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Marine Hazard Assessment of Soluble and Nanostructured Forms of the Booster Biocide DCOIT in Tropical Waters

Fernando Perina [1,*], Cristiane Ottoni [2,3], Juliana Santos [2], Vithória Santos [1,4], Mariana Silva [2], Bruno Campos [2], Mayana Fontes [2], Debora Santana [2], Frederico Maia [5], Denis Abessa [2] and Roberto Martins [1]

1 CESAM-Centre for Environmental and Marine Studies and Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
2 Biosciences Institute, São Paulo State University (UNESP), São Vicente 11330-900, SP, Brazil
3 LEAF-Linking Landscape, Environment, Agriculture and Food Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal
4 Institute of Advanced Sea Studies (IEAMAR), São Paulo State University (UNESP), São Vicente 11330-900, SP, Brazil
5 Smallmatek—Small Materials and Technologies, Lda, 3810-075 Aveiro, Portugal
* Correspondence: perinafc@ua.pt

Abstract: The encapsulation of antifouling compounds, such as DCOIT (4,5-Dichloro-2-octylisothiazol-3(2H)-one), in mesoporous silica nanocapsules (SiNC) has recently been demonstrated to be an eco-friendly alternative to decrease biocide toxicity towards marine non-target species. However, the lack of information on the chronic effects of such nanomaterials on non-target tropical species is critical for a more comprehensive environmental risk assessment. Thus, the present study aimed to assess the chronic toxicity and hazard of the soluble and encapsulated forms of DCOIT on neotropical marine species. Chronic tests were conducted with six ecologically relevant species. No effect concentration (NOEC) values were combined with NOEC values reported for tropical species to assess the hazard using the probabilistic approach to derive each predicted no effect concentration (PNEC). The SiNC-DCOIT was three- to ten-fold less toxic than soluble DCOIT. Probabilistic-based PNECs were set at 0.0001 and 0.0097 µg DCOIT L^{-1} for the biocide soluble and nanostructured forms, respectively. The immobilization of DCOIT into SiNC led to an 84-fold hazard decrease, confirming that the encapsulation of DCOIT into SiNC is a promising eco-friendly alternative technique, even in a chronic exposure scenario. Therefore, the present study will contribute to better management of the environmental risk of such innovative products in the tropical marine environment.

Keywords: antifouling biocides; nanomaterials; environmental regulation

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1. Introduction

Biofouling is one of the most impressive and successful ecological successions worldwide [1]. It corresponds to the settlement, growth, proliferation, and succession of micro- and macrofoulers on partially or fully immersed surfaces evolving in a complex manner, as an ecological succession process, which begins with the development of a bacterial biofilm, followed by the settlement of ephemeral algae, perennial algae, and animal species [1]. Such a process is particularly challenging on human-made structures (e.g., ship hulls and fuel systems, marine sensors; biofilms on drinking water systems and medical devices), causing problems in different fields (e.g., maritime transportation; aquaculture, healthcare; water treatment) when it is not controlled [1–4]. Such problems may include an increase of roughness, weight, shear stress, drag force and maintenance costs in ships, buoys, or other floating structures, leading to excessive fuel consumption and greenhouse gas emissions or structural failure (e.g., sink) [2,5]. Maritime industry activity also has indirect consequences, such as bioinvasions by non-indigenous species transported in ship hulls

and marine chemical pollution caused by the leaching from protective coatings (e.g., antifouling agents, metals), but also ship engines (e.g., airborne particulate matter associated with polycyclic aromatic hydrocarbons, metals, nitric oxide, sulphur dioxide) or sanitary wastewater (e.g., pharmaceuticals) [6,7]. Not surprisingly, fouling-free surfaces is one of the most critical steps towards the reduction of maritime industry environmental impacts (e.g., reduce greenhouse gas emissions, chemical contamination, or non-indigenous species introduction) [5] aiming at accomplishing several United Nations Sustainable Development Goals, particularly the SDG 14 (conservation and sustainable use of the oceans), but also 13 (climate action), 6 (clean water) or 12 (sustainable consumption and production) [8].

Current and emerging approaches to manage biofouling on submerged marine structures include mechanical abrasion, high pressure jets, bubble streams and power washing, desiccation, heat, ultrasound, laser radiation, autonomous and remotely operated cleaning systems, sprays, innovative biomimetic antifouling (AF) surfaces, superhydrophobic or superhydrophilic nonbiocidal coatings, hydrolysis-based coatings and, the most-used worldwide, biocidal coatings [3,9–11]. Biocidal coatings include AF ingredients that have been evolving over time. Previous generations of biocides included metals (e.g., Cu) and very efficient organotins (e.g., tributyltin-TBT) that were banned in 2008, but still pose environmental risks to the aquatic biota due to their physicochemical properties and illegal trade and use in some regions across the globe [7,12]. A new generation of organic or organometallic biocides (e.g., dicopper oxide; copper or zinc pyrithione (ZnPT/CuPT); diuron; chlorothalonil; cybutryne or Irgarol® 1051; DCOIT (4,5-Dichloro-2-octylisothiazol-3(2H)-one)) emerged as an environmentally safer alternative than the former generation, promising a broad-spectrum activity and lower ecotoxicity, solubility in seawater, bioaccumulation and persistence in the environment comparing with TBT-related biocides [13]. Nevertheless, several studies have shown that most current AF biocides are toxic, very, or extremely toxic towards non-target marine species, causing a wide variety of acute and/or chronic effects in non-target organisms [14–20], and posing critical environmental risk to demersal and benthic species worldwide [13,21–23]. Consequently, a number of countries have increasingly adopted stricter regulations on the use of such chemicals, in particular, cybutryne, which was already banned to be used as active ingredient in AF paints in the EU (since 2017 [24]), USA (after 2023 [25]), and other countries, due to its toxicity, including coral bleaching, and persistence in the environment [26]. To solve this issue and avoid the repetition of past mistakes, recent research has focused on the replacement of current biocides by natural or naturally inspired antifoulants, such as fatty acid amides [27], peptides [28], algae extracts [29], or bacterial quorum-sensing inhibitors [30], enzymes [31] and toxins [32]. Despite their anti-fouling efficacy and environmentally friendly properties, the industrial scale-up process is difficult, complex, time-consuming, and expensive [33].

Alternatively, modifications of the availability of current biocides have gained a lot of attention in recent years. Basically, active ingredients are encapsulated in stimuli-responsive engineered nanomaterials (ENM), such as silica mesoporous nanocapsules (SiNC), layered double hydroxides, polyurea microcapsules or clay nanotubes [34–39]. As AF additives, immobilized biocides are control-released from the ENM to the coating's matrix, upon specific triggers (e.g., pH changes, presence of water or chlorides), decreasing the undesired biocidal leaching to the seawater in the early stages of the coating with undeniable economic and environmental benefits [34,36]. Amongst the several recently developed AF nanoadditives, the nanostructured form of DCOIT into silica mesoporous nanocapsules (SiNC-DCOIT) has been demonstrated to be an efficient and eco-friendly alternative to the current state-of-the-art booster biocide, lowering DCOIT short-term toxicity [36,40–42] and environmental hazard on temperate organisms [43]. In fact, SiNC-DCOIT exposure causes less toxicity than DCOIT on non-target marine species (e.g., *Perna perna* gametes, EC_{50} = 0.063 and 8.6 mg DCOIT L^{-1} for DCOIT and SiNC-DCOIT, respectively [41]; microalgae *Isochrysis galbana*, IC_{50} = 0.032 and 6.84 mg DCOIT L^{-1}, for DCOIT and SiNC-DCOIT, respectively [36]). In terms of hazard assessment, the predicted no-effect concentration (PNEC) for SiNC-DCOIT in temperate waters is 0.01 mg L^{-1} [43], and for DCOIT was

recently set on 0.0004 mg L^{-1} in global waters [13], based on the most conservative approach. However, a full understanding of the chronic effects of SiNC-DCOIT and their hazardousness in tropical environments is still lacking, and is critical for a global and more comprehensive environmental risk assessment of such innovative AF nanoadditive. Therefore, the present study aimed to assess the chronic toxicity of both soluble and encapsulated forms of DCOIT (DCOIT and SiNC-DCOIT) on neotropical marine species as well as at assessing the chronic hazard of both conventional and nanostructured AF additives in tropical environments.

2. Materials and Methods

2.1. Chemical Compounds

DCOIT (CAS nr. 64359-81-5) was purchased from Sigma-Aldrich (São Paulo, Brazil). Nanomaterials (SiNC; SiNC-DCOIT) were supplied by Smallmatek, Lda. (Aveiro, Portugal).

2.2. Nanomaterial Characterization

Morphological and Structural Characterization

Morphological characterization of ENM size and shape, were performed using a transmission electron microscope (TEM) LEO 906E (Zeiss, Germany), operated at 200 kV. Sample preparation was done by dripping colloidal dispersions of ENM onto carbon-coated copper grids (Sigma Aldrich—Merck, São Paulo, Brazil) [44].

2.3. Environmental Behavior and Ecotoxicity Assessment

2.3.1. Preparation of Solutions/Dispersions in Natural Seawater

Stock solutions/dispersions were prepared in natural seawater (salinity 33 ± 2) filtered through a 0.22 µm microporous membrane filter and dispersed in an ultrasonic bath (Kondontech cd-4820, São Carlos, Brazil) at 40 kHz, for 30 min. Serial dilutions of the stock dispersions were then prepared according to the selected concentrations for each tested organism (pls. cf. next sub-sections) and sonicated again for 15 min immediately before the exposure test. For each toxicity test, salinities were adjusted by adding milli-Q water.

2.3.2. Environmental Behavior of Nanomaterials in Natural Seawater

The environmental behavior of SiNC and SiNC-DCOIT in natural seawater (pls cf. previous section) was assessed using three concentrations (0.001, 0.1 and 10 mg L^{-1}; n = 1) mimicking the exposure tests (pls. cf. next section). The hydrodynamic particle size (DLS), zeta potential (ζ) and polydispersion index (PdI) were performed using the Dynamic Light Scattering equipment Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK).

2.3.3. Marine Ecotoxicity

Ecotoxicological tests were carried out using six neotropical marine species representing four trophic levels including decomposing organisms: bacteria (*Vibrio parahaemolyticus*) and fungi (*Penicillium citrinum*); primary producer: microalgae (*Chlorella minutissima*); primary consumer: crustaceans (*Nitokra* sp.); and secondary consumer: echinoderms (*Echinometra lucunter* and *Mellita quinquiesperforata*).

Antibacterial and Antifungal Activity

The bacterial and fungal strains were acquired from the Culture Collection of the Biosciences Institute, São Paulo State University (UNESP). The minimum inhibitory concentration (MIC) of the soluble and nanostructured forms of the booster biocide (DCOIT and SiNC-DCOIT) and the unloaded ENM were determined in a miniaturized bioassay in 96-well plates [45]. The microbial concentration of the inoculum used in the bioassays was defined as 1.0×10^5 cells mL^{-1} for the Gram-negative marine bacterium *V. parahaemolyticus* and 1.0×10^5 spores mL^{-1} for the marine fungus *P. citrinum* IB-CLP11. For each compound, 20 µL of colloidal dispersion was added to the wells at concentrations ranging from 1 to 10,000 µg DCOIT L^{-1} (nominal concentrations). After 24 h of incubation at 37 °C, a volume

of 10 µL of 0.01% resazurin solution was added to all wells. The microplates were one more time maintained at 37 °C for 24 h in a biological oxygen demand incubator incubator (Novatecnica, NT 703, São Paulo, Brazil). The MIC, defined as the lowest concentration of compounds required for the absence of microorganism growth, was then determined. To confirm the antimicrobial activity (minimum lethal concentration—MLC), 10 µL of each well from the MIC assay were inoculated in a Petri dish containing TSA (TSB + 1.5% agar) for bacteria, and PDA for fungi. The Petri dishes were incubated at, 37 °C for 24 h for bacteria, and 30 °C for 96 h for fungi [45,46]. After the incubation period, compounds that inhibited 100% of the microorganisms' growth were considered bactericides/fungicides. All MIC and MLC assays were performed in triplicate.

Microalgae Growth Inhibition Toxicity Tests

The marine microalgae *C. minutissima* was acquaired from the Aidar & Kutner Culture Collection (BMAK—"*Banco de Microrganismos*" Aidar & Kutner), at the Oceanographic Institute, University of São Paulo, Brazil. The microalgae stock was cultured in 250 mL Erlenmeyer flasks containing 50 mL Guillard medium, with salinity 35, under a cool white fluorescence light illumination (18 W LED tube lamps each, with a color temperature of 6500 K), with an intensity of 70 µphotons m^{-2} s^{-1}, for 12 h cycles of light and dark, at 23 ± 1 °C, for 5 days. The microalgae assay was carried out based on the ABNT NBR16723 standard method [47] and Dupraz et al. [48]. Briefly, 180 µL of fresh cultures of *C. minutissima* in the exponential growth phase, at an initial concentration of 10^4 cells mL^{-1} were added into 96-well microplates. A 20 µL of DCOIT, SiNC-DCOIT and SiNC solutions/dispersions with nominal concentration ranging from 1 to 10,000 µg DCOIT L^{-1} (final exposure concentrations, i.e., previously adjusted to the dilution) were individually pipetted for each compound (n = 8). The peripheral wells were filled with sterile water to reduce the effect of evaporation [49]. Growth inhibition was determined from the average absorbance of eight wells compared with the controls, measured by optical density (OD) using a UV-Vis spectrophotometer (Multiskan GO 283 Microplate, Thermo Fisher Scientific, Waltham, MA, USA), at 680 nm, every 24 h. The regression equation used to convert optical density (x) into cell density (y) was: $y = 3^{-6}x - 0.0015$ ($R^2 = 0.99$), determined from a standard calibration curve presented in the supplementary material (Figure S1). Growth inhibition was then calculated after 96 h of exposure, according to the average specific growth rate.

Long-Term Chronic Toxicity Tests Using Crustaceans

Copepods were obtained from laboratory stock cultures of the Biosciences Institute, São Paulo State University (UNESP), and kept in 1 L flasks with saline water (salinity 17 and 30), fed twice a week with dissolved fish food (1:4; food: distilled water) at a controlled temperature (25 ± 2 °C) and photoperiod (16:8 h; light: dark) with water renewal every 15 days. The long-term chronic exposure test on microcrustacean *Nitokra* sp. followed the ABNT NBR 16,723 standard method [50]. In brief, three replicates containing 20 mL of test solutions, prepared as previously described, were set at nominal concentrations of 0.01, 0.1, 1, 10, 100 µg L^{-1} of SiNC, soluble DCOIT and SiNC-DCOIT (as µg DCOIT L^{-1}). Subsequently, five ovigerous females were transferred from the stock culture to each test vial and fed with dissolved fish food a single time during the experiment. The experiment was maintained for seven days at 25 ± 2 °C and with a photoperiod of 12 h:12 h in a BOD incubator (Novatecnica, NT 411D, São Paulo, Brazil). After the exposure period, 1 mL of formaldehyde (4%) buffered with borax and Rose-Bengal dye (0.1%) was added to resume the experiment and to preserve and facilitate the prole identification. The number of nauplii and copepodites observed in each treatment was compared with the control group offspring to assess the chronic effects. Offspring and adults were counted to determine the fertility rate.

Short-Term Chronic Toxicity Tests Using Echinoderms

Adult individuals of *E. lucunter* were collected at rocky shores Ilha de Palmas (Santos, Brazil), transported to the laboratory, and kept in tanks with seawater under controlled conditions for acclimation (24 h). The short-term chronic exposure tests with *E. lucunter* following the ABNT NBR15350 standard method [51]. Briefly, around 600 recently fertilized eggs (in each replicate) were exposed to the studied substances in test tubes containing 10 mL of test solutions/dispersions. The concentrations ranged from 0.01 to 1 μg L^{-1} for DCOIT and SiNC, and from 0.33 to 100 μg DCOIT L^{-1} for SiNC-DCOIT (dilution factor of 3).

Adult individuals of *M. quinquiesperforata* were collected in the subtidal zone of sandy beaches at the coast of São Paulo (São Vicente, Brazil). The short-term chronic exposure tests with *M. quinquiesperforata* followed the EPS 1/RM/27 [52] guideline, with adaptations proposed to the species [53,54]. Approximately 500 fertilized eggs were transferred to the glass test tubes containing 10 mL of test solutions/suspension. Nominal concentrations of DCOIT ranged from 0.01 to 1 μg L^{-1}, and for SiNC-DCOIT from 0.33 to 33.3 μg DCOIT L^{-1} (dilution factor of 3 for both biocides forms). For SiNC, nominal concentrations ranged from1.3 to 808 μg L^{-1} (dilution factor of 5). Four replicates per treatment and a negative control (filtered seawater, salinity 35) were prepared. Both echinoderm experiments were maintained at 25 ± 2 °C and with a photoperiod of 12 h:12 h in a BOD incubator (Novatecnica, NT 411D, São Paulo, Brazil).

Statistical Analyses

For each species, no observed effect concentrations (NOEC) were derived from One-way Analysis of Variance (ANOVA) followed by the Dunnett test, whenever significant differences between the treatments and the control were noticed ($p < 0.05$). GraphPad Prism software v6.0 was used to calculate the IC/EC_{50} values after using the nonlinear regression equation that best fit the data, considering the R^2 value, the absolute sum of squares, and the 95% confidence intervals.

2.4. Hazard Assessment

The hazard assessment relied on the determination of the predicted no-effect concentration ($PNEC_{marine}$) of each tested chemical on marine tropical waters through probabilistic and deterministic approaches following the European Technical Guidance Document (TGD) on risk assessment [55]. Short- and long-term chronic ecotoxicological data from this study and literature [17,18,56–58] was used to determine the chronic predicted no-effect concentration (PNEC) values of the tested compounds in the tropical environment.

The probabilistic, also referred as statistical, PNEC was estimated through the species sensitivity distribution (SSD) method using a log-normal distribution, from which the hazardous concentration at 5% (HC_5) was derived through the software ETX 2.1. Then, the statistical PNEC was obtained through the ratio between the HC_5 value and an appropriate assessment factor, considering the associated uncertainty regarding the whole dataset for each chemical following the recommendations of the TGD [55]. The assessment factors were 1 for DCOIT and 3 for SiNC-DCOIT following the database size and representativity of sensitive life stages.

The deterministic PNEC was calculated through the quotient between the lowest available NOEC value divided by a conservative assessment factor following the recommendations of the TGD [55]. In this case, the assessment factors were 10 for DCOIT and 50 for SiNC-DCOIT considering the existence of three long-term NOEC values from marine microalgae, crustaceans, and fish for DCOIT and two long-term NOEC values from marine microalgae and crustaceans for SiNC-DCOIT, in addition to long-term NOEC values (>2) from other additional marine taxonomic groups.

3. Results

3.1. Nanomaterials Characterization and Environmental Behavior

Figure 1 shows the images of tested ENM, obtained through transmission electron microscopy (TEM). Both ENM present a spherical and regular shape, with an average size between 112 and 136 nm. Unloaded silica mesoporous nanocapsules have a core shell and porous structure (Figure 1a).

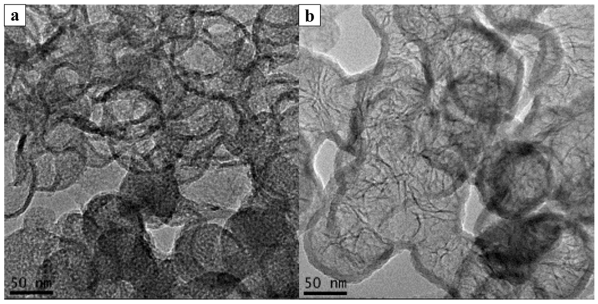

Figure 1. Transmission electron microscopy of tested nanomaterials: (**a**) unloaded silica mesoporous nanocapsules (SiNC); and (**b**) silica mesoporous nanocapsules loaded with DCOIT (SiNC-DCOIT).

The hydrodynamic diameter obtained through DLS (Table 1) indicated that the size of the ENM varied between 202.67 and 231.11 nm, while the determined PdI was within a range of 0.44–0.58, indicating an average dispersity. The zeta potential (ζ) values measured in natural seawater are positive and the dispersions are unstable (30 mV > ζ > −30 mV), with the exceptions of SiNC at 0.001 mg L^{-1} (Table 1).

Table 1. Determination of hydrodynamic particle size (DLS), zeta potential (ζ) and polydispersion index (PdI) on natural seawater dispersions of unloaded silica mesoporous nanocapsules (SiNC) and silica mesoporous nanocapsules loaded with DCOIT (SiNC-DCOIT) at different concentrations.

Nanomaterial	Concentration (mg L^{-1})	DLS (nm)	(ζ)	PdI
SiNC	0.001	203.37	30.90	0.45
	0.1	210.33	27.23	0.50
	10	226.21	29.34	0.55
SiNC-DCOIT	0.001	202.67	12.15	0.58
	0.1	206.12	11.26	0.44
	10	231.11	13.93	0.52

3.2. Ecotoxicity Assessment

The estimated MIC, MLC, NOEC, LOEC and E/IC_{50} values are summarized in Table 2. DCOIT is the most toxic compound for all tested species (lowest NOEC = 0.03 µg DCOIT L^{-1}). Apart from the tested bacteria and fungi (NOEC ≤ 100 µg L^{-1}), the nanostructured form of DCOIT is 3 to 10-fold less toxic than the soluble form.

Both forms of the booster biocide (DCOIT and SiNC-DCOIT) presented bacteriostatic (MIC) and bactericidal (MLC) effects for *V. parahaemolyticus*, at concentrations of 1000 µg DCOIT L^{-1} and 10,000 µg DCOIT L^{-1}, respectively (Table 2). SiNC did not compromise the viability of the *P. citrinum* selected for the study (Table 2). The fungicidal effect on *P. citrinum* was observed only for DCOIT at 10,000 µg L^{-1}, confirming the lower toxicity of SiNC-DCOIT compared to the soluble form (Table 2).

The effects of SiNC, DCOIT, and SiNC-DCOIT on the growth rate of *C. minutissima* are shown in Table 2. Significant effects were observed at 1000 µg L^{-1} of SiNC. Regarding the soluble and nanostructured forms of DCOIT, significant effects were observed at 50 µg DCOIT L^{-1} and 500 µg DCOIT L^{-1}, respectively. The estimated EC_{50} of SiNC for *C. minutissima* was 2240 µg L^{-1}. SiNC-DCOIT (IC_{50} = 625 µg DCOIT L^{-1}) presents lower toxicity than its soluble form DCOIT (IC_{50} = 40 µg L^{-1}).

Table 2. Minimum inhibitory concentration (MIC), minimum lethal concentration (MLC), no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), median effective concentration (EC_{50}), median lethal concentration (LC_{50}), and median inhibitory concentration (IC_{50}) values for species exposed to unloaded silica mesoporous nanocapsules (SiNC), the soluble (DCOIT) and nanostrucutured (SiNC-DCOIT) forms of the anti-fouling biocide DCOIT. "nd": not determined. The 95% confidence interval of L/EIC_{50} values are presented in brackets (95% CI).

Species	SiNC (μg L^{-1})					DCOIT (μg L^{-1})					SiNC-DCOIT (μg DCOIT L^{-1})				
	MIC	**MLC**	**NOEC**	**LOEC**	**E/IC_{50} (95%IC)**	**MIC**	**MLC**	**NOEC**	**LOEC**	**E/IC_{50} (95%IC)**	**MIC**	**MLC**	**NOEC**	**LOEC**	**E/IC_{50} (95% IC)**
Nitokra sp.	–	–	1	10	12.8 (3.22–50.7)	–	–	0.1	1	0.322 (0.10–1.09)	–	–	0.1	1	1.27 (0.60–2.69)
E. lucunter	–	–	100	>100	no observed effect	–	–	0.03	0.1	0.162 (0.12–0.22)	–	–	3.33	1	13.5 (8.44–21.7)
M. quinquiesperforata	–	–	6.5	32.3	57.6 (26.0–127.7)	–	–	0.1	0.33	0.166 (0.10–0.28)	–	–	0.33	1	8.12 (3.13–21.0)
V. parahaemolyticus	nd	nd	nd	nd	nd	10^3	$\geq 10^4$	100	10^3	nd	10^3	$\geq 10^4$	100	10^3	nd
P. citrinum	nd	nd	nd	nd	nd	10^3	10^3	100	10^3	nd	10^3	$\geq 10^4$	100	10^3	nd
C. minutissima	nd	nd	nd	10^3	2240 (nd)	nd	nd	nd	50	40 (nd)	nd	nd	nd	500	625 (nd)

The long-term exposure revealed that DCOIT exhibited higher toxicity to *Nitokra* sp. than the other tested chemicals (Figure 2a). The estimated EC_{50}-7d values were set at 0.32 and 1.27 µg DCOIT L^{-1} for DCOIT and SiNC-DCOIT, respectively (Table 2). Although the EC_{50} of DCOIT was about an order of magnitude lower than the estimated for SiNC-DCOIT, the NOEC and LOEC for both substances were 0.1 and 1 µg DCOIT L^{-1}, respectively (Table 2). The unloaded nanomaterial (SiNC) was the compound that least affected the number of offspring produced by *Nitokra* sp. (Figure 2a). The EC_{50}-7d estimated for SiNC was 12.8 µg L^{-1}.

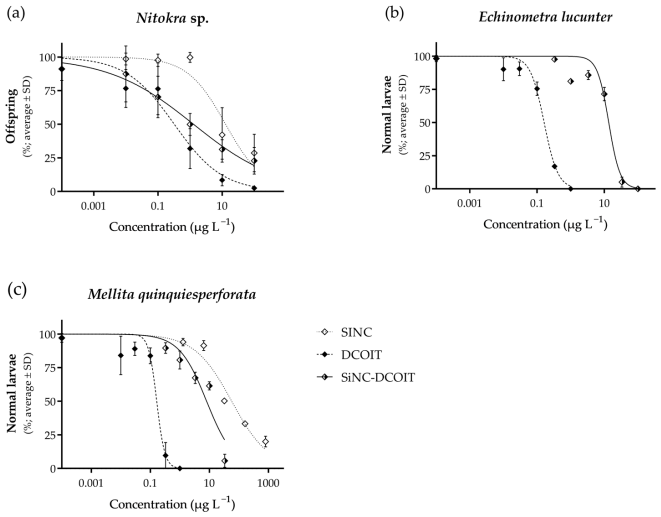

Figure 2. Chronic effects of SiNC, DCOIT and SiNC-DCOIT on offspring of *Nitokra* sp. copepods (**a**); embryo-larval development of sea urchin *E. lucunter* (**b**); and sand dollar *M. quinquiesperforata* (**c**). Concentrations of SiNC-DCOIT are presented in µg DCOIT L^{-1} in all treatments.

SiNC did not significantly affect the *E. lucunter* embryo-larval development at the range of tested concentrations (LOEC > 100 µg L^{-1}). The effects of soluble and nanostructured forms of the booster biocide DCOIT are presented in Figure 2b. The soluble form of DCOIT was two orders of magnitude more toxic than the nanostructured form since the IC_{50}-48h values estimated were 0.16 and 13.5 µg DCOIT L^{-1}, respectively. Moreover, DCOIT significantly inhibits the normal development of *E. lucunter* at concentrations as low as 0.1 µg L^{-1} (Table 2).

The effects of the studied substances on the sand dollar *M. quinquiesperforata* embryo-larval development are presented in Figure 2c. The IC_{50}-36h values were estimated at 0.16 µg L^{-1} for free DCOIT and 7.04 µg DCOIT L^{-1} for SiNC-DCOIT. Like the other species in the present study, the unloaded SiNC presented the lowest toxicity to *M. quinquiesperforata*, among the studied substances, which IC_{50}-36h was estimated at 57.6 µg L^{-1}.

3.3. Hazard Assessment

The NOEC values estimated in the present study and those reported for tropical species in the literature are summarized in Table 3. The hazard endpoints estimated through statistical and deterministic approaches are summarized in Table 4. Regarding the statistical approach, derived hazardous concentrations at 5% (HC_5) are 0.00012 and 0.02902 µg DCOIT L^{-1} for the soluble and nanostructured forms of DCOIT, respectively. The $PNEC_{marine}$ is set on 0.00012 and 0.00967 µg DCOIT L^{-1} for DCOIT and SiNC-DCOIT, respectively (Table 4), demonstrating that the encapsulation technique decreased the DCOIT's hazard by 80. The deterministic approach was even more conservative setting PNEC values 7.5-fold and 5 orders of magnitude lower than the statistical method for both SiNC-DCOIT and DCOIT, respectively (Table 4). Using this approach, SiNC-DCOIT is six orders of magnitude less hazardous than DCOIT.

Table 3. No observed effect concentration (NOEC) values of soluble and nanostructured forms of the antifouling biocide DCOIT (referred as DCOIT and SiNC–DCOIT, respectively) estimated for marine tropical species (2 cosmopolitan species known for tropical waters were also considered). na —NOEC data not available.

Taxa	Species	Life Stage	Exposure Time	DCOIT (μg L^{-1})	SiNC-DCOIT (μg DCOIT L^{-1})	Reference
Bacteria	*Vibrio parahaemolyticus*		48 h	100	100	This study
Fungi	*Penicillium citrinum*		48 h	100	100	This study
Microalgae	*Chlorella minutissima*		96 h	≤10	≤100	This study
	Phaeodactylum tricornutum		72 h	3	1	[36]
	Skeletonema costatum		72 h	0.06	na	[56]
Macroalgae	*Fucus serratus*	Zygotes	72 h	8	na	[18]
Ascidiacea	*Ciona intestinalis*	Embryo	48 h	17	na	[17]
Crustaceans	*Nitokra* sp.	Ovigerous female	7 d	0.1	1	This study
	Mysidopsis juniae	Juvenile	7 d	3	1.83	[42]
Mollusk	*Americamysis bahia*	Juvenile	28 d	0.63	na	[57]
	Perna perna	Embryo	48 h	1	0.064	[41]
Echinoderm	*Echinometra lucunter*	Embryo	48 h	0.03	3.33	This study
	Mellita quinquiesperforata	Embryo	36 h	0.1	0.33	This study
Fish	*Anthocidaris crassispina*	Embryo	32 h	0.00000001	na	[58]
	Cyprinodon variegatus	Embryo	35 d	6	na	[57]

Table 4. Environmental hazard of soluble and nanostructured forms of the antifouling biocide DCOIT (referred as DCOIT and SiNC–DCOIT, respectively) in tropical marine waters. HC_5: hazardous concentrations at 5% (and respective 95% confidence intervals $CI_{95\%}$); $PNEC_{probab}$: probabilistic predicted no effect concentration.

	Probabilistic/Stastical Approach			Deterministic Approach
Tested Chemical	**HC_5**	**$CI_{95\%}$**	**$PNEC_{marine}$**	**$PNEC_{marine}$**
DCOIT (μg L^{-1})	0.00012	0.000001-0.003186	0.00012	1×10^{-9}
SiNC-DCOIT (μg DCOIT L^{-1})	0.02902	0.000235-0.219391	0.00967	0.00128

4. Discussion

4.1. Nanomaterials Characterization and Environmental Behavior

Recent studies [39] have highlighted that the controlled release of biocides mediated by ENM is an outstanding feature for developing a new generation of simultaneously eco-friendly and efficient nanoadditives for marine coatings. In this context, the characterization of ENM is fundamental since it allows relating their structural characteristics with the potential effects on the environment [59].

The present results agree with previous studies in which unloaded mesoporous silica nanocapsules (SiNC) tended to present higher absolute ζ values than those loaded with DCOIT [36]. According to Kaczerewska et al. [60], positive values of ζ are associated with the traces of the cationic surfactant cetyltrimethylammonium bromide (CTAB) used in the synthesis of SiNC. Previous studies have also indicated the spherical and regular shape of SiNC, with a core shell and porous structure [61], as well as the larger particles in the encapsulated form SiNC-DCOIT, compared to unloaded SiNC [36,60]. Ruggiero et al. [62] suggested that the size of the biocide molecule shapes the size of the surfactant micelles and, consequently, the resulting size of encapsulated biocide in SiNC.

Due to the mutability present in manufacturing processes, monodispersion of the products is rarely obtained; even so, it is ideal that the PdI is close to zero [63]. Saman et al. [64] characterized SiNC and observed its cauliflower-shaped clusters. According to that study, this phenomenon occurs due to the adsorption capacity of these nanomaterials. These clusters form voluminous particles that directly influence polydispersity and stability.

In fact, recent studies have also highlighted that SiNC dispersions in distilled water have a PdI of 0.68, a zeta potential of 32.8 mV and a hydrodynamic size of 157 nm with a secondary peak centered at 731 nm which shows the heterogeneity of the dispersions with a high tendency to form large nanomaterial aggregates [61]. However, the hydrodynamic sizes of SiNC and SiNC-DCOIT were lower than previously reported at the same concentrations in artificial saltwater [36]. Figueiredo et al. [36] reported larger hydrodynamic values in similar concentrations of both SiNC and SiNC-DCOIT dispersed in filtered artificial saltwater, which may signalize eventual physicochemical differences of both media, including the presence of organic matter in the natural seawater tested in the present study. Organic molecules in natural seawater are known to adsorb onto the particle surfaces, providing a barrier to aggregation [65].

Recent studies indicated that both soluble and nanostructured forms of the booster biocide DCOIT have high efficacy in preventing marine fouling [36,41]. Michailidis et al. [66] highlighted that DCOIT covalently interacts with the SiNC surface, allowing this interaction to provide antifouling properties for coating formulations, extending their performance. Additionally, the nanoencapsulation of DCOIT in silica controls the biocide's leaching and reduces its toxicity to non-target species [36].

4.2. Ecotoxicity Assessment

Studies that associate biocidal antifouling paints are deeply linked to potential changes in the structures of microbial communities; however, the role of fungi in these processes is still unclear. Dobretsov et al. [67] detected the marine filamentous fungi *Aspergillus tubingensis*, *Aspergillus terreus*, *Alternaria* sp., *Aspergillus niger*, *Cladosporium halotolerans* and *Cladosporium omanense* in antifouling paints that contain copper in their formulation. According to them, the high tolerance to copper (3.8 μg cm^{-2} day^{-1}) signals the adaptability of these organisms to the metal and is a challenge in the formulation of this type of paint. Ruggiero et al. [62] also highlighted the tolerance of meristematic fungi to two natural biocides, zosteric acid sodium salt and usnic acid, encapsulated in silica nanosystems.

According to the results obtained for microorganisms, the encapsulated DCOIT shows less toxicity than DCOIT in the soluble form. Such findings are aligned with the well-known broad-spectrum activity of DCOIT, against bacteria, fungi, and algae, but also hard fouling [68,69]. In the other hand, Maia et al. [34] evaluated the antimicrobial effect of SiNC and SiNC-DCOIT on the photobioluminescent recombinant bacterium *E. coli*. According to these authors, there was a significant and immediate decrease in the light emission of the bacteria after exposure to DCOIT. However, the inactivation occurred more slowly indicating a gradual release of DCOIT from the SiNC, which release profile was demonstrated later by Figueiredo et al. [36]. In this sense, Aidarova et al. [70] reported an increase in inhibition of the microorganism growth (*Aspergillus niger*, *Aspergillus awamori* and *Bacillus cereus*) by the nanostructured form of DCOIT in comparison with the soluble form. The authors concluded that free biocide loses its activity more quickly, while the encapsulated biocide is released gradually, retaining 60% of its activity after 15 days of seeding. In contrast, the effect of DCOIT was decreased by about 48% after a storage period of five days [70]. The comprehensive ecotoxicological assessment on temperate species, by Figueiredo et al. [36], showed that SiNC-DCOIT is systematically less toxic than DCOIT for microorganisms, such as the bacteria *Aliivibrio fischeri*, the microalgae *Isochrysis galbana* or *Nannochloropsis gaditana*. The authors estimated EC_{50} values for DCOIT and SiNC-DCOIT of 299 and 459 μg DCOIT L^{-1} respectively, for *A. fischeri*, 32 and 37,400 μg DCOIT L^{-1}, respectively, for *I. galbana* and 35 and 590 μg DCOIT L^{-1}, respectively, for *N. gaditana*. Such results agree with the effects of soluble and nanostructured DCOIT on the microalgae *C. minutissima* recorded in the present study. Recent studies also indicated a microalgae growth inhibition upon the exposure to silica-based nanocomposites containing DCOIT [71].

The chronic effects of DCOIT on invertebrates are poorly understood; however, this study suggests that its encapsulation on SiNC decreases the biocide's chronic toxicity on tested invertebrate species. Ovigerous females of *Nitokra* sp. exposed to soluble DCOIT exhibited a

significant decrease in the number of produced offspring even at environmentally exposure concentrations while the DCOIT nanoencapsulation reduced its toxicity by four-fold over the offspring of *Nitokra* sp. These results corroborate other studies in which SiNC-DCOIT reduced lethal short-term effects on non-target species, such as crustaceans from temperate regions [36]. Likewise, Jesus et al. [42] investigated the toxicity of SiNC-DCOIT on the tropical microcrustacean *Mysidopsis juniae*, demonstrated that mysids were more sensitive than temperate species, and concluded that SiNC-DCOIT showed less toxicity than soluble DCOIT. Also, the authors found that SiNC-DCOIT chronic exposure caused no significant effects on the length and weight of *M. juniae* (NOEC = 1.83 μg DCOIT L^{-1}). However, soluble DCOIT at 6 μg L^{-1} induced a significant weight reduction [42]. Chronic effects were also observed on *Acartia tonsa* egg production, exposed to soluble DCOIT for 48 h, which was assigned to the lethality of females during the exposure period, highlighting the importance of our findings in assessing fecundity effects caused by DCOIT [72].

In the short-term experiments, the differences between the toxicity of DCOIT and SiNC-DCOIT were more pronounced. For *E. lucunter*, the EC_{50} of SiNC-DCOIT was two orders of magnitude lower than the EC_{50} estimated for soluble DCOIT. For *M. quinquiesperforata*, the immobilization of DCOIT in SiNC presented a toxicity decrease of about 50-fold over the embryo-larval development, similarly to the 54-fold decrease of the embryotoxicity on the temperate sea urchin *Paracentrotus lividus* [36]. In fact, sea urchin development is more susceptive to damage because continued mitotic cell division is subject to interactive processes among xenobiotics and DNA and may be associated with changes in gene transcription [73,74]. Thus, the high difference between toxicity of nanostructured and soluble forms of DCOIT may be related to the biocidal controlled release from the nanocapsules during the exposure period [36].

Despite SiNC toxicity being lower among the tested chemicals, the effects caused by this nanomaterial were higher than expected since silica is regarded as an inert element. Previous studies [35,36,41] also demonstrated that SiNC exposure cause toxicity against several other marine species, which has been attributed to the cationic surfactant CTAB used in the synthesis of SiNC that remains in the capsules. A recent study synthesized new SiNC with the alternative surfactant 1,4-bis-[N-(1-dodecyl)-N,N-dimethylammoniummethyl]benzene dibromide (QSB2-12) [75]. Toxicity studies indicated that SiNC synthesized with CTAB is significantly more toxic than SiNC synthesized with QSB2-12 [60] which was assigned to the lower toxicity of QSB2-12 [75]. Therefore, a future incorporation of DCOIT on such new nanocontainers could improve more the environmental performance of SiNC-DCOIT.

4.3. Hazard Assessment

There is a lack of information about hazard and measured environmental concentrations (MEC) data of DCOIT in tropical waters, in contrast with temperate marine environments [76]. DCOIT appears to be more hazardous for tropical species since the probabilistic PNEC value calculated in this study was 20-fold lower than probabilistic PNEC derived for temperate marine waters [43]. This can be linked to the sensitivity of species from tropical and temperate regions, and their abiotic conditions, affecting the species' metabolic rate and their response of the xenobiotics [77,78].

The dataset of NOEC values available for DCOIT, and specially for SiNC-DCOIT, was amplified with the present study which was key to derive both the deterministic and probabilistic PNECs. According to our findings, the immobilization of DCOIT into SiNC decreased the hazard of DCOIT by 84-fold compared with the soluble DCOIT using the probabilistic PNEC approach. Such decrease reflects the extremely toxicity of DCOIT towards non-target species. Among the NOEC values reported in the literature, echinoderm species are more sensitive to DCOIT with the lowest NOEC value being as low as 10^{-8} μg L^{-1} in the case of the sea urchin *Anthocidaris crassispina* [58]. In contrast, the lowest NOEC value available in the literature for SiNC-DCOIT refers to the bivalve species *Perna perna* with a NOEC value of 0.064 μg L^{-1} [41]. The deterministic approach, the most conservative approach to derive the PNEC values in this study, indicated a more expressive hazard reduction. In this sense, PNEC for SiNC-DCOIT is

six orders of magnitude higher than the estimated PNEC for DCOIT, influenced by the lowest chronic NOEC available in the literature. The lower hazard of nanostructured DCOIT may be explained by their slow biocidal release capacity in time and environmental behavior in seawater, which occurs gradually through diffusion by predefined stimuli [34].

More studies on long-term exposure and sub-chronic effects (e.g., biomarkers) are needed to address the hazard of such innovative antifouling nanomaterials. Nevertheless, our results highlight that tropical species are more sensitive than those temperate zones and indicate that environmental hazard and risk assessments based on NOEC values of long- and short-term toxicity tests, considering species from different biogeographic regions, were more appropriate to derive realistic PNEC values, adjusted for the ecological specificities of each marine region.

5. Conclusions

DCOIT chronic toxicity and environmental hazardousness were reduced following an immobilization into silica mesoporous nanocapsules. This first chronic environmental hazard assessment brought essential contributions to better understand and manage the environmental risks of such innovative products, particularly in tropical areas. However, further studies are still needed to address their sub-cellular and bioaccumulation effects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15061185/s1, Figure S1: Standard curve of calibration (absorbance x cell density) - *Chlorella minutissima*.

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