31.9 % |
| 3-C-03-22 | undiluted | No | No | 0.345 | 3.3 % | 0.0 | n/a |
| 3-O-7-21 | undiluted | Yes | No | 0.539 | 7.9 % | 70.2 | 27.3 % |
| 2-O-7-21 | undiluted | Yes | No | 0.484 | 3.4 % | 39.9 | 44.9 % |
| 1-O-7-21 | undiluted | Yes | No | 0.477 | 3.5 % | 35.9 | 49.0 % |

**TAB 9 enzyme immunoassays with yeasts for the detection of androgens or molecules with similar stimulation (androgenic activity)**



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