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First evaluation of the threat posed by antifouling biocides in the Southern Adriatic Sea

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The CARISMA project (characterization and ecological risk analysis of antifouling biocides in the Southern Adriatic Sea) aims to appraise the quality of the Southern Adriatic Sea between Italy (Apulia region) and Albania and, in particular, the impact due to the use of biocidal antifouling coatings. Under this project, a preliminary survey at the main hot spots of contamination (*e.g.* ports and marinas) was conducted at the end of the nautical season in 2012. Chemical seawater analyses were complemented with ecotoxicological assays and the results were analyzed by principal component analysis (PCA). As expected, PCA splits the Albanian and Italian ports, according to the different degrees of contamination indicated for the two countries by the experimental data, highlighting the most critical situation in one port of Apulia. In addition, in order to assess the potential adverse ecological effects posed by antifouling agents (*i.e.* tributyltin (TBT)–irgarol–diuron) on non-target marine organisms, hazard quotients (HQ) were calculated. The results showed a low risk posed by irgarol and diuron whereas the probability of adverse effects was high in the case of TBT.

Environmental impact

The consumption of non-toxic antifouling paints is far from being widespread, making it impossible to avoid the use of toxicant-based paints. Due to TBT persistence, its presence is still recorded, even if its use is banned. Alternative biocides (irgarol, diuron) are widely distributed in seawaters, with possible harmful effects even at low concentrations. In the coastal areas of the Southern Adriatic Sea, we evaluated their occurrence, the effects by ecotoxicological assays, and the hazard by ERA. The results were analyzed by the PCA statistical method. This work gives an example of how the combined use of different and complementary methodologies allows a deep and robust interpretation of the data, thus permitting the capture of different aspects of the system.

Introduction

Antifouling (AF) paints are routinely used to prevent the unwanted attachment of living organisms to the submerged surfaces of ships, boats and aquatic structures; they act by releasing effective biocides from the coated surface.

Actually, the biocides employed in AF paint formulation are made of herbicides and fungicides with a wide range of action against a number of organisms. AF paint biocides need to be toxic to fouling organisms on the ship hull but this toxicity is not completely lost once AF paints are released into the water column, where toxicity also affects non-target species.

Formulations containing organotin (OT) compounds (*e.g.* tributyltin, TBT) were the most successful compounds against biofouling and were extensively used in 70% of the world's fleet. Unfortunately, TBT exhibits detrimental impacts on sea life, causing for example imposex, *i.e.* the development of male characteristics in female gastropods.[1]

Therefore, in the 1980s some European countries introduced restrictions on using TBT-based paints and an ultimate global ban was enforced in 2008 by IMO (International Maritime Organization) for all vessels.

Many studies have involved surveys on TBT distribution in the water column, sediments, and biota.[2,3] In particular, the environmental half-life of TBT in seawater was estimated to be in the order of weeks.[2,3] Measurements taken prior to restrictions on TBT use in antifouling paints have shown levels higher than 500 ng L^{-1} in North American and European marinas. In recent investigations, it has been reported that TBT concentrations have generally declined, rarely exceeding 100 ng L^{-1}, even if hot spots have been reported[4–7] especially in those countries where IMO restrictions have not been applied.

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Alternative products were then developed by paint manufacturers, usually containing a Cu(I) compound as the main biocide, mixed with one or more organic compounds called booster biocides: chemical agents that enhance the formulation making it effective also against copper-resistant organisms.

Irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) and diuron (3-(3,4-dichlorophenyl) 1,1-dimethylurea) are two herbicides used worldwide in AF paints which exhibit toxicity to aquatic plants by blocking electron transport within chloroplast membranes and inhibiting photosynthesis.[8] Irgarol has a half-life of 100–350 days,[9] and diuron is stable in the water column, with <1% reduction in 42 days.[9] As a consequence, they reach significant concentrations in the aquatic environment, as demonstrated by several surveys in the last decade and exert a continuous influence on the marine ecosystem with possible damage especially to seagrass, micro- and macro-algae.[8]

These biocides are widespread worldwide as reported in the literature. In Europe, irgarol was in the range of <1–338 ng L^{-1}.[9–11] Moreover, concentrations of irgarol up to 1816 ng L^{-1} were detected in the Eastern coastal areas of USA;[12] up to 304 ng L^{-1} in California;[13] up to 4200 ng L^{-1} in Asia (Singapore),[14] up to 4800 ng L^{-1} in South America (Brazil)[15] and up to 6 ng L^{-1} in Australia.[16]

With regard to diuron, levels as high as 2000 ng L^{-1} were found in Europe (Spain),[17] 1540 ng L^{-1} in Japan;[18] 2160 ng L^{-1} in Australia,[16] and 7800 ng L^{-1} in Brazil,[15] whereas lower levels, down to 68 ng L^{-1}, were detected in California.[19]

Consequently, these AF biocides need to be monitored in order to assess the possible environmental damage related to their use.

This study was conducted within the project named "CARISMA" (characterization and ecological risk analysis of antifouling biocides in the Southern Adriatic Sea), funded by the Ministry of Foreign Affairs, which aims to appraise the quality of the portion of Adriatic Sea between Italy (Apulia region) and Albania and, in particular, the impact due to the use of antifouling paints. Unlike Italy, Albania is characterized by a low maritime traffic, mainly due to fishery and marine transport of goods and passengers, whereas recreational boating is negligible. Hence a low environmental loading is expected for AF agents.

A preliminary survey was carried out on the occurrence of selected contaminants from antifouling paints in ports along the coasts of Apulia (Italy) and Albania.

As far as we know, no monitoring data of organic booster biocides are available for Albanian marine waters whereas previous studies have been carried out in Italy (*e.g.* Di Landa *et al.*).[20]

The sampling strategy was limited to ports and marinas because they can represent the worst scenario for the exposure of marine organisms to AF biocides. In fact, such sites are usually characterized by intense boat traffic and by a conformation that does not favour water exchange, so the contaminants tend to accumulate reaching levels higher than those in the open sea.

Seawater and biota (sea urchins) samples were collected before the end of summer, when boating activity is still intense and the contamination from AF paints is expected to be significant. It must be said that although water is subject to large and fast temporal variations, it is useful to get information on contamination "at the time". It is particularly true when the considered compounds can be regarded as persistent organic pollutants. On the other hand, biota analyses can reflect the effects of water quality over a period of time.

The environmental concentrations of diuron, irgarol and OT compounds have been determined along with the ecotoxicological effects. Actually, chemical analysis is not enough to evaluate the toxic effects or to characterize contaminated sites and it needs to be complemented with biological methods that assess the toxicity of biologically available contaminants,[21] even those not considered or detected by chemical analyses.[22]

In addition, chemical contaminants rarely affect organisms as single substances, but rather cause adverse effects as diverse mixtures.[23,24]

The ecotoxicological approach is generally based on a battery of bioassays with organisms belonging to several species since a sensitive species to all environmental contaminants does not exist.[25,26] The use of a combination of assays and/or organisms increases the ecological reliability and easy interpretation of results, and offers a powerful tool to assess the potential bias of individual organisms as well as the mode of action of contaminants.

Moreover, Ecological Risk Assessment (ERA) has been accomplished for assessing potential adverse ecological effects posed by antifouling agents (*i.e.* TBT–irgarol–diuron) to non-target marine organisms in the studied area: the high-risk or low-risk situations can be identified by the estimation of the numerical hazard quotient (HQ). Although the quotient method does not permit quantification of the actual risk posed by a single contaminant, it provides an efficient and inexpensive tool to identify those chemicals of potential ecological concern.

In this study quotients have also been used to integrate the risks of multiple chemical stressors.[27]

Generally, to integrate different data and to extrapolate maximum information, multivariate statistical methods, such as principal component analysis (PCA), are used.[28] PCA is a well-known unsupervised pattern recognition technique to extract, rationalize, and visualize all useful information from the dataset.[29]

Therefore, for a wide analysis allowing a good understanding of the site characteristics and a reliable evaluation of their environmental quality, in this study, PCA was applied to chemical and ecotoxicological data as well as to the calculated HQ values.

The combined use of diverse and complementary methodologies such as PCA and ERA enables a deep and robust interpretation of the data, allowing us to capture different aspects of the studied system.

Materials and methods

Study areas

In Italy three medium-to-large-size ports were selected, Manfredonia (MN), Trani (TR) and Margherita di Savoia (MDS), which are located in the northern Apulia region. This region is

situated at the south-eastern tip of the Italian peninsula and neighbours Albania across the Adriatic Sea, with distance ranging from 72 to 290 km. The port of Manfredonia handles the traffic of ferries, commercial ships, fishing boats, and pleasure craft while Trani and Margherita di Savoia mainly host fishing boats and pleasure craft.

Despite the Albanian coastline length being 472 km, there are only a few important ports, which are intended for freight and passenger traffic as well as mooring of fishing vessels, while recreational boating is still very poorly developed.

Samplings were carried out in the three main Albanian ports: Durres (DR), Vlora (VL) and Shengjin (SH). Durres is currently hosting 78% of the maritime trade at the national level and this is also a key location for transit networks and passenger ferries. Shengjin houses mainly fishing vessels and Vlora is made up of two distinct ports, one dedicated to the traffic of goods and passengers, and the other one to fishing boats. Only the latter one was sampled in this preliminary campaign.

Fig. 1 shows the selected Italian and Albanian sampling locations. The geographical coordinates and the main information on each port are reported in Table 1.

Sample collection

Water and biota samples were collected in Italy and Albania in September 2012.

At each port, samplings were carried out in the middle of the basin and the inner part (*i.e.* quay), to evaluate the potential spatial changes.

A glass-sampler probe (International PBI, Milan, Italy) was submerged to a depth of 0.5 m below the sea surface and seawater samples were collected in pre-cleaned 1 L glass bottles. All the containers were additionally rinsed with seawater before sample collection.

The aqueous samples for analysis of OT compounds were acidified *in situ* with 0.8 mL 37% HCl per liter. Where available sea urchins (*Paracentrotus lividus*) were collected. Additional seawater samples were taken at two "reference" sites (approximately one mile away from TR and VL) where the contamination was presumably negligible.

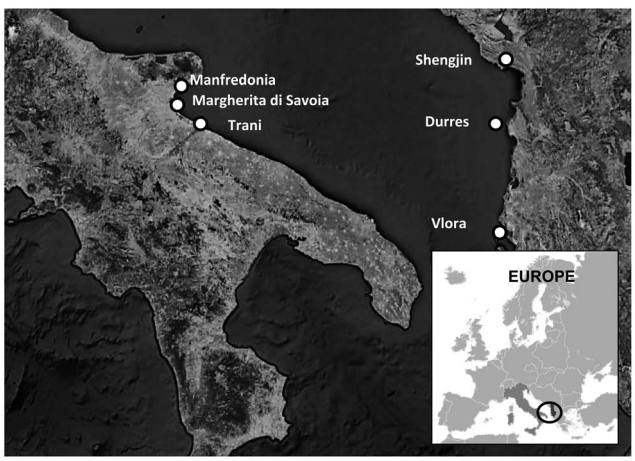

Fig. 1 Italian and Albanian sampling locations.

With regard to transport, sea urchins were wrapped in towels soaked in seawater and stored at 4 °C, together with water samples, until arrival at the laboratory where urchins were immediately processed. All water samples were stored in a fridge at 4 °C until being analyzed.

In addition standard water quality measurements of temperature (T, °C), conductivity (mS cm^{-1})/salinity, pH and dissolved oxygen (DO, % saturation) were performed *in situ*, using a portable multi-meter (Multiline P4, WTW, Wissenschaftlich-Technische Werkstätten 82362 Weilheim, Germany).

Booster biocide analysis

The analytical procedure used to determine the biocides levels in seawater was based on that reported by Di Landa *et al.*[20] with some modifications. Briefly, the isolation and preconcentration of the target compounds from aqueous samples (500 mL), previously filtered at 0.45 μm, were carried out by solid phase extraction (SPE) columns with a polymeric stationary phase (LiChrolut EN 200 mg, Merck, Darmstadt, Germany). The extracts were evaporated to dryness and added with an appropriate amount of isotope-labelled internal standard (atrazine-d5). Then a suitable solvent was supplemented and a final volume of 0.5 mL was obtained. Hence the concentration factor of our sample preparation was 1000.

Analysis of biocides was performed using a Series 200 HPLC (Perkin Elmer, Norwalk, CT) coupled to an API 150 EX single quadrupole mass spectrometer (MS) with a TurboIonspray – electrospray ionization source (ESI) (Applied Biosystems Sciex, Foster City, CA). The chromatographic separation of the analytes was achieved with a C18 HPLC column using a linear gradient elution.

The determination of AF biocides was carried out using positive electrospray ionization (ESI+) under Selected Ion Monitoring (SIM) mode: two or three ions (quantification and confirmation ions) were selected for each compound.

The positive identification of target compounds in real samples was based on the retention time and the simultaneous presence of quantification and confirmation ions: the deviation of the relative intensity of these recorded ions should not exceed ±10% with respect to that observed in the authentic standards, and the retention time should not deviate more than 2.5%.

Quantitative determination was performed using constructed calibration curves with standard mixtures of known concentrations (2.5, 5.0, 10, 20, 50, 100, 200, 500, and 1000 μg L^{-1} of each compound and 200 μg L^{-1} of atrazine-d5). For real samples with a low concentration of irgarol (<2.5 ng L^{-1}) a linear calibration curve within the range of 0.5–20 μg L^{-1} was used for quantification.

Reliable measurements were ensured including a calibration check sample both at the start (low level of AF biocides) and at the end (high level of AF biocides) of each batch of 4 unknown water samples. The accuracy of the calculated concentration in the calibration check sample should be within 90–110%. Moreover both procedural and reagent blanks were analyzed in order to check for carryover. Every real sample was prepared and analyzed in duplicate and the percent deviations of AF

Table 1 Description of sampling sites

Sampled ports	Abbreviation	Position	Site description	Berths
Italy				
Manfredonia	MN1	41°37′27.94″N–15°55′13.490″E	Port; marina	365
Manfredonia quay	MN2	41°37′30.73″N–15°54′53.600″E		
Margherita di Savoia	MDS1	41°23′17.19″N–16°08′2.770″E	Marina; fishery port	200
Margherita di Savoia quay	MDS2	41°23′02.63″N–16°07′55.190″E		
Trani	TR1	41°16′51.399″N–16°25′17.234″E	Marina; fishery port	550
Trani quay	TR2	41°16′44.966″N–16°25′10.346″E		
Trani reference[a]	TRref	41°17′30.000″N–16°26′6.000″E		
Albania				
Shengjin	SH1	41°48′42.900″N–19°35′17.400″E	Fishery port	28[b]
Shengjin quay	SH2	41°48′49.320″N–19°35′11.400″E		
Durres	DR1	41°18′22.800″N–19°27′19.740″E	Port	98[b]
Durres quay	DR2	41°18′10.440″N–19°27′14.100″E		
Vlora	VL1	40°29′4.800″N–19°25′58.200″E	Fishery port	61[b]
Vlora quay	VL2	40°29′3.300″N–19°25′51.720″E		
Vlora reference[a]	VLref	40°28′25.920″N–19°24′38.640″E		

[a] Blank seawater samples collected at the 1 mile offshore site. [b] Registered fishery vessels.

chemicals found in replicate samples were from 1% to 14% and from 1% to 21% for irgarol and diuron, respectively.

As certified reference materials do not exist for AF biocides in seawater, some recovery experiments using fortified samples were performed. With this aim "reference samples" were used and matrix spike (100 ng L^{-1}, $n = 5$) gave recoveries as high as 104 ± 4% and 107 ± 7% for diuron and irgarol, respectively. Similar recoveries were obtained for fortified seawater samples ($n = 5$) at 50 ng L^{-1} (111 ± 3% and 104 ± 5% for diuron and irgarol, respectively).

The limits of detection (LODs) for the analytical procedure were determined using fortified seawater samples and calculated as the analyte concentration that gave a signal-to-noise ratio of 3. LODs were 0.2 ng L^{-1} (irgarol) and 1 ng L^{-1} (diuron).

OT compounds analysis

1 liter water samples (pH adjusted to 2 at the moment of sampling) were added with an appropriate amount of a solution of ^{119}Sn-enriched butyltin compounds (an isotopically enriched solution of monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT)) as a procedure/quantification standard, and allowed to equilibrate for 15 min with occasional agitation.

The extraction was performed using a separatory funnel with at least 2 aliquots (30 mL) of a 0.03% tropolone solution in dichloromethane (to improve the extraction efficiency of the monosubstituted species), and the organic phase was collected through anhydrous sodium sulphate. The organic phase was evaporated using a rotary evaporator down to a final volume of 1 mL at the temperature of 30 °C. The organic extract was transferred into a vial, added with 2 mL of hexane and 1 mL of isooctane (as a keeper solvent) and then evaporated almost to dryness under a gentle stream of nitrogen. The organotin compounds were pentylated by the Grignard reagent and then were extracted twice with 1 mL of hexane. The extract was concentrated and purified on a silica gel column. After lowering the volume to 0.5 mL, 1 μL of the final solution was injected for GC-MS-SIM analysis and the organotin quantitative determination was based on the isotope dilution method.

Method limits of detection as Sn were 0.3 ng L^{-1} for TBT and 0.5 ng L^{-1} for DBT and MBT.

All the analyses were carried out by the same operator. Certified reference material, coastal sediment (IRMM BCR 462) and fortified blank seawater samples (TBT, DBT and MBT spiked at 10 ng L^{-1} and 100 ng L^{-1} as cation) were used for the validation of the procedures. The analysis of the reference material ($n = 3$) showed a good performance as TBT and DBT (MBT not certified) results overlapped the certified values ± their uncertainty and recoveries from fortified blank seawater samples ($n = 5$) were: TBT 92 ± 21%, DBT 87 ± 23%, and MBT 82 ± 24% (10 ng L^{-1}) and TBT 96 ± 10%, DBT 92 ± 13%, and MBT 86 ± 20% (100 ng L^{-1}). The GC-MS determination was done in a single run for all the samples including blanks and BCR 462.

Ecotoxicological test battery

Toxicity tests were carried out on sampled seawaters in triplicate.

The bioassay battery consisted of tests with four different species representing different trophic levels: algae *Dunaliella tertiolecta*, bacteria *Vibrio fischeri*, crustacean *Artemia salina*, and echinoids *Paracentrotus lividus*.

***D. tertiolecta* test.** The chronic test was carried out as reported by Mecozzi *et al.*[30] The culture medium for algal growth was prepared according to the ISO protocol.[31] Bioassays were performed using serial dilutions (1 : 2) of the seawater sample. Artificial seawater (ASW)[32] was used for dilution of the samples. The samples were placed in sterilized glass flasks, in triplicate. An algal suspension at a concentration of 1×10^6 cell per mL

was prepared. The purity of the algal stock was verified by examining a subsample under a microscope (400×) for contamination by micro-organisms. Then an aliquot of algal suspension was added to each replicate to reach the final concentration of 1×10^4 cell per mL.

Culture medium has been utilized as the negative control (6 replicates) and zinc as the reference toxicant ($ZnSO_4$). The test flasks were placed in a thermostatic chamber at 20 °C with a light source in the 7000–8000 lux range for 72 h. The cell density of each sample was measured after 72 h by using a Burker chamber. EC50 was calculated for each sample; where not possible, the growth inhibition percentage was estimated.

***A. salina* test.** *A. salina* cysts were hatched by using the procedure described in APAT-IRSA.[33] The encysted organisms were first hydrated in a volume of artificial seawater (Istant Ocean 3% m/v) for 1 h at 25 °C at 3000–4000 lux. Then the cysts were incubated for 24 h in the dark at the same temperature. The acute toxicity test (96 h) was conducted according to APAT-IRSA.[33] Ten nauplii were transferred into a beaker with 40 mL of the sample. Each sample was tested in triplicate. The negative control consisted of 6 replicates of artificial seawater. The reference toxicant ($K_2Cr_2O_7$) was also tested as the positive control. The treatments were incubated at 25 °C with a light regime of 14 : 10 h light–dark. No food was provided during the exposure. Every 24 h the number of the live individuals was recorded. The effect percentage for each sample was calculated with respect to the control. The test was valid when the control mortality did not exceed 10%.

***V. fischeri* test.** The *Vibrio fischeri* luminescence inhibition test was carried out with seawater samples. *V. fischeri* was exposed to 9 serial dilutions (1 : 2) and to a negative control Microtox diluent (NaCl 2%). The luminescence decrease was evaluated after an exposure of 15 and 30 min. The luminescence was measured using a Microbics Model 500 Toxicity Analyzer according to the manufacturer's instructions (Microbics Corporation). Reference toxicant tests were periodically conducted with phenol to determine the sensitivity of the test organisms over time and to identify potential sources of variability. The results were expressed as the luminescence inhibition percentage with respect to the control and, where possible, as EC50.

***P. lividus* test.** The spermiotoxicity test was performed, as reported by Manzo *et al.*,[34] on seawater samples. Spawning was induced by injection of 1 mL of 0.5 M KCl. Eggs were collected by placing under each female a beaker containing artificially filtered (Ø 0.45 μm) seawater (ASTM, 1994),[32] while the sperm was collected "dry", *i.e.* directly from the surface of each male using a micropipette, and was stored on ice. 10 μL of concentrated sperm were diluted in 10 mL of sample. The solution was incubated for 30 min at room temperature, and then 50 μL of the exposed sperm were added to 10 mL of artificial seawater containing not exposed eggs. Experimental wells were incubated at 18 °C for 20 min. Three replicates were carried out for each sample. The reference toxicant ($CuSO_4$) was also tested as the positive control. The fertilization rate was determined using a sample of 100 eggs. The effect percentage for each sample was calculated with respect to the control. The acceptability of test results was fixed at a fertilization rate of 70% in control tests.

Data analyses. Data were expressed as mean ± standard deviation. Data from the algal growth inhibition test were analyzed by the inhibition concentration (ICp) approach[35] to determine EC50 values, with their respective 95% confidence limits.

The results were always recorded as the effect percentage with respect to the control by using the Abbott's formula.

Ecological risk assessment (ERA)

In the present study the ERA procedure, developed by US-EPA and described in detail in the Guideline for Ecological Risk Assessment,[27] was applied. It consists of a three-stage methodology (Fig. 2): (1) problem formulation; (2) analysis (*i.e.* exposure and effect characterization); (3) risk characterization.

Problem formulation. In the problem formulation the assessment endpoints are selected and the conceptual model for characterizing the risk is prepared. In the estimation of the risk associated with the occurrence of irgarol, diuron and TBT in the Adriatic Sea, the conceptual model was based on the hypothesis that the use of AF paints on submerged structures contributes to the release of these substances. Thus, the assessment consists of determining whether these chemical stressors might have adverse effects towards the marine ecosystem.

Analysis. The aim of this step is to determine how exposure to stressors is likely to occur (*i.e.* exposure characterization) and what are the possible adverse ecological effects that may occur upon exposure to these stressors (*i.e.* effect characterization).

(1) The exposure characterization is based on the TBT, irgarol and diuron measured environmental concentrations (MEC) obtained by the site-specific monitoring survey in the coastal areas of the Apulia region (Italy) and Albania.

(2) The toxicity data-set used in the effect characterization for irgarol and diuron consisted of toxicity values related to

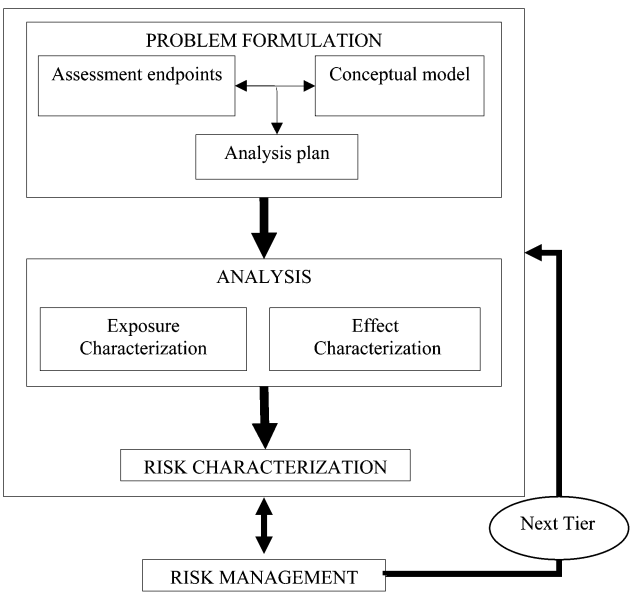

Fig. 2 Framework of ecological risk assessment.

phytoplanktonic and macrophyte species[20] that are expected to be more sensitive to herbicides than other aquatic organisms.

For TBT, instead, the saltwater chronic toxicity data for zooplanktonic and filter feeder species are used, as reported by Hall *et al.*[36] By using the most sensitive group of species, it is possible to obtain conservative effect benchmarks to characterize the risk and then by protecting the good health of sensitive species, the entire ecosystem can be defended.

The effect benchmarks or the predicted no-effect concentrations used in the risk estimation are calculated according to the probabilistic method proposed by Solomon *et al.*,[38] and Hall *et al.*[37] In this study, for each contaminant, the 5th percentile values have been evaluated. This benchmark of effects represents a value protective of 95% of the considered species from adverse effects. The US-EPA endorses the use of the 5th percentile for risk assessment.[38]

Risk characterization. The evaluation of the ecological risk was performed by calculating the numerical hazard quotients (HQ), obtained as a ratio between a predicted or measured exposure concentration (MEC/PEC) of the stressor, and a reference value of the toxicant, considered as the no-effect predicted concentration (PNEC).

In this study, the point risk estimate posed by antifouling agents was obtained by comparison of the MEC of irgarol, diuron and TBT with the corresponding 5th percentile used as the toxicity effect benchmark. If the exposure concentration is equal to or exceeds the effect concentration, the resulting value is equal to or greater than one, and an ecological risk is suspected.

Moreover, even if the toxicity of a chemical mixture may be different from the toxicity of individual constituents, the assumption of additivity may be applicable when the modes of action of chemicals in a mixture are similar.[27] Since irgarol and diuron are both photosystem II inhibitors, the hazard quotient associated with their mixture (HQ_m) can be computed by summing the values of the HQ relative to the individual substances.[39] Once again, an HQ_m >1 suggests an ecological risk and/or the need for additional analysis or data collection.

Principal component analysis (PCA)

PCA is a multivariate method that transforms a number of (possibly) correlated original variables into a smaller number of new uncorrelated variables called principal components, which are obtained as linear combinations of the original variables. The first principal component (PC1) is required to have the largest possible variance. The second component (PC2) has the largest remaining variance and each succeeding component accounts for as much of the remaining variability as possible.[40] The loss of information is given by the sum of the percentage of information associated with the excluded PCs.

In this study, PCA was performed on the aqueous samples considering a data matrix having as many objects as the sampling points (middle port and quay). For details, 12 sample points were taken into account: two for each of the six harbours (namely MN, MDS, TR, SH, DR, and VL). With reference to the variables, the data matrix has as many variables (10 overall) as the chemical data (concentrations of diuron, irgarol and TBT), the ecotoxicological results (percentage effect for *D. tertiolecta*, *V. fischeri P. lividus* and *A. salina*) and the risk analysis results (HQ, one for each sampling point).

Since variables were very different (chemical, ecotoxicological and risk responses) and they were not measured using the same scale units, they were autoscaled (standardized) prior to analysis so as to be treated with equal importance. Autoscaling was executed by first performing a mean column-centering and then dividing by the standard deviation (again, per column). The PCA results were reported as the simultaneous graphical representation (biplot) of the objects (scores plot) and variables (loadings plot).

Loadings represent the contribution of each original variable to the new PCs, and vary between −1 and +1: the greater the absolute value of the loading the more representative the associated variable.[41] Variables exhibiting high loading values for the same PC are considered closely related to each other.

Multivariate statistical analysis of the dataset was performed using the R-based chemometric software of the Italian chemometrics society[42] and the package PARVUS. Concentrations below the detection limit were substituted by one half of the detection limit.[43] These arbitrary values are called "censored" values and when few of these values are employed in the variables used for PCA, only a little bias in the results is expected.

Results and discussion

The characterisation of the sites included a survey of selected AF agent levels in seawater samples from ports and marinas of the Southern Adriatic Sea and the evaluation of their ecotoxicological effect.

The results are summarized in Table 2.

Occurrence of booster biocides in seawater

The two most persistent booster biocides, diuron, and irgarol, were monitored in seawater.

Diuron always exhibited higher concentrations than irgarol in both Italy and Albania where the diuron–irgarol concentration ratios ranged from 1.3 to 87.7 in Apulia and from 3.6 to 145.1 in Albania.

As diuron is also employed as a herbicide in agriculture and weed control, it is frequent that estuaries and runoff receptor coastal waters also contain this compound.[20]

Diuron was detected in all the surveyed Italian ports, with concentrations in the range of 12.4–583.5 ng L^{-1}, and a mean value of 193.9 ng L^{-1}, thus indicating a widespread contamination in the Apulia coastal waters.

MN resulted to be the least polluted port. Contrary to what usually observed, at TR the levels (68.9 ng L^{-1}) near the quay were much lower than those at the centre of the port (448.7 ng L^{-1}), probably because of a particular pattern of the currents and/or of a contamination source nearby. At MDS, the very high concentration (583.5 ng L^{-1}) found in the inner channel was due to the high density of moored boats and to a very

Table 2 Physical, chemical and ecotoxicological data for Italian (Apulia) and Albanian ports

	Sampling stations					
Variables	Manfredonia	M. di Savoia	Trani	Shengjin	Durres	Vlora
Organic compounds in seawater						
Diuron (ng L^{-1})	12.9	16.5	448.7	1.9	78.8	28.8
Diuron[a] (ng L^{-1})	12.4	583.5	68.9	8.4	93.9	33.3
Irgarol (ng L^{-1})	10.0	0.6	5.1	<0.2	0.8	8.5
Irgarol[a] (ng L^{-1})	8.9	14.7	16.1	0.5	0.7	9.3
TBT (ng L^{-1}, as cation)	76.0	12.0	24.0	5.0	24.0	34.0
TBT[a] (ng L^{-1}, as cation)	105.0	110.0	22.0	22.0	24.0	44.0
Physical parameters						
T (°C)	28.5	27.3	26.7	26.0	25.4	26.7
O_2 (% saturation)	81	39	65	84	85	108
Salinity	34.9	36.6	36.6	37.7	38.3	38.1
pH	7.95	7.81	7.87	7.88	8.04	8.00
Ecotoxicological assays[c]						
A. salina (% effect)	17(2.3)	13(4.1)	18(3.2)	11(2.6)	10(1.0)	4(2.3)
A. salina[a] (% effect)	23(3.5)	6(2.4)	16(2.8)	26(2.9)	15(2.2)	4(2.6)
D. tertiolecta (% effect)	66(0.6)	79(1.2)	75(1.4)	99(0.7)	85(1.1)	88(1.8)
D. tertiolecta[a] (% effect)	76(0.7)	100	62(1.5)	87(1.7)	85(0.9)	88(1.2)
P. lividus (% effect)	34(1.9)	43(1.8)	34(2.4)	27(2.2)	43(1.4)	47(2.9)
P. lividus[a] (% effect)	47(2.2)	76(2.7)	32(3.3)	37(2.9)	40(3.1)	40(1.4)
V. fischeri (% effect)	−31.6	−19.6	29.9	−39.0	−11.0	−31.2
V. fischeri[a] (% effect)	2.7	−34.4	−24.8	−44.7	−25.0	−33.0
Risk analysis						
HQ						
Diuron	0.004	0.005	0.144	0.001	0.025	0.009
Diuron[a]	0.004	0.187	0.022	0.003	0.030	0.011
Irgarol	0.053	0.003	0.027	0.001[b]	0.004	0.045
Irgarol[a]	0.047	0.078	0.085	0.003	0.003	0.049
TBT	25.333	4.000	8.000	1.667	8.000	11.333
TBT[a]	35.000	36.667	7.333	7.333	8.000	14.667

[a] Quay. [b] A value derived by a concentration arbitrarily set equal to one half of the detection limit. [c] Mean values ± SD.

poor water exchange in contrast to the middle of the port (16.5 ng L^{-1}), which is free of berths and connected to the open sea.

Average concentrations of diuron in the ports of Apulia were comparable with those reported by other authors (<7 and 366 ng L^{-1})[44,45] but lower than those measured elsewhere in the world (up to 2.160 ng L^{-1})[46] and higher than the levels recorded in Japan, (mean 84 ng L^{-1})[47] and in Gran Canaria's coastal areas in 2007–2009 (2.3–203.6 ng L^{-1}).[10,48]

Diuron was detected in all Albanian water samples, too. Diuron levels (1.9–93.9 ng L^{-1}) were lower than those observed in Italy, except for MN and SH, exhibiting comparable amounts of herbicides.

SH was the least contaminated port (1.9 and 8.4 ng L^{-1}) and DR was the most polluted one (78.8–93.9 ng L^{-1}). This result is not surprising since DR is the main Albanian port, with a high degree of boating activities and a rather closed conformation.

On an international scale, Albanian concentrations were similar to the diuron amounts detected in Seto Inland Sea, Japan (10–62 ng L^{-1})[49] and in California (<2–68 ng L^{-1}).[19,50]

In all seawater samples, from the ports of Apulia, irgarol was detected in the range of 0.6–16.1 ng L^{-1} (on average 9.2 ng L^{-1}).

Comparable amounts of irgarol were measured in Maizuru Bay, Japan, (2–18 ng L^{-1})[47] whereas lower levels were observed in the coastal waters of the Chagos Archipelago, Indian Ocean (<1–8 ng L^{-1}), in 2006.[51] However, usually, the concentrations found in the samples from Apulia were considerably lower than those detected in ports and marinas worldwide, where levels up to 1300 ng L^{-1} have been achieved.[52,20]

Albanian marine waters showed even lower irgarol concentrations (<0.2–9.3 ng L^{-1}, on average 3.3 ng L^{-1}) than those detected in Italy and, in one sample from SH, the irgarol level was below the detection limit (<0.2 ng L^{-1}). VL is more densely populated by fishing boats compared to the other two ports, and was the only sampled site exhibiting concentrations comparable with an Italian port (MN).

Irgarol levels in the samples collected from Albanian ports were similar to those found by Guitart *et al.*,[51] in the Indian Ocean, but they were lower than most of the levels reported in the literature, as illustrated above.

Unlike the ports of Apulia, the Albanian ones are characterized by basins with good water circulation, hence both irgarol and diuron concentrations were quite similar in the samples collected from the quayside and the centre of the basin.

Occurrence of OT compounds in seawater

Despite the total ban of TBT-based paints, TBT was still a commonly encountered contaminant[53] and it was detected in all the water samples collected from both Albanian and Italian coastal areas.

The highest TBT concentrations were observed when samples were collected near the quayside in both a large commercial port (MN2) and a little marina (MDS2).

Apulia's coastal area seemed to be more contaminated with TBT (range 12–110 ng L^{-1} as cation) than Albanian selected sites (range 5–44 ng L^{-1} as cation), but the investigated area needs to be enlarged to obtain a more complete picture. The degradation products, DBT and MBT, were found only where the TBT concentration was higher than 40 ng L^{-1} as Sn, as was the case of Apulia's coastal area. The results are in agreement with recent studies on the marine environment,[53] where maximum concentrations in water rarely exceed 100 ng L^{-1}.

The recent legislation developed in Europe (Water Framework Directive, 2000/60/EC, WFD) provides that good chemical status is reached for a water body when it complies with the Environmental Quality Standards (Directive on EQS, 2008/105/EC) for all the priority substances and other pollutants listed in the EQS directive. In particular, TBT is identified as priority hazardous substance and if we compare the TBT monitoring data obtained in this work with the maximum allowable concentration (MAC-EQS) of 1.5 ng L^{-1} as cation, TBT levels were always not negligible and hence its contamination is still an environmental issue.

Therefore there is an urgent need for a careful evaluation of the TBT source in the water column: the presence could be due to the occurrence of contaminated sediments where organotin compounds are retained and persist for years with the possibility to be re-suspended or it could also be due to the use of "illegal" TBT-based paints from illicit markets.

Ecotoxicity

Among all the battery tests applied, the growth inhibition test with the marine algae *D. tertiolecta* showed always the highest effects. In fact, EC50 values have been obtained for this test only (Fig. 3).

V. fischeri always showed biostimulation, with the only exception of TR1, where a 30% effect has been recorded. The *A. salina* test recorded an effect percentage less than 20 for all the samples, while the spermiotoxicity test with *P. lividus* evidenced higher toxicity values, even if always with an effect lower the fifty percent. Conversely, in the case of MDS2, the toxic effect obtained with sea urchins was quite similar to the results obtained with the algal test.

On the basis of all the test results, the toxic effects resulted higher for Albania than for Apulia, with the highest toxicity registered in SH1 (99%) and in MDS2 (100%).

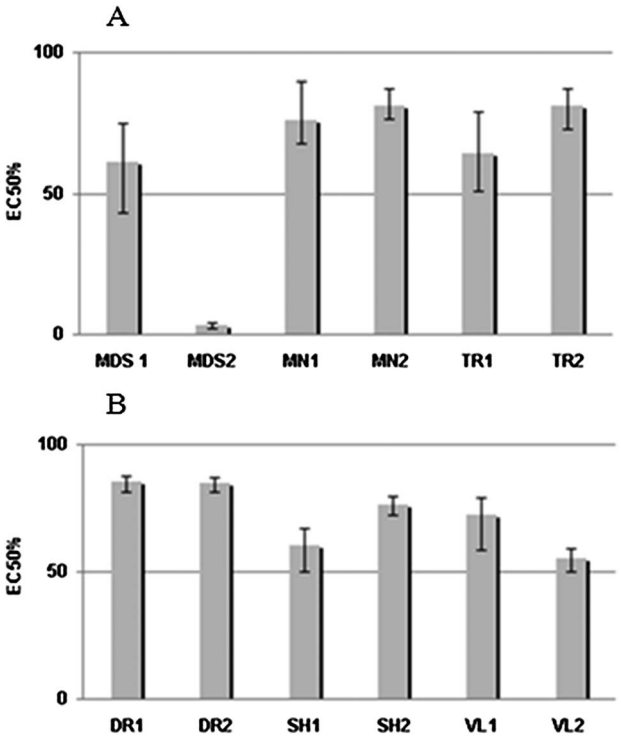

Fig. 3 EC50 (% sample ± CL) resulted from the algal growth inhibition test for (A) Apulia and (B) Albania seawaters. The acronyms for the ports/marinas are explained in Table 1.

As for the distribution of the toxic effect inside the port itself, we evidenced the highest values in the inner part (quay) of the Italian ports while this trend was never evidenced in Albania, where, according to chemical analyses, the results were quite similar for both sampling points. Only at SH, higher effects have been evidenced in the outer part of the port.

Fig. 3A shows the results of the algal test with the diluted seawater samples from Apulia. The highest toxicity has been registered in MDS2 with an EC50 of 3% of the sample, one order of magnitude lower than MDS1, the outer sampling point. Slight differences between the two MN sites with values over 50% (of sample) have been reported, while an opposite trend of toxicity can be noted at TR, exhibiting the highest toxicity in the outer sampling point (TR1).

The results of the algal test with Albanian samples are shown in Fig. 3B. The highest toxicity (as EC50) has been registered for VL2 (55% of sample). A similar value has been obtained for SH1 (60%). For both DR samples similar values have been measured (around 85%).

The used test battery allowed us to do a preliminary screening of the ecotoxicological status of the studied area. In fact, the employed species responded differently to the investigated samples.

The algal toxicity assay had the highest frequency of maximum toxicity identification and therefore it was the most sensitive test. This peculiar sensitivity could be easily ascribed to the type of test and to the endpoint. In fact the algal inhibition growth test foresees a chronic exposure (72 hours).[31] This

high sensitivity was evidenced also in other studies utilizing different matrices.[54,55] Marine algae are highly diffused in coastal ecosystems[56] so they are particularly exposed and susceptible to contaminants associated with anthropogenic pollution. Furthermore, algae have been shown to be more sensitive to toxicants than fish or invertebrates.[57]

The toxicity of the samples collected near the quay can be attributable to the high concentrations of contaminants released by antifouling paints as some authors have reported.[58,20] The differences evidenced for the Italian samples between the two sampling points (quay and middle-port) were probably due to a low hydrodynamic current in the vicinity of the quay[20] and/or to a local pollution source. This was not evident in the Albanian sites, where contamination deriving from antifouling activities is lower.

Sea urchins are among the main marine organisms expected to be exposed to several kinds of pollutants, comprising antifoulants.[59] Their gametes and embryos are often utilized to assess the toxicity of chemical compounds,[34,60] due to their sensitivity and availability. The spermiotoxicity test with sea urchin *P. lividus* showed a moderate sensitivity. In fact, in the evaluation of the seawater toxicity, only once the measured toxic effect was similar to that obtained with the algal test (MDS2) (Fig. 3A).

The bioluminescence test with *V. fischeri* utilizes well established and standardized protocols and, due to the simplicity and rapid nature of the test, it is commonly used worldwide. This assay is a useful tool also for screening and ranking of a large number of samples and then it allows quick identification of the areas of concern. Often a biostimulating effect was observed. This phenomenon can be explained by low bioavailable concentrations of chemicals producing the hormetic effect. Biostimulation was noticed by some authors on several organisms.[61,62]

Marine crustacean *A. salina* is extensively used in ecotoxicology, due to the commercial availability of dried cysts from which live test organisms can be hatched at will. However, there is still a lack of clear information about its sensitivity. A correlation between *A. salina* and fish sensitivity, for some organic compounds, has been recently reported.[63] The acute test with this crustacean was the least sensitive, showing always the lowest toxic effects. A longer exposure time could surely improve the sensitivity of the test.

Ecological risk assessment

The estimated 5th percentile values from toxicity data were 189 ng L^{-1}, 3126 ng L^{-1} and 3 ng L^{-1} for irgarol, diuron and TBT, respectively. On the basis of these values, it was possible to evidence that the highest sensitivity has been shown for TBT, while among herbicides, the plant species were more sensitive to irgarol than to diuron.

The HQ interval values of 0.001–0.187, 0.001–0.085 and 1.67–36.67 were obtained for diuron (Fig. 4), irgarol (Fig. 5) and TBT (Fig. 6), respectively. These data suggest that in all the investigated ports and marinas, a negligible risk was posed by irgarol and diuron, taken individually. The HQ_m computed for a mixture of irgarol and diuron was still lower than 1 (Fig. 7).

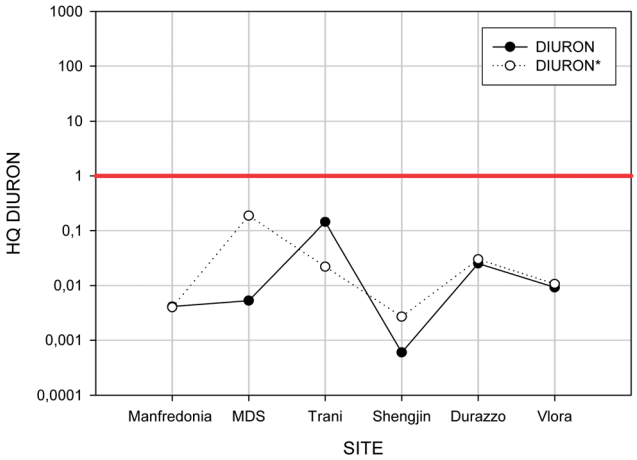

Fig. 4 HQ values of diuron in the Italian and Albanian ports. The acronyms for the ports/marinas are explained in Table 1.

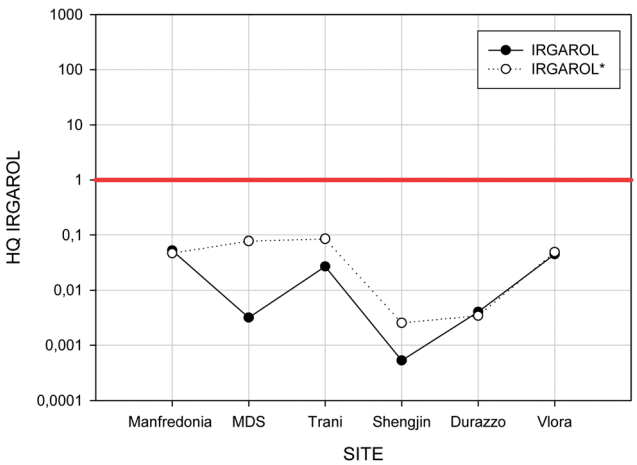

Fig. 5 HQ values of irgarol in the Italian and Albanian ports. The acronyms for the ports/marinas are explained in Table 1.

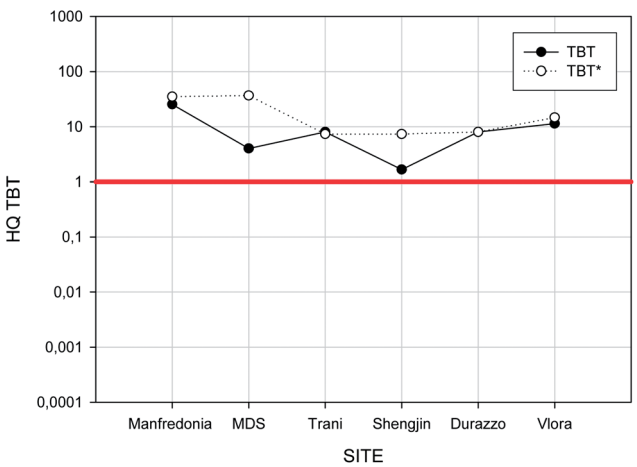

Fig. 6 HQ values of TBT in the Italian and Albanian ports. The acronyms for the ports/marinas are explained in Table 1.

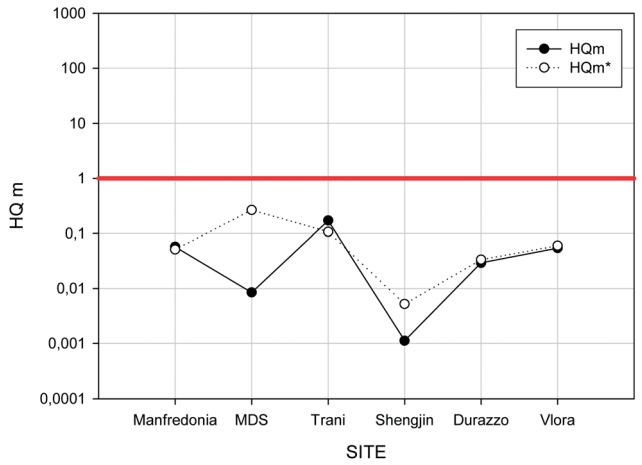

Fig. 7 HQ_m values of a mixture of irgarol and diuron in the Italian and Albanian ports. The acronyms for the ports/marinas are explained in Table 1.

For TBT, instead, the individual HQ values were always greater than 1: it means that even if TBT has been banned, deleterious effects on aquatic exposed organisms can still be exerted.

In addition, considering the physicochemical properties of the studied antifouling biocides, the sediments may present higher concentrations, and consequently a different risk.

Principal component analysis

The PCA rationalized the original dataset into two principal components, which together explained about 66% of the total variance in the original data (Fig. 8).

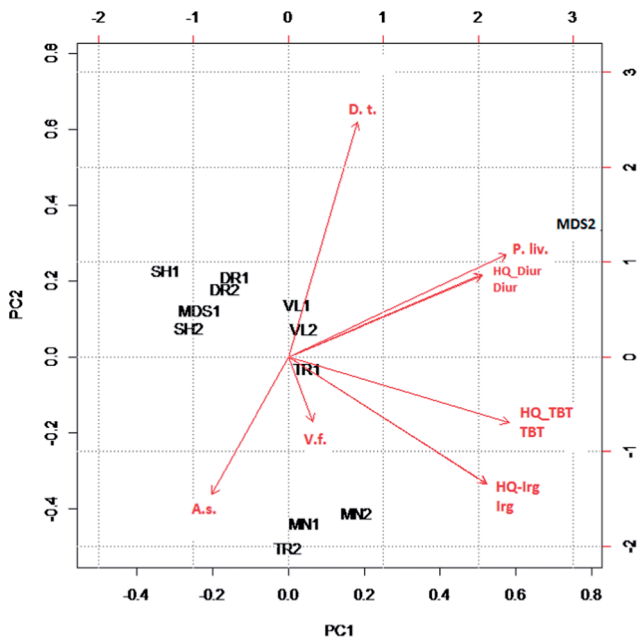

Fig. 8 Loadings are indicated by red arrows; the corresponding scales are at the bottom and on the left of the plot. The score scales are reported on the top and on the right of the plot. The acronyms for the ports/marinas are explained in Table 1.

PC1 explained about 45% of the total variance and indicated the risk posed by AF agents diuron, irgarol and TBT to the marine ecosystem, since the variables with the highest positive loadings on PC1 were the biocide concentrations and their relative HQs. Therefore, sampling stations showing high scores for PC1 should be identified as the most polluted, with the highest risk to aquatic life, as was the case of MDS2.

The PC1 *vs.* PC2 scores plot highlighted a significant difference between MDS2 and all the other sampling stations, according to chemical, ecotoxicological and ERA findings.

The two sampling points for each port were always close to each other in the plot, indicating that they provide similar information. The only two exceptions were TR and MDS where a great difference in diuron concentrations was detected between the two sampling points, as reported above.

PC2, accounting for 21% of the variance, contributed to separate objects (*i.e.* sampling points), mainly on the basis of some ecotoxicological effects (*D. tertiolecta* and *A. salina*). In the PC1–PC2 plot, the Italian sampling sites, except for MDS1 and TR1, were clearly set apart from the Albanian ones right along the PC2 axis; this suggests that ports in Apulia differ from those in Albania more for the recorded ecotoxicological effects than for the different degrees of AF contamination.

Toxic effects on *P. lividus* seem to be associated with the AF concentrations and related HQs (all showing positive values on the PC1 axis). On the other hand, the low loadings observed on PC1 for *A. salina, D. tertiolecta* and *V. fischeri* suggest that results on these organisms are not significantly correlated with chemical concentrations and HQ values. A weak correlation between the risk posed by irgarol and the toxic effect detected for *A. salina* is hypothesized on the basis of loading values on PC2.

Considerations on chemical, ecotoxicological, risk and statistical analyses results

Chemical analyses showed a different extent of contamination by the investigated AF agents between Albania and Apulia, the latter exhibiting higher levels for all biocides, as expected since the marine traffic in Albania is much lower. Similarly, PCA found different information for water samples coming from the two countries, leading to a separation of the Albanian ports from the Italian ones except for MDS1 and TR1, where the levels of contamination were comparable to the Albanian ones.

However, HQs calculated by ERA indicated that, in both countries, the risk posed by irgarol and diuron to aquatic organisms was always low, even when their mixture was considered. Conversely, a risk was determined for TBT.

This biocide was detected in all the sampling sites and, in particular, MN2 and MDS2 have shown very high concentrations of TBT (>100 ng L^{-1} as cation). It is worth noting that MDS2 was a hotspot for all the investigated AF biocides.

In agreement with the chemical results, the growth inhibition test with the marine algae *D. tertiolecta* showed the highest effect (100%) right at MDS2 as well as the spermiotoxicity test with sea urchin *P. lividus*, for MDS2, was about twice (76%) the effect observed in all the other sampling stations (32–47%).

The findings of chemical and ecotoxicological analyses were confirmed by statistical analysis, PCA, which showed a much more critical situation for MDS2 compared to all the other sampled sites.

In contrast to the results of chemical analyses, the algal test, which is the most sensitive, highlighted slightly higher toxic effects in Albania (on average 86.8%) than in Italy (on average 76.3%). This result suggests the presence of contaminants not taken into account by chemical analyses. In particular, it should be noted that SH, despite being the least contaminated port by the three monitored biocides, was among the sites exhibiting the highest toxic effects for algae *D. tertiolecta* (99% effect) and the major response from bacteria *V. fischeri* in bioluminescence tests (−44.7%).

Spatial distribution assessment of irgarol, diuron and TBT evidenced concentration differences between the quay and the centre of the basin in Italy, except at MN, while in Albania the spatial variability was rarely observed.

In agreement with chemical findings, ecotoxicological bioassays evidenced higher toxic effects in the quay than in the middle of the basin in the ports of Apulia, whereas in Albania similar values were obtained for samples collected from the two sampling points.

Moreover, PCA found a remarkable similarity between samples collected from the quay and the middle-port in Albania and at Manfredonia. Once again, this is in accordance with chemical and ecotoxicological results.

MDS was the port where the greatest concentration differences between the two monitored points (quay and middle-port) were observed for the three AF biocides, with very high levels in the quay. However, the pattern distribution was not always similar for the investigated AF agents, thus suggesting that the concentration changes were due to the proximity of pollution sources in addition to the dynamics of the currents.

For example, at MN, both irgarol and diuron exhibited comparable levels in the two sampling points, while TBT showed a higher concentration in the quay. At TR, the opposite was true: diuron and irgarol, to a much lesser extent, showed a spatial variability, while TBT did not. In particular, for diuron, a much higher level was found in the centre of the port than in the quay, while in all the other ports the opposite was always observed for all the assessed AF agents.

Similarly, the ecotoxicological test showed that TR2 was less toxic than TR1 where a 30% toxic effect for *V. fischeri* was recorded while a biostimulation was observed for all the other sampling points.

Again, at SH, diuron and TBT exhibited higher levels in the quay while irgarol was present in comparable amounts in the two sampling points. Conversely, higher toxic effects have been found at SH1, once again suggesting the presence of not analysed toxicants.

By comparison between the HQs and the bioassay results (Table 2 and Fig. 3–7), it can be speculated that toxic effects found in Italian (Apulia) and Albanian ports, where a potential risk (TBT HQ > 1) was estimated, may be related to the presence of TBT.

Moreover, even if negligible risks from diuron and irgarol are expected on the basis of HQ values, the contribution of these biocides to the overall observed toxicity cannot be excluded when they act in a complex environmental matrix.

The major toxic effects were highlighted for samples from MDS2 where the highest HQ values were also observed for all antifoulants. In particular, the diuron HQ value was one order of magnitude higher than diuron HQs obtained for the other ports. In contrast, in TR and MN, bioassays (mainly the algal test) highlighted high effects while irgarol and diuron HQ values were always lower than 0.1.

With reference to the Albanian sites, the lowest HQ values were determined for SH where, instead, the highest effect (100%) from the toxicity test battery was recorded. On the other hand, VL2 showed the highest HQ values for irgarol and diuron corresponding to the highest toxic effect registered for algae as EC50 (Fig. 3).

Conclusions

In this work the pollution from antifoulants and the ecotoxicological effects were assessed for selected ports and marinas along the Italian (Apulia) and Albanian coasts. Physicochemical and ecotoxicological data were obtained and two different methods, ERA and PCA, were employed to manage this heterogeneous dataset in order to get a good insight into the environmental quality of the areas under investigation.

The chemical characterization showed that the coastal areas in Albania were less polluted than in Apulia, especially with regard to irgarol and diuron.

However, a low contamination by irgarol was found in both Albanian (on average 3.3 ng L^{-1}) and Italian (Apulia, on average 9.2 ng L^{-1}) coastal waters.

Greater differences for diuron concentrations were observed between Apulia and Albania, with average values of 193.9 ng L^{-1} and 40.85 ng L^{-1}, respectively.

Diuron exhibited higher concentrations than irgarol in all ports and marinas monitored in this study, except for MN where the levels of the two herbicides were comparable.

Surprisingly, TBT was detected in all the sampling sites, always exceeding the maximum allowable concentration (MAC) of 1.5 ng L^{-1} as cation, indicated by the Directive on Environmental Quality Standards.

In contrast to the results of the chemical analyses, the algal test, which is the most sensitive, highlighted slightly higher toxic effects in Albania (on average 86.8%) than in Italy (on average 76.3%).

Spatial patterns of irgarol, diuron and TBT in seawater were assessed, showing a variation of concentration between the quay and the middle-port in the Italian ports and a quite homogenous contamination inside Albanian sampling locations.

On the basis of the measured concentrations for the three AF biocides, the risk for the local marine ecosystem has been determined by calculating the HQ values. It was found that irgarol and diuron did not pose a risk to aquatic organisms while the likelihood of adverse effects was found for TBT.

Although TBT has been banned for years from the market of antifouling paints, it is still present at levels of concern that pose a serious risk to the health of marine organisms.

Therefore further investigation is really needed on the TBT presence in marine waters in order to determine more accurately the extent of contamination and its associated risk as well as to understand the possible sources of this dangerous biocide.

Acknowledgements

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