Venezia2021

Programma di ricerca scientifica per una laguna “regolata”

Linea 2.1

Qualità del sedimento lagunare a supporto della sua gestione sostenibile

2.1.3.2

Report finale sullo studio della frazione ultrafine

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1. Introduction

The marine-coastal environment is nowadays constantly subjected to numerous environmental stressors, including the accumulation in sediments of contaminants, and their possible mobilization[1]. This phenomenon may be more pronounced in the fragile ecosystem of Venice lagoon, generally characterized by an irregular bathymetry (average water depth = 1 m), with extensive shallow water areas, where there is a greater probability of sediment resuspension, due to factors of both natural (e.g. wave motion generated by the wind) and anthropogenic (wave motion of boats and fishing activities) origin[2–4]. The role of the sediment is therefore twofold: i) sink for chemical species present in the overlying water column and ii) site of processes that may lead to their release in the water column, both in soluble form and in association with particulate matter. When resuspension events occur, sediment-associated chemicals, including dangerous pollutants, may become available for water column organisms, possibly causing detrimental effects. In shallow water systems as the Venice lagoon, resuspension is known to involve mainly the fine fraction of sediments, with the pollutant-enriched finest particles remaining in suspension for longer times. Moreover, besides acting as possible carriers of pollutants, finest particles themselves could interfere with the vital functions of aquatic organisms, due to the unique properties exhibited by particles below a certain size.

In this perspective, the objective of task 2.1.3.2 was the in-depth study of the finer fraction of the lagoon sediment, in order to understand its role in determining the overall quality of this compartment. Going beyond the usual definition of fine fraction (< 63 µm), the study focused on the finer particles with an average diameter approx. <4 µm, with the aim to disclose the role that the intrinsic physico-chemical properties of the suspended particulate matter - in particular its particle size - may have in influencing its toxicity for target aquatic organisms.

Specifically, task 2.1.3.2 aimed at isolating the finer fraction of the resuspended sediment and to investigate its influence on the overall sediment quality by performing tailored ecotoxicological testing on organisms living in salt water. To this purpose, existing methods have been optimised or new procedures had to be specifically developed, both for particulate phase isolation and ecotoxicity testing, since most previous studies took into account mostly the sediment or the fraction < 63 µm as a whole or referred to the freshwater environment.

2. Experimental design

The study required the development of a novel procedure for post-sampling treatment and for the proper isolation of the finer fractions of the resuspended surface sediment, without altering their properties. The procedure was optimized using artificial marine water (AMW), to obtain suitable fine particle suspensions for ecotoxicity testing with marine organisms. To further investigate the possible toxic effects exerted by fine particles *per se*, surface sediments were also treated by an *ad hoc* procedure aimed at removing the pollutant-bearing phases, followed by the isolation of the finer fractions of the obtained material.
Ecotoxicity assay methods as well had to be adapted and optimized for the application to each particular type of samples.

All the details about the methods used for characterization of sediment fractions and for ecotoxicity testing are reported in the Materials and Methods section.

To meet the task objective, the work has therefore been structured as follows:

1. selection of sampling sites in the Venice lagoon with different environmental characteristics, to collect the surface sediment samples;
2. critical evaluation of current methods and/or protocols available in the literature for the separation of the finer fractions of sediment/soil, and preliminary experiments on lagoon sediment samples;
3. based on the information gathered from the literature and on preliminary results obtained, development of an experimental procedure for post-sampling treatment and for the extraction, separation and characterization of the finer fractions of the resuspended sediment;
4. development of a procedure to remove the major pollutant-bearing phases from the surface sediment and to isolate the finer particle fractions of the obtained material
5. optimization of the methods for ecotoxicity testing on the different types of samples obtained by the developed separation procedures;
6. assessment of acute toxic effects of the all the different isolated fractions by Aliivibrio fischeri (Microtox® test) and further evaluation of the effect of fine particles *per se* by *Acartia tonsa* (larval development test) and by *Mytilus galloprovincialis* (embryotoxicity assay).

2.1 Surface sediment sampling sites

Surface sediment (0-5 cm) was collected at three different sampling sites of the Venice Lagoon (Figure 1) selected on the basis of past and current knowledge on the trophic status, pollution trends and sediment grain-size, as well as on sediment resuspension and sedimentation rates in the lagoon.[1-8] The three selected sampling sites were: 1) San Giuliano (SG, 45° 27’ 46.2” N, 12° 17’ 22.6” E) and 2) Fusina (FU, 45° 24’ 53.4” N, 12° 16’ 15.6” E), which are located closer to the mainland, to rivers inflow and to the industrial area of Porto Marghera, and are thus affected by various anthropogenic sources of pollutants. The SG site is also characterized by reduced water renewal and high macroalgal biomass, while no macroalgal biomass and high water turbulence is observed at the FU site, due to the proximity of Malamocco-Marghera canal, a water way for cargo ships reaching the Porto Marghera industrial zone.[5] These sampling sites were selected also, in accordance with Venezia 2021 line 3.1, on the basis of the very high content of the fine fraction (<63 µm) in sediments. 3) San Nicolò (SN, 45° 26’ 35” N, 12° 23’ 17.3” E), which is instead located close to the Lido inlet, a typical area influenced by its proximity to the Northern Adriatic Sea in terms of pH, salinity, with high water exchange and a higher distance from pollution sources. Sampling was carried out on February 4th and May 25th, 2020.

Figure 1. Location of sampling sites in the Venice lagoon: San Nicolò (SN, 45° 26’ 35” N, 12° 23’ 17.3” E), San Giuliano (SG, 45° 27’ 46.2”N, 12° 17’ 22.6”E) and Fusina (FU, 45° 24’ 53.4” N, 12° 16’ 15.6” E).

2.2 Ecotoxicity assays

The investigation of ecotoxicological impact of the fine fractions of the collected surface collected was conducted by involving the following bioassays: a) bacteria (*Alivibrio fischeri*), b) calanoid copepod (*Acartia tonsa*) and c) mollusk bivalve (*Mytilus galloprovincialis*). The use of organisms from different taxa allow to evaluate the impact of fine particles on the organism in relationship to different evolutive level, complexity of structure and dimension.

The bioluminescence inhibition assay with *A. Fischeri* (Microtox® test)[6] has been extensively used to assess the quality of the lagoon sediment. In this work, since the procedures used to separate the fine sediment fractions allowed to obtain different types of samples (such as dispersions, solutions, dry particles), the toxic effects were evaluated by different procedures of the bioassay, that is the Microtox® Solid-Phase test[6] (modified according to Volpi Ghirardini et al., 1998, 2009)[7,8] and the Whole Effluent Test,[6] depending on the specific sample. As mentioned above, the various procedures have been adapted and modified for the specific fine particle samples obtained in this study. The detailed description of the different ecotoxicological tests employed is reported in the Materials and Methods section.

3. Materials and methods

3.1 Extraction of the finer fractions from the sediment of the Venice lagoon: a literature search

The set up for the mechanical extraction of the finer fractions from sediment of the Venice lagoon was developed based on available methods reported in the literature. The most suitable extraction procedures to obtain the different fractions were selected and a summary of the different methods is reported in the result section. This literature search was performed by querying the Scopus database: natural colloids, surface sediment, size distribution. This analysis identified 5 relevant studies focused on the extraction of the natural colloids from surface sediment.

3.2 Surface sediment characterization

The particles size distribution of the different fractions from the original sample in the water-based solutions (deionized water and artificial marine water, i.e., DW and AMW), obtained after a separation procedure developed *ad hoc*, were calculated by means of laser diffraction techniques. In detail, the laser diffractometer Mastersizer 3000 (Malvern Instruments, Malvern, UK) and the Dynamic Light Scattering (DLS) multi-angle Nicomp ZLS Z3000 (Particle Sizing System, Port Richey, FL, USA) were used. The DLS analysis was performed with an optical fiber set at 90° scattering angle (W = 25 mW and λ = 639 nm) for at least 5 min to obtain a robust average statistic of the aggregate size of the particles. All the results are expressed in volume-based average hydrodynamic diameter and represent the average of at least three independent measurements. Moreover, Scanning Electron Microscopy (Nova NanoSEM 450, FEI, Eindhoven, The Netherlands) coupled with Energy Dispersive X-ray Spectrometry (Bruker Quantax-200 EDS, Bruker Nano GmbH, Berlin, Germany) and X-Ray Powder Diffraction (X'Pert PRO XRPD, Malvern Panalytical Ltd, United Kingdom) techniques were employed to determine the size range, the morphology and the crystalline structure of the surface sediment < 63 µm as well as its sub-fractions.

3.3 Ecotoxicity assays

Alivibrio fischeri - Microtox®

The Microtox® test with *A fischeri* was performed by using lyophilized bacteria, reconstitution solution, cuvettes, filters, and tubes for the Solid Phase Test produced by Modern Water Inc. (New Castle, DE, United States) and purchased from Ecotox LDS srl (Milan, Italy). A Microtox analyzer M500 (Azur Environmental) was used to read light emissions. The reactivation of lyophilized bacteria (Microtox Acute Reagent, Modern Water, Lot No. 17E4123) was carried out by adding ultrapure distilled water (Reconstitution Solution, Modern Water) to the acute reagent vial; the cuvette with the bacterial dispersion was placed in the appropriate analyzer well at 4 °C during the assay time of the biological assay.

For the Solid Phase Test, solid fine fractions obtained from the surface sediment as explained in the next section were dispersed in Artificial Marine Water (AMW)[9] as diluent for evaluating the toxicity of these particles. The Microtox® Solid-Phase test was performed according to the Azur Environmental protocol,[6] following modification of the procedure.[7,8] The test slurry was obtained adding and mixing for 10 minutes an amount of 200 mg of dry particles in 20 ml of the AMW. Two replicate series of seven test tube were prepared adding 1.5 ml diluent solution (AMW) in six tubes for replicate. A 3 ml volume of the resulting solid phase dispersion were introduced in the empty test tubes and from these 1.5 ml was sequentially transferred six time to other tube to dilute the slurry by a factor 2 (1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64). The final slurry volume in tube was 1.5 ml for each dilution and two additional tubes for replicate were used as negative control filling with diluent solution. Test started inoculating 20 µL of bacterial dispersion

into the slurry dilution and control tube. After a 20 minutes incubation time at 15 °C under orbital mixing and 5 minutes without mixing to allow the settling of the solid phase, each dilution of the sample and the controls with bacteria were filtered to separate liquid by solid phase. The liquid phase, then, was transferred to cuvette and light emission was measured in the Microtox analyzer 500 to evaluate inhibition effects due to tested samples and calculate EC_{50} parameter. Actual concentration of suspended solids in the solid phase dispersion was estimated oven drying 1.5 ml of slurry in five replicates. Results were reported as EC_x in $g \cdot L^{-1}$ of solid materials dispersed in AMW.

Furthermore, to test all the different dispersions previously prepared, solid phase test was applied directly to three replicates of dispersion after four dilutions (1:1, 1:2, 1:4, 1:8) without slurry preparation. Each dispersion replicate was further split in two replicates. Results were reported as EC_x in % of original undiluted fine particle suspension.

In addition to the Microtox® test on the dispersions, this assay was carried out also on the resulting solution, after taking out the solid particles by centrifugation. In this case, the test has been performed following the procedure for liquid samples denominated 100% test or WET, Whole Effluent Test.[6] Inhibition of the natural bioluminescence (at 490 nm) of *A. fischeri* has been measured after 30 minutes of exposure at 15 °C to the solution. AMW was used as a negative control and dilution medium. Positive control was performed using a $ZnSO_4$ solution as reference toxicant and following the procedure reported ISO 11348-3 standard method for liquid samples using freeze-dried bacteria.[10]

Acartia tonsa - Larval Development Ratio (LDR)

The larval development test with *A. tonsa* was performed according to the procedure reported in Picone et al.[11,12] but modified and adapted for the exposure to solid particle dispersion. The solid dispersions were not applied directly to the eggs at the start of the test but after the development in nauplii at day-1. Furthermore, in order to maintain in suspension, the solid particles tests were conducted in an Orbital shaker (set to an adequate rotation speed which permitted to maintain dispersion without the damage of organisms). The particles were kept in suspension by orbital movement of the tubes placed in a horizontal position, in such a way as to keep the dispersions in constant oscillation along the axis. All tests were performed in five replicates for five solid particle dispersion concentration (1, 0.5, 0.25, 0.125 and 0.063 gL^{-1}), using the 40 ml glass tubes. Briefly, the test started on day-0 by adding a known number of newly released eggs (up to 30) to a 40 mL glass tube containing 15 mL of 20% saline medium. On day-1, 15 mL of particle dispersion was added to each tube (with the exception of negative control added of fresh medium) and the tubes were posed in agitation on the Orbital Shaker until the end of the assay, after a 24h + 72h of exposure to solid dispersion. Larvae were fed on day-0 and day-1 with 150 µL of a concentrated (> 6×10^4 cell mL^{-1}) mixture of *T. suecica, T. lutea* and *P. lutheri* obtained by centrifuging cultured algae per 5 minutes at 4,000 g. Test vessels were then maintained for four days in a thermostatic room (at 20 ± 1°C, with a 16-h light 8-h dark photoperiod. The test ended on day-4 when approximately 40% of the larvae in negative controls reached the copepodite-I stage. The ratio of nauplii to copepodites was determined staining the tubes content with 0.5-mL of Lugol's solution. The test solution was then filtered through a mixed cellulose ester filter with gridlines (diameter 47-mm, porosity 0.45-µm), and all the larvae and unhatched eggs were counted under a dissecting microscope. Dissolved oxygen (DO) and pH were measured on day-0 in one beaker per each concentration, before the inoculation of the eggs, and on day-5, before staining with Lugol's solution. The 20‰ salinity culture medium was used as a negative control.

Endpoint was evaluated as reduction of the Larval development ratio (LDR). LDR is the ratio between copepodite-I larvae and the total number of early stages (nauplii plus copepodite-I larvae) recovered at the end of the test:

$$LDR = \frac{copepodites}{nauplii + copepodites}$$

Mytilus galloprovincialis - embryotoxicity test

Mature Mediterranean mussels were sampled on unimpacted sites in the Venice Lagoon. Tests were performed according to the procedure reported in Volpi Ghirardini et al.,[13] with some modifications. Briefly, mussels were induced to spawn by thermal stimulation, alternating temperature cycles (30 minutes) at 18 °C and 28 °C. Spawning females were individually placed in 300 mL glass beaker and let to spawn for at least 30 minutes; then the suspension of eggs was filtered through a 100 µm mesh sieve, to remove debris and impurities. Spawning males were all collected in a crystallizing dish and allowed to spawn for 15 minutes before filtering the sperm suspension using a 32 µm mesh sieve. Filtered eggs collected from each female were fertilized with 2 mL of the sperm suspension collected from males, then fertilization was allowed for 2-h. Segmentation status was checked under an inverted microscope (mod. DM-IL, Leica Microsystems, Wetzlar, Germany), and only eggs collected from females with the best fertilization rate were poured into a 1 L graduated cylinder. Zygote density was determined by counting four subsamples of 100 µL under an inverted microscope; fertilized eggs were then added to test fine solid particle dispersion to obtain a final density of 60-70 eggs mL^{-1} in 7 mL of testing solution. Incubation of zygotes was performed in the dark, for 48 h at a temperature of 18 °C. All tests were performed in triplicate for four solid particle dispersion concentration (1, 0.5, 0.25 and 0.125 $g \cdot L^{-1}$), using the 7 ml glass tubes. Larval development was estimated by counting 100 larvae per replicate under an inverted microscope, by distinguishing between normally developed prodissoconch-I larvae (D-shaped) and abnormal or retarded larvae (abnormal prodissoconch I larvae, trochophore larvae, gastrulae).[14] In order to maintain solid particles in suspension, some modifications to the original methods to allow the continue resuspension of material were applied; the tests were performed with two different procedures by using an Orbital Shaker and a customized Overhead Mixer (Rotax, Velp Scientific). The Overhead Mixer was set up at 2 rpm. The results obtained by setting up these two procedures were compared to evaluate the impact of different resuspension dynamics on the larval development.

4. Results

4.1 Separation of the finest fractions of the lagoon sediment

The finest fractions of the resuspended surface sediment were isolated after a deep investigation of the available literature. Therefore, a first step of the research activity was focused on a critical survey of the state of the art through a literature search on the methods for isolating the fine fractions of the sediment. Table 1 summarizes the information collected, divided according to: purpose of the study, investigated matrix, post-sampling matrix treatment, extraction procedure steps and results obtained. In general, after sample drying and sieving, the methods outlined in Table 1 are related to the resuspension of the solid sample in water, followed by sedimentation, agitation and/or mixing, and possibly centrifugation. The duration for each step varies according to the investigation objectives of each study. In addition to the sediment matrix, the literature search was also extended to the methods applied to extract the finer fraction of soil[7–10] in order to obtain further information potentially useful for the purpose of task 2.1.3.2. Since the available procedures for soils are comparable to those described for sediments, both as regards the post-sampling treatment of drying and sieving and for the extraction phases, they were not included in Table 1.

Table 1. Summary of the information collected from the literature about the separation of the fine fraction of the sediment.

Aim	Investigated matrix	Post-sampling matrix treatment	Separation steps	Results	Reference
Isolation and characterization of natural colloidal material from the Fox River (USA)	Surface sediment of the Fox river (USA)	Air drying + sieving at 63 µm	1. Resuspension of 1 g in 1 L water 2. Mixing 3. Settling (2 min) 4. Ultrasonic bath (24 h)	Particle size distribution by laser diffraction: < 2 µm	Tulve and Young 1999[15]
• Characterization of colloids isolated from sediments to determine their dimensional distribution at various depths and to understand changes in electrical conductivity compared to concentration • Study of colloids mobility in unconsolidated sediments	Sediment to a 8.75 m depth of the Mahi river (India)	Air drying + sieving at 2 mm	1. Resuspension of 100 g in 500 ml deionized water 2. Stirring overnight 3. Centrifugation at 25 RCF (15 min)	Particle size distribution by laser diffraction: between 20 and 300 nm	Murali et al.,[16]
Evaluation of the adsorption of organic compounds and toxic metals on isolated colloidal particles and study of the transport property before and after adsorption	Sediment to a 8.75 m depth of the Mahi river (India)	Air drying + sieving at 2 mm	1. Resuspension of 200 g in 500 ml deionized water 2. Stirring overnight 3. Centrifugation overnight at 25 RCF 4. Flocculation with 0.5 M $CaCl_2$ 5. Drying at 30 °C	Particle size distribution by laser diffraction: between 10 and 200 nm	Murali et al.,[17]
Guidance for preparing suspended particulate matter samples in water to perform ecotoxicity tests	Marine dredged material	Sieving at 2 mm	1. Resuspension to sediment-to- unfiltered water from the sample site 1:4 ratio (w/v) 2. Stirring (30 min) 3. Settling (1 h) 4. Siphoned out the supernatant	n.d.	USACE & EPA[18]

Aim	Investigated matrix	Post-sampling matrix treatment	Separation steps	Results	Reference
Characterization of the sediment from the Tresse Est/Tresse Nuovo channel of the Venice lagoon before excavation activities	Surface sediment + suspended particulate matter	1. Washing with deionized water 2. Treatment with H_2O_2 (20 vol) 3. Oven drying (50 °C) 4. Sieving at 1 mm	1. Resuspension (no indication of the solid:liquid ratio) 2. Settling (no indication of time) 3. Siphoned out 10-15 ml 4. Ultrasonic bath before analysis (5 min)	Particle size distribution by laser diffraction: 18% in volume < 4 µm	Final results by DAIS within the APV-Corila agreement[19]

The information collected in Table 1 were used for setting-up an experimental procedure with the dual purpose of:

- extracting as much finer fraction as possible from the samples selected as displayed in Figure 1 (chapter 4);
- altering as little as possible the geomorphological and biological characteristics of the original sample for subsequent ecotoxicological testing.

Taking into account the information summarized in Table 1, a general scheme of the method developed is displayed in Figure 2.

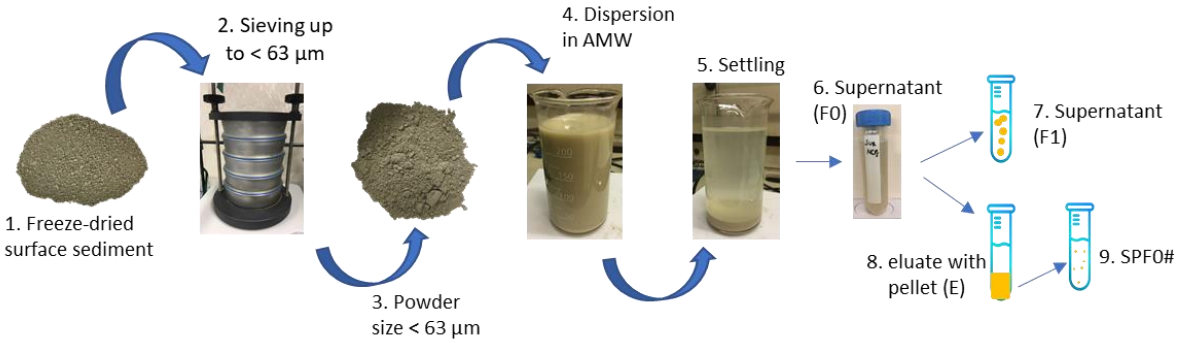

Figure 2. Separation procedure of the finer fraction of the surface sediment: 1) Freeze-drying of surface sediment; 2) Sieving up to < 63 µm; 3) Finer fraction powder with a particles size < 63 µm; 4) Finer fraction dispersion in AMW, stirring for 1h; 5) Settling for 30 min, siphoning out of the supernatant (named F0); 6) F0 dispersion; 7) F1 dispersion, after centrifugation of F0 at 221 relative centrifugal force (RCF) for 8 minutes; 8) Eluate (E), after centrifugation at 12229 relative centrifugal force (RCF) for 10 min, with pellet on the bottom; 9) the pellet was then extracted and resuspended in new AMW and named SPF0#.

In detail, the surface sediment was initially wet sieved with mesh <2 mm to remove the coarse material. The extraction method then involved the freeze-drying of the sample, followed by dry sieving with mesh below 63 µm. Afterwards, in a preliminary version of the extraction method, the sediment fraction <63 µm (more than 90% as determined by weighing) was dispersed in deionized water (DW) at 1:10 (w/v) solid:liquid ratio, mixed for 1 h with a magnetic stirrer and allowed to settle for 1 h. However, whereas the ecotoxicological tests on the finer fraction had to be carried out on target species living in salt water, DW was replaced by artificial marine water (AMW, according to ASTM D1141-98[9]), which required to reduce the sedimentation time from 1 h to 30 min. Indeed, it is recognized from literature that the presence of salts in water leads to a greater destabilization of a colloidal system,[14,20] favoring coagulation, agglomeration and sedimentation processes. As can be seen from Figure 3, the experiments performed in AMW highlighted the occurrence of these processes, with a general increase of agglomerates of more than 2.5 times.

Furthermore, after 30 min sedimentation, the remaining supernatant was siphoned out to obtain a dispersion named F0; then, according to the literature, F0 was centrifuged at 221 relative centrifugal force (RCF) for 8 minutes to obtain a further dispersion with lower size range (named F1) and both the dispersions were characterized by light scattering diffraction techniques for determining the particle size. Finally, F0 was centrifugated at 12229 relative centrifugal force (RCF) for 10 min to obtain the resulting solution (named E) for elemental analysis. Data, expressed as the mean particles size of the F0 and F1 fractions, are reported in Figure 4a and 4c, from which it could be observed the effective difference in particles size of the two fractions ($F0_{mean}$ between 4 and 5 µm and $F1_{mean}$ between 1.5 and 3 µm), without a significative difference from the samples collected in February and May 2020. $F0_{mean}$ data were obtained by Mastersizer 3000, which allowed to characterize particles in dispersions with a diameter ranging from 100 nm to 10000 µm but the amount needed is of the order of magnitude of grams, while $F1_{mean}$ data were obtained by DLS Nicomp Z3000, which can detect particle size between 10 nm up to a few microns, also

with very low numbers of particles in dispersion. Since a much larger amount of F0, compared to F1, was obtained through the separation method developed, a focus on hydrodynamic diameter distributions of only F0 fractions is depicted in Figure 4b and 4d, which showed very similar distributions among the samples collected and between the two periods of sampling, highlighting the goodness of the method developed in terms of the separation efficiency.

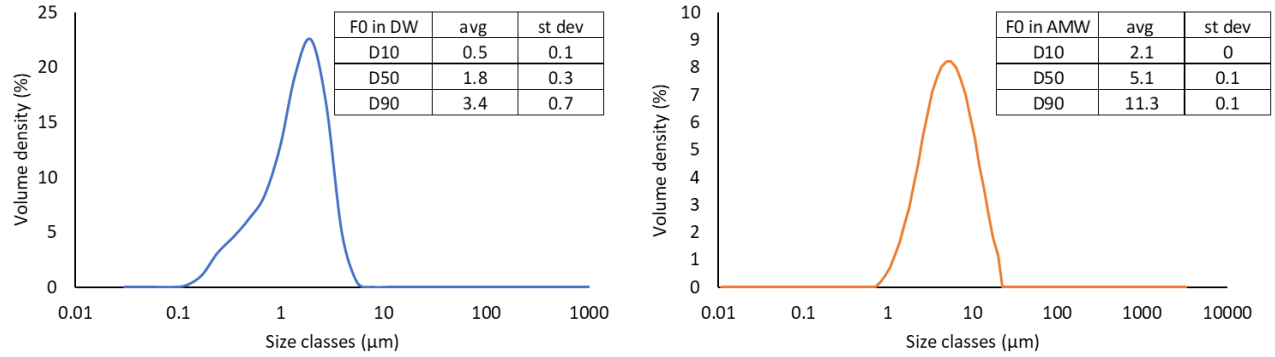

Figure 3. Comparison of hydrodynamic size distribution (by volume) of the finer fraction (F0) extracted from the surface sediment, by applying the extraction method herein proposed in a) deionized water, b) artificial marine water. The data, obtained through the Mastersizer 3000 laser diffractometer, represent the average of three independent measurements. D_{10} = 10th percentile; D_{50} = 50th percentile; D_{90} = 90th percentile.

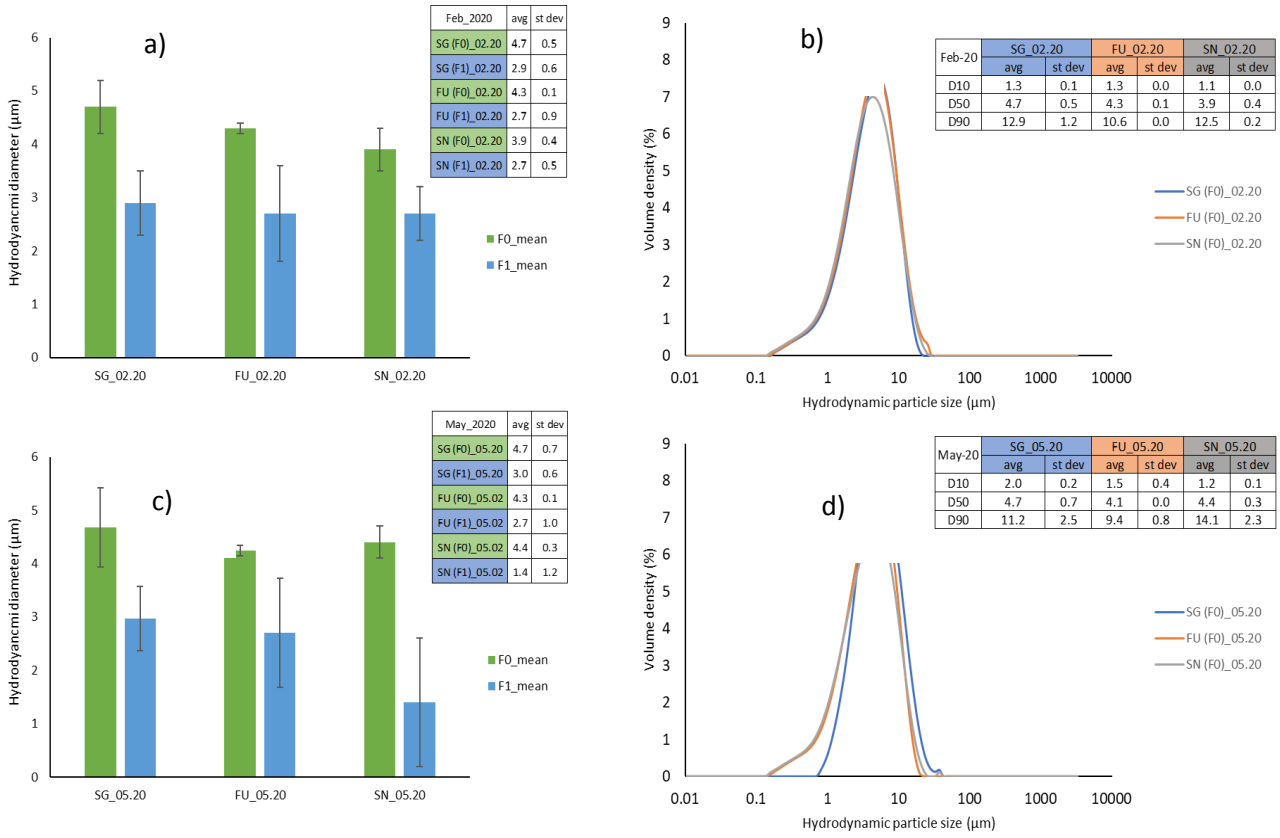

Figure 4 – Comparison of the mean particles size of F0 and F1 fractions (a and c) and of the hydrodynamic diameter distributions of only F0 fractions (b and d). F0 and F1 were obtained from the surface sediment collected at San Giuliano (SG), Fusina (FU) and San Nicolò (SN) monitoring sites on February 4th and May 25th, 2020. D_{10} = 10th percentile; D_{50} = 50th percentile; D_{90} = 90th percentile.

In addition to the colloidal characterization of the samples, the size range, morphology and crystalline structure of the solid fraction < 63 µm, obtained by siphoned out the particles, centrifuged at 6146 g for 15 min and dried overnight at 50 °C, were determined by SEM-EDS and XRD techniques (Figure 5). SEM analysis showed the presence of grains in the size range of few microns, i.e. 1–10 µm, with irregular shapes (some elongated, while others a more squared form). The EDS analysis in Figure 6 revealed a highly inhomogeneous sample, constituted by O (60%) and C (28%) as the main elements, and Mg, Al, Si, K, Ca, Fe with a minor content, ranging from 4.4 to 0.2% (moles percentage, Table 2).

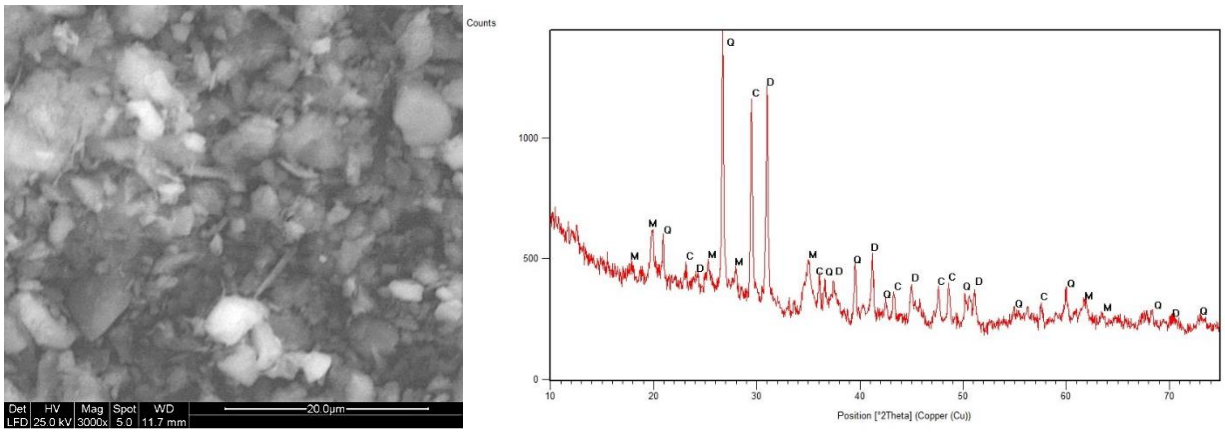

Figure 5. SEM image (left) and XRD spectrum (right) of the fraction < 63 µm. Q = quartz, D = dolomite, C = calcite, M = mixed silicate.

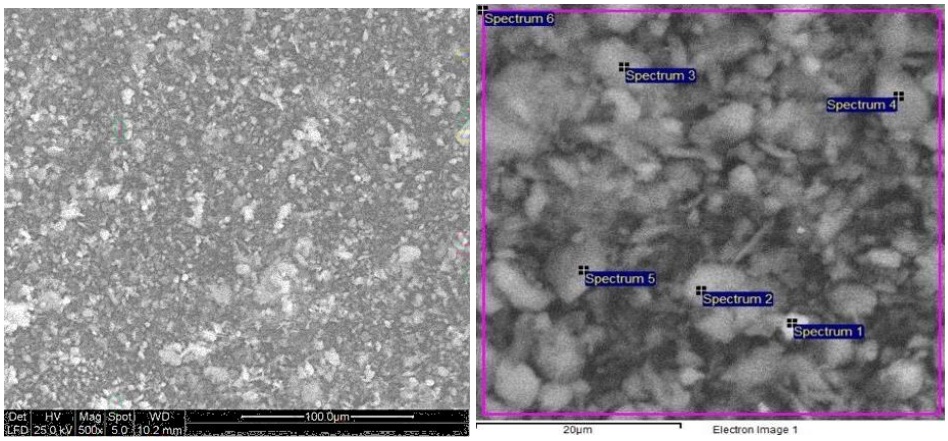

Figure 6. SEM micrographs of the fraction < 63 µm (left) and map of the EDS analysis (right). The length of the particles was between 2.3 and 6.2 µm, with an average of 4.2 ± 1.3 µm, while the width was between 1.7 and 5.7 µm, with an average of 3.2 ± 1.3 µm

Table 2. Normalized moles percentages of elements detected in the fraction < 63 µm by SEM-EDS.

	C (%)	O (%)	Mg (%)	Al (%)	Si (%)	K (%)	Ca (%)	Fe (%)
Sectrum 1	27.1	60.5	1.6	2.1	3.7	0.2	0.9	4.0
Spectrum 2	27.1	61.8	1.0	1.1	2.5	0.2	6.2	0.1
Spectrum 3	21.4	63.6	0.8	1.8	11.0	0.4	0.8	0.2
Spectrum 4	25.7	60.1	6.0	0.9	2.1	0.1	4.9	0.2
Spectrum 5	30.9	55.8	5.1	1.1	2.2	0.1	4.5	0.2
Spectrum 6	33.3	55.4	1.3	2.0	5.0	0.4	2.2	0.4
Average	**27.6 ± 4.1**	**59.5 ± 3.3**	**2.6 ± 2.3**	**1.5 ± 0.5**	**4.4 ± 3.4**	**0.2 ± 0.1**	**3.3 ± 2.3**	**0.9 ± 1.5**

4.2 Ecotoxicity assessment of the finest fractions of the lagoon sediment

The ecotoxicity of four different types of sub-samples obtained from the surface sediment samples collected on February 4th and May 25th 2020, was assessed using *A. fischeri*. According to the method proposed in Figure 2, starting from the original freeze-dried sample, sieved at 63 µm and dispersed in AMW in a ratio of 1:10 g/ml, the investigated fractions were as follows:

- fraction isolated according to the extraction method in Figure 2, corresponding to the supernatant (F0)
- fraction isolated from F0, according to the extraction method in Figure 2, after centrifugation at 221 rcf for 8 min (named F1)
- eluate, containing only the liquid phase, after centrifugation of F0 at 12298 rcf for 10 min (named "E")
- solid fraction isolated after centrifugation of F0 at 12298 rcf for 10 min and re-dispersed in fresh AMW with the same initial volume (named "SPF0#")

The ecotoxicity of each fraction isolated was investigated for F0, F1 and SPF0# on *A. fischeri* according to the Microtox® Solid Phase protocol,[6] and for E by the 100% test protocol (WET or Whole effluent toxicity)[6].

The inhibition of bioluminescence curves (effect %) at different concentrations for the different fractions obtained are reported in Figure 7 (for F0), Figure 8 (for F1), Figure 9 (for E) and in Figure 10 (for SPF0#). The bioassays were carried out on three fine-dispersion replicates, obtained performing three time the fine fraction separation procedure to different aliquots of the same sample. The obtained dispersion samples were divided into two further replicates. The bioassay results represented the average of 6 replicas.

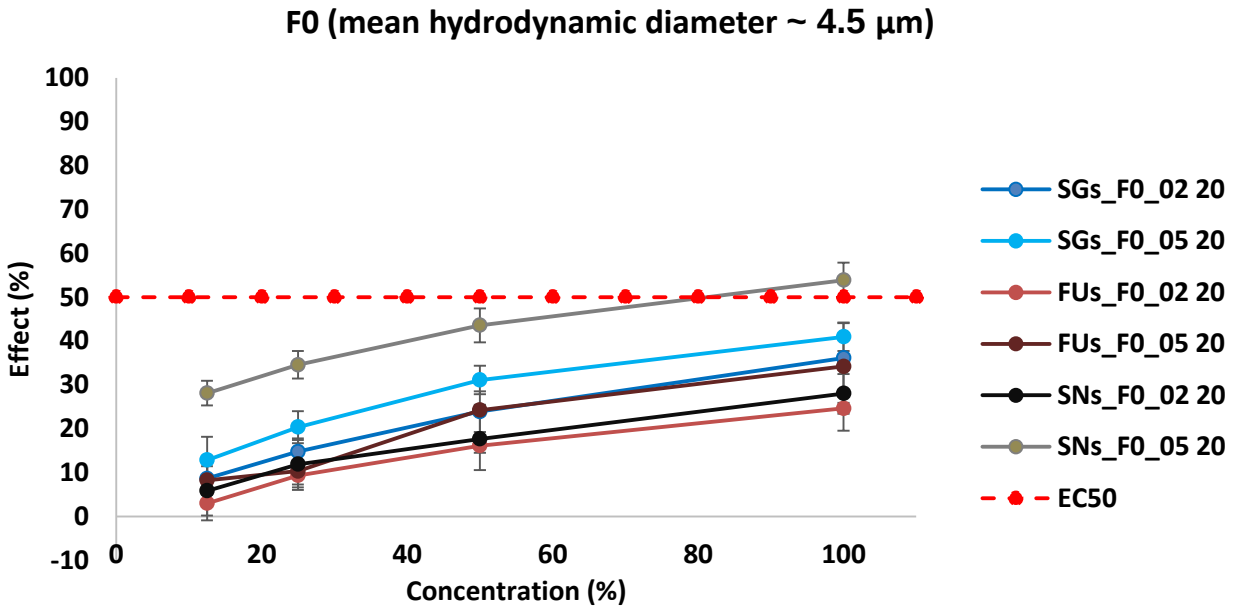

Figure 7. Inhibition of bioluminescence (effect %) of the F0 fraction at different concentrations tested (i.e., 100% of the initial concentration, 50%, 25% and 12.5%), corresponding to the sampling of the February 4th and May 25th 2020, for each monitoring site.

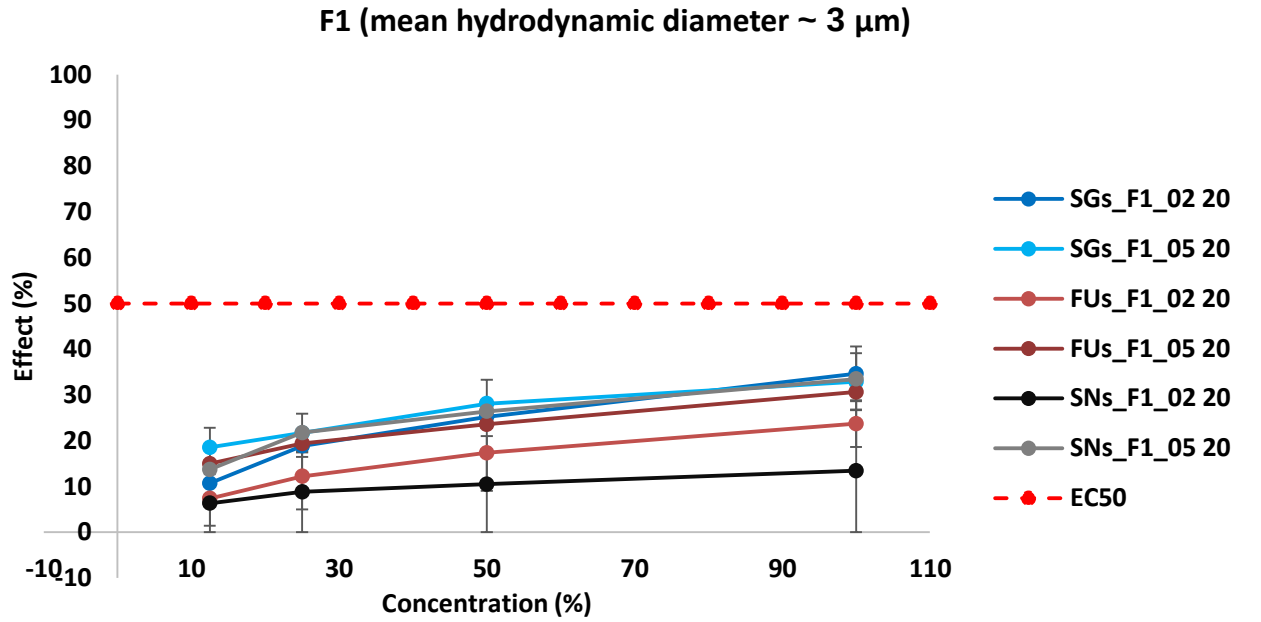

Figure 8. Inhibition of bioluminescence (effect %) of the F1 fraction at different concentrations tested (i.e., 100% of the initial concentration, 50%, 25% and 12.5%), corresponding to the sampling of the February 4th and May 25th 2020, for each monitoring site.

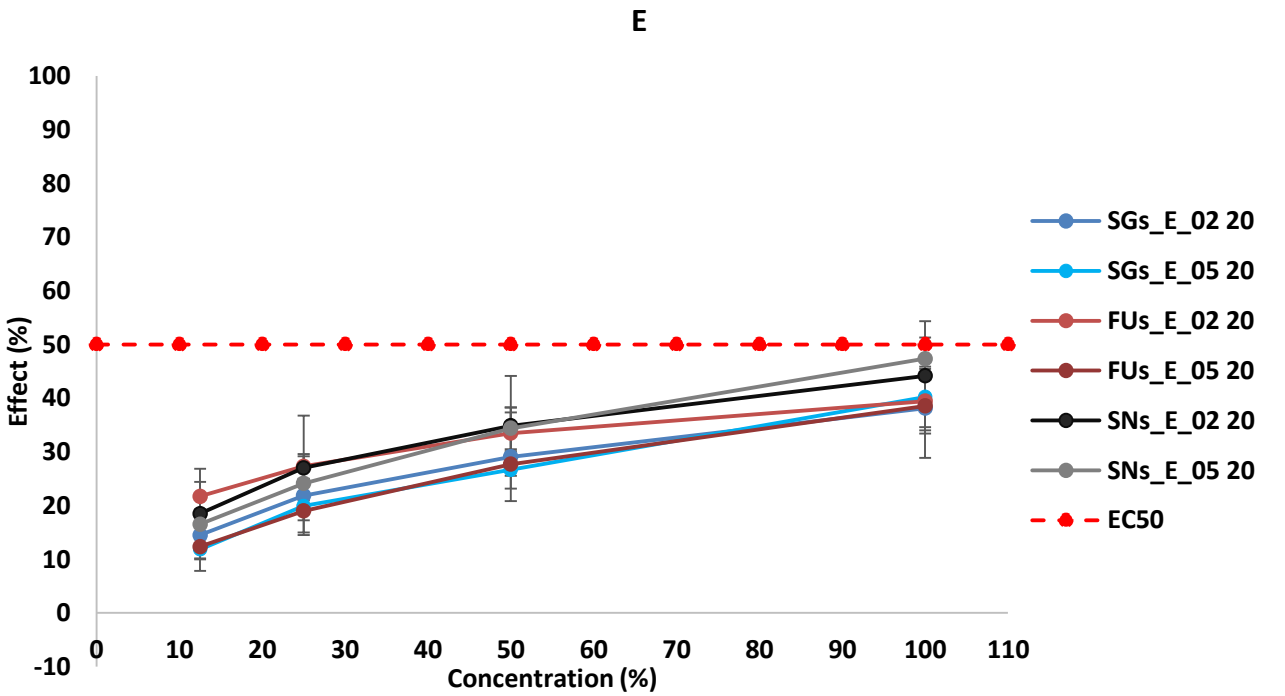

Figure 9. Inhibition of bioluminescence (effect %) of the E fraction at different concentrations tested (i.e., 100% of the initial concentration, 50%, 25% and 12.5%), corresponding to the sampling of the February 4th and May 25th 2020, for each monitoring site.

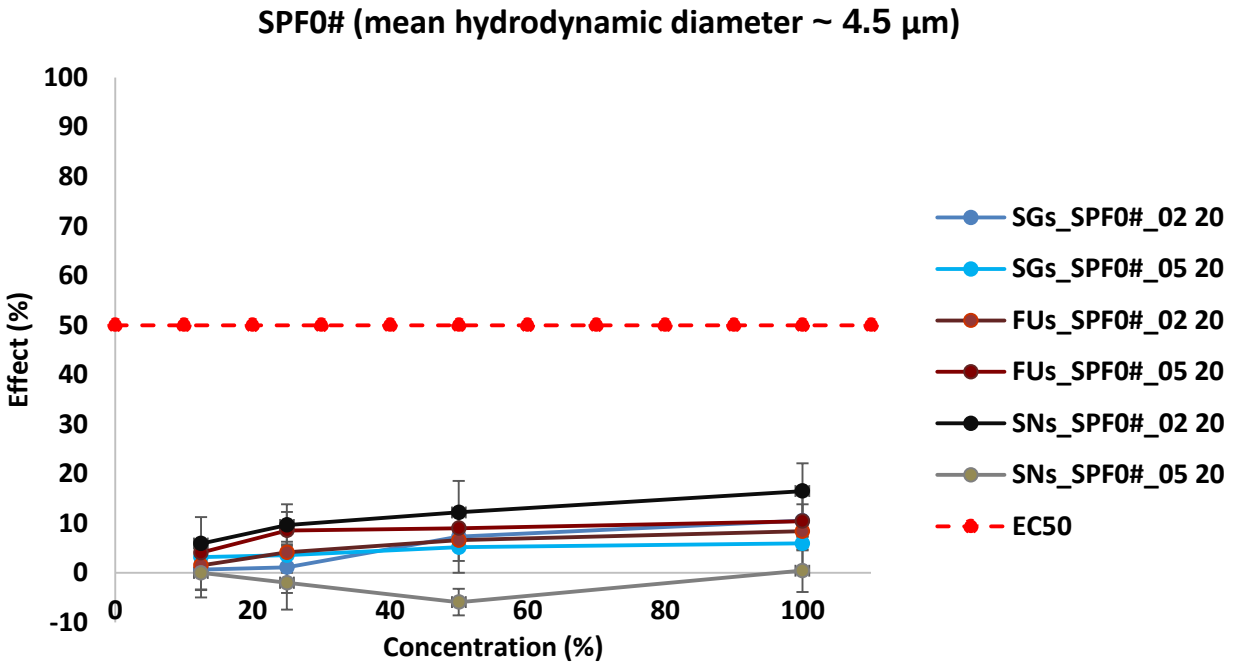

Figure 10. Inhibition of bioluminescence (effect %) of the SPF0# fraction (i.e., isolated F0 by centrifugation and further dispersion in fresh AMW) at different concentrations tested (i.e., 100% of the initial concentration, 50%, 25% and 12.5%), corresponding to the sampling of the February 4th and May 25th 2020, for each monitoring site.

In general, these data showed that the suspended particles (contained in F0, F1 and SPF0#, but not in E) would provide a modest contribution to the total inhibition. Indeed, the only EC_{50} exceeding value corresponded to the F0 fraction of San Nicolò (SNs_F0_05 20). However, the trends displayed in Figures 7-10, with higher effect % for E with respect to the other fractions tested, could suggest that the adverse effect occurred would mainly be due to contaminants present in the liquid phase. An alternative way of displaying these data is depicted in Figures 11 and 12, by which the comparison of the inhibitory effects on the luminescence of *A. fischeri* with the same percentage concentration between the fractions F0 and E and between the fractions F0 and SPF0# is highlighted. The graphs on the right of both Figures clearly highlighted that the solid phase re-dispersed in fresh AWM (i.e., SPF0#) did not show any adverse effect. This may be due both to a low concentration of solid particles in F0 and F1, below the dosage needed to severely inhibit the luminescence of *A. fischeri*, as well as to a release of contaminants from these solid particles, which migrated into the liquid phase. Indeed, the graphs on the left side showed that the samples (empty dots) are almost always above the red dashed line, which corresponds to equivalent inhibitory effects. To better discriminate whether the size of the finer fraction would be the main responsible of the bioluminescence inhibition, a further investigation has been carried out and described in the next paragraph, by testing contaminants-free finer particles.

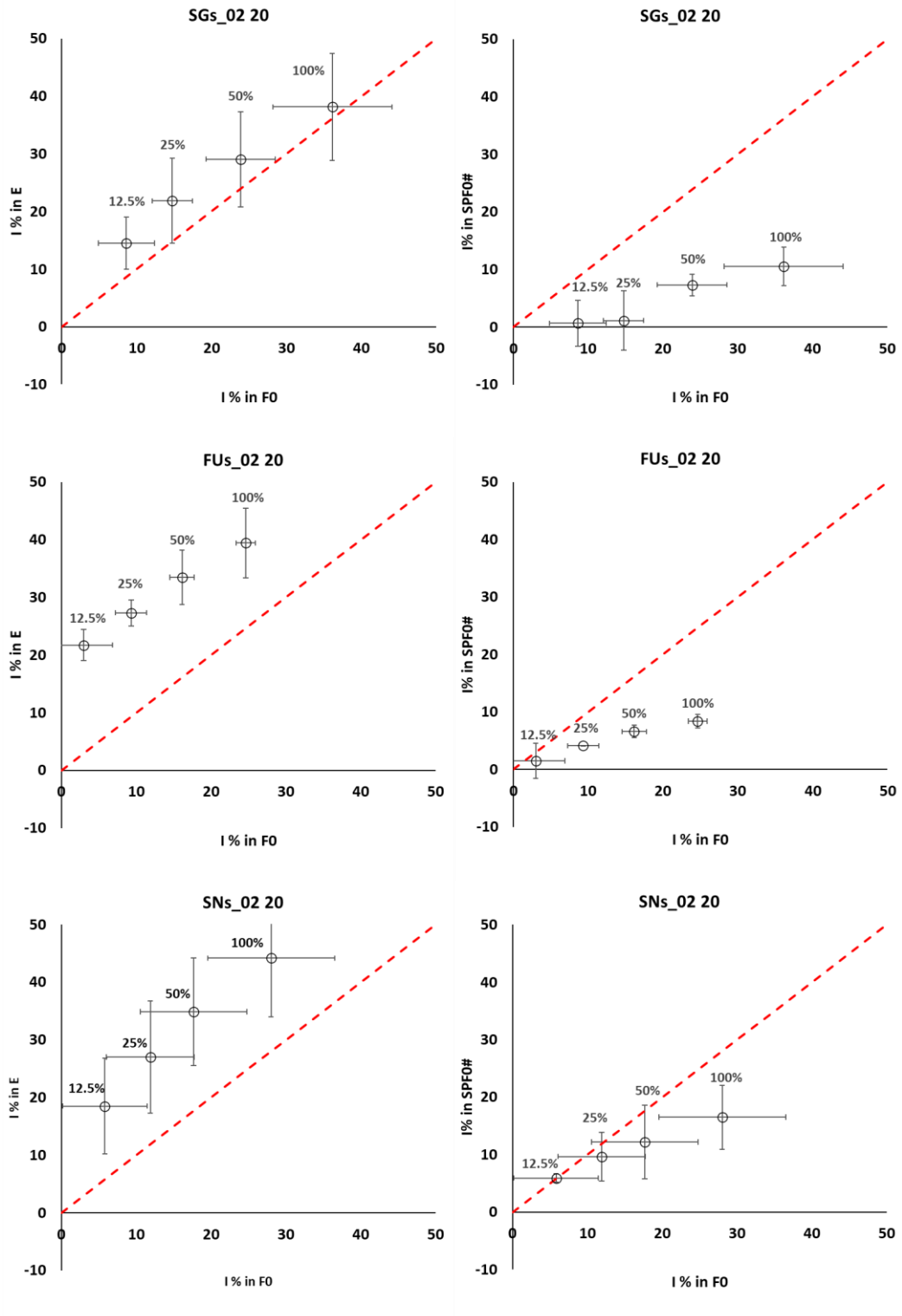

Figure 11. Comparison of inhibitory effects at the same concentration (as percentage), for the F0 fractions (supernatant) vs. E (eluate from centrifugation of the supernatant) on the left side and for F0 vs. SPF0# on the right side. The red dashed line indicates equivalent inhibitory effects and allows for easier visual comparison. The data is referred to the samples collected on February 4th 2020.

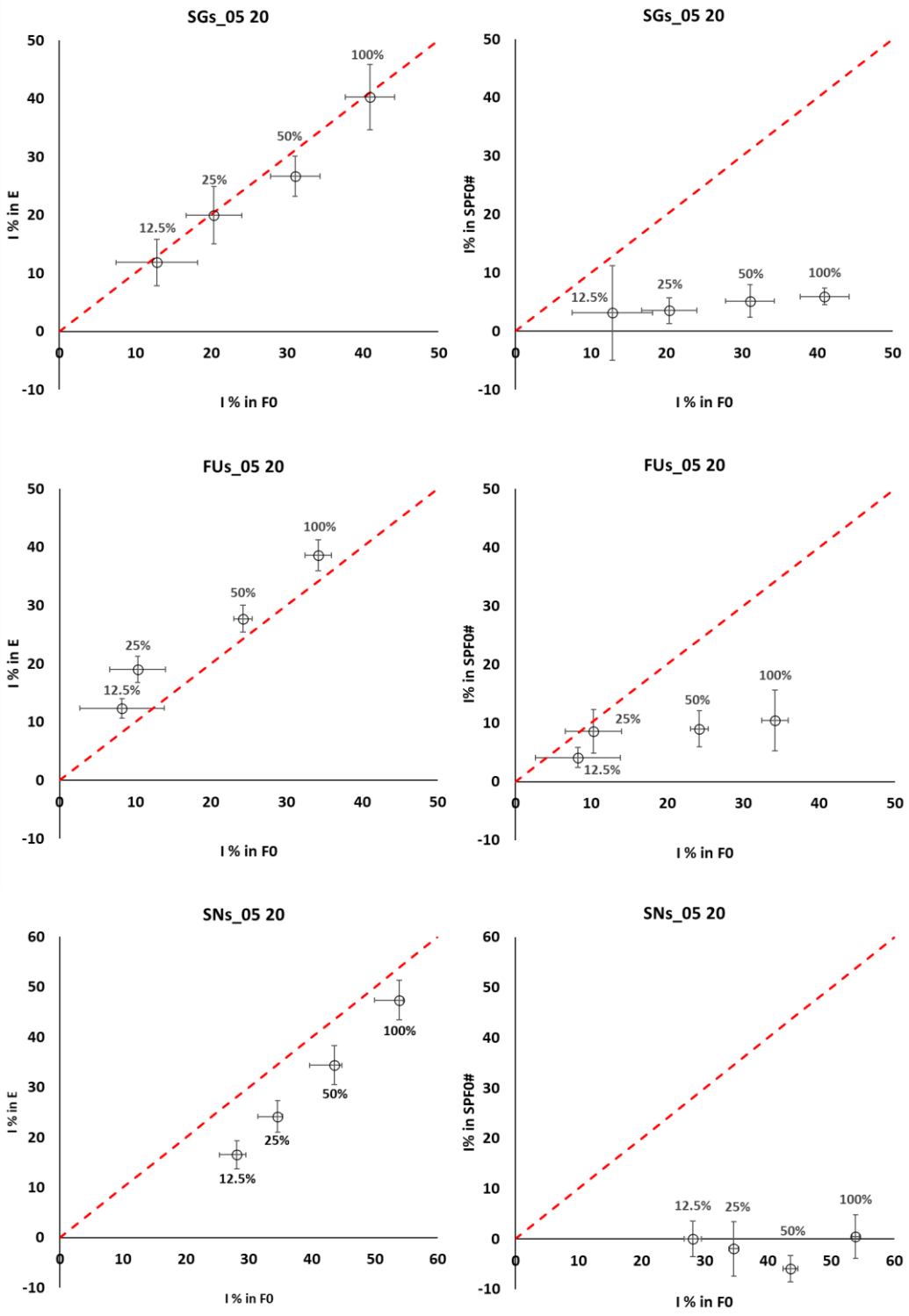

Figure 12. Comparison of inhibitory effects at the same concentration (as percentage), for the F0 fractions (supernatant) vs. E (eluate from centrifugation of the supernatant) on the left side and for F0 vs. SPF0# on the right side. The red dashed line indicates equivalent inhibitory effects and allows for easier visual comparison. The data is referred to the samples collected on May 25th 2020.

4.3 Ecotoxicity assessment of the contaminants-free finer particles

This specific research activity focused on developing a procedure to obtain a mostly mineralogical material from the surface sediment collected as displayed in Figure 1, for a potential comparison with solid suspended particles to better highlight the role of particle size in inducing adverse effects on targeted organisms. One of the main objectives was to obtain, from the original samples, a suitable amount of the finer fraction, needed for the dosage of ecotoxicological tests. To achieve this aim, this activity included a treatment of the original surface sediment samples with an *ad hoc* procedure, followed by the isolation of different fine fractions, which should present a similar size range (4-5 µm as mean) of the fractions obtained for the method in Figure 2 (i.e., F0, F1 and SPF0#).

Surface sediment treatment

As a first step, the surface sediment collected at San Giuliano and Fusina monitoring sites underwent different steps as displayed in Figure 13. San Nicolò site was not considered due to the low amount of the fraction <63 µm compared to the total (about 2% by weight of the total), which would have required the treatment of huge quantities of sediment as it is. The sample treatment involved the dispersion of the original surface sediment sample as it is (ratio of approx. 100 g per liter of solution) in: 1) deionized water; 2) hexametaphosphate + $NaCO_3$ solution (respectively 35 g and 7 g per liter); 3) 0.5 M NaOH solution; 4) Technical acetone; 5) 20% H_2O_2 solution and 6) 0.5 M Na-EDTA solution and 0.5 M citric acid, buffered at pH 6. This pre-treatment approach was conceptualized and adopted with the aim to obtain a progressive removal of the interstitial fluid (steps 1 and 2, with use of hexametaphosphate to enhance the sediment particles dispersion), the organic phase (steps 3, 4 and 5) and charged elements and compounds. After these steps, the material underwent a final washing with distilled water. Furthermore, a commercially available diatomaceous earth was involved in the same treatment procedure and used for the following application together with the other obtained materials (FU and SG). The diatomaceous earth used was a food grade product, DiatomPest® (Diatom Retail LTD, Leicester, UK), with certified approval for use in feed and organic systems (Food Standards Agency, UK; EU Feed Additive) in order to test a potentially uncontaminated material (<0.1% of crystalline silica declared). According to the manufacturer, this material has a mean composition of 80% to over 90% silica (SiO_2), 2%-4% of alumina (principally clay minerals) and 0.5%-2% of hematite.[20] Preparation of fine fraction of diatomaceous earth in relevant amount allowed, moreover, to have enough test materials for the set up and explorative trials of the bioassays. In addition, also a well grain-size characterized kaolin, cleaned by NaOH washing procedure, was adopted for comparison purposes, as suggested in Badetti et al.[21]

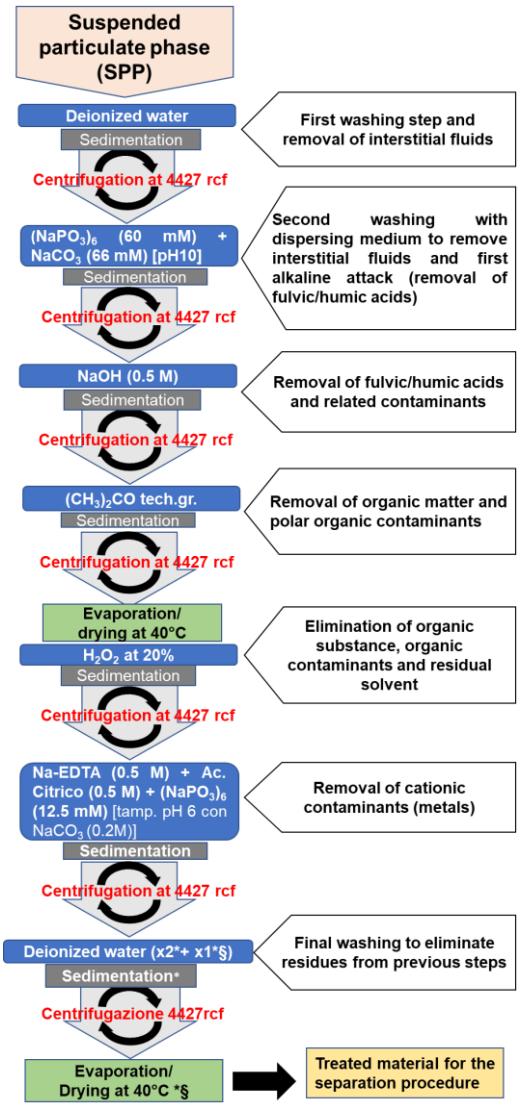

Figure 13. Steps of the treatment for food-grade diatomaceous earth and for the surface sediment collected from the Venice lagoon.

Separation of treated surface sediment in different grain-size sub-samples

After the sample treatment procedure, as displayed in Figure 14, the material was isolated from the water-based solution with the following steps:

1. dispersion and mixing of the treated material in deionized water in a coupled cylinder container equipped with taps at different heights;
2. recovery of the unsettled materials after 1 h from dispersion by sieving at 20 µm of the solids remaining in suspension (Ø < 20 µm - Light Fraction, LF);
3. preparation of the Light fraction as dry powder by means of centrifugation and drying of LF at temperature < 50 °C;
4. recovery of the settled fraction after 1 h and processing of recovered materials and further washing to separate further two different grain-size fractions by wet sieving on 20 µm. Recovery of the sieve-passing fraction after centrifugation and drying at temperatures below 50 °C (Ø < 20 µm - Heavy Fraction, HF);
5. recovery of the over screen fraction and drying at temperatures below 50 °C (Ø > 20µm – Silt and Sand, SS).

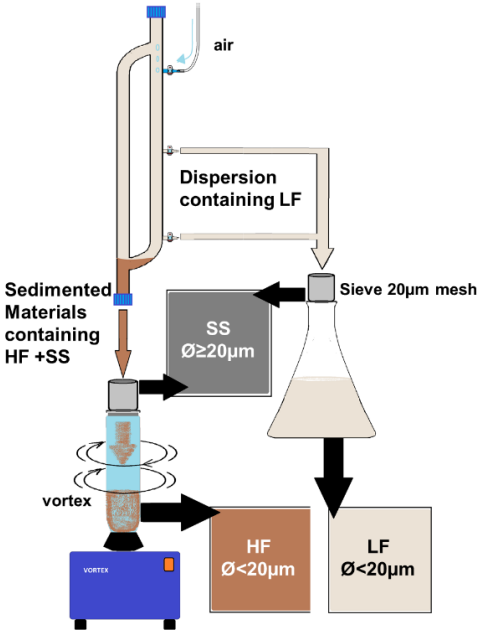

Figure 14. Scheme of the procedure used to isolate the fractions from the solution.

The actual isolation of the finer fractions from the original samples has been verified by laser diffraction via the MasterSizer 3000 laser granulometer of both wet sieved samples at 20 µm and diatomaceous earth. The results are shown in Figure 15.

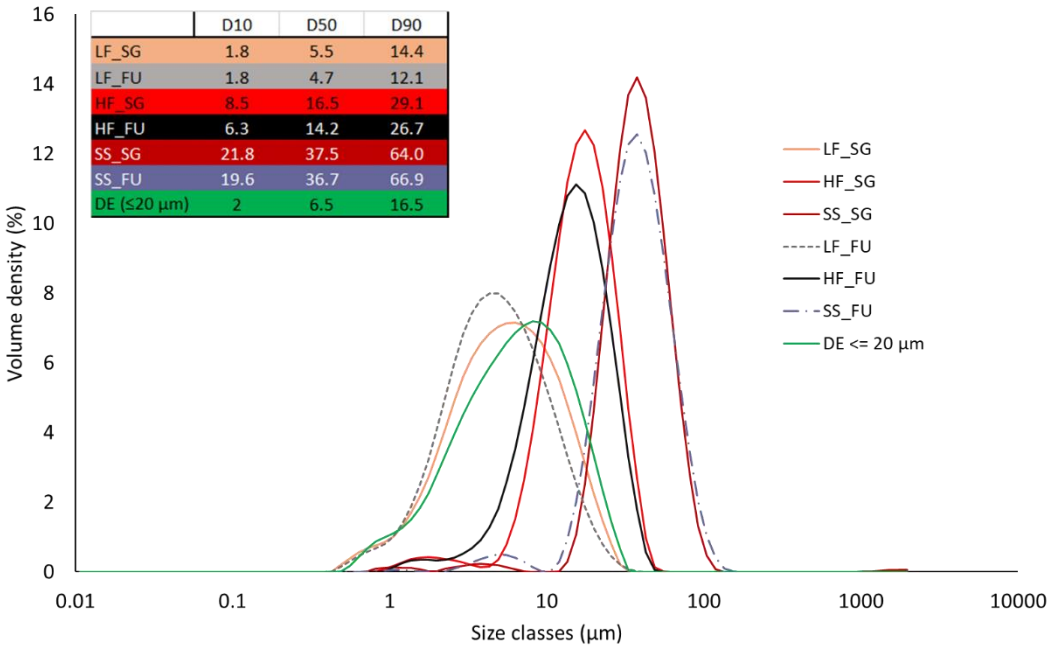

Figure 15. Volume-based size distribution of the treated fractions of the surface sediment samples collected at San Giuliano (SG) and Fusina (FU) sieved at 20 µm and of the food-grade diatomaceous earth (DE).

The results in Figure 15 showed that LF presented a hydrodynamic diameter distribution comparable to the F0 fraction (data shown for comparison in Figure 4d), obtained by separation method developed, while HF showed bigger size values. The size distributions data of SS fractions confirmed that, for both the samples collected at San Giuliano and Fusina, actually only 10% of the population (D10) is lower than 20 µm. Finally, the diatomaceous earth sample showed a dimensional distribution between 1.96 (10^{th} percentile) and 16.5

µm (90th percentile), therefore comparable to the dimensional distributions of LF and the fraction F0, thus allowing its use as a potential reference sample for subsequent ecotoxicity tests.

Physicochemical characterization of sub-samples

The finest (LF) and the largest fractions (SS) obtained through the method showed in Figure 14 have been characterized by scanning electron microscopy (SEM) and by X-ray diffraction (XRD) to evaluate, respectively, the effective separation in terms of size as well as if and how a structural modification of the various fractions took place, after following the treatment steps described in Figure 13. The SEM images of the different fractions from the surface sediment collected at San Giuliano and Fusina, as well as the diatomaceous earth, are shown in Figures 16-17-18. A clear separation in terms of size range can be observed by comparing Figure 16a (SG_LF) with Figures 14b and 14c (SG_SS), as well as Figure 17a (FU_LF) with Figures 17b and 17c (FU_SS). Moreover, Figures 18a and 18b confirmed the size range of the diatomaceous earth, which is similar to that of the finer fractions LF obtained through the method in Figure 14.

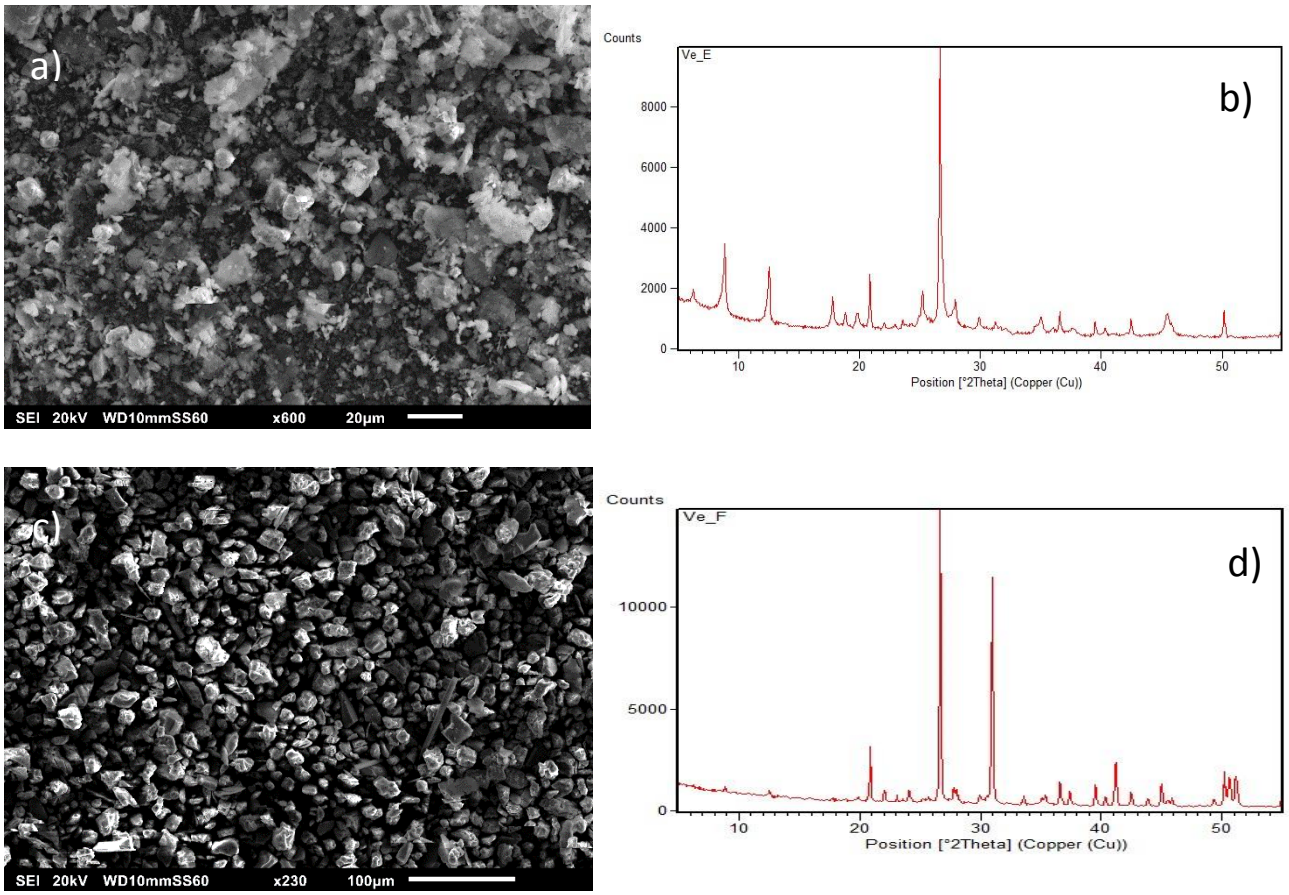

Figure 16. SEM images and XRD spectra of San Giuliano (SG) samples: a) SEM image of LF (Ø < 20 µm); b) XRD spectrum of LF; c) SEM image of the largest fraction SS (Ø > 20 µm); d) XRD spectrum of SS.

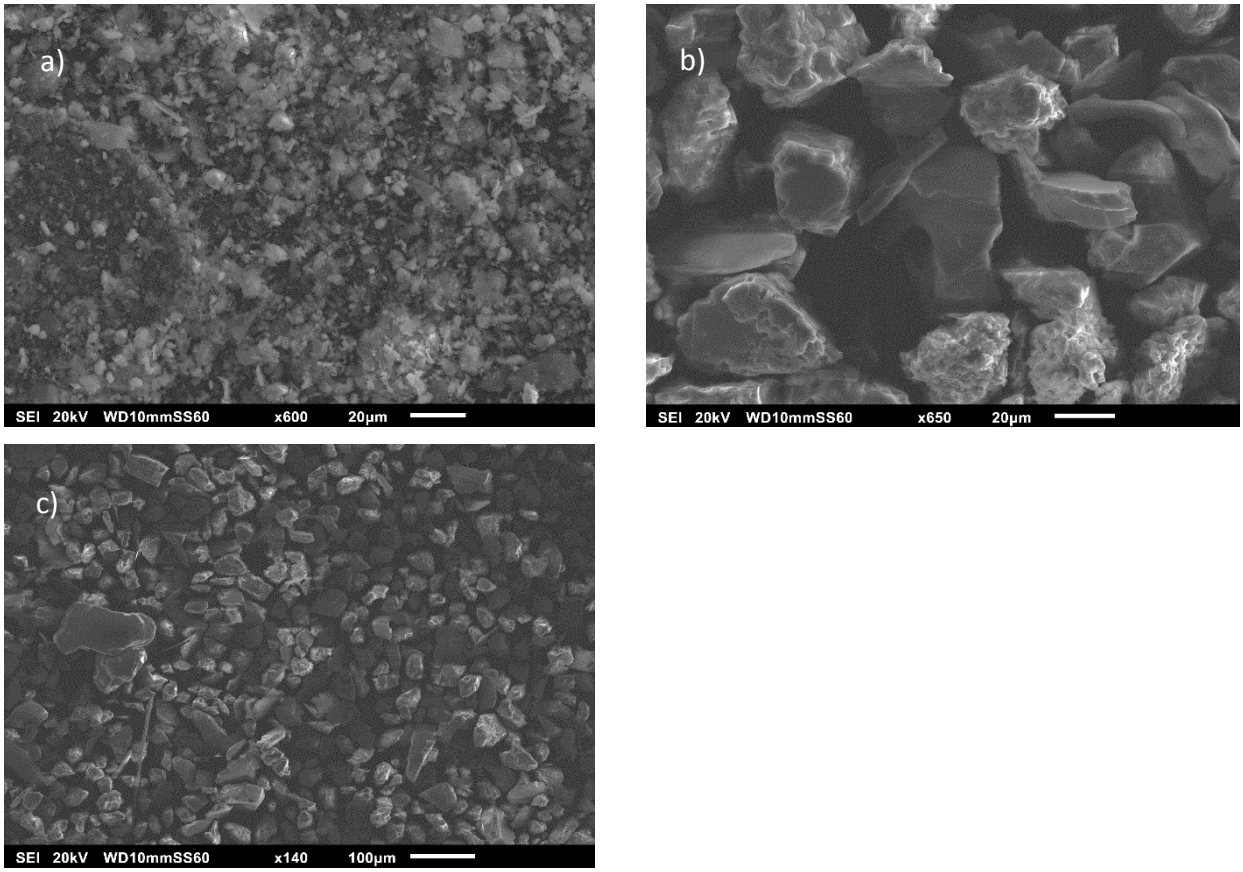

Figure 17. SEM images of a) LF (Ø < 20 µm) and of b-c) largest fraction SS (Ø > 20 µm) at two different magnifications, both from Fusina (FU) monitoring site.

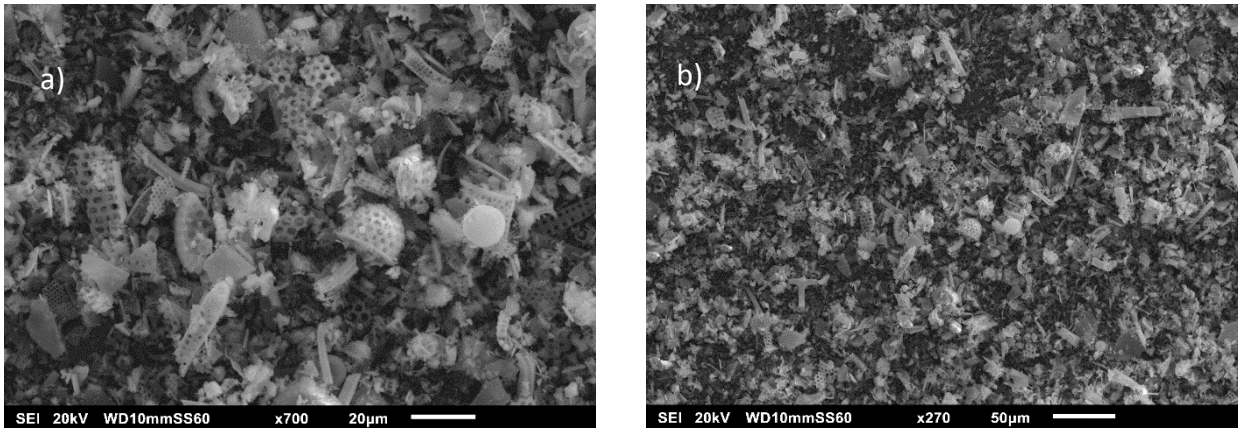

Figure 18. SEM images of the food grade diatomaceous earth (DE) at a) 700x and b) 270x.

Ecotoxicity assays of the treated finer fractions of the lagoon sediment

The ecotoxicity of the different fractions of the treated material, obtained from the original surface sediment samples of the Venice lagoon, was assessed on *A. fischeri, A. tonsa* and *Mytilus galloprovincialis*. Methods and protocols applied are described above. The results from the *A. fischeri and A. tonsa* assays are summarized in Table 3, showing the EC_{20} and EC_{50} values ($g \cdot L^{-1}$). Data on *Mytilus galloprovincialis* (Table 4) are only partial due to the high complexity of the bioassay design. To help in reading, the results of *A. fischeri* and *A. tonsa* are also depicted in Figure 19 and Figure 20.

Table 3. Summary of the results of the particle size of the different dispersed fractions in AMW and the relative EC_{20} and EC_{50} values, obtained by means of the toxicity tests conducted with *A. fischeri* and *A. tonsa*. D_{10}, D_{50} and D_{90} refer to the 10^{th}, 50^{th} and 90^{th} percentiles of the size distribution. Food-grade diatomaceous earth (DE) and kaolinite (KA) were used as reference materials for the bioassays; SG = sample collected at the San Giuliano monitoring site; FU = sample collected at the Fusina monitoring site. The value > 1 refers to an undetected toxicological effect, using 1 $g \cdot L^{-1}$ as the maximum dose for LDR tests with *A. tonsa*.

Parameters / tested organisms	**Metrics**	**DE**	**KA**	**SG**			**FU**		
		<20 µm	**<5 µm**	**LF (<20 µm)**	**HF (<20 µm)**	**SS (20≥ µm)**	**LF (<20 µm)**	**HF (<20 µm)**	**SS (20≥ µm)**
Hydrodynamic diameter distribution	D10-**D50**-D90 **(µm)**	2.0-**6.5**-16.5	**1.5**±0.2	1.9-**5.5**-14.4	8.5-**16.5**-29.1	21.8-**37.5**-64.0	1.8-**4.7**-12.1	6.3-**14.2**-26.7	19.6-**36.7**-66.9
Alivibrio fischeri (Microtox Solid Phase Test)	**EC20 (g/L)**	**0.39** (0.35-0.43)	**0.56** (0.51-0.61)	**0.52** (0.46-0.60)	**10.20** (8.85-11.7)	**24.35** (23-25.8)	**0.50** (0.44-0.57)	**7.71** (7.33-8.11)	**12.0** 11.4 12.7
	EC50 (g/L)	**1.01** (0.94-1.09)	**1.49** (1.39-1.61)	**1.28** (1.15-1.41)	**35.01** (30.3-40.6)	**69.40** (64.5-74.6)	**2.16** (1.96-2.38)	**25.10** (23.4-27.0)	**28.6** 27.3 29.9
Acartia tonsa (Larval Development Rate)	**EC20 (g/L)**	**0.12** (0.06-0.24)	**0.21** (0.11-0.40)	**0.22** (0.01-0.65)	**>1**	**>1**	**0.51** (0.41-0.62)	**0.25** (0.08-0.82)	**>1** - -
	EC50 (g/L)	**0.24** (0.17-0.34)	**0.26** (0.21-0.33)	**0.24** (0.12-0.47)	**>1**	**>1**	**0.65** (0.53-0.79)	**0.59** (0.29-1.19)	**>1** - -

Table 4. Summary of the results of the particle size of the different dispersed fractions in AMW and the relative EC_{20} and EC_{50} values, obtained by means of the toxicity tests conducted with *Mytilus galloprovincialis*. D_{10}, D_{50} and D_{90} refer to the 10^{th}, 50^{th} and 90^{th} percentiles of the size distribution. Food-grade diatomaceous earth (DE) and kaolinite (KA) were used as reference materials for the bioassays; SG = sample collected at the San Giuliano monitoring site; FU = sample collected at the Fusina monitoring site. The samples with > 1 refer to an undetected ecotoxicological effect, considering 1 $g \cdot L^{-1}$ as the maximum dose for LDR tests with *M. galloprovincialis*.

Parameters / tested organisms	**Metrics**	**DE**	**KA**	**SG**			**FU**		
		<20 µm	**<5 µm**	**LF (<20 µm)**	**HF (<20 µm)**	**SS (20≥ µm)**	**LF (<20 µm)**	**HF (<20 µm)**	**SS (20≥ µm)**
Hydrodynamic diameter distribution	D10-**D50**-D90 **(µm)**	2.0-**6.5**-16.5	**1.5**±0.2	1.9-**5.5**-14.4	8.5-**16.5**-29.1	21.8-**37.5**-64.0	1.8-**4.7**-12.1	6.3-**14.2**-26.7	19.6-**36.7**-66.9
***Mytilus galloprovincialis* - Embriotoxicity (Orbital Shaker)**	**EC20 (g/L)**	**0.21** (0.16-0.28)	**>1**	**0.21** (0.14-0.32)			**0.49 (0.38-0.63)**		
	EC50 (g/L)	**0.86** (0.72-1.02)	**>1**	**0.56** (0.43-0.74)			**1.17 (0.94-1.46)**		
***Mytilus galloprovincialis* - Embriotoxicity (Overhead Mixer[Rotax])**	**EC20 (g/L)**	**0.005** (0.001-0.190)		**0.03** (0.02-0.06)			**0.02**		
	EC50 (g/L)	**0.36** (0.16-0.79)		**0.16** (0.12-0.21)			**0.11**		

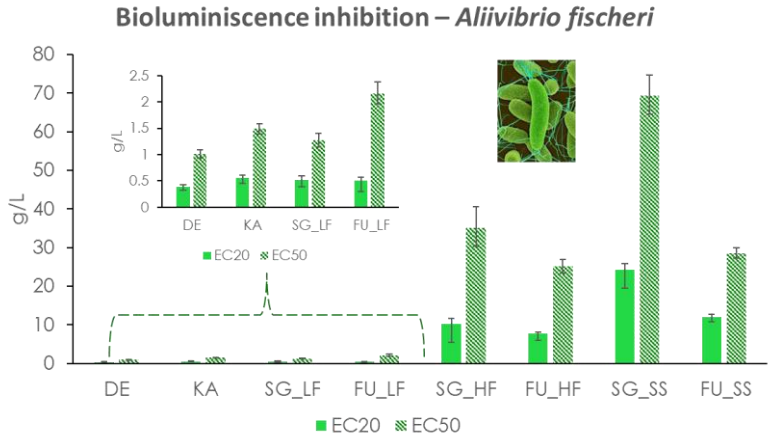

Figure 19. EC_{20} and EC_{50} values of acute toxicity assays with *A. fischeri*. DE = food grade diatomaceous earth; KA = kaolinite; SG = sample collected at the San Giuliano monitoring site; FU = sample collected at the Fusina monitoring site; LF = Light Fraction; HF = Heavy fraction; SS = Silt and Sand. Since the values of DE, KA, SG_LF and FU_LF were much lower than the others, a zoom-in graph is also displayed.

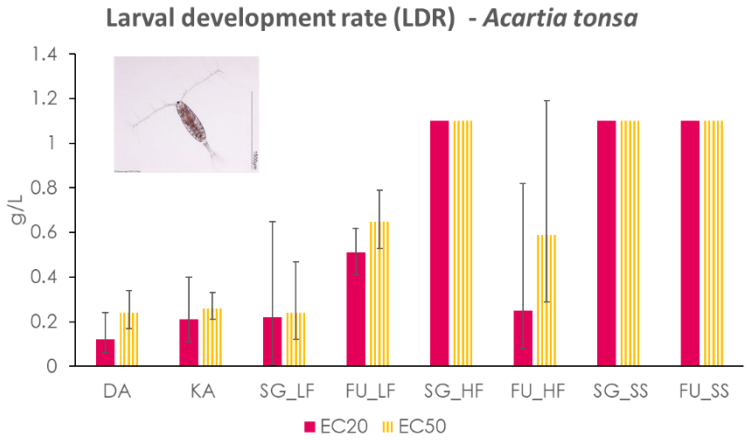

Figure 20. EC_{20} and EC_{50} values of acute toxicity assays with *A. tonsa*. DE = food grade diatomaceous earth; KA = kaolinite; SG = sample collected at the San Giuliano monitoring site; FU = sample collected at the Fusina monitoring site; LF = Light Fraction; HF = Heavy fraction; SS = Silt and Sand. The bars higher that 1 and without a standard deviation represent the samples without an ecotoxicological effect observed, considering 1 $g \cdot L^{-1}$ as the maximum dose tested for LDR.

According to these ecotoxicity results, the following considerations can be drawn:

1. the contaminants-free fractions of the lagoon samples (SG and FU), obtained by the sample handling procedure illustrated in Figure 14, showed EC_{20} and EC_{50} values very similar to those obtained for the reference materials kaolinite and diatomaceous earth. The only exception is represented by the assays with *M. galloprovincialis*, for which, as shown in Table 4, the results refer to two different methods: one based on the Orbital Shaker, as used in LDR, and the other implying a more striking effect by using an Overhead Mixer. Moreover, as explained above, for such bioassay results for HF and SS are not available while for kaolinite are only partially available (i.e., only for the Orbital Shaker method), thus not allowing to perform a complete assessment.
2. EC_{20} and EC_{50} values were always (i.e., for *A. fischeri* and *A. tonsa*) significantly lower for the finest fractions tested (LF, median around 5.5 µm) with respect to HF (median: 15 µm) and SS (median: 37 µm).

To conclude, even if the obtained results support the hypothesis that the particle size could play a role in inducing adverse effects to both the target organisms tested, i.e., *A. fischeri* and *A. tonsa*, It is worth to notice that the tested concentrations used for these ecotoxicity bioassays were approx. one order of magnitude higher (grams vs. milligrams) compared to those found in the monitoring sites considered (SG and FU) and therefore the role of the finer fraction could not be that relevant for the two species tested. However, further studies with other target organisms could support these conclusions but, as we have seen with the mussel, the development of bioassays protocols for this purpose is particularly complex.

5. Final remarks

Sediment resuspension, especially in shallowest environments as lagoons, beside causing the release of chemicals that may induce a negative impact to water column organisms, could also increase the probability of interactions between the aquatic organisms and the resuspended finer particles. However, the toxicity related to the particle size on aquatic organisms is still poorly investigated. In this context, this study provided novel information about the role played in shallow water systems by the finer fractions of sediment. After developing an experimental procedure for post-sampling treatment and for the extraction, separation and characterization of the finer fractions of the lagoon sediment, recently published (Brunelli et al., 2022)[22], the investigation of the ecotoxicological impact of the fine fractions of the lagoon sediment was conducted with the most sensitive life stages of lagoon organisms: a) bacteria (*Alivibrio fischeri*), b) calanoid copepod (*Acartia tonsa*) and c) mollusk bivalve (*Mytilus galloprovincialis*). The results highlighted the key role of the particle size in potentially inducing adverse effects to the different marine organisms tested.

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