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Programma di ricerca scientifica per una laguna “regolata”

Linea 2.1

Qualità del sedimento lagunare a supporto della sua gestione sostenibile

D2.1.2.2

Risultati dei bioassays

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Sommario

1 Aims of the activity

This document reports the results of the bioassays performed on sediments obtained from 1m sediment cores collected in Venice lagoon channels in November 2020 (sampling campaign I) and March 2021 (sampling campaign II). For each sampling campaign, six sediment cores (1m in length from the bottom of the channel) were collected in five sampling sites along the Vittorio Emanuele III channel and one sampling site in the San Felice channel chosen as a reference site for Venice lagoon channels (Figure 1).

The ecotoxicological characterisation of the sediment was performed by using a suite of four bioassays:

- acute toxicity test on the whole sediment with amphipods, according to the standard guideline ISO 16712 (ISO 2005), using the mortality measured after 10 days of exposure as an endpoint. This acute test aims at identifying potential hot spots of contamination that can jeopardise the survival of amphipods in the short term. The test provides insights concerning effects due to a) the more labile contaminants b) the contaminants associated with the oxides/hydroxides of Fe and Mn (potentially soluble in the interstitial waters), and metals and organics associated with organic matter and amorphous sulphides, which can be ingested by detritivorous organisms and therefore remobilized during the digestive process.
- sub-chronic toxicity test on water-sediment interface (SWI) with copepods (Picone et al. 2018a) using the larval development of *Acartia tonsa* as an endpoint, following exposure of 6 days. This sub-chronic toxicity test on SWI aims at identifying the effects related to the release of sediment-bound contaminants into the water column, with consequent possible effects on the zooplankton community. The test is a complementary procedure to the acute test with amphipods, but with an higher sensitivity to sediments characterised by a medium-low level of contamination. The larval development test with copepods mainly measures the effects due to the more labile contaminants, associated with the Fe and Mn oxides/hydroxides, and present in the aqueous phase. The test is also quite sensitive to ammonia and sulphur compounds (Picone 2006; Picone et al. 2018a).
- sub-chronic toxicity test on elutriate with bivalve molluscs (Volpi Ghirardini et al. 2005b), using the larval development of *Mytilus galloprovincialis* as an endpoint, following an exposure of 48 hours. The test aims at identifying the medium-term effects related to the more labile and easily extractable fraction of contaminants from sediments.
- chronic toxicity test with amphipods (Picone et al. 2018b), using a set of sub-lethal endpoints including growth rate and achievement of sexual maturity in females, after 28 days of exposure to the sediment. As the acute test, the chronic test measures effects due both to the more labile contaminants associated with the oxides/hydroxides of Fe and Mn and to metals and organics adsorbed on organic matter and amorphous sulphydes. Compared to the acute test, however, it allows for obtaining more relevant information from an ecological point of view, since chronic exposure to contaminants is a more frequent condition in natural environments than acute exposure, and the sub-lethal effects are of greater interest for long-term conservation of ecosystem functionality.

Figure 1. Sampling site location within the Venice Lagoon.

2 Methods

2.1 Acute toxicity test with amphipods

2.1.1 Aims of the activity

Amphipods are a relevant component of the marine and lagoonal benthic infauna and play a relevant role in the benthic trophic web being both recyclers of the detritus and food for many vertebrates and predatory invertebrates. They are widely used at international and national level as biological indicators since they are quite sensitive to sediment associated contaminants, particularly organic pollutants (Reichert et al. 1985; Plesha et al. 1988; Ciarelli et al. 1997; McCready et al. 2005). Nonetheless, the acute effects on amphipods are generally associated with high levels of contamination, so that this bioassay is considered a good method for identifying hot-spots of contamination.

The bioassay was performed using the allochthonous species *Grandidierella japonica* (Fam. Aoridae), since, during the sampling of test species in our reference area, the adults of this species were dominant as compared with the local ones *Corophium orientale* and *Monocorophium insidiosum* (Fam. Corophiidae) usually used in previous studies in the Venice Lagoon (Picone et al. 2008, 2016, 2018b). The allochthonous species is, however, internationally recognised as a good bioindicator for performing sediment toxicity tests (Ingersoll 1995; Hiki et al. 2019; Lee et al. 2022), and it is also included in the ISO standard protocols as a recommended test species (ISO 2005). The literature data confirm that *G. japonica* has a sensitivity to trace elements and ammonia very similar to that of the autochthonous species *C. orientale* and *M. insidiosum* (Table 1), suggesting that using the allochthonous species does not affect the results of the tests nor generate a discontinuity with the existing data.

Table 1. Cd and ammonia 96h-LC_{50} with 95% confidence limits reported in literature for *C. orientale*, *M. insidiosum*, and *G. japonica*.

Chemical	**Species**	**96h-LC_{50} (95% c.i.)**	**Reference**
Cd	*C. orientale*	3.30 mg L^{-1} (0.87 - 5.80)	Picone et al. (2008)
		2.91 mg L^{-1} (2.09 - 3.73)	Onorati et al. (1999)
	G. japonica	1.17 mg L^{-1} (0.94 - 1.46)	Hong and Reish (1987)
		3.14 mg L^{-1} (2.36 - 4.10)	Kohn et al. (1994)
	M. insidiosum	1.27 mg L^{-1} (0.88 - 1.83)	Hong and Reish (1987)
Ammonia	*C. orientale*	126 mg L^{-1} (114 - 138)	Picone et al. (2008)
	G. japonica	155 mg L^{-1} (133 - 204)	Kohn et al. (1994)

2.1.2 Lethality bioassay with Grandidierella japonica

The 10 d whole sediment bioassay were performed following the ISO standard method (ISO, 2005), the only modification being the number of amphipods used per replicate (25 instead of 20) and the number of replicates (4 instead of 5). The specimens of *G. japonica* were sampled in the Northern Lagoon of Venice, by using a stack of sieves with mesh size 1000 µm and 500 µm. Only the young adult amphipods passed through the 1000 µm and retained by the 500 µm screen were selected for the transport in the laboratory.

After their arrival, the organisms were gradually acclimated to the test conditions (T=15 °C; salinity=32 psu; pH=7.5–8.5) under constant aeration and continuous light (500–1000 lx) using native sediment as substrate. Acclimation of amphipods to toxicity bioassay temperature and salinity occurred at a maximum rate of 3 °C and 3 psu per day. Once the test salinity and temperature were reached, the organisms were kept under those conditions for at least 72 h before testing. The acclimation period was no longer than 5 days. Temperature, pH, salinity and ammonia of the overlying water were checked at the beginning and at

the end of the bioassays in all the test chambers. The artificial seawater used as dilution water was prepared according to ASTM (2004) in Milli-Q® purified water (Millipore, Bedford, MA, USA).

According to the intra-laboratory QA/QC procedure, a negative control with native sediment and a positive control test (water only exposure) with copper (Cu) as reference toxicant were carried out in parallel with each session of sediment testing.

2.1.3 Statistical analysis

Analysis of the variance (ANOVA) and Dunn's *post-hoc* test were used to check for differences among treatments. Normality and homogeneity of variance were verified using Kolmogorov-Smirnov's and Levene's test respectively ($\alpha = 0.05$). When normality and homogeneity of variance conditions were not met, Kruskal–Wallis test was performed to check for significant differences. Median lethal Concentration (LC50) with 95% confidence limits for the reference toxicant were calculated using the Trimmed Spearman–Karber method v1.5. All statistical analyses were performed using Stat-Soft® Statistica v7.0.

2.2 Sub-chronic toxicity test on water-sediment interface (SWI) with copepods

2.2.1 Aims of the activity

Calanoid copepods play a key role in marine and brackish food webs, due to their position as grazers for phytoplankton and consumers of detritus and their role as food for zooplankton and fish. Consequently, a decline of copepods may lead to critical changes at a community and ecosystem level.

In this context, although calanoid copepods are mostly planktonic, contaminated sediments may act as a secondary source of contaminants for the water column. Resuspension phenomena induced both by natural (i.e. wind, tidal currents) and by anthropogenic (i.e. dredging) driving forces may facilitate the release of the contaminants into the overlying water, allowing for their interaction with planktonic species. In this compartment they can impact calanoids copepods, which are documented as one of the most sensitive planktonic component (Drira et al. 2017). In the Venice lagoon the calanoid copepod *Acartia tonsa* was widely studied both for its diffusion, dynamic and role in the planktonic community (Bianchi et al. 2003; Camatti et al. 2019), and as bioindicator for ecotoxicity assessment (Picone et al. 2018a). At national level toxicity testing using *Acartia tonsa* as bioindicator species are included in recent legislations for sediment quality assessment (Ministerial Decree n. 173/2016).

The main goal of this activity is thus to assess whether sediment-bound pollutants may represent an hazard for the zooplankton. In particular, the larval development ratio (LDR) test on sediment-water interface aims to verify whether contaminants released from sediments and accumulated on the sediment-water interface may significantly affect the larval development of the copepods, from the egg to the copepodite-I stage. Copepod larvae, although planktonic, have negative buoyancy and tend to accumulate into the bottom of the water column. Consequently, in shallow-water ecosystems, they could be relevantly exposed to the contaminants accumulated onto the sediment-water interface.

2.2.2 Sample preparation

The day before the beginning of the test, 3.5 g of wet sediment were transferred into a 100 mL glass beaker filled with 80 mL of 20‰ salinity medium (ISO 2015) and fully homogenised by using a glass rod. The experimental unit was then allowed to equilibrate for 24-h at 20°C before starting the test. Each sediment was tested bulk (no dilutions) and in six replicates.

A formulated sediment was used as a negative control, prepared by mixing the following materials:

- Quartz sand (980g) (Merck);
- Kaolin (10g) (Sigma-Aldrich);
- Sphagnum peat (10g), as organic substrate.

The obtained dry formulated sediment had the following composition: 0.4% coarse sand (>500µm), 48.5% medium sand (250-500 µm), 48.3% fine sand (63-250 µm), 0.6% silt (4-63 µm), 0.6% clay (1-4 µm), and 1% organic matter.

The dry formulated matter was then homogenised by using a tumbler (Rotax, Velp Scientifica Srl) for 48-h, at 10 rpm. At the end of the homogenisation period, artificial seawater at a salinity of 20‰ (ISO 2015) was added, to obtain a substrate with 25% moisture. After the addition of the seawater, the formulated sediment was mixed for 24-h at 10 rpm and left to age for 7 days at 15°C.

2.2.3 A. tonsa *culturing*

Adult specimens of *A. tonsa* were purchased from Guernsey Sea Farms Ltd, Port Vale, Guernsey, United Kingdom. In-house laboratory cultures were started by adding 800-900 freshly released eggs to 1.8-L of a 20‰ salinity culture medium prepared according to ISO 16778 (ISO 2015). The cultures were kept at 20 ± 1°C in a climatic chamber with a 16-h light and 8-h dark photoperiod and under continuous aeration. The food, consisting of a mixture of three marine flagellates (*Tetraselmis suecica*, *Pavlova lutheri* and *Tisochrysis lutea*), was provided four times per day through a timer-controlled peristaltic pump. All algal clones were cultured in Guillard's F/2 medium (Guillard and Ryther 1962), at 20 ± 1°C, under continuous aeration and 16:8 light:dark photoperiod.

The eggs were removed daily from cultures by siphoning off the medium from the bottom of the culture flask and then filtering it through two sieves with mesh sizes of 170-µm and 50-µm, respectively. Adult copepods are retained by the 170-µm mesh sieve and then are reintroduced in the culture. Eggs and nauplii passed through the 170-µm sieve but are retained by the 50-µm sieve, so they could be collected and stored separately. Each culture was maintained for testing for up to 6 weeks.

2.2.4 Larval development ratio test (test LDR)

The test on the sediment-water interface is an adaptation of the test on water samples (Picone et al. 2018a).

Briefly, the test started on day-0 by adding a known number of newly released eggs (up to 80) to a 100 mL glass beaker 3.5 g (wet weight) of sediment and 80 mL of artificial seawater at a salinity of 20‰. This was done by 1) collecting the eggs from the culture as reported above, 2) filtering aliquots of egg suspension (100-200 µL) through a cellulose filter, 3) counting under a dissecting microscope all the eggs recovered on the filter, 4) inoculating them into test beakers using a disposable sterile syringe, and 5) checking under a dissecting microscope for eggs remained attached to the filter. Six replicates per each sediment sample and twelve for the negative control (formulated sediment) were used.

Test vessels were then maintained for six days in a thermostatic incubator (FOC 215E, Velp Scientifica, Milan, Italy) at 20 ± 1°C, with a 16-h light 8-h dark photoperiod and under a LED illumination to minimise the ultraviolet (UV) emission and avoid photolysis. Hatched larvae were fed on day-0 and day-2 with 100 µL of a concentrated (> 6 x 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.

Exposures ended on day-5 when approximately 40% of the larvae in negative controls reached the copepodite-I stage (Andersen et al. 2001). The ratio of nauplii to copepodites was first determined in one control replicate after exactly 5-d by staining the beaker's content with 0.5 mL of Lugol's solution (100 g L^{-1} KI, 50 g L^{-1} I2, 100 g L^{-1} trichloroacetic acid). Lugol's solution kills, stains, and preserves unhatched eggs,

nauplii and copepodites for microscopic analysis. The test suspension 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 (Stemi SV 6, Zeiss). If the first control contains 40% or more copepodites, the test was finished and also the content of the other beakers was fixed by adding 0.5 mL of Lugol's solution. Otherwise, the test was run for one additional hour before another control was sacrificed. All unhatched eggs, nauplii and copepodites recovered on the mixed cellulose ester filter were counted under a dissecting microscope to calculate the larval development ratio (LDR), namely 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 (Eq. 1).

Dissolved oxygen (DO) and pH were measured on day-0 in one beaker per concentration, before the inoculation of the eggs, and on day-5, before staining with Lugol's solution.

$$\text{Eq. 1} \quad LDR = \frac{n.copepodites}{n.nauplii+n.copepodites}$$

2.2.5 Statistical analysis

Statistical differences between samples and negative controls were assessed by using one-way ANOVA and Tukey's *post-hoc* test.

2.3 Sub-chronic toxicity test on elutriates with bivalve molluscs

2.3.1 Aims of the activity

The larval development of bivalve molluscs is one of the more critical phases of their life cycle. The planktonic larvae may take up contaminants through contact with dissolved contaminants (ions and molecules) or via ingestion of contaminated particulate matter and food (microalgae and detritus).

The objective of this experimental activity is therefore to determine whether the contaminants present in the elutriates extracted from sediment cores collected in the Venice lagoon may interfere with the first stages (48- hours) of the larval development of the Mediterranean mussel (*Mytilus galloprovincialis*), which lead from the zygote to the prodissoconch-I larva, also called D-shape due to the shape of its shell.

2.3.2 Sample preparation

Elutriates were prepared according to the protocol proposed by Volpi Ghirardini et al. (2005a). Briefly, wet sediment and artificial seawater (ASTM 2004) were mixed at a ratio 1:4w/v (w=sediment dry weight; v=water volume) and stirred for 24 h at 230 rpm using a Jar Test (mod. JLT6, Velp Scientifica, Milan, Italy) at 4 °C in the dark. The slurry was allowed to settle for 60 min at 4 °C and then the supernatant was centrifuged at 15000g for 15 min using a refrigerated centrifuge at 4 °C (Beckman Coulter, Fullerton, CA, USA). After centrifuging, elutriate samples were stored in polyethylene (PE) containers without filtering and then frozen at −18 °C for toxicity testing.

When the adult mussels for testing were available, the elutriate samples were thawed and then transferred to microplates with 24 wells of 3 mL. Each well served as an experimental unit. Six different concentrations were tested for each sample, including the undiluted sample (100% concentration) and five serial dilutions (50%, 25%, 12.5%, 6.25%, and 3.125%). Natural seawater (NSW) filtered with cellulose nitrate filters with a porosity of 0.2 µm was used as the dilution water. After filtering, the NSW was kept at 4°C in amber glass bottles. Each sample and dilution was tested in triplicate.

2.3.3 *Toxicity testing with* M. galloprovincialis

Adult organisms of *Mytilus galloprovincialis* were sampled during their reproductive period close to Malamocco artificial reef (natural population). They were used for testing after an acclimation period in lab aquaria at a temperature of 10°C and fed with *Tetraselmis suecica*.

The test was performed according to the procedure reported in Volpi Ghirardini et al. (2005b), with minor modifications. Mussels were induced to spawn following thermal stimulation, by alternating water baths at 18°C and 28°C every 30 minutes. Spawning females were individually isolated in 300 ml glass beaker and allowed to release their eggs for 30 minutes, then the egg suspension was filtered through a 100 µm mesh sieve to remove impurities, faecal pellets and detritus. Males were collectively placed into a 500 ml crystallising dish and allowed to spawn for 15 minutes before filtering the sperm-cell suspension by using a 32 µm mesh sieve. The eggs collected from each female were then fertilised by adding 2 ml of the filtered sperm-cell suspension and the early development stages (polar body appearance and first segmentation) were monitored under an inverted microscope (mod. DM-IL, Leica Microsystems, Wetzlar, Germania). Only the eggs collected from females displaying the highest fecundation rate were selected for the test and then collected and poured into a 1 liter glass cylinder. Zygotes density was determined by counting four times 100 ml aliquots, then the fertilised eggs were added to the test solutions to obtain a final density of 60-70 eggs ml-1 in 3 ml of testing solution.

The incubation was performed at 18°C, for 48 hours under dark conditions. All the tests were performed in triplicate, using sterile polystyrene microplates with 24 wells, each filled with 3 ml of testing solution. The larval development has been quantified by counting 100 larvae per replicate under the inverted microscope, by discriminating normally developed prodissoconch-I larvae (D-shaped larvae) from abnormal prodissoconch-I larvae and delayed larval stages (trochophore larvae, gastrulae) (ASTM 2004).

A positive control using Cu (using copper sulphate as standard) as a reference toxicant was performed together with the test on the surface waters, to verify the sensitivity of the testing organisms and allow for data comparison among different tests.

2.3.4 *Statistical analysis*

All toxicity data were calculated as “normalized percentage of success” (S), calculated as the percentage of normally developed D-shaped larvae in the sample relative to the percentage of normally developed larvae in the control.

For the samples which provided a percentage of success lesser than 50%, the results were also reported as Effective Concentration 50 (EC_{50}). All data were subsequently reported as Toxicity Units (TU), calculated as (100−S)/50 or 100/EC_{50} respectively. EC_{50}s with 95% confidence limits for reference toxicants and elutriate tests were calculated using the Trimmed Spearman–Karber method v1.5.

2.4 Chronic toxicity test with amphipods (*Monocorophium insidiosum*)

2.4.1 *Aim of the activity*

The goal of the tests performed with *M. insidiosum* is the assessment of the long-term effects of surface sediments on survival, growth and reproductive success of the amphipods, after 28 days of exposure. The test was performed according to the procedure outlined in Picone et al. (2018b) by exposing the amphipod to the bulk sediment.

2.4.2 Sampling and holding of amphipods

The amphipods were sampled in the Venice Lagoon, in a brackish pond located inside a sandy-silt salt marsh of the northern Lagoon (45°27'30.47"N, 12°26'44.75"E). Surface sediments were hand collected using a shovel and then wet sieved *in situ* using a set of screens with mesh size of 1000, 500 e 250 µm. The juvenile amphipods (< 2.3 mm length), passed through the 500 µm sieve and retained by the 250 µm sieve, were collected for the chronic test. The young-adult amphipods (2.3 – 5.0 mm length), passed through the 1000 µm sieve and retained by the 500 µm sieve, were collected for performing the positive control test according to ISO standard (ISO 2005). During sampling, a 5 liter aliquot of native sediment was also collected, to be used as substrate for amphipod holding before testing, and as negative control for sediment testing.

In the laboratory, the amphipods were transferred to 3-L glass tanks filled with native sediment and natural seawater collected in the sampling area. The holding tanks were then placed into a climatic room, at a temperature of 15°C and under a photoperiod 16:8 light:dark (Picone et al. 2018b).

2.4.3 Toxicity testing

The chronic sediment test was performed according to Picone et al. (2018b). The 28-d whole-sediment test was performed in triplicate, using 1,000 ml glass beaker as experimental unit, each with 30 juvenile amphipods. In each of the experimental units, juvenile amphipods were exposed to 200 ml of sediment and 600 ml of filtered natural seawater. The exposure was performed under 16:8 light:dark cycle, continuous aeration and semi-static conditions, with water renewal on days 7, 14 and 21. Overlying water temperature, salinity and pH were measured at the beginning of test (day-0), prior to each water renewal and at the end of exposure. Amphipods were fed *ad libitum* with a mix of three marine flagellate microalgae (*Pavlova lutheri, Tetraselmis suecica* e *Tisochrysis lutea*) in order to maintain a thin layer of algae on sediment surface.

On day-0, 30 randomly selected juvenile amphipods were individually measured under a dissecting microscope at ×10 magnification to establish starting conditions (t = 0) for length. On day-28, the content of each beaker was sieved through a 250 µm mesh sieve and then poured into a sorting tray. Surviving amphipods isolated from each replicate were then counted, individually measured at the nearest 0.1 mm (from tip of the rostrum to end of the telson) and sexed. Females with fully developed oostegites, ovigerous females and females carrying embryos in brood pouch were all regarded as mature. Length measurements were performed on amphipods fixed with a 2% formalin. Newborn amphipods eventually produced in each replicate were separately collected and counted (clearly discernible from adults by their smaller size).

The QA/QC procedure for the chronic test provided for the use of a negative control, represented by native sediment, and a positive control test (water only exposure) with copper (Cu) as reference toxicant. In the case of the test with the reference toxicant, the test was performed using young adults selected as reported in section 0.

2.4.4 Statistical analyses

Survival of juvenile amphipods after 28-d was reported as mean of 3 replicates per sample. Growth rate of juvenile amphipods was expressed as daily length increment (µm individual^{-1} d^{-1}). Daily length increment within each experimental unit was calculated for the pooled population (males and females together). Daily increments were expressed as difference between length at t = 28 d and length at t = 0 d, divided by the total exposure time (28 d).

Attainment of female maturity after 28-d of exposure to test sediments was calculated as percentage of mature females on surviving females in each replicate.

Analysis of the variance (ANOVA) and Dunn's *post-hoc* test were used to check for differences among treatments. Normality and homogeneity of variance were verified using Kolmogorov-Smirnov's and Levene's test respectively ($\alpha = 0.05$). When normality and homogeneity of variance conditions were not met, Kruskal–Wallis test was performed to check for significant differences. Median lethal Concentration (LC50) with 95% confidence limits for the reference toxicant were calculated using the Trimmed Spearman–Karber method v1.5. All statistical analyses were performed using Stat-Soft® Statistica v7.0.

3 Results and discussion

3.1 Acute mortality test with *G. japonica*

3.1.1 QA/QC

Two different batches of amphipods were used for testing the sediment samples; consequently, for each batch was performed a test with the reference toxicant (Cu) following a 96h water-only exposure. The amphipods collected in december 2020 provided an EC_{50} of 0.69 mg L^{-1} (95% confidence interval 0.52 – 0.92), while the batch sampled in march 2021 provided an EC_{50} of 1.63 mg L^{-1} (1.48 – 1.97). These values agree with the literature data concerning the autochthonous species *M. insidiosum* (Prato et al. 2006), underlining that the use of the allochthonous species did not compromise the sensitivity of the procedure.

The survival in the negative control performed with natural seawater was always > 90%.

3.1.2 Environmental samples

Four sediment samples collected during the December 2020 campaign evidenced a significant reduction in survival compared to both the negative control (native sediment) and the reference sample (site VI) (Figure 2). Samples I and IV, in particular, were characterised by survival of less than 50% after normalisation to negative control survival, underlining the exposure to sediments that severely affect the amphipods also after short-term exposure. Less marked, but equally relevant, were the effects observed in samples II and V, for which the survival normalised to control was 61% and 54%, respectively.

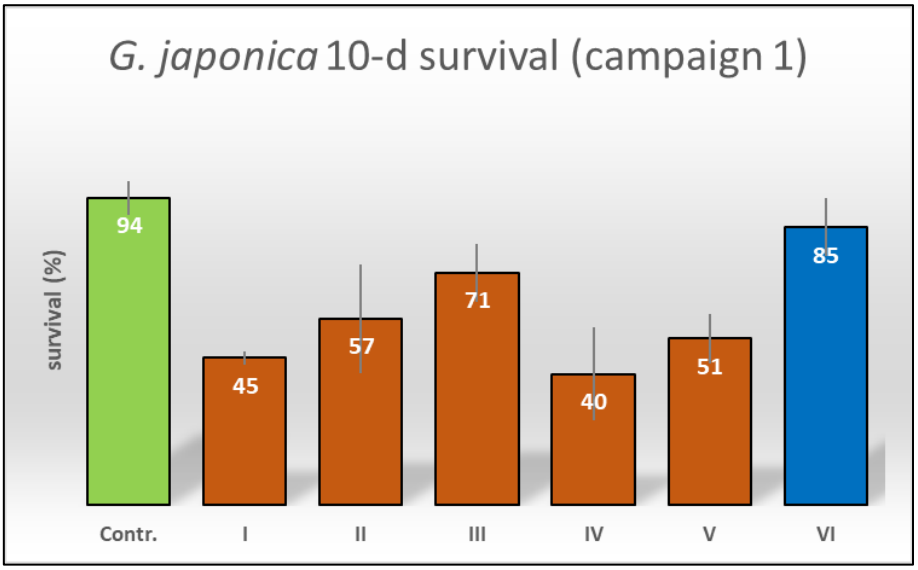

Figure 2. Survival of G. japonica in the sediment samples collected during the December 2020 campaign (campaign 1). Error bars represent the standard error.

In the March 2021 campaign, the toxic effects of sampled sediments were less marked than in the winter campaign. However, also for the second campaign three sediments (samples II, IV, and V) showed a significant reduction in the amphipod survival as compared with the negative control and reference sample, confirming the trend observed in the first campaign. Sample I, although characterised by relevant mortality, resulted not significantly different from the control and reference samples due to the variance observed among replicates.

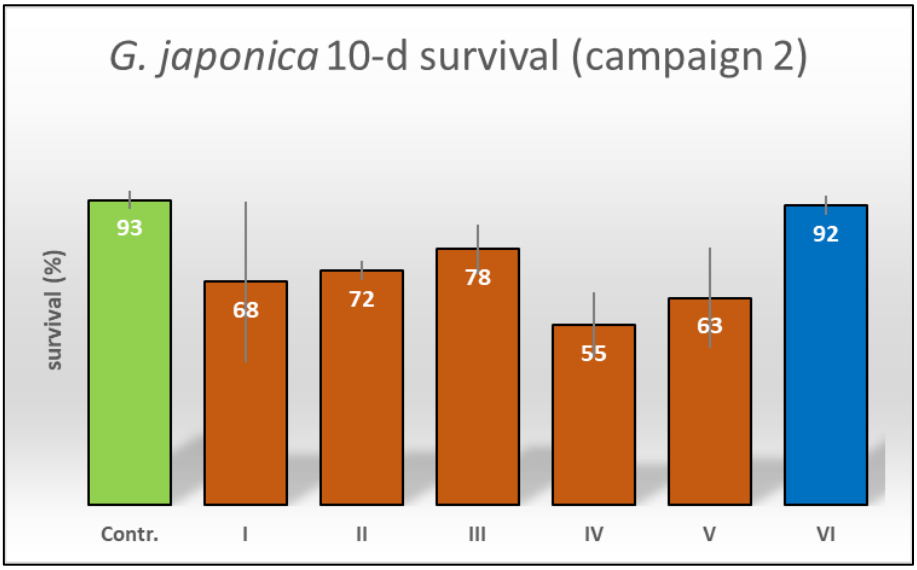

Figure 3. Survival of G. japonica in the sediment samples collected during the March 2021 campaign (campaign 2). Error bars represent the standard error.

Using the toxicity scores available for the autochthonous species for Venice Lagoon sediments (Picone et al. 2008), the toxicity of the analysed samples toward *G. japonica* might be summarised as follows (Figure 4):

- In the first campaign, sample VI can be classified as a sample with the absence of toxicity, sample III as a medium toxicity sample, while all the other samples (I, II, IV, and V) should be classified as samples with high toxicity.
- In the second campaign, samples III and VI can be classified as samples with an absence of toxicity, samples I, II, and V as medium toxicity samples, while sample IV should be classified again as samples with high toxicity.

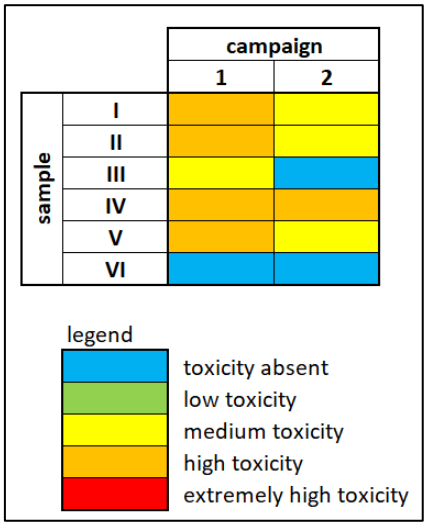

Figure 4. Toxicity classification for the sediment samples tested with amphipod (lethality test)

3.2 Sub-chronic toxicity test on water-sediment interface (SWI) with copepods

3.2.1 QA/QC

The tests on sediment samples were performed in two distinct testing sessions, using two different parental groups. The two tests provided an EC_{50} of 371 µg L^{-1} (95% confidence interval: 109 – 633 µg L^{-1}) and 263 µg L^{-1} (95% confidence interval: 118 – 583 µg L^{-1}), respectively. Both values fell within the acceptability criteria reported by ISO (2015) for tests performed with copepods cultured at salinity 20‰. In both tests, the ratio copepodites:total larvae met the acceptability criteria of 0.5±0.2 (ISO 2015).

3.2.2 Environmental samples

In the campaign 1, stimulation was observed of the larval development in samples II and III, while in samples I, IV and V the larval development of the copepods was inhibited by the exposure to the sediment. However, in the case of samples I and IV the inhibition was significant as compared to sample VI, chosen as the reference sample, but not as compared with the formulated sediment (control) (Figure 5). The stimulation detected in samples II and III should be treated carefully, since it might have been induced by contaminants that induce moulting and metamorphosis in crustaceans (endocrine disruption).

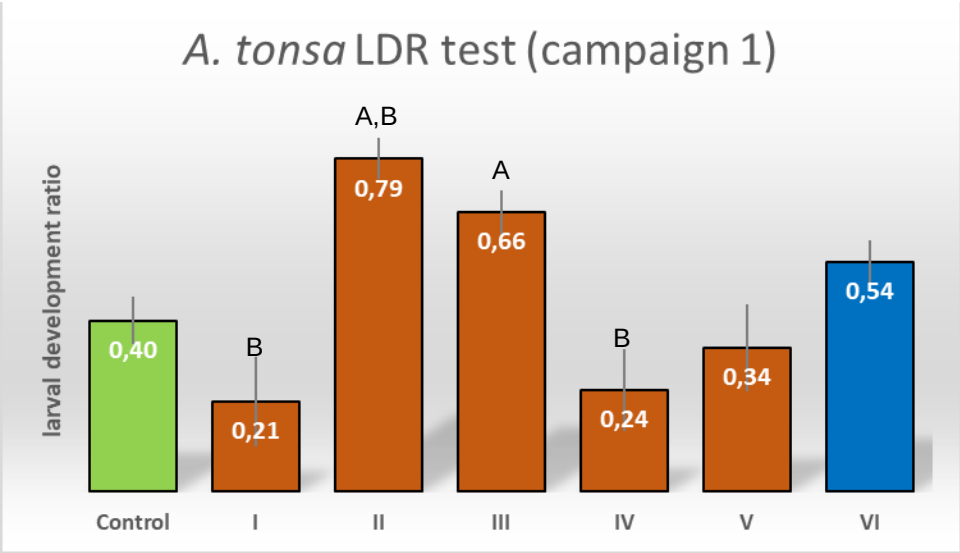

Figure 5. Larval development of A. tonsa after 6 days of exposure to the sediments collected during the December 2020 campaign (campaign 1). Error bars represent the standard error. A = significant difference compared to negative control (formulated sediment). B = significant difference as compared with the reference sediment (VI).

The trend observed for campaign 2 was completely different from the previous. The only sample providing a a significant effect compared to the control (formulated sediment) was indeed the sample VI, while for all the other the other samples there was no significant difference as compared to control. Obviously, all the samples resulted

resulted significantly different from sample VI (

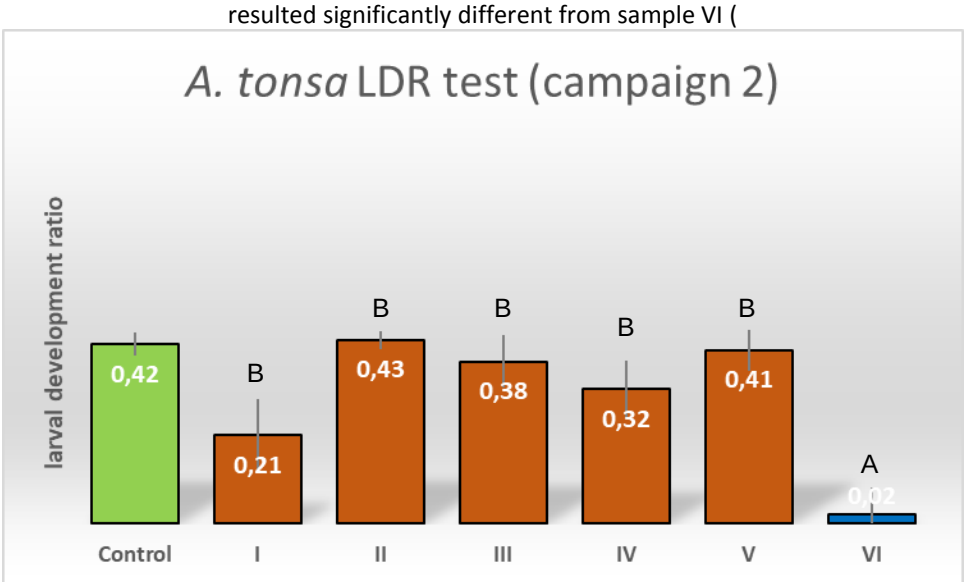

Figure 6).

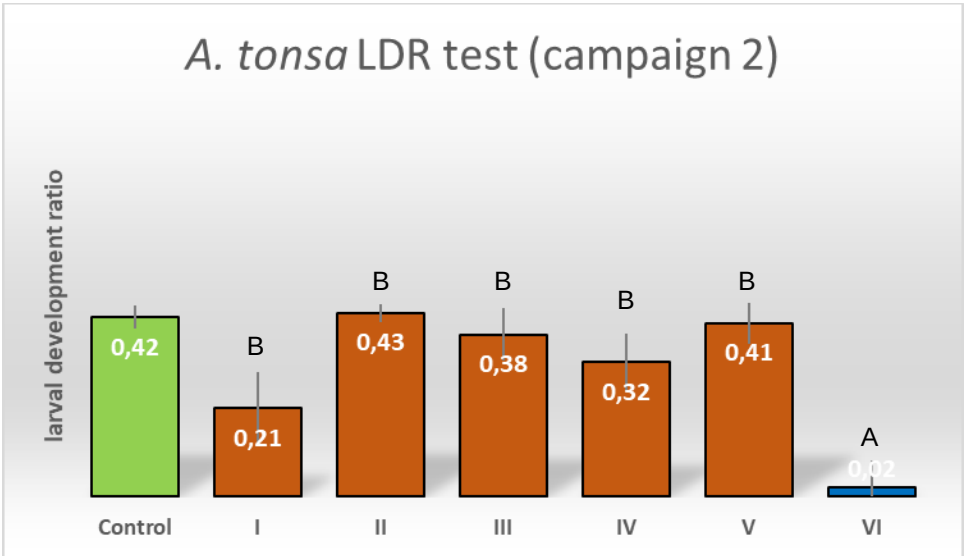

Figure 6. Larval development of A. tonsa after 6 days of exposure to the sediments collected during the March 2021 campaign (campaign 2). Error bars represent the standard error. A = significant difference compared to negative control (formulated sediment). B = significant difference as compared with the reference sediment (VI).

According to the toxicity classification proposed for the sediment of the Venice Lagoon tested with the larval development test with *A. tonsa*, based on the inhibition percentage compared to the control (Picone et al. 2018a), it was observed that sample V was the only to elicit negligible effects in both the campaigns, while sample I was the only to be toxic in both cases. For the other samples, it was advised a relevant variation in the response. The general lower toxicity observed in campaign 2 is consistent with the data obtained with *G. japonica.*

		% of inhibition campaign	
		1	2
sample	I	47%	51%
	II	-95%	-3%
	III	-63%	10%
	IV	41%	25%
	V	16%	3%
	VI	-34%	95%

legend
toxicity absent
possible toxicity
toxic sample

Figure 7. Toxicity classification for the sediment samples tested with the larval development test with A. tonsa.

3.3 Sub-chronic toxicity test on elutriates with bivalve molluscs

3.3.1 QA/QC

The test with the reference toxicant provided an EC_{50} of 23 µg L^{-1} (22 - 24), in agreement with the acceptability range of the intralaboratory control chart (15 - 32 µg L^{-1}) (Volpi Ghirardini et al. 2005b). In the negative control with NSW, the percentage of normally developed D-shape larvae was 94%, according to the acceptability criterion reported in Volpi Ghirardini et al. (2005) (> 80%).

3.3.2 Environmental samples

All the tested samples caused a significant inhibition of the larval development of the Mediterranean mussel compared to the negative control (natural seawater). However, a clear gradient of effect was observed among the samples and the results were consistent between the two sampling campaigns (Figure 8). The lowest rate of abnormal larvae was observed in samples I and VI, while the other samples exhibited a marked reduction of the larval development starting from the concentration 25% elutriate.

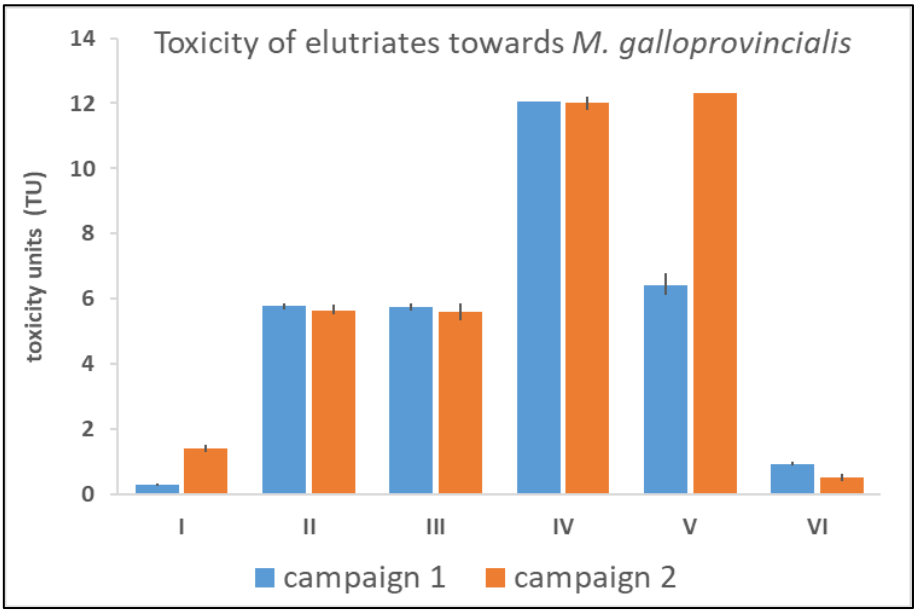

Figure 8. Toxicity of the elutriates towards the larval development of M. galloprovincialis. Data are reported as toxicity units. Error bars represent the 95% confidence interval associated with the TU.

According to the toxicity scores developed for the larval development test with *M. galloprovincialis* applied to elutriates prepared from sediments collected in the Venice Lagoon (Losso et al. 2007), the toxicity of the analysed samples might be summarised as follows (Figure 9):

- Sample I is characterised by negligible effects in campaign 1, while its toxicity is medium in campaign 2.
- Sample VI is characterised by low toxicity toward the larval development of *M. galloprovincialis* in both the campaigns
- Samples II, III, IV, and V are characterised by high toxicity in both campaigns.

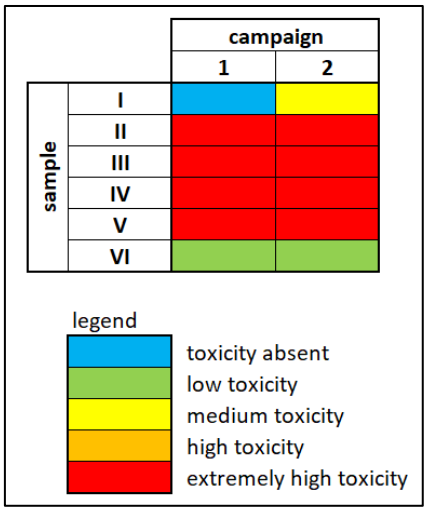

Figure 9. Toxicity classification for the elutriate samples tested with the larval development test with M. galloprovincialis.

3.4 Chronic toxicity test with amphipods

3.4.1 QA/QC

Two different batches of amphipods were used for testing the sediment samples. The amphipods collected in December 2020 provided an EC_{50} of 0.89 mg L^{-1} (95% confidence interval 0.72 – 0.99), while the batch sampled in March 2021 provided an EC_{50} of 0.57 mg L^{-1} (0.43 – 0.68). These values agree with the literature data concerning the autochthonous species *M. insidiosum* (Prato et al. 2006). The survival in the negative control performed with natural seawater was always > 90%.

3.4.2 Environmental samples

Survival

In campaign 1, the survival of juvenile *M. insidiosum* was significantly affected by the exposure to the sediments in samples I and IV compared to control (native) sediment and reference site (sample VI). Data concerning sample V were missing due to a shortage of amphipod which did not allow for the testing of all the samples (Figure 10).

In campaign 2, the trend was the same as observed in campaign 1: the sample I and sample IV significantly affected the survival of *M. insidiosum*, while no effects were observed in the other sediment except sample V, which exerted significant lethality on *M. insidiosum* (Figure 11).

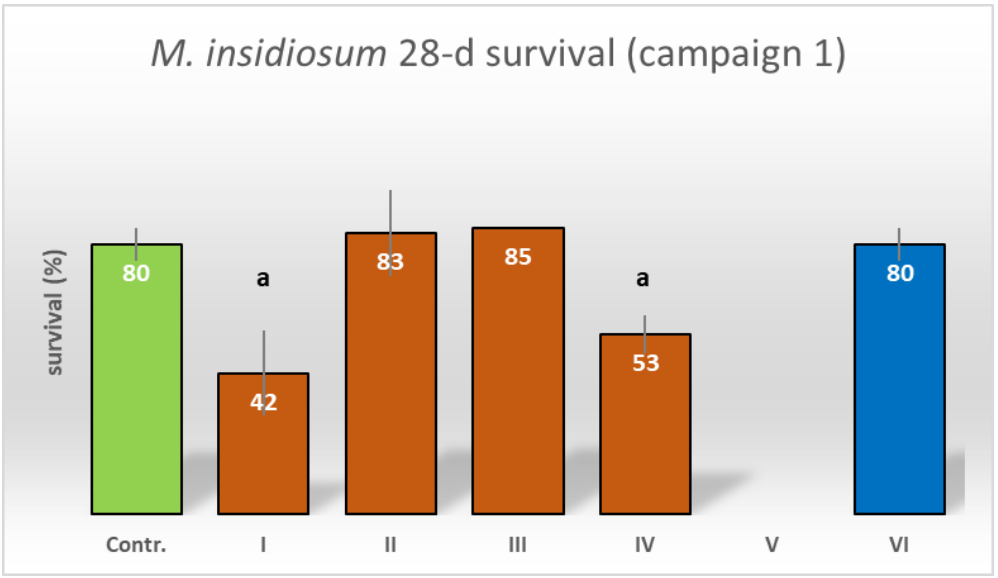

Figure 10. Survival of juvenile M. insidiosum in the sediment samples collected during the December 2020 campaign (campaign 1). Error bars represent the standard error. Letter "a" indicates sample with mortality significantly higher than both control (native sediment) and reference sediment (sample VI).

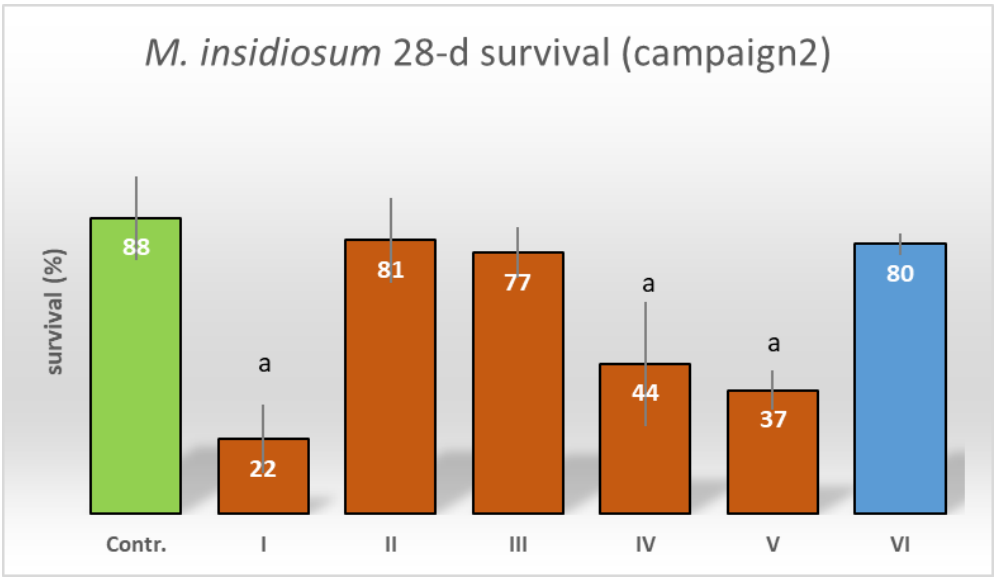

Figure 11. Survival of juvenile M. insidiosum in the sediment samples collected during the March 2021 campaign (campaign 2). Error bars represent the standard error. Letter "a" indicates sample with mortality significantly higher than both control (native sediment) and reference sediment (sample VI).

Growth rate

In campaign 1, the sample I and sample IV confirmed their unsuitability for *M. insidiosum* by reducing significantly also its growth rate (Figure 12). No differences were observed among the other samples. However, it should be noted that the growth rates observed for this overwintering generation are somewhat lower than the value reported in the literature for specimens of the same species during the spring and autumn reproduction periods, both in the Venice Lagoon (62 µm d^{-1}; Picone et al. 2018a) and in the North Sea (37–71 µm d−1; Nair and Anger, 1979).

In campaign 2, the trend was completely different. The growth rate measured in the control (native sediment) was significantly higher than the growth rate observed in all samples (Figure 13). Furthermore, in the control the growth rate was within the typical values from the reproductive period (Nair and Anger 1979; Picone et al. 2018b); conversely, in all the other samples, the growth rate was lower than the value observed for the December 2020 campaign, evidencing dystrophic conditions for the amphipods.

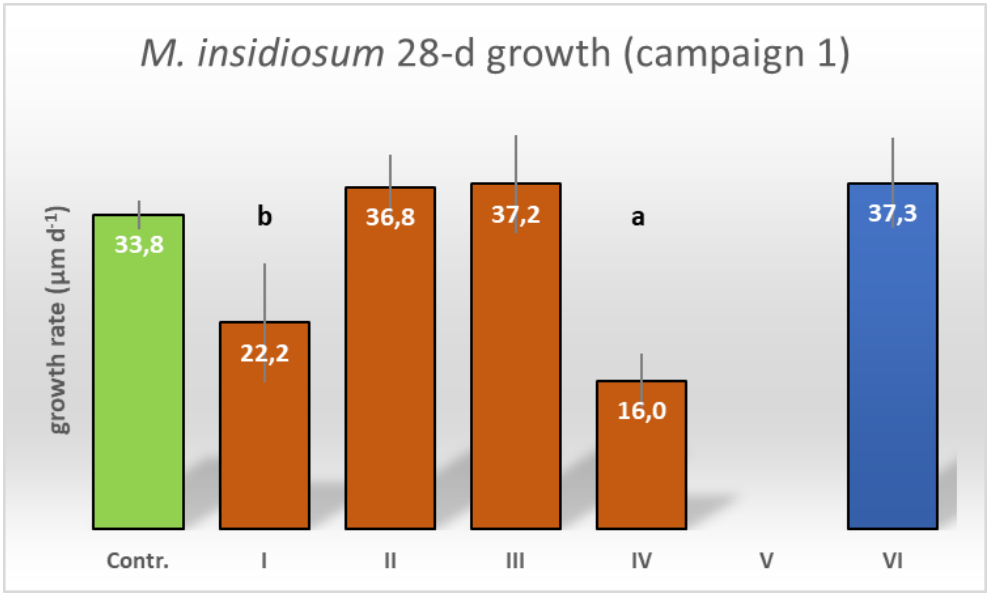

Figure 12. Growth rate of juvenile M. insidiosum in the sediment samples collected during the December 2020 campaign (campaign 1). Error bars represent the standard error. Letter “a” indicates sample with growth rate significantly lower than both control (native sediment) and reference sediment (sample VI), while letter “b” indicate significant differences respect to control only.

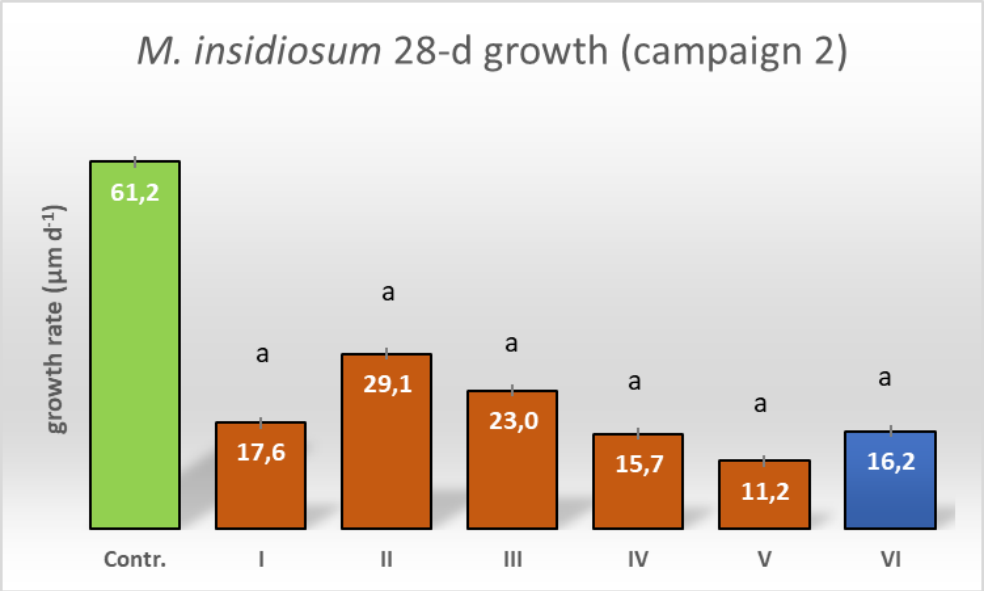

Figure 13. Growth rate of juvenile M. insidiosum in the sediment samples collected during the March 2021 campaign (campaign 2). Error bars represent the standard error. Letter “a” indicates sample with growth rate significantly lower than control (native sediment).

Attainment of sexual maturity

The maturation of females was the endpoint most affected by the exposure to the sediments. Both in campaign 1 and campaign 2, all the tested samples were characterised by a ratio of mature females significantly lower than in the control with the native sediment (Figure 14 and Figure 15). The reproduction occurred only in the control sediment, campaign 2, with a mean of 2.3 offspring per female. These results suggest that the tested sediments caused a significant delay of the reproduction of the amphipods, possibly due to 1) a retard in the attainment of the minimum size necessary for the reproduction, and 2) the need to invest metabolic energy in the detoxification.

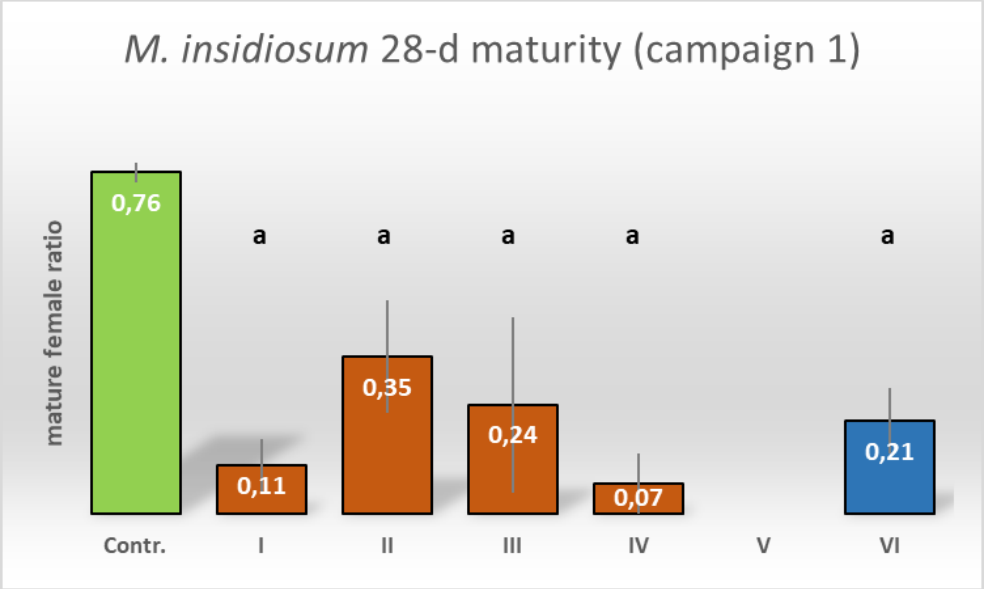

Figure 14. Ratio of mature M. insidiosum females in the sediment samples collected during the December 2020 campaign (campaign 1). Error bars represent the standard error. Letter "a" indicates sample with growth rate significantly lower than control (native sediment).

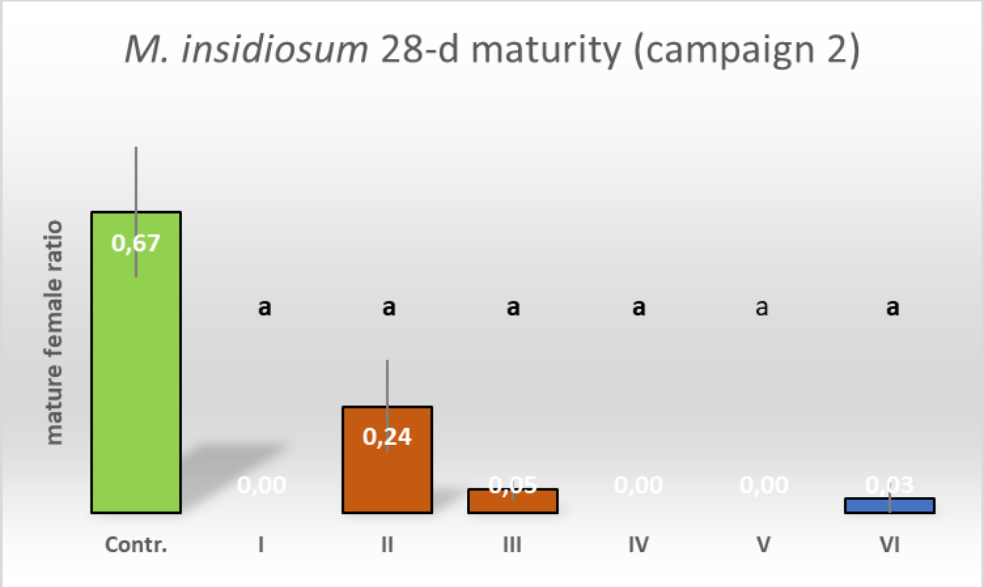

Figure 15. Ratio of mature M. insidiosum females in the sediment samples collected during the March 2021 campaign (campaign 2). Error bars represent the standard error. Letter "a" indicates sample with growth rate significantly lower than control (native sediment).

3.4.3 Overall assessment of the effects on M. insidiosum

The scarcity of toxicity data concerning the chronic effects on amphipods did not allow for the development of a scoring procedure to assess the overall effects of the sediment on the life cycle of *M. insidiosum*. In the lack of specific toxicity thresholds or limits, a difference of 20% between the controls and the test and reference sediments may be accepted as a conservative threshold for identifying sediments with toxicity not environmentally relevant, although in chronic tests greater differences can occur between the controls and the test sediments without being distinguishable (Chapman and Anderson 2005). The

effects can then be considered as “major” when a statistically significant reduction of >50% occurs in the considered endpoints (Chapman and Anderson 2005).

The assessment of the effects according to this conservative scheme is reported in Figure 16. What can be inferred is a diffused toxicity of the tested sediments, with clearly higher effects caused by sediments collected in campaign 2, especially as concern the growth of the amphipods, while lethality and maturation of females showed minor differences between the two campaigns, although effects in campaign 2 tend to be more marked.

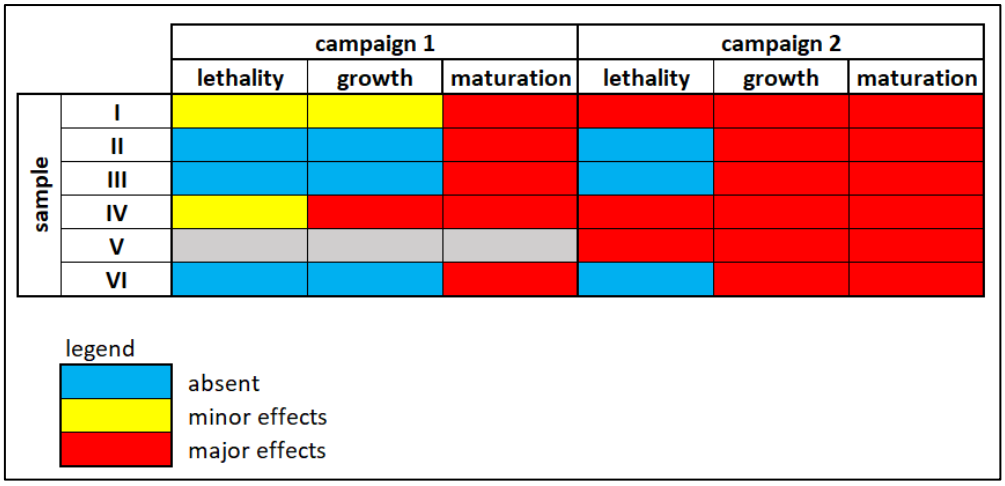

Figure 16. Toxicity classification for the sediment samples tested with the chronic test with M. insidiosum.

3.5 Integrated assessment

To integrate the results of the toxicity tests with the chemical analyses of the sediment, a factor analysis (FA) was performed with the aim to explore possible correlations between the two lines of evidence. Principal components analysis (PC) on standardized data was used as the extraction procedure in FA, to reduce the dimensionality of the original set of variables. FA factor rotation was performed using the varimax raw routine of the statistical software StatSoft Inc. Statistica 7.0. Graphical representation of factor scores was used to help identify relationships between the new variables (factors) and data collected for each of the analysed samples (Riba et al. 2003; Picone et al. 2016). Only factors accounting for at least 5% of the total variance of the original dataset were taken into consideration. Due to the lack of chronic data, the sample V/1 was not included in the analysis.

The original dataset contained the following 37 variables:

- fraction F1 (bioaccessible and labile fraction) for V, Cr, Ni, Cu, Zn, As, Cd, and Pb;
- fraction F2 (acid reducible fraction, bound to Fe/Mn oxy-hydroxides) for V, Cr, Ni, Cu, Zn, As, Cd, Hg, and Pb;
- fraction F3 (oxidizable fraction, bound to organic matter and sulfide) for V, Cr, Ni, Cu, Zn, As, Cd, Hg, and Pb;
- sum of polycyclic aromatic hydrocarbons (ΣPAHs);
- sum of polychlorinated biphenyls (ΣPCBs);
- sum of polychloro-p-dibenzodioxins and furans (ΣPCDD/F);
- sum of C<12 hydrocarbons;
- sum of C>12 hydrocarbons;
- mortality of *G. japonica* after 10-d lethality test (GJ);
- toxicity units (TU) for the larval development test with *M. galloprovincialis* (MG);
- inhibition of larval development for the LDR test with *A. tonsa* (AT);

- mortality (MI-mort), inhibition of the growth rate (MI-growth), and inhibition of sexual maturity (MI-sex) for the chronic test with *M. insidiosum.*

For trace elements, fractions F1, F2, and F3 were kept separated since they were not intercorrelated (Spearman correlation: $p > 0.05$) while fraction F4 was not considered for the FA due to its low bioavailability. Fraction F1 for Hg was not considered since all data were below method quantification limits.

As concern organic pollutants, the sum of PAHs was used since all the isomers were significantly correlated (Spearman correlation: $R > 0.8$; $p < 0,05$). Similarly, the sum of PCBs and PCDD/F were used rather than single compounds since strong and significant correlations among the congeners were observed (Spearman correlation: $R > 0.8$; $p < 0,05$).

Hexachlorobenzene (HCB) and organotin compounds were excluded from the analysis due to a large number of non-detect data (> 83% for HCB and > 90% for organotins).

FA identified 4 factors able to explain 88% of the original variability of the data, of which only 3, however, related the exposure to contaminants with the biological effects. The rotated factor loadings are reported in Table 2. Only variables with loadings greater than 0.4 were considered as those associated with factors: a loadings greater than 0.4 (DelValls et al. 1999) corresponds to an associated explained variance over 30% and approximates Comrey's cut-off of 0.55 for a good association between an original variable and a component (Comrey 1973). Factor scores are reported in Figure 17.

Table 2. Summary of rotated factor loadings for the variables of the original dataset. In red are highlighted scores higher than 0.4, while in yellow are marked scores lower than − 0.4.

	Factor 1	Factor 2	Factor 3	Factor 4
ΣPCB	0,71647	0,021839	-0,369706	-0,280136
ΣIPA	0,89193	-0,167951	-0,373678	0,001399
ΣPCDD/F	0,93285	-0,195576	-0,101688	0,031638
C < 12	0,29356	-0,035414	-0,431829	-0,168221
C > 12	0,87237	-0,251814	-0,304709	0,122651
V (F1)	0,90562	0,274030	-0,133733	0,235423
V (F2)	0,80352	0,501495	0,247615	-0,033863
V (F3)	0,60906	0,524893	0,186984	0,509893
Cr (F1)	0,59770	0,685801	0,271861	-0,043295
Cr (F2)	0,86694	0,458567	0,051515	-0,131792
Cr (F3)	0,75105	0,423487	0,141197	0,427322
Ni (F1)	-0,20368	-0,095211	-0,936004	-0,083087
Ni (F2)	0,35128	0,846333	0,002058	-0,340383
Ni (F3)	0,54100	0,814262	0,053012	0,191501
Cu (F1)	0,12027	0,661394	0,309653	0,016379
Cu (F2)	0,80812	0,420530	-0,143100	0,288629
Cu (F3)	0,89934	0,384053	0,087839	0,169309
Zn (F1)	0,86784	0,234225	-0,016484	0,180659
Zn (F2)	0,95322	0,092771	0,150138	0,209560
Zn (F3)	0,93571	0,154818	0,134440	0,253261
As (F1)	0,38967	0,814586	-0,066257	0,282680
As (F2)	0,13981	0,869118	0,078130	0,267196
As (F3)	-0,34922	0,791075	0,183159	0,132408
Cd (F1)	-0,15524	0,884008	0,091145	-0,001163
Cd (F2)	0,92293	0,086130	0,098390	0,346037
Cd (F3)	0,91705	0,170389	0,177359	-0,195800
Hg (F2)	0,53229	0,092230	0,055830	0,807237
Hg(F3)	0,06166	-0,358870	-0,767184	-0,348637
Pb (F1)	0,70082	0,267351	-0,454286	0,303802
Pb (F2)	0,83347	0,302256	-0,081505	-0,298498
Pb (F3)	0,71858	0,311133	0,140533	0,552804
GJ	0,19527	0,003993	-0,761084	0,222933
AT	-0,07584	0,281170	-0,006496	0,023885
MG	0,81617	-0,263147	-0,413009	0,044495
MI-mort	0,12272	0,524783	-0,235252	0,172598
MI-growth	0,48863	0,381437	0,054927	-0,025462
MI-sex	0,21035	0,231514	0,091053	0,080484

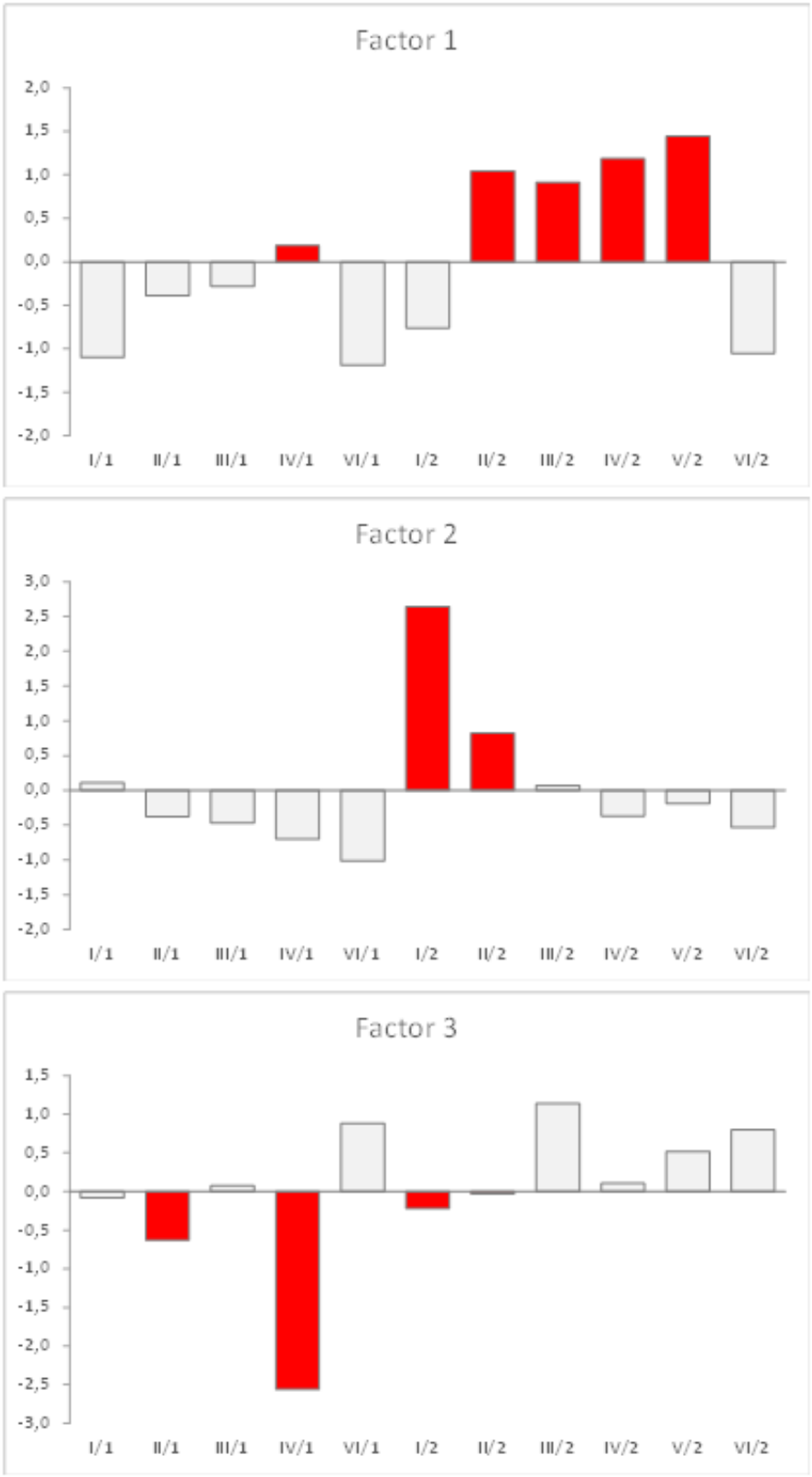

Figure 17. Estimated factor scores. The bar plots show the relative score for the 3 identified factors relating chemistry and ecotoxicology. For a given sample within each factor, higher scores highlight stronger relationships among the combined variables. In red are highlighted the scores with higher significance.

Factor 1 explained 51.4% of the variance of the original dataset and positively related the concentration of PAHs, PCBs, PCDD/F, long-chain alkanes, and several fractions of trace elements (but excluded As and labile fractions of Ni, Cu, Cd) with impairment of the larval development in mussels and retard in the growth of *M. insidiosum.* Factor 1 is characterised by a relevant positive score in sample IV, in both campaigns (**IV/1, IV/2**), and in samples II, III, and V in campaign 2 (**II/2, III/2,** and **V/2**). The effects observed in these samples toward mussel larval development and amphipod growth after chronic exposure may be then related to the contamination that characterises these sediments, typical of industrial areas. Accordingly, in previous studies (Picone et al. 2016), significant effects on the larval development of bivalves (*C. gigas*) were correlated with concentrations of PAHs, PCBs, and trace elements such as Cd, Cu, Hg, Pb and Zn in surface sediments collected in the industrial area (Tresse and Canale Industriale Nord). Notably, this correlation was confirmed only in the industrial area and its neighbourhoods, corroborating the results obtained with the sediment cores collected in the Vittorio Emanuele III channel. As concern amphipods, long term exposure to sediment (i.e. 28 days or more) may result in impairments in the growth due to the presence of Cu and PAHs in the sediments; nevertheless, effect concentrations reported in the literature are often higher than the sediment concentrations reported for the investigated cores. As an example, Lotufo et al. (2016) observed a consistent decrement of *Leptocheirus plumulosus* growth at a total PAHs concentration of 2.6 mg kg^{-1} and provided an EC_{20} of 1.05 mg kg^{-1} PAHs, clearly higher than the maximum PAHs concentration observed in the tested sediments (0.60 mg kg^{-1} PAHs, in sample II/1). Conversely, Marsden (2002) observed a significant decrease in length of male and immature *Paracorophium excavatum* with increasing Cu concentration from 5 to 46 mg kg^{-1} dw and in this case the range of effect concentrations bracket the environmental concentration of total Cu observed in our samples. The species-specific sensitivity of amphipods makes these comparisons inconclusive since benchmark data for *M. insidiosum* are not available. Nonetheless, previous studies performed in the Venice Lagoon confirmed that the growth rate of *M. insidiosum* tends to decrease with increasing concentrations of PAHs, PCBs, and total concentrations of Pb, Zn, Cu and As (Picone et al. 2018b), with the highest reduction observed in the area of Tresse. Furthermore, the observed correlation between the F3 fraction of several elements and growth rate inhibition underlines those toxic effects could be also related to the ingestion of sediment-bound or particulate bound trace elements, as already noted by Gale et al. (2006) for *Melita plumulosa*.

Factor 2 accounted for 20.9% of the original dataset variance and related the mortality of amphipods after the chronic exposure with the exposure to trace elements, in particular the bioavailable and labile fraction (F1) of Cr, Cu, As, and Cd, the fraction bound to Fe/Mn oxyhydroxides (F2) of V, Cr, Ni, Cu, and As, and the fraction bound to organic matter and sulphide (F3) of V, Cr, Ni, and As. This factor indicates that the more bioavailable fractions of toxic elements such as Cu, As, and Cu may have exerted a lethal effect on amphipods after long-term exposure to sediments, in particular in samples I of the second campaign (**I/2**) and, to a lesser extent, sample II of the same campaign (**II/2**). Lethality in amphipods, both after acute (10-d) and chronic exposure (28-d or more) is usually associated with hot spots of contamination, especially for sediments contaminated with crude oils (Scarlett et al. 2007a, b). Previous studies in the Lagoon did not report lethality of *M. insidiosum* after long term exposure; nevertheless, the available dataset concerning long term effects is too scant to provide adequate information concerning the long-term toxicity of the sediments.

Factor 3 relates with negative scores the effects on survival of *G. japonica* and the larval development of *M. galloprovincialis* with the sediment concentrations of short-chain alkanes (C < 12), labile fractions (F1) of Ni and Cd, and the fraction bound to organic matter and sulphide (F3) of Hg. The factor explains the 9,0% of the variance of the original dataset and assumes relevant weight only in samples II/1 and IV/1, and to a lesser extent in sample **I/2**. The association between labile Ni and larval development of mussels was already observed in sediments of the Vittorio Emanuele II channel (Corami et al. 2020), suggesting that the correlation highlighted by FA may be plausible. Similarly, also a correlation with total Hg and Pb was observed for the sediment of the industrial area (Picone et al. 2016), as well as in factor 1 for Pb, confirming the consistency of the association between these elements and the effects on larval development. Conversely, correlation with the C < 12 alkanes was never observed in previous studies. Acute effects on amphipods were barely observed in the lagoon of Venice (Picone et al. 2008) and in previous studies

associations with sediment contamination were not evidenced (Picone et al. 2016). In any case, the association is of interest since it underlines both that sediment-bound metals (Hg, in this case) and alkanes may affect the survival of amphipods.

Factor 4 relates only geochemical variable, thus is not of concern for the integrated assessment chemistry-ecotoxicology.

4 Conclusions

The set of bioassays used for assessing the toxicity of the sediments collected in Vittorio Emanuele III and San Felice channels evidenced a diffused toxicity in all the analysed samples, although with an evident gradient.

Acute effects on amphipod survival were observed only in sediments of Vittorio Emanuele III canal. The integrated analysis evidenced that contaminant of potential concern for the acute test with the amphipods are short-chain alkanes (C < 12), labile fractions (F1) of Ni and Cd, and the fraction bound to organic matter and sulphide (F3) of Hg. In particular, this association was observed in samples **II/1** and **IV/1**, and to a lesser extent in sample **I/2.**

Conversely, effects on the early-life stages of bivalves were clearly evident in Vittorio Emanuele III canal, while copepods were affected in both San Felice and Vittorio Emanuele III canals. However, the multivariate analysis allowed the identification of some possible causal association between delayed larval development in mussels and sediment chemistry only in Vittorio Emanuele III canal, while significant associations were not observed for the copepod test. In the case of mussels, impairments in the larval development were positively related with the concentration of PAHs, PCBs, PCDD/F, long-chain alkanes, and several fractions of trace elements in a single sample of the campaign (**IV/1**) and several samples of the campaign (**II/2, III/2, IV/2** and **V/2**). This association was the more diffused in the study area and confirmed the results of previous studies performed in the Venice Lagoon, highlighting the strong impact of organic micropollutants and bioavailable fraction of trace elements on the larval development of bivalves. A further, less frequent association was also observed with short-chain alkanes (C < 12), labile fractions (F1) of Ni and Cd, and the fraction bound to organic matter and sulphide (F3) of Hg, similarly to that observed for the acute effects on amphipods (samples **II/1** and **IV/1**). Also in this case, the association was already observed in previous studies. The effects on copepods in both canals may have been due to the release of chemicals from the sediment-water interface; however, from the analysis, it was not possible to identify any potentially causal correlation between chemicals and effects.

Chronic effects on amphipods, especially as concern the sub-lethal endpoint, evidenced that the tested sediments impair both survival and growth of the amphipods. Effects on survival were observed in the samples with the highest concentrations of short-chain alkanes (C < 12), labile fractions (F1) of Ni and Cd, and the fraction bound to organic matter and sulphide (F3) of Hg (samples **II/1** and **IV/1**). Conversely, effects on growth rates were more marked in sediments with high concentrations of PAHs, PCBs, PCDD/F, long-chain alkanes, and several fractions of trace elements (but excluded As and labile fractions of Ni, Cu, Cd), namely in samples **IV/1**, **II/2, III/2, IV/2** and **V/2**. Although effects on growth and attainment of sexual maturation cannot be discriminated, since the maturation of *M. insidiosum* occurs after reaching a critical size, it cannot be excluded that the mixture of contaminant could interfere both with the somatic growth and the reproduction of the species. Factors inhibiting growth and attainment of sexual maturity in sample VI/2 (San Felice canal) were not identified.

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