CNR
IRBIM
ISTITUTO PER LE
RISORSE BIOLOGICHE
E LE BIOTECNOLOGIE
MARINE

Report:

EVALUATION ECO-TOXICOLOGICAL OF WATERS ORIGINATE FROM "LIMENET PLANT" OF AUGUSTA

In accordance with the operational purposes, on 17 April 2025, a total of ten (10) samples of water treated from the experimental plant at the port of Augusta were transferred from Limenet S.r.L Società Benefit to the Institute for Biological Resources and Marine Biotechnology (IRBIM)-CNR headquarters in Messina. Characteristics and quantities of the samples (**Figure 1**) are shown in **Table 1**. The samples, once arrived at the laboratory, were stored at 10±1°C waiting to be analyzed.

Figure 1 – Photograph of some of the bins containing water samples under study at the IRBIM-CNR Messina Headquarters.

Nome Campione	Limenet	%	pH	Temp. (°C)	Volume (~ L)
Acqua di Mare			8,11	19,8	20
30 Mare			8,11	19,4	30
19,34 Mare	0,66	3,3	8,21	19,8	20
29,01 Mare	0,99	3,3	8,26	19,6	30
18,66 Mare	1,34	6,7	8,33	19,7	20
28 Mare	2	6,7	8,29	19,5	30
46 Mare	4	13,3	8,39	19,4	30
17,34 Mare	2,66	13,3	8,33	19,5	20
22,11 Mare	7,89	26,3	8,18	19,7	30
14,74 Mare	5,26	26,3	8,16	19,6	20

Table 1 – List and characteristics of the experimental samples analyzed in this study.

Messina
Headquarters Via
S. Raineri, 86
98122 - Messina, IT
+39 090 6015411
C.F. 80054330586 - P.IVA 02118311006
www.irbim.cnr.it
protocollo.irbim@pec.cnr.it

Ancona
Largo Fiera della Pesca, 2
60125 - Ancona, IT
+39 071 2078826

Mazara del Vallo
Via Vaccara, 61
91026 - Mazara del Vallo, IT
+39 0923 948966

Awl
Via Pola, 4
71010 - Lesina, IT
+39 0882 992702

In order to evaluate a possible toxicity of the waters under analysis, a series of ecotoxicological analyzes were planned by applying a battery of assays (bacterial bioluminescence, mortality and viability of crustaceans, micro-algal growth and spermiotoxicity of mussels on the liquid phase) such as to be able to determine the impact, if present, of marine waters after treatment with the Limenet experimental system on the marine trophic chain. In order to have a more accurate overview, analyses of water were carried out following different levels of treatment.

1. MATERIALS AND METHODS

1.1 Preparation of samples in the study

The water samples, before being subjected to eco-toxicological analyses, were brought back to room temperature. After about an hour, the waters subjected to the same treatment but present in different containers, were unified so as to have samples as homogeneous as possible.

1.2 Bacterial Bioluminescence Test (MicroTox)

The acute toxicity test on *Vibrio fischeri* was conducted following the method described in the UNI EN ISO 11348-3 guideline.

The assay is based on the ability of this marine bacterium to be bioluminescent under optimal conditions. A decrease in the light emitted is therefore attributable to toxic effects of the tested sample. In these preliminary tests, the Microtox® Model 500 automated system (M500; Alco Automation Pte Ltd) with frozen bacteria of *Vibrio fischeri* strain NRRL-B- 11177 (stored at -20±1°C).

The test was carried out by exposing an aliquot of bacteria to the samples (treatment water) under study. In order to assess the real toxicity of the matrices leaving the treatment plants, no dilutions of the samples themselves were made; therefore, the possible toxicity of the matrices under study was evaluated at the maximum and real concentration.

At the beginning of each test, the frozen bacteria were reactivated by adding 1000 µL of a specific restorative solution and leaving them to incubate at a temperature of 5±1°C for 40 minutes. At the end of the incubation period, the reconstituted bacterial suspension was diluted in an appropriate solution (150 µL of experimental reagent in 1350 µL of Microtox ® SPT diluent).

After 20 minutes from the preparation of the diluted reagent, an aliquot equal to 100 µL of bacterial suspension was added to each tube (such as to present an amount of microbial cells equal to about 106 mL-1 cells). The tubes were incubated at 15±1°C for 20 minutes and luminescence was measured after a 10-minute acclimatization period in the incubation wells. The inhibition of bioluminescence was analyzed by performing light emission measurements after exposure of the bacteria for 15 and 30 minutes to the sample to be analyzed, comparing it with that of bacteria exposed to the negative control (represented only by seawater without treatment).

1.3 Acute Toxicity Test with *Artemia salina*

The acute toxicity test carried out with *Artemia salina* was carried out according to official indications (APAT IRSA-CNR protocol, 2003).

Reactivation of the cysts was performed approximately 48 hours prior to the start of the assay. To this end, an amount of cysts equal to about 100 mg was poured into a Petri dish (Ø, 9 cm) in which 20 ml of a suitably prepared marine (sterile) solution (NaCl, 26.4 g; KCl, 0.84 g; $CaCl_{2\times H_2O,}$ 1.67 g; $MgCl_{2\times 6H_2O,}$ 4.6g; $MgSO_{4\times 7H_2O,}$ 5.58 g; $NaHCO_3$, 0.17 g; HBO3, 0.03
g per litre, pH=7.8). The cysts were incubated in the presence of light (3,000-4,000 lux) for about one
(1) hour at 25 ± 1°C. Subsequently, the cysts were incubated in the dark (25 ± 1°C) for 24 hours. After the incubation period, the hatched larvae (nauplie) were transferred to a new Petri dish filled with 20 mL of marine solution (sterile) and incubated, further, for 24 hours.

At the end of the incubation period and the reactivation of the cysts, the juvenile forms of *Artemia salina* (10 nauplii in the larval stage) were used to carry out two trials. The first test was carried out by transferring the organisms filled with 20 mL of the different solutions under study into plates with a diameter of 9 cm. The second test the organisms were transferred to beakers with a volumetric capacity of 50 mL filled with the different solutions under study (experimental volume 40 mL). For both tests in question, each concentration was tested in duplicate.

Both petri dishes and beakers were closed with parafilm and kept at a constant temperature of 25 ± 2°C with an illumination cycle of 14:10 light:dark. Every 24 hours from the start of the incubation period, the organisms present in the systems were examined to assess their viability (%, of living and dead organisms). All organisms that were motionless for more than 10 seconds under the microscope's field of observation were considered dead.

1.4 Bioassay with *Phaeodactylum tricornutum*

The algal assay method has been updated in the UNI ISO 10253 (2006) standard which provides for the use of *Phaeodactylum tricornutum* Bohlin.

The principle of the test consists in exposing a pure algal culture in the exponential growth phase for several generations at known sample concentrations, under standardized physicochemical conditions and with a defined and homogeneous supply of nutrients. At the end of the incubation period, the algal growth in the sample is compared with that of the control (seawater without treatment).

The mother cell cultures were kept in an appropriate growth medium with periodic renewals to maintain (the same) in the exponential growth phase. Starting from the parent culture, a pre-culture with a cell density between 2 x 103 and 10^4 mL-1 cells was prepared 2 - 4 days before the start of the test and incubated under the same conditions as for the test. The cell density achieved by the pre-culture was then evaluated immediately before use, for the preparation of the inoculum culture with defined cell density.

An aliquot of the inoculum culture was then added to the study waters together with an appropriate amount of concentrated culture medium. The solution thus obtained had a cell density of about 8 x 10^3 mL-1. The cells thus prepared were distributed (in duplicate) in sterile disposable 6-well plates and incubated for 72 hours at 20 ± 2°C, with continuous lighting (white light) with an intensity between 7,000-8,000 lux.

Natural seawater without treatment (samples referred to as "Seawater", Table 1), was used as a negative control. In parallel, a positive, highly toxic control was carried out using potassium dichromate (K2Cr2O7) as a reference toxic matrix was established to control the procedure and sensitivity of the test. At the end of the predetermined incubation period, the algal growth of each replicate was determined through light microscope readings [Axioplan 2 Imaging (Zeiss) microscope (Carl Zeiss, Thornwood, NY, USA)].

1.5 Mytilus galloprovincialis *spermiotoxic test*

Specimens of *Mytilus galloprovincialis* are purchased from an authorized dealer and transported to the laboratory dry, wrapped in a damp cloth, inside a thermal container.

Subsequently, the molluscs were cleaned of debris and epibiotic organisms (by manual scraping of the shell), quickly rinsed under running water and housed for
5 days at the "Mesocosmi Laboratory/Plant" of the IRBIM-CNR in Messina (tanks with a volumetric capacity of 100 liters with continuous water flow, temperature of 19±1°C).

After the housing period, the breeding animals of *Mytilus galloprovincialis* (10 organisms) were placed in experimental tanks (volumetric capacity of 25 litres) filled with the different waters under study. Each tank has been equipped with an independent ventilation system. After 3 days of conditioning in experimental systems, gametes were induced to be released by applying a thermal stimulation protocol.

The individuals were placed dry at a temperature of 4°C for 3-4 hours and then transferred to aquarium-type tanks (with a volumetric capacity of 5 liters) containing the

different waters under study at the experimental temperature of 28±1°C (heated using two electric heaters and an aerator to facilitate movement). From the moment almost all the organisms opened the valves and resumed filtration, 30 minutes were waited; after this period, the organisms were transferred to additional tanks (with a volumetric capacity of 5 liters) containing the experimental waters under study at a temperature of 18±1°C. The producing organisms were promptly removed to be placed individually in 250 ml beakers containing 200 ml of experimental water. Subsequently, a total of 100 ml of sperm solution was prepared, obtained from at least two different males, and the mobility of the male gametes was observed under the light microscope.

2. RESULTS

2.1 Bacterial Bioluminescence Test (MicroTox)

Analyses of possible toxicity of the water treated with the Limenet system were carried out by evaluating the bacterial biluminescence test (MicroTox). Inoculums of the bacterium *V. fisheri* were incubated, in accordance with the reference methodology, for 15 and 30 minutes with the matrices under study. As shown in **Table 2**, the evaluation of the reduction in brightness (toxicity index) did not have any of the experimental waters (subjected to different treatments) under study for anyone.

Ecotossicità con *Vibrio fischeri* (Microtox)							
ID campione	**Composizione**	**Descrizione campione**	**Tipo di matrice**	**EC50 15'**	**EC50 30'**	**% effetto campione TOTO a 15'**	**% effetto campione TOTO a 30'**
1	SW	liquido	liquido	>100	>100	no	no
2	3.3	liquido	liquido	>100	>100	no	no
3	6.7	liquido	liquido	>100	>100	no	no
4	13.3	liquido	liquido	>100	>100	no	no
5	26.3	liquido	liquido	>100	>100	no	no
Metodica		UNI EN ISO 11348-3:2019					

Table 2 – Results of the toxicity of *V. fischeri* during exposure (15 and 30 minutes) of treated water at different concentrations (3.3, 6.7, 13.3 and 26.3) with the Limenet system; the negative control was carried out using samples of untreated water (SW).

2.2 Acute Toxicity Test with *Artemia salina*

Raw data from the acute toxicity test with the crustacean *A. salina* are shown in **Table 3 and Figure 3**. As indicated in the "Materials and Methods" section, after appropriate preparation the organisms were exposed (for 24, 48 and 72 hours) to water treated with different concentrations of Limenet product in two different experimental tests (**Figures 4 and 5**). In none of the experiments carried out were any particular variations, intended as an increase in the mortality index, observed at the different experimental times (**Figure 3**).

		SW			3,3			6,7			13,3			26,3	
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
I prova	10	10	10	10	10	10	10	9	9	10	10	10	10	10	10
II prova	10	10	9	10	10	10	10	10	10	10	10	10	10	9	9

Table 3 –Crude results of the viability/mortality of the crustacean *A.salina* during exposure (for 24, 48 and 72 hours) to

different waters treated with the Limenet system. the negative control was carried out using samples of untreated water (SW). Test I made of petri dishes; Test II made of baeker.

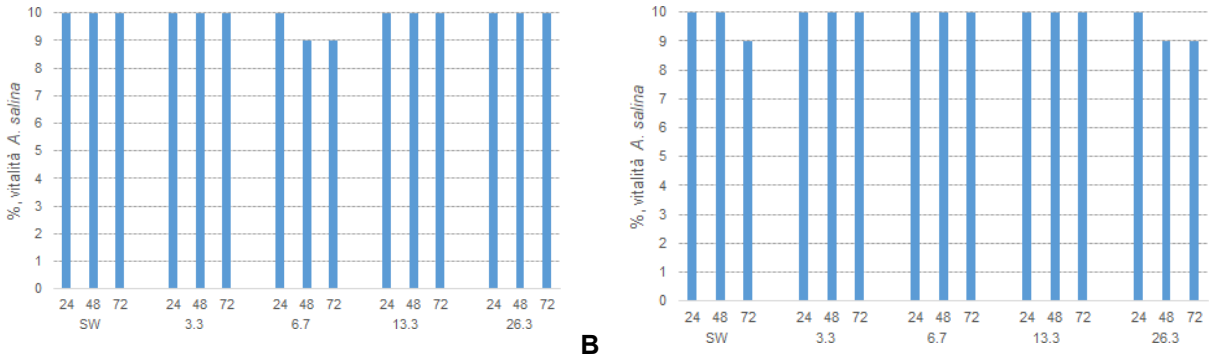

Figure 3 – Raw results of the first (A; made in Petri dishes) and second (B; made in baeker) experiment on the viability/mortality of the crustacean *A. salina* during exposure (for 24, 48 and 72 hours) to the different waters treated with the Limenet system. Data expressed as an absolute percentage. the negative control was carried out using samples of untreated water (SW).

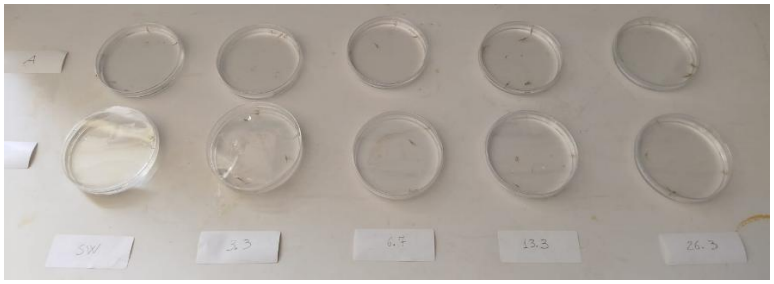

Figure 4 – Image of the experimental systems carried out during the execution of the "I Trial" for the observation of the vitality/mortality index of the crustacean *A. salina* exposed to the waters under analysis.

Figure 5 – Image of the experimental systems carried out during the execution of the "II Test" for the observation of the vitality/mortality index of the crustacean *A. salina* exposed to the waters under analysis.

2.3 Bioassay with *Phaeodactylum tricornutum*

Evaluation of the growth of the diatom *Phaeodactylum tricornutum* was observed by cultivation in mineral soil with the addition of the experimental waters under study. Initial inoculations

(cell concentration of approximately 8 × 103 mL-1 cells) were incubated in multi-well plates for 72 hours under controlled conditions. After the incubation period, a constant cellular increase of about 1 logarithmic unit could be observed for all the experiments under study (**Figure 6**). This increase was uniform both in the control experiments (carried out with untreated seawater) and in the experiments with water subjected to different treatments.

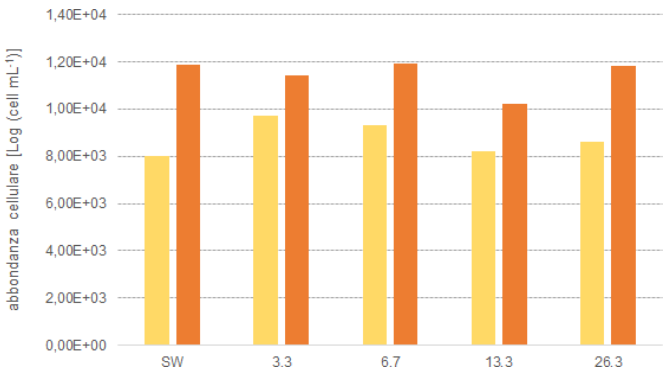

Figure 6 – Evaluation of the growth of *Phaeodactylum tricornutum* during the experimental water exposure tests.

2.4 Spermiotoxic thesis of *Mytilus galloprovincialis*

Following the exposure of mussel specimens (*Mytilus galloprovincialis*; common mussel) to the experimental waters under study, the possible toxic effect of the mussels was evaluated by evaluating the mobility of the male gametes (sperm). In all the conditions observed, sperm motility was not altered, remaining similar to that present in the control experiment (untreated seawater). A value of 100% sperm motility was attributed to all experimental conditions.

3. DISCUSSIONS and CONCLUSIONS

Results obtained from the eco-toxicological tests carried out in this study did not reveal an apparent toxicity of the water treated with the Limenet methodology. As indicated by the protocols of the Higher Institute for Environmental Protection and Research (ISPRA), the eco-toxicological tests have been developed on a battery of target organisms with different evolutionary levels (bacteria, crustaceans, micro-algae and mussels). For none of the chronic toxicity assays carried out in accordance with official protocols, a form of toxicity or total or partial inhibition towards the organisms or gametes under study was observed. The data obtained from the water subjected to Limenet treatment showed the same behavior as samples of untreated water which served as a negative control, thus excluding a toxicity of the same towards the bioindicator organisms in use.

Possible research developments could be oriented towards the realization of a series of molecular and immunohistochemical tests aimed at evaluating the possible genetic response of selected organisms exposed to water after treatment.

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Dr. Simone Cappello

Researcher, IRBIM-CNR Messina Campus

Dr. Maria Genovese

Researcher, IRBIM-CNR Messina Campus