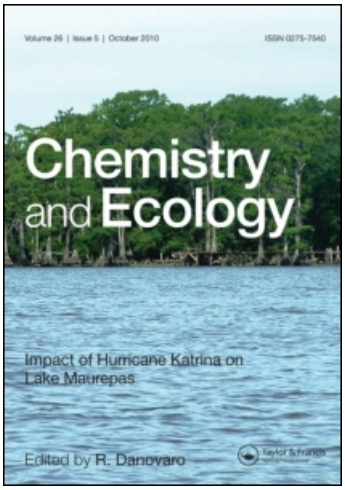

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Standardization of laboratory bioassays with *Balanus amphitrite* larvae for preliminary oil dispersants toxicological characterization

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The Italian National regulations on oil-dispersants use (D.D. 23 December 2002) require for these products to pass several laboratory screenings before they can be applied in oil-spill clean-up. Although legislation recommend the use of the American mysid shrimp *Americamysis bahia*, for laboratory toxicity testing, there is growing interest in employing local marine crustacean species more representative than *A. bahia*, in quantifying the risk of significant harm to Mediterranean ecosystems. The aim of this study (in the framework of the National Project 'Taxa Project', supported by the Italian Ministry for the Environment and Territory) is to improve new specific bioassays for assessing acute or sublethal responses to oil dispersants using the larval stages of the sessile crustacean *Balanus amphitrite*. The bioassays were standardized using sodium dodecyl sulphate (SDS) as toxic reference compound. Results of acute toxicity (48 h LC_{50}, 7.49 mg l^{-1}) and behavioural tests (7 d EC_{50}, 7.79 mg l^{-1}) with barnacle larvae showed that their susceptibility to SDS could be comparable with that of *A. bahia* (96 h LC_{50}; 6.6 mg l^{-1}). Therefore, a *B. amphitrite* bioassay could be proposed to replace the *A. bahia* bioassay in a standardized toxicological screening of new products for oil-pollution remediation technologies in the Mediterranean Sea.

Keywords: Oil pollution; Dispersants; SDS; *Balanus amphitrite*; Crustacean; Toxicity tests

1. Introduction

Each year, many millions of tonnes of crude and refined oil enter the marine environment as a result of anthropogenic sources such as oil spills. Detrimental effects of offshore oil spills usually occur on shoreline and shallow subtidal areas. There are many possible technical responses to an oil-spill situation [1], including bioremediation through hydrocarbon-degrading microorganisms [2–5] and application of surfactants (oil dispersant) to oil-contaminated zones [6]. Surfactants can be either chemically synthesized (synthetic) or microbially produced (biosurfactant). Synthetic surfactants may be cationic, anionic, non-ionic, and amphoteric, although only the anionic and the non-ionic surfactants have been used as oil dispersants [7]. Dispersants are designed to reduce the interfacial tension between oil and water, and to increase both the

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concentration of oil in the water column and the potential of biodegradation by creating small oil micelles dispersed into the water column [8].

Previous studies showed that surfactants can determine important biological injuries, including inactivation of key enzymes such as esterases and phosphatases, disruption of normal cell function by alteration of membrane permeability, interruption of cellular respiration, or membrane lysis [9]. The biological impact of oil dispersant on marine ecosystems is traditionally assessed on the basis of data gathered from acute toxicity tests. The common international parameters used to evaluate toxicity of a chemical are median lethal/effective concentration (LC_{50}, EC_{50}), no observed effect concentration (NOEC), and lowest observed effect concentration (LOEC): the first is the concentration of a compound that is effective (mortality or other effects) on half the test organisms [10, 11] in short-term tests (24, 48, or 96 h) [9], and the second and the third parameter are useful to estimate, respectively, the concentration of a compound not effective and the lowest concentration of a compound effective on test organisms.

Knowledge on the toxicity of dispersants comes largely from laboratory studies. Only in a few cases have systematic studies been carried out on the dispersants at a spill. Apart from certain oil companies and institutes having developed their own tests, no common standard method for testing the effectiveness of dispersants has been developed yet. The United States Environmental Protection Agency (EPA) has carried out tests to measure the effectiveness and toxicity of several commercialized dispersants [12].

Many experiments have been conducted on the toxicity of commercial dispersants on different target species [13–20], but no data are available for commercial products commonly used in Italy.

Recently, a new regulation (D.D. 23/12/2002) of the Italian National legislation on oil-spill dispersant use and application, provides guidelines to perform assays to validate commercial oil dispersants, before they can be used in oil-spill clean-up operations in marine environments. Although the marine crustacean *Americamysis bahia* [21] has been recommended as a target organism for toxicity assessment, this species is not indigenous in the Mediterranean Sea. Therefore, considerable problems, in terms of both ecological relevance and availability of test organisms occur, because Italian legislation restricts the importation of foreign species. The use of this organism, indeed, might not be representative, in terms of ecological significance, in monitoring the Mediterranean region.

As a consequence, the Italian Ministry for the Environment and Territory supported a national research project (the Taxa Project) to develop and standardize alternative ecotoxicological bioassays using marine crustaceans living in the Mediterranean Sea, in order to replace the above-mentioned non-indigenous species.

Within this project, our laboratory proposed the use of different larval stages of the crustacean *Balanus amphitrite* [22]. This organism has been chosen for different reasons: it is found worldwide, it is easily available and simple to rear, and it plays an important role in the coastal ecosystem. Moreover, barnacles colonize coastal areas, where the influence of contaminants (dispersed oils and oil dispersants) is heavy. Furthermore, other authors [22, 23] have also proposed *B. amphitrite* larval stages to assess the toxicity of several oil dispersants commonly used in Hong Kong waters in settlement inhibition assays.

Since the Taxa Project did not require tests on new or dispersants already on the market in the first step, the use of *B. amphitrite* in laboratory assays has been preliminarily validated and standardized using a reference toxic compound. For this reason, sodium dodecyl sulphate (SDS) has been suggested. In particular, the aims of the study are:

(1) The standardization of acute bioassay (48 h larval immobilization test) and behavioural bioassay (7 d settlement inhibition test) using *B. amphitrite* larval stages (nauplii and

cypris). The susceptibility of the two tests is indicated using the anionic surfactant (SDS) as a toxic reference compound; indeed, the toxicity of dispersants is generally attributed to the effects of their surface-active components on biological membranes [24].

(2) The susceptibility evaluation of this cosmopolitan organism larval stages compared with the American crustacean *A. bahia* commonly used in this kind of tests [25, 26]. In this preliminary study, two different toxicological end-points were validated for use in further studies on several dispersant products representative of the Italian and European markets.

2. Materials and methods

2.1 *Reference toxicant*

SDS ($CH_3(CH_2)_{11}OSO_3Na$) is an anionic surfactant capable of emulsifying lipids and low-surface-tension aqueous solutions. It can be produced by sulphation of lauril alcohol followed by neutralization with sodium carbonate. Because of its surfactants properties, it is often used as a detergent in the textile industry, in lipid and protein electrophoretic dissociation, and in determining the molecular weight of proteins. SDS was selected as a reference compound in toxicological tests, because the toxicity of dispersants is generally attributed to the effects of their surface-active components on biological membranes [24]. The Italian law on oil-spill dispersant use and application (D.D. 23 December 2002) advocated the use of SDS as a standard reference compound in all toxicological bioassays.

SDS was purchased from Sigma Aldrich (St. Louis, MO). It is commercially available in water-soluble (10% w v^{-1}) white crystals, flakes, or dusts. Stock solutions of SDS were prepared in ASW (Instant Ocean® Artificial Sea Water 37‰) as the carrier solvent.

2.2 *Culture of barnacle larvae*

Larvae were obtained from laboratory cultures of a brood stock of *Balanus amphitrite*. Twenty to 30 adult barnacles (averaged basal diameter 1 ± 0.4 cm) were reared in 700 ml beakers containing aerated, filtered (0.45 μm pore size, Millipore) natural sea water (FNSW) at 20 ± 1 °C, with a 16 h:8 h light:dark (L:D) cycle. The barnacles were fed every day with *Artemia salina* (20 larvae ml^{-1}), and *Tetraselmis suecica* (2×10^6 cells ml^{-1}). The sea water was changed three times per week, and barnacles were periodically rinsed with clean water to remove any epibionts or debris. Twenty beakers of adults, reared under these conditions, produced nauplii for the assays throughout the year. Nauplii (II stage) were collected and reared in 500 ml beakers on *T. suecica* (5×10^5 cells ml^{-1}) in sterile FNSW (0.22 μm pore size, Millipore), until they reached the cyprid stage. The beakers were gently aerated at 28 ± 1 °C with a 16 h:8 h L:D cycle. Newly metamorphosed cyprids (0-d-old larvae) were filtered in FNSW and directly used in settlement assays [27, 28].

2.3 *Acute bioassay*

After emission from adults, larvae (II stage nauplii) were collected and filtered, then used directly in acute toxicity testing. Static tests with 20–25 nauplii per test condition were used to determine the LC_{50} and EC_{50} of the toxicant, in 24-well plates containing 4 ml of test solution. Test solutions were prepared in ASW just before carrying out the tests.

A preliminary test with SDS was made to assess the optimum concentration range: 10 logarithmic concentrations were prepared, from 0.1 to 1000 mg l^{-1}. Afterwards, in order to obtain a definitive LC_{50}, new tests were made using the following concentrations of SDS: 0, 1.25, 2.5, 5, 10, 20, and 40 mg l^{-1}. Each test was made in four replicates for each concentration. Plates were then sealed and incubated at 20 ± 1 °C 16 h:8 h L:D cycle for 48 h. Immobilization was assessed every 24 h by counting the number of nauplii that did not show any movement for 10 s. Bioassays were repeated three times.

2.4 *Behavioural bioassay*

In this assay, the inhibition of settlement of *B. amphitrite* cyprids was evaluated after 7 d of toxic exposition, according to Wu *et al.* [22]. Cypris larvae were obtained from nauplii culture using the method described by Faimali *et al.* [28]; about 20 cyprids were placed in 24 well plates containing 2 ml of test solution.

The SDS concentrations tested were 0, 1.25, 2.5, 5, 10, 20, and 40 mg l^{-1}; four replicates of each concentration were prepared. Plates were sealed and incubated at 20 ± 1 °C (16 h:8 h L:D cycle) for 48 h. After 7 d of incubation, settled, attached, and dead organisms were counted. Three repetitions were carried out.

2.5 *Statistical analysis*

Trimmed Spearman Karber method was used to calculate LC_{50} and EC_{50} values with 95% confidence limit (CL). Dunnet test was used to calculate LOEC values.

A two-way ANOVA was used to test for differences in the percentages among different test repetitions for both acute and non-lethal bioassays. Prior to the analysis, the homogeneity of variance was verified using Cochran's test, and if necessary, data were Sqrt $(x + 1)$ transformed to remove heteroscedasticity.

A Student–Newman–Keuls (SNK; $P < 0.01$) test was used for an a posteriori comparison of means [29] to test for any differences in the effects (naupliar immobilization and settlement inhibition) obtained at each tested concentration, compared with that obtained in the relative control.

3. Results

3.1 *Acute bioassay*

The differences in experimental conditions between the two kinds of assays (acute and behavioural), the object of this work, are summarized in table 1. The end-point values obtained in toxicity testing using various crustacean species and values obtained in this study using *B. amphitrite* are compared in table 2.

The average of the naupliar immobilization percentage related to the three repetitions (A, B, and C) of 48 h acute toxicity test (figure 1) showed a reduction in this end-point proportional to the increase in SDS concentration; complete immobility of all organisms was observed, starting from the concentration of 20 mg l^{-1}. The Dunnet test fixed the LOEC at 5 mg l^{-1}. The median lethal concentration values and confidential limits (LC_{50} ± 95% CL) of the different bioassay

Table 1. Experimental conditions for the acute and behavioural toxicity tests conducted with the II stage nauplii and cyprids larvae of *B. amphitrite*.

Test conditions	Immobilization	Settlement inhibition
Larval stage	Nauplii II	Cypris
Test type	Static	Static
Temperature	20 ± 0.5 °C	28 ± 0.5 °C
Light conditions	1400 lux (wide spectrum fluorescence lights)	1400 lux (wide spectrum fluorescence lights)
Photoperiod	16 light:8 dark	16 light:8 dark
Salinity	37 ‰	37 ‰
Feeding	None	None
Dissolved oxygen	Over 60% of saturation value	Over 60% of saturation value
Test solution volume	4 ml	2 ml
Dilution water	Artificial (Instant Ocean)	Artificial (Instant Ocean)
Dilution factor	Preliminary = 10; final = 0.5	Preliminary = 10; final = 0.5
Number of concentrations	6 + Ctr	6 + Ctr
Number of larvae in test solution	20–30	≤20
Number of replicates	Three each concentration and Ctr	Three each concentration and Ctr
Exposition time	48 h	7 d
Number of test repetitions	Three with different batches, timing operator	Three with different batches, timing operator
End-points	Mortality/Immobilization: LOEC, LC_{50} (mg l^{-1})	Larval settlement (metamorphosis): LOEC, EC_{50} (mg l^{-1})
Statistical data treatment	Trimmed Spearman-Karber Method, Dunnet test	Trimmed Spearman–Karber Method, Dunnet test
Test acceptability criteria	Survival: Ctr (24 h) ≥ 80% Ctr (48 h) ≥ 70%	Settlement: Ctr (3 d) ≥ 60% Ctr (7 d) ≥ 80%

Note: Ctr = control test.

repetitions (A, B, and C) were 8.13 (7.39–8.95) mg l^{-1} and 7.76 (7.00–8.60) mg l^{-1}, 6.59 (5.96–7.28) mg l^{-1}, respectively. The average of the three LC_{50} values was 7.49 ± 0.80 mg l^{-1}. Two-way ANOVA revealed that there were no differences between repetitions ($F = 2.46$; $P = 0.094$) consistently with the concentrations (concentration × repetition: $F = 0.59$; $P = 0.84$).

Table 2. Median lethal concentration values (mg l^{-1}) of SDS toxicity towards aquatic crustaceans.

Organisms	$LC_{50\,24\,h}$	$LC_{50\,48\,h}$	$LC_{50\,96\,h}$	$LC_{50\,7\,d}$	Reference
Gammarus palustris	41.2				[37]
Ceriodaphnia dubia		48.4			[38]
Daphnia magna	45.8	19.1			[39]
	28.8				[34]
		16.2			[40]
		14.5			[40]
Artemia salina	19.1				[41]
Artemia salina	18				[42]
Daphnia similis	11.5				[43]
Neomysis americana			7.2		[25]
Balanus amphitrite		7.49			This study
Americamysis (Mysidopsis) bahia				9.3	[26]
Americamysis (Mysidopsis) bahia			6.6		[25]
Allorchestes compressa			3.6		[44]
Temora stylifera		3			[45]
Acartia lillgeborgi		2.6			[45]
Ampelisca abdita			2.6		[7]
Eurytemora affinis			2.6		[25]

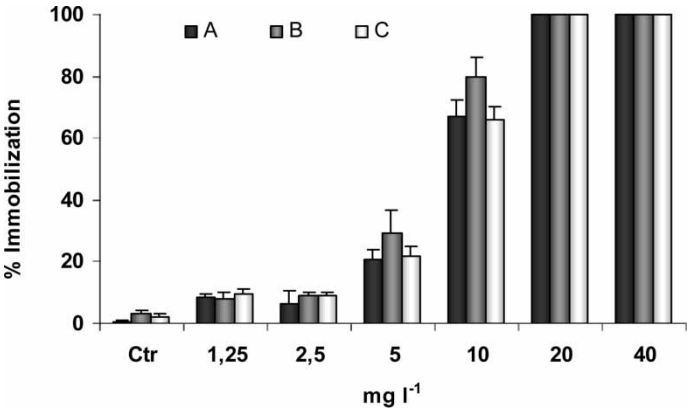

Figure 1. Acute toxicity test with *B. amphitrite* stage II nauplii. Immobilization percentage (M ± S.E., $n = 3$) of nauplii with increasing SDS concentration after 48 h. Histograms A, B, and C are repetitions of toxicity tests performed with different batches and timing by different operators. Ctr is the control test without toxicant.

3.2 *Behavioural assay*

Increasing concentrations of SDS resulted in a significant reduction in settlement after 7 d of SDS exposure (figure 2). Percentage of settlement inhibition presented a trend similar to that of the acute toxicity test. A clear effect of the reference toxic compound SDS could be observed, starting from the concentration of 5 mg l^{-1}. The EC_{50} values (±95% CL) of settlement inhibition assay after 7 d of exposition to SDS were 7.83 (6.74–9.10) mg l^{-1} for repetition A, 6.99 (5.89–8.30) mg l^{-1} for repetition B, and 8.57 (6.91–10.64) mg l^{-1} for repetition C. The EC_{50} average of the three repetitions was 7.79 ± 0.79 mg l^{-1}. The LOEC was 10 mg l^{-1} for each bioassay. Two-way ANOVA revealed that there were no differences between repetitions ($F = 0.93$; $P = 0.4$) consistently with the concentrations (concentration × repetition: $F = 0.67$; $P = 0.76$).

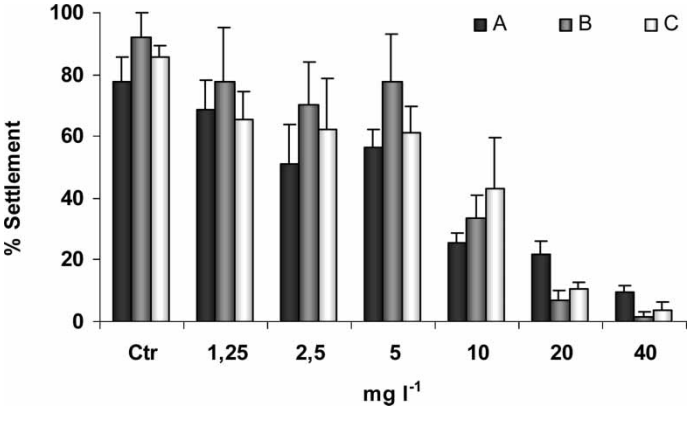

Figure 2. Behavioural test with cypris larvae of *B. amphitrite*. Settlement percentage (M ± S.E., $n = 3$) of cypris with increasing concentration of SDS after 7 d. Histograms A, B, and C are repetitions of behavioural tests performed with different batches and timing by different operators. Ctr is the control test without toxicant.

4. Discussion and conclusion

It was found that wind and sea conditions influence the use of oil dispersants. In fact, suitable field conditions cause a decrease in dispersant concentration under the detection limit in a relatively short time [24, 30–31]. The laboratory conditions during acute toxicity tests generally do not reflect the complexity of chemical dynamics that occur in the field. In particular, aquatic organisms are generally exposed to constant test concentrations for up to 96 h, resulting in laboratory mortalities that may exceed those expected in the field during short-term events such as spills. To obtain a more accurate estimation of environmental hazard associated with oil-spill response chemicals, shorter exposure periods (<96 h) in laboratory tests are likely to minimize any overestimation of potential toxicity of dispersant in the field [32].

For these reasons, the 48 h acute toxicity test results (LC_{50}) were compared with 7 d EC_{50} values estimated from the settlement inhibition test, to evaluate their applicability as a more rapid, yet reliable and more sensitive toxicity screening test for experimental dispersants.

The toxicity assay with a mortality end-point showed an unexpected result: LC_{50} values are similar to those obtained using the behavioural assay (settlement inhibition) previously proposed for dispersant products by Wu *et al.* [22], indicating that these two tests have the same sensitivity towards SDS.

Moreover, the acute test yielded significant responses (LOEC) during the exposure time close to the persistence time in the marine environment for this kind of compound (surfactants), which is considered to be less than few milligrams per litre within a few hours [33].

The larval stages used in mortality tests are obtained directly from an adult stock, while cypris larvae are obtained after feeding nauplii with microalgae for at least 5–7 d under controlled conditions. The acute toxicity tests performed on nauplii are easier to perform than the behavioural tests on cypris. However, to validate these preliminary results, the same comparison between these two kind of bioassay will have to be repeated in the presence of both anionic and cationic oil dispersant commercial products.

Few data concerning the effect of oil dispersants towards aquatic organisms belonging to different taxa, using SDS as reference toxic compound, are available in the literature. A comparison of end-point values, considering exposure times, showed how our data are significantly higher (>5 mg l^{-1}) or significantly lower (<10 mg l^{-1}) than the values recorded in previous studies for several crustacean species (see table 2 and references therein), whereas the results for *B. amphitrite* are very similar to the data on *Americamysis (Mysidopsis) bahia* with longer exposure times (4–7 d). It is interesting to note the lower susceptibility of daphnids than cirripeds to this compound. These data are in contrast to the usual relationship between these two organisms (see table 3): daphnids were usually found to be more sensitive than barnacles towards several toxic compounds. Sandbacka *et al.* [34], comparing surfactant toxicity between fishes and *Daphnia magna*, pointed out that the *Daphnia* assay was more susceptible than fish

Table 3. Comparison of susceptibility (EC_{50}) to cadmium, sea nine 211 (antifouling biocide) and methomyl (carbamate pesticide) of *Daphnia magna* and *B. amphitrite* larval bioassays (concentration are in mg l^{-1}).

Chemical	Organisms	End-point	Exp. time	EC_{50}	Reference
Cadmium	*Balanus amphitrite*	Mortality	1 d	1.36	[46]
		Immobilization	1 d	1.39	[47]
	Daphnia magna	Mortality	2 d	0.32	[48]
Sea Nine 211	*Balanus amphitrite*	Mortality	2 d	0.03	[49]
	Daphnia magna	Mortality	2 d	0.004	[50]
Methomyl	*Balanus amphitrite*	Immobilization	1 d	>10.24	Unpublished data
	Daphnia magna	Mortality	2 d	0.028	[50]

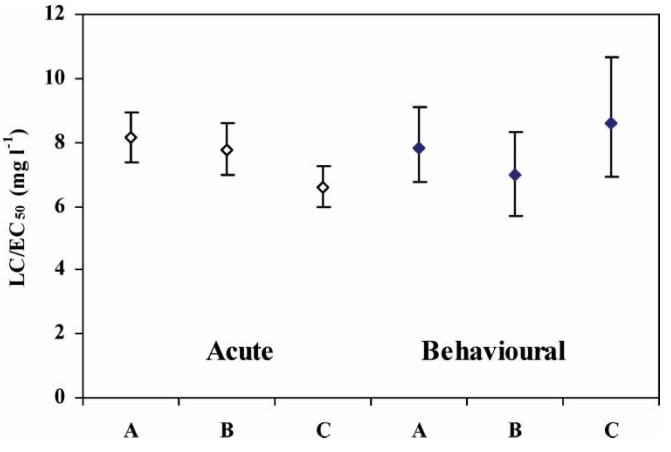

Figure 3. Comparison of median-lethal/effective concentrations of three repetitions of acute and behavioural toxicity tests (A, B, and C) using SDS as the toxic reference compound. Data symbols represent the LC/EC_{50} with 95% confidence limits.

cellular tests for all tested chemicals, except anionic surfactants. Other authors [35, 36] have found a peculiar susceptibility of *Daphnia magna* to cationic surfactants. From the comparison of literature data with results obtained in the present study, barnacle larvae appear to be more sensitive than daphnids when exposed to an anionic surfactant like SDS.

The results of this preliminary screening confirmed the applicability of *B. amphitrite* larvae as a biological model for toxicity studies on compounds used in hydrocarbon pollution remediation in marine environments (oil dispersants). In particular, both the performed bioassays (acute and behavioural) showed the same susceptibility of *A. bahia*, the only crustacean species that, according to Italian decree, should be used in dispersant toxicity bioassays.

Moreover, this study indicated the absence of any susceptibility difference between acute and behavioural bioassay with larvae of *B. amphitrite*; furthermore, the acute bioassay is easier, faster, more representative and less expensive than tests with cyprids (figure 3).

Therefore, *B. amphitrite* bioassays could be proposed as a potential substitute of *A. bahia* bioassay in a standardized toxicological screening, in order to meet requests of the Italian and international laws for validation of commercial oil dispersants used in oil-pollution remediation technologies.

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