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Validating an In-House Method for Assessing Effluent Discharge Toxicity Using *Acartia tonsa* in the Black Sea

Elena Bisinicu [1], George Harcota [1,2], Valentina Coatu [3] and Luminita Lazar [3,*]

[1] Ecology and Marine Biology Department, National Institute for Marine Research and Development "Grigore Antipa", 300 Mamaia Blvd., 900581 Constanta, Romania; ebisinicu@alpha.rmri.ro (E.B.); gharcota@alpha.rmri.ro (G.H.)
[2] Doctoral School of Ecology, University of Bucharest, 050663 Bucharest, Romania
[3] Chemical Oceanography and Marine Pollution Department, National Institute for Marine Research and Development "Grigore Antipa", 300 Mamaia Blvd., 900581 Constanta, Romania; vcoatu@alpha.rmri.ro
* Correspondence: llazar@alpha.rmri.ro

Featured Application: This research on the marine copepod *Acartia tonsa* focuses on its application as a bioindicator in ecotoxicological studies. Specifically, it evaluates the effects of a reference toxicant on the survival of *A. tonsa* to validate an in-house method (protocol) for testing in the Black Sea. By exposing *A. tonsa* to varying concentrations of the 3,5-dichlorophenol (reference toxicant) in controlled conditions and analyzing survival rates to determine the lethal concentration (LC50), the study provides benchmarks for assessing the environmental impact of effluent discharges. These findings aim to enhance the accuracy and consistency of environmental monitoring and risk assessment in the Black Sea, ensuring data reliability and reducing methodological discrepancies.

Abstract: The marine copepod *Acartia tonsa* is an important bioindicator in ecotoxicological studies due to its sensitivity to pollutants and ecological importance. This research evaluates the effects of reference toxicant on the survival of *A. tonsa*, aiming to validate an in-house method that assesses the sensitivity and reliability of the species for testing in the Black Sea. *A. tonsa* individuals were exposed in controlled laboratory conditions to varying 3,5-dichlorophenol (reference toxicant) concentrations, with their survival rates monitored over 48 h. Survival data were analyzed to determine the lethal concentration (LC50), a metric for assessing toxicity. The derived LC50 values offer important benchmarks for assessing the environmental impact of different effluent discharges in the Black Sea region. The outcomes will contribute to improving the accuracy and consistency of environmental monitoring and risk assessment practices in the Black Sea region, ensuring that variations in toxicity data are attributable to environmental factors rather than methodological discrepancies.

Keywords: methodological consistency; 3,5-dichlorophenol; ecotoxicology; lethal concentration (LC50); copepods; Black Sea

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1. Introduction

Toxicity tests are key tools for biomonitoring and assessing the risk of chemical substances released into the environment. Protocols for organism culture and bioassays with species from various trophic levels and taxonomic groups are essential for understanding the potential impacts of environmental pollution. Thus, improving standardization and developing new standardized protocols for bioassays with ecologically relevant species are key priorities in ecotoxicology [1].

Identifying model species in marine ecotoxicological studies is particularly important. These species should be abundant, widely distributed, ecologically relevant, and experimentally manageable in the laboratory. They should aid in elucidating pollutants' acute, chronic, and developmental toxicity. Copepods fulfill all these criteria, and their potential in ecotoxicology has long been recognized [2]. An increased understanding of

copepod physiology, monitored through endpoints such as survival, development, growth, fecundity, respiration, and swimming behavior, is essential. This knowledge can help predict how natural copepod populations will respond to environmental changes in the context of oil and gas exploration and production [3,4].

Acartia tonsa was selected as the model species for this study due to its extensive usage in ecotoxicological research, ecological importance, and high sensitivity to environmental pollutants. Widely utilized in ecotoxicology, it offers a strong foundation of scientific literature and standardized protocols, enabling reliable comparisons across studies [5]. Ecologically, *A. tonsa* plays a crucial role in marine and estuarine ecosystems as a primary consumer, influencing the food web and serving as an indicator of ecosystem health [6–8]. Its high sensitivity to pollutants, even at low concentrations, makes it an ideal species for detecting and assessing toxic effects in aquatic environments, providing early warning signals of ecological disturbances [3,9]. These factors collectively make *A. tonsa* a critical species for environmental research and the assessment of ecological risks [1].

The Black Sea is a unique marine environment characterized by specific climatic conditions and distinct ocean currents, contributing to its ecological sensitivity [10]. The region experiences limited water exchange with other seas, leading to a stratified water column where the deeper layers are often oxygen-deprived [10–12]. This stratification, combined with the sea's semi-enclosed nature, makes the Black Sea particularly vulnerable to ecological disturbances [13,14]. The ecosystem is highly sensitive to changes, and even minor environmental shifts can have significant consequences for marine life [15–17]. Potential environmental stressors in the Black Sea include pollution from agricultural runoff, industrial discharges, and heavy maritime traffic, all of which pose substantial risks to the balance of this ecosystem [18,19]. These factors make the Black Sea an area of concern for environmental research, necessitating careful monitoring and assessment to protect its ecological integrity.

In recent years, the interest and development in oil and gas exploration and production have increased significantly in the Romanian Black Sea region [20,21]. Covering 22,000 square kilometers and reaching depths beyond 1000 m, the Romanian offshore area is divided into various perimeters [22]. Some of these perimeters have been allocated to operators for exploration, development, and production activities [21,22]. While current exploration primarily targets shallower waters (up to 1000 m deep), there is also a focus on developing new plays represented by slope and basin-floor fans located further offshore [21,23]. As a result, there is a growing concern about the potential environmental impact of chemicals discharged during oil and gas production on the Black Sea [21].

Apart from the international and European legislation for the discharge in the natural receivers, in Romania, the applicable national legislation is the regulations regarding the establishment of pollutant loading limits of industrial and urban wastewater when discharged into natural receivers [24]. This document lists chemical substances with maximum allowable concentrations for discharge and also stipulates that, for unlisted substances, dedicated studies should be performed to establish the discharge limits. Thus, the need for ecotoxicity studies derives from the lack of maximum admissible limits for discharge into the Black Sea of the concentrations of chemical substances used by operators from the applicable legislation in force, as well as the requirement for the user to present them when issuing the water authorization. Otherwise, the potential risks associated with unregulated chemicals may go unnoticed, posing significant threats to human health and the environment.

Copepods are essential trophic links between primary producers and consumers in aquatic systems. By grazing on phytoplankton and serving as prey for fish, they play a key role in the bioaccumulation and biomagnification of toxic pollutants within aquatic food webs [25–27].

The calanoid copepod, *Acartia* (*Acanthacartia*) *tonsa* Dana, 1849, is a widespread species found in temperate regions, typically abundant in coastal and estuarine areas [28] of the Indian, Atlantic, and Pacific Oceans, as well as in the Malay Archipelago, the Baltic Sea, and

the Sea of Azov [29]. In the Black Sea, it has been recorded since the early 1970s [7,30–32], and in the Mediterranean since 1986 [32]. Despite its widespread presence, *A. tonsa* is considered a non-indigenous species in the Mediterranean Sea, having been introduced in the 1980s via ballast water [9].

The International Organization for Standardization (ISO) recommends using *A. tonsa* for assessing the lethality and toxicity of various contaminants because it is sensitive to different types of contaminants and toxins [33,34] and is considered a valuable model organism for studying the effects of pollutants on marine zooplankton communities [35].

Since the 1970s, research has increasingly focused on the physiology, reproduction, and feasibility of multi-generation rearing of *A. tonsa* [8,36,37]. Additionally, *A. tonsa* has been widely used as a model organism in ecotoxicology studies to evaluate the quality of marine environments and detect hazardous compounds [38–40].

Currently, *A. tonsa* is included in international standardized protocols for acute, semi-chronic, and chronic bioassays for environmental risk assessment in marine coastal areas [1,33,34,41]. Recently, it has also been incorporated by the Italian Ministry of the Environment (DM, 173/2016) [42] and the Government of Canada [43] in tests for evaluating sediment toxicity. These bioassays involve either direct exposure of eggs to sediment or incubation in sediment-derived aqueous solutions (e.g., elutriate or pore water) and assess specific endpoints such as egg hatching success and larval viability. Furthermore, a chronic endpoint, the reduction of *A. tonsa* fecundity, has been proposed for assessing the impact of emerging contaminants like nanomaterials on adult copepods [6].

Copepods of the genus *Acartia* inhabit numerous coastal and offshore environments, where they are often among the most abundant zooplankton species. Multiple species can coexist simultaneously, but typically, winter species alternate with summer ones. Local populations of Acartiidae can form due to the highly variable conditions of temperature, salinity, and food availability [32].

In the Black Sea, two predominant species are present most of the year: the permanent eurythermic *Acartia clausi* and the more recent warm-water species *A. tonsa*, which has replaced the stenothermic *Acartia latisetosa* since the 1970s [7]. *A. tonsa* is a thermophilic species that produces resting eggs. Consequently, the *A. tonsa* community remains dormant during the cold season in the Black Sea. The hatching of these resting eggs is triggered by rising temperatures in the spring and summer [7]. Both species are tolerant of salinity changes, with *A. tonsa* capable of enduring a wide salinity range of 2–33 PSU [44]. *A. tonsa* has a wide salinity and oxygen concentration tolerance, while *A. clausi* is more stenohaline and extremely sensitive to hypoxia [45]. *A. tonsa* is adapted to high food concentrations, which are common in nearshore environments and estuaries [31,46].

This study aims to evaluate the use of the calanoid copepod, *A. tonsa*, as a model organism in marine ecotoxicology, with a strong emphasis on in-house protocol validation. This research focuses on adapting the bioassay protocols (ISO 14669:1999) [33] for the Black Sea features, using *A. tonsa*, while introducing necessary protocol deviations to account for specific regional conditions. The study validates the robustness and accuracy of the revised protocols with a conclusive test on the reference substance 3,5-dichlorophenol conducted in 2023.

2. Materials and Methods

The Black Sea, with an average salinity ranging from 17 to 18 g/L, exhibits a pronounced stratified structure characterized by distinct biogeochemical layers. The hydrographic pattern consists of low-salinity surface waters from river inflows overlaying deeper, high-salinity waters of Mediterranean origin. A well-defined and persistent pycnocline exists between these layers, limiting vertical mixing to 100–150 m depths. The oxic layer extends to approximately 50 m, coinciding with the euphotic zone and the boundary of the pelagic habitat. This layer supports robust biological processes, with high oxygen concentrations around 300 µM, subject to seasonal variations in nutrients and organic matter from rivers and coastal areas. The mean thermohaline state of the Black Sea is influenced by the

balance of water and salinity (surface dilution and salinification by Mediterranean inflow), while air–sea heat exchange drives seasonal variability. In the coastal areas, the oxycline, marking a distinct oxygen gradient, begins at depths of 70–100 m. Below this, the suboxic layer is found at depths of 100–130 m, characterized by reduced oxygen levels (<10 μM) and increasing hydrogen sulfide concentrations. Further down, the anoxic layer starts at depths of 150–200 m, devoid of oxygen except for sulfate-reducing bacteria sustained by hydrogen sulfide [47–49].

To determine the optimal conditions for maintaining cultures, we adapted the ISO 14669 (1999) [33] requirements to align with the specific conditions typical of the Black Sea environment, as follows:

- Salinity: 11–18 PSU, reflecting the salinity of the Black Sea based on data from NIMRD;
- Temperature: 20 °C ± 3 °C;
- Lighting: Up to 2200 lux;
- Oxygen Saturation: Greater than 80%;
- pH: 8 ± 0.5.

2.1. *Acartia tonsa* Culture

Natural seawater (SW), collected from a site far from known pollution sources, filtered through filters with a porosity of 0.45 μm (Millipore) using a vacuum pump in a recirculation system to remove suspended matter, was used for *Acartia* cultures. Before the experiment, the water underwent analysis for salinity, dissolved oxygen (DO), pH, nutrients, and contaminants to check the ambient conditions and absence of contamination. Temperature, salinity, DO, and pH measurements were conducted using a Seven Excellence Mettler Toledo instrument (N.V. Mettler-Toledo S.A., Zaventem, Belgium) calibrated before each use. Nutrient and contaminant analyses followed international seawater analysis methods [50–54].

A. tonsa eggs (Figure S1) from the market (Recifathome, France, [55]) were inoculated into 500 mL flasks containing 250 mL of dilution water and kept at 20 ± 2 °C with a 16:8 light photoperiod and gentle aeration (1 bubble/s) (Figure S2) for 48 h, as specified by the producer.

After inoculation and hatching (after 48 h), microscopic analysis was performed to assess the number of nauplii, which were transferred to 4 L flasks with 3 L of dilution water, maintained with gentle aeration (1 bubble/s) at 20 ± 2 °C under a 16:8 light photoperiod (Figure S3).

After reaching copepodite stage I, the cultures were moved to a temperature-controlled room at 20 ± 2 °C. The cultures were observed as they progressed to copepodite I- V and the adult stage (Figure 1) by microscopic analysis every 3 days.

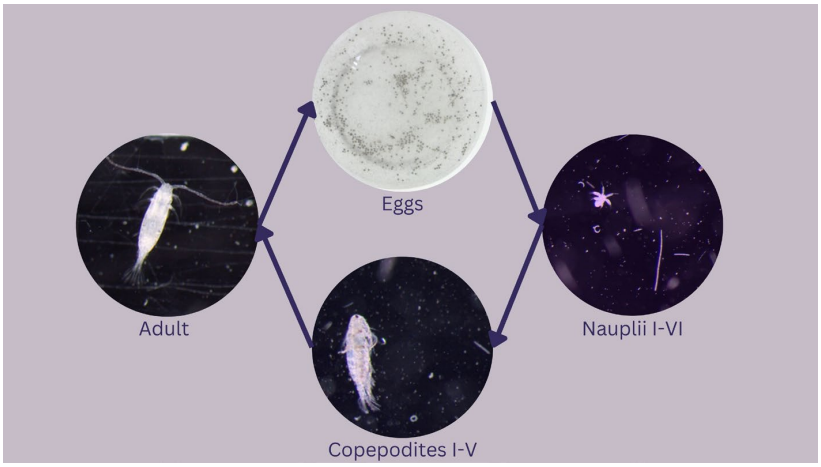


Figure 1. Schematic representation of *A. tonsa* development stages (original photos).

The culture water was renewed at each evaluation. The old water was passed through a series of different sieves (60–180 µm mesh size), depending on the copepod's development stage.

2.2. Cultures of Chryptomonas baltica—Feed for A. tonsa Cultures

The production of *Cryptomonas baltica* (Karsten) Butcher, 1967 original *Rhodomonas baltica*) [56] microalgae (Figure S4) cultures, as a food source for copepods, were also ensured. The scientific name *R. baltica* has recently undergone a taxonomic revision and is now recognized as *Cryptomonas baltica*. We will refer to the organism as *C. baltica* to align with current taxonomic standards in this text. However, since many of the references and much of the existing literature still use the name *R. baltica*, including ISO 14669 (1999) [33], we will acknowledge this synonym where applicable. This dual approach ensures that our discussion is consistent with both contemporary taxonomy and historical research.

C. baltica from the market (Aquacopa, Germany [57]) was cultivated in a sterile medium to prevent bacteria or other microalgae contamination.

According to ISO 14669 (1999) [33], the optimal conditions for cultivating *C. baltica* are well-defined. The ideal temperature is 20 °C with a tolerance of ± 3 °C. Lighting should be up to 10,000 lux, preferably using cool white light. A controlled photoperiod of 16 h of light followed by 8 h of darkness is recommended. Strong oxygenation is necessary to ensure continuous movement of the microalgae. The pH should be maintained at 8 ± 0.5.

To obtain the microalgae culture, *C. baltica* was acclimatized to the environmental conditions of the Black Sea. Seawater (SW) was filtered using 0.22 µm filters and autoclaved for 40 min at 121 °C. The microalgae cultures were grown in sterilized glass containers with volumes ranging from 50 to 4000 mL (Figure S5) [58].

Culture stocks were replanted at high peak density (dark brown color) through subsequent replanting, increasing the volume with sterile seawater and culture medium. Cultures of *C. baltica* were maintained by adding Walnes culture medium [59] (Table S1), considering the volume of water used for the culture.

The microalgae were concentrated by centrifugation in 50 mL test tubes with threaded caps at 2000 rpm for 20 min. After centrifugation, the supernatant was removed, and the centrifuged biomass (the food) was carefully transferred to a labeled sterile container. The food should be stored in the refrigerator for up to one week.

The density of the microalgae after centrifugation was determined using a hemocytometer under a microscope (Figure S6).

A. tonsa individuals were fed daily, with the microalgae culture density (number of microalgae cells/mL) adjusted according to the number of individuals in the culture. The calculation formula of the food volume for the organisms in the culture is:

$$FV = \frac{2 * 10^7}{D.a.} \times \frac{N}{50}$$

where FV = food volume, $D.a.$ = density of microalgae in the culture (cells/mL), N = number of individuals in the copepod culture, 50 = maximum number of copepods in one liter of culture, and $2 * 10^7$ = total amount of food recommended by ISO 14669:1999 [33] (cells/mL).

2.3. Testing

Nominal concentrations for the 3,5-dichlorophenol reference toxicant were selected based on LC50 data from ISO 14669:1999 [33].

The study includes several treatment groups (Table 1), each with specific goals to assess the toxicological impact of the reference toxicant on *A. tonsa*.

- Control (0.00 mg/L): Establishes baseline conditions using seawater, serving as a reference to compare toxicant effects.
- 0.10 mg/L: Tests the minimum concentration where toxicant effects begin, aiming to identify the sensitivity threshold.

- 0.32 mg/L: Assesses moderate toxicant exposure, expecting noticeable biological impacts.
- 1.00 mg/L: Evaluates higher exposure levels, anticipating significant adverse effects or increased mortality.
- 3.20 mg/L: Tests high concentrations, expecting severe impacts, including near-complete mortality.
- 10.00 mg/L: Determines the maximum impact, likely resulting in 100% mortality.

Table 1. Test design.

Treatment Group	Concentration mg/L	Description	Number of Organisms *
1 (Control)	0.00	SW	20
2	0.10	SW and reference toxicant	20
3	0.32		20
4	1.00		20
5	3.20		20
6	10.00		20

* Five organisms per replicate.

Collectively, these groups allow for a detailed analysis of the toxicant's effects across concentrations, supporting the research objective of understanding its environmental impact and establishing safe exposure levels.

Following the ISO guidelines [33], each experimental replicate (N = 4) consisted of 5 organisms, leading to a total of 20 organisms per treatment group.

A reference stock solution was prepared one day before testing. To make this solution, 0.1 g of 3,5-dichlorophenol was added to SW in a 100 mL calibrated glass flask and mixed on a stirrer plate at 500 RPM for 10 min, resulting in a stock solution of 1 g/L 3,5-diclorophenol.

A dilution series of the reference toxicant (0 mg/L, 0.1 mg/L, 0.32 mg/L, 1 mg/L, 3.2 mg/L, and 10 mg/L) was prepared by diluting the stock solution with SW in six 500 mL calibrated flasks, labeled from C0 to C5. The flasks were filled with 0 mL, 0.5 mL, 1.6 mL, 5 mL, 16 mL, and 50 mL of stock solution, respectively (Table S1).

The test chambers consisted of 50 mL cell culture flasks made of polystyrene, closed with screw caps made of polypropylene to minimize contamination, evaporation, and volatilization. Five copepods, at copepodites V and adult stages, were transferred via pipette (Figure S7) to each chamber containing 50 mL of the test solution with headspace.

Test chamber positions in the test area and organism assignments were determined using a randomized [60] block design (Figure S8). Each test chamber was labeled with species initials, treatment group, and replicates (Figure S8).

Before the experiment began, light intensity was checked using an HD 2302.0 Delta OHM light meter.

The exposure duration was 48 ± 1 h. Observations for immobilization were performed and recorded every 24 ± 1-h. Immobilization was defined as the lack of swimming ability within 10 sec after gentle agitation of the test chamber.

Temperature and lighting conditions were monitored daily. A photoperiod of 16 h of light and 8 h of dark was maintained in the exposure area, provided by LED lamps.

Water quality parameters, temperature, pH, salinity, and dissolved oxygen were measured at the beginning and end of the experiment.

The results will be considered as valid if the following conditions are satisfied together:

(a) the dissolved oxygen concentration at the end of the test is greater than or equal to 4 mg/L;
(b) the percentage mortality of the controls is less than or equal to 10%;

(c) the toxicity of the reference chemical is within the documented range [33].

Upon completion of the study, all test organisms were discarded, and the entire experimental process was stopped.

2.4. Data Analysis

Data analyses were conducted using PRIMER v. 7.0 [61]. The data was utilized in its raw form without any normalization or transformation. Shade plots were generated to represent the intensity and distribution of the data, employing a gradient of shades to denote varying levels of intensity across the dataset. Color scales were carefully chosen to accurately reflect these variations, providing a precise and interpretable visual representation of the intensity of the data. Shade plots were used to visualize the concentration's effect on copepods' survival, providing a detailed graphical representation of the data. These plots represent data in a two-dimensional format where different shades or colors indicate varying levels of a particular parameter [62]. They effectively illustrated the variations in concentration levels across the 24-h and 48-h exposure periods, facilitating a clear comparison and interpretation of the results. Shade plots are a valuable tool in data analysis, offering a clear and concise way to visualize complex datasets and draw meaningful insights from them [62].

Statistica® 14.0.1.25 [63] was used for statistical analysis to evaluate copepod mortality across different concentrations of the toxicant. A Kruskal–Wallis test was conducted to determine whether there were significant differences in mortality between the experimental groups at 24 h and 48 h, with results considered statistically significant if $p < 0.05$. Data were expressed as mean ± standard error (SE), and the variability was assessed using the standard deviation (SD) to reflect potential differences in mortality trends over time and concentration levels. GraphPad Prism version 10.0.0 [64] was used to calculate the mean cumulative percent mortality of copepods with standard error of the mean (SEM).

For each concentration, the data were pooled from the replicates, and the percentage mortality after 24 h and 48 h in relation to the total number of copepods used was calculated. The 48-h LC50 was calculated using a sigmoid function with the LC50 calculator tool from AAT Bioquest [65].

3. Results

3.1. Water Quality Check

The seawater used for the experiment was checked for quality. Before the experimental run, temperature, salinity, dissolved oxygen (DO), pH, nutrients, and contaminants were measured to confirm ambient conditions and the absence of contamination. All parameters were in the accepted range for the water quality (Tables S3–S7).

3.2. A.tonsa Testing and Evaluation

For the copepod cultures, parameters such as temperature, pH, oxygen, and salinity were also periodically monitored (Figure S9). The pH ranged from 7.37 to 7.69, and the salinity varied from 14.75 PSU to 15.83 PSU. The test organisms were cultured at a similar salinity (±3 PSU) for at least 7 days before the test, maintaining salinity levels between 14.75 PSU and 15.83 PSU.

The light intensity was measured in five points (Figure S9), with values between 1259 and 2200 lux, and was constant throughout the experiment, falling within the protocol's stipulated range of up to 2200 lux.

The box plot illustrates mortality trends across different toxicant concentrations, showing a stronger response at 48 h, with significant differences confirmed by the Kruskal–Wallis test and p-values < 0.05 for both time points, emphasizing the significant differences between the toxicant's dose- and time-dependent impact (Figure 2). In the control group, copepod mortality remains consistently low over 24 and 48 h. At lower concentrations of 3,5-dichlorophenol (0.1 mg/L and 0.32 mg/L), mortality gradually rises, with minor time-dependent variations. At an intermediate concentration (1 mg/L), mortality increases

more notably, particularly after 48 h, indicating a potential delayed or accumulative effect. At higher concentrations (3.2 mg/L and 10 mg/L), mortality reaches its maximum within both time frames (Figure 2).

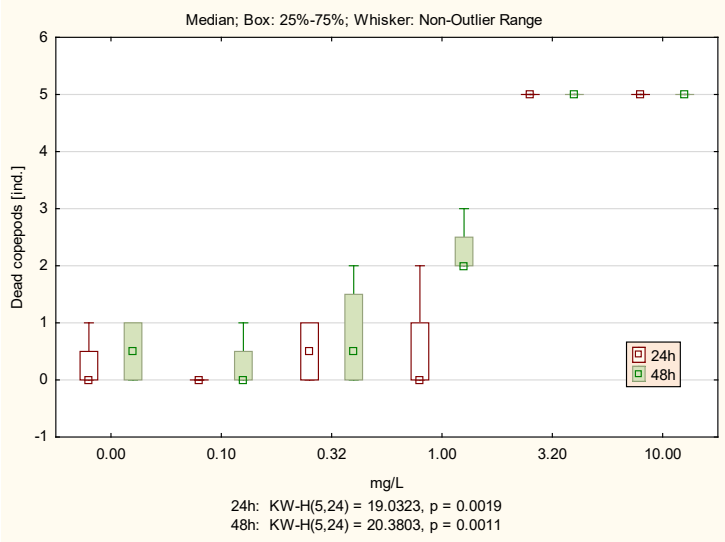


Figure 2. Box plot of copepods mortality per reference toxicant concentration and time exposure.

In the control group C (0.0), mortality is low, indicating that environmental factors likely did not cause significant death and validating the experiment (Figure 3). As the concentration increases, mortality gradually rises, with more pronounced effects at 48 h, suggesting time-dependent toxicity. At higher concentrations (3.2 and 10 mg/L), mortality reaches 100%, indicating that these concentrations are lethal to copepods within 24 h. (Figure 3). The error bars (SEM) reflect variability in the data, showing that while some variation exists, the trends are consistent and indicate dose-dependent mortality (Figure 3). This suggests that the substance is toxic to copepods in a concentration- and time-dependent manner.

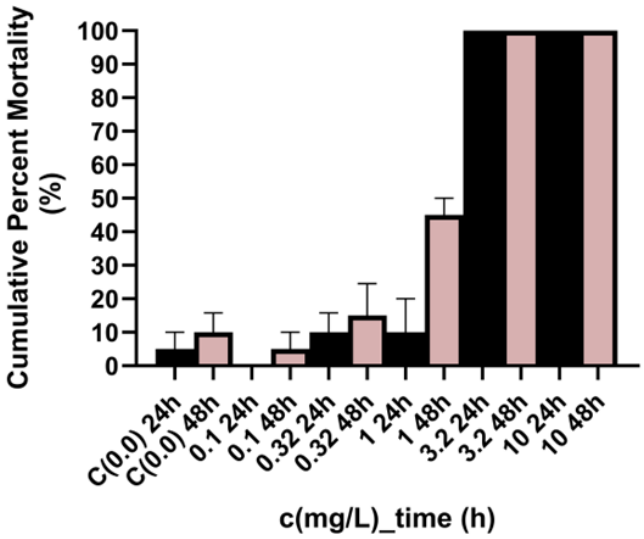


Figure 3. Mean cumulative percent mortality of copepods with standard error of the mean (SEM).

The LC50 graph illustrates the dose-response relationship for the reference toxicant affecting *A. tonsa* (Figure 4). At very low concentrations (around 0.01 mg/L), the effect is minimal, below 20%, and as the concentration increases, particularly between 0.1 mg/L and 1 mg/L, the effect rises noticeably, reaching near maximal levels (close to 100%)

around 10 mg/L. The LC50, where 50% of the maximal effect is observed, is 1.15 mg/L (Table 2). This graph demonstrates that low doses have minimal effects, moderate doses show increasing effects, and high doses achieve maximum effect

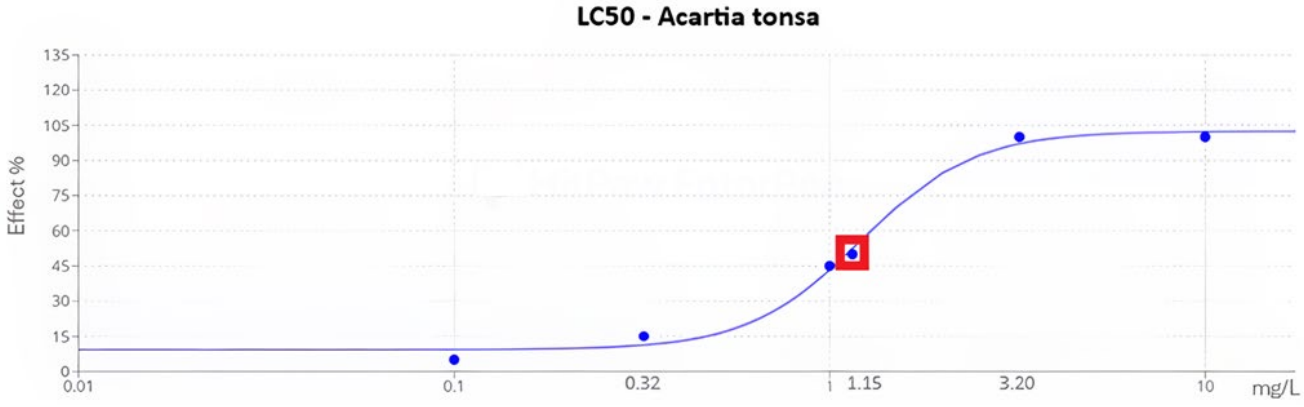


Figure 4. LC50 for the effect of reference toxicant (3,5-dichlorophenol) on *Acartia tonsa* (red square—LC50).

Table 2. Endpoint—48-h LC50.

Species	3,5-dichlorophenol	
	48 h LC50 (mg/L)	Concentration (mg/L) for 20% to 80% Mortality
Acartia tonsa—ISO [33]	0.5–1.5	1.0
Acartia tonsa—test value	1.15	1.0

Increasing concentrations of the toxicant result in progressively greater effects over time. The highest concentration (10 mg/L) caused maximal effect at 24 h, while lower concentrations show milder effects, with some increasing over time (e.g., 1 mg/L). The control group, as expected, shows a negligible effect (Figure 5).

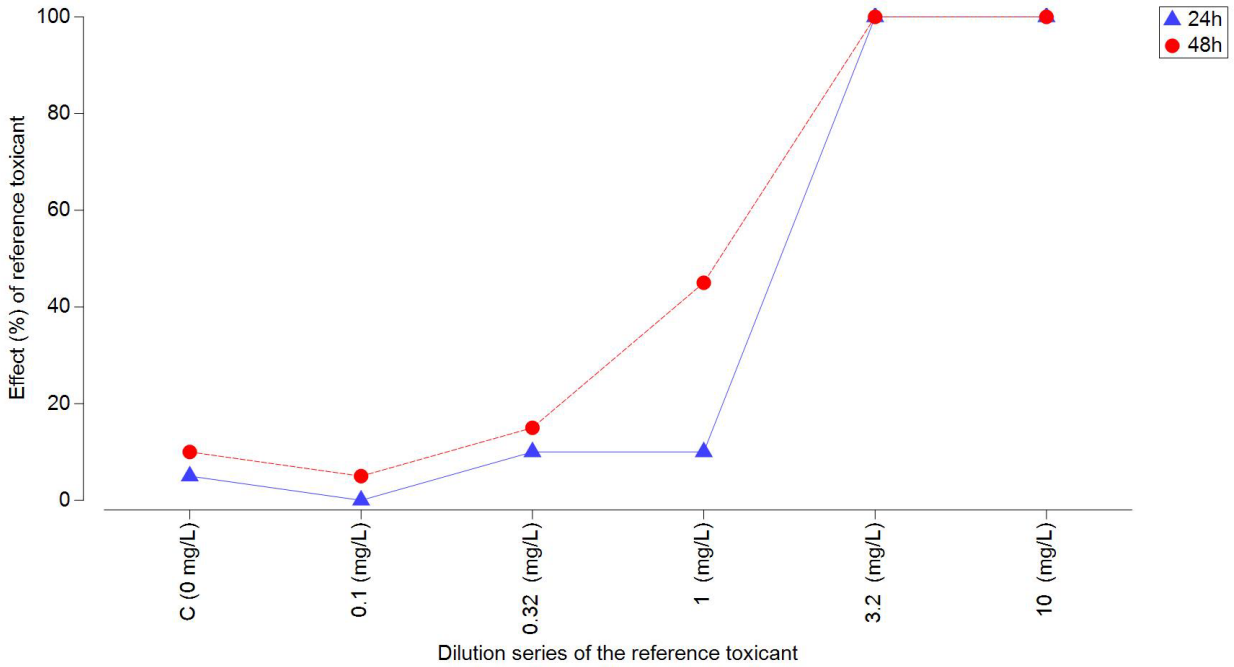


Figure 5. Effect (%) of reference toxicant concentrations.

Concentrations of 3.2 mg/L and 10 mg/L exhibited high effects across both time points (24 h and 48 h) showing a significant effect, with colors moving towards black, indicating 100% effect (Figure 6). Conversely, concentrations 0.1 mg/L and C (control) showed low effects, suggesting that the 0.1 mg/L concentration does not significantly differ from the control. The 0.1 mg/L concentration effects remained consistently low across both time points. A notable time-dependent change was observed for the concentration of 1 mg/L, which transitioned from a lower effect at 24 h to a higher one at 48 h, indicating that this concentration is a threshold where the substance starts to have a notable impact (Figure 6).

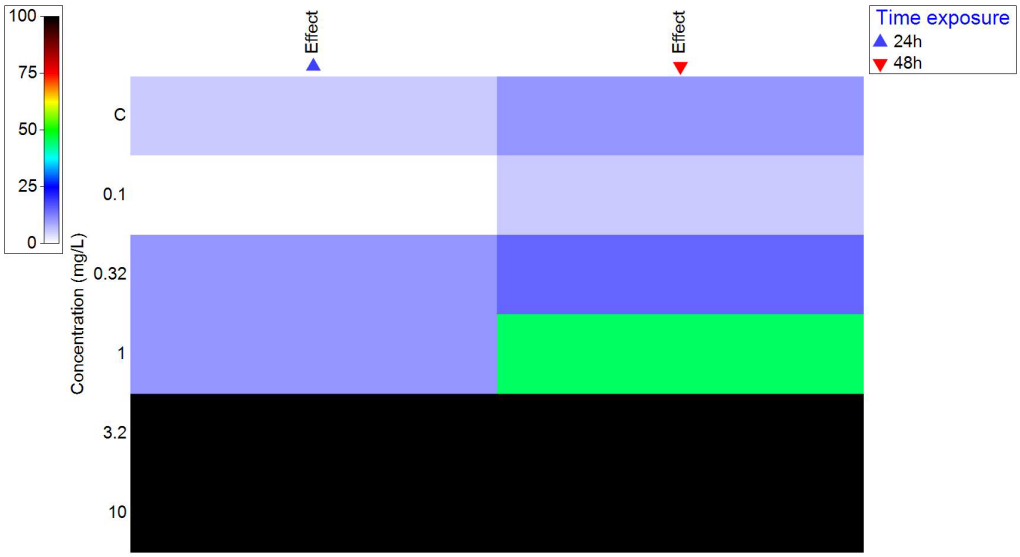


Figure 6. Effect (%) of reference toxicant concentrations for 24 and 48-h exposure of *Acartia tonsa*.

This shade plot effectively illustrates the relationship between the concentration of the reference toxicant, the duration of exposure, and the effect percentage. It demonstrates a clear dose-response relationship, where higher concentrations and longer exposure times lead to more significant effects.

Water quality measurements were conducted to assess the impacts of substance concentration on various parameters before and after the experiment. The parameters were measured immediately after mortality was recorded, leading to some missing values, as certain concentrations were measured at 24 h while others were at 48 h.

The temperature, pH, and dissolved oxygen levels were within the stipulated range of the revised protocol (temperature of 20 ± 2 °C, pH of 8.0 ± 0.5, and dissolved oxygen saturation above 80%) (Table S8). Salinity ranged from 15.95 PSU to 16.97 PSU, being characteristic to the Black Sea features.

4. Discussion

4.1. Insights from A. tonsa Test

As a primary consumer, *A. tonsa* feeds on phytoplankton and serves as a key food source for fish species [7,30]. Its population trends directly reflect the broader environmental conditions, such as nutrient availability, pollution levels, and impacts from climate change [45,46,66]. A decline in *A. tonsa* populations could indicate problems like eutrophication or water quality degradation, signaling a need for stronger conservation measures [66–69]. Conversely, stable populations suggest effective management of these issues.

Monitoring *A. tonsa* is essential for assessing the success of conservation strategies and ensuring the long-term sustainability of the Black Sea ecosystem.

This study was considered acceptable as the mortality (10%) in the control *A. tonsa* and the dissolved oxygen concentration at the end of the test (8.65–9.28 mg/L) were in the range stipulated by the acceptability criteria of the protocol.

Protocol acceptability criteria state that the percentage mortality of the controls should be less than or equal to 10%, the dissolved oxygen concentration at the end of the test should be greater than or equal to 4 mg/L, and the toxicity of the reference chemical should be within the documented range.

Our study found that the sensitivity of copepods to 3,5-dichlorophenol is consistent with the range stipulated in ISO standard, specifically with an obtained value of 1.15 mg/L. This value aligns with the expected sensitivity thresholds, indicating that the copepods exhibit a measurable response to 3,5-dichlorophenol exposure at this concentration. Such findings are critical for environmental monitoring and risk assessment, as they validate the use of copepods as bioindicators for aquatic toxicity. The alignment with ISO standards also enhances the reliability of our results, suggesting that our methodological approach is robust and can be replicated in other studies for consistent outcomes.

The data indicates that the environmental parameters (temperature, salinity, pH, dissolved oxygen (D.O.), and oxygen saturation) were in the protocol range across different treatments (Table S7). Any slight variations observed were within acceptable limits and did not impact the validity of the results. Over time, observed trends, such as a decrease in temperature and pH, were consistent across all treatments, including the control. This consistency suggests that any changes in the experiment's outcomes are likely due to the treatments rather than environmental fluctuations.

Therefore, the results were validated as the environmental conditions were maintained within a narrow and acceptable range across different treatments and time points. Minor variations observed do not significantly impact the overall experiment, ensuring that the treatment effects are the primary variable influencing the outcomes. Maintaining these controlled conditions makes the experiment's findings reliable and can be attributed to the treatments rather than extraneous environmental factors.

4.2. Protocol Deviations

Before the in-house validation of the protocol in 2023, multiple tests with the reference toxicant were conducted between 2019 and 2022. Experimental inconsistencies were identified, necessitating deviations from the original methodology stipulated by ISO [33]. The final test, conducted in 2023, confirmed the validity of the revised protocol. These adjustments were prompted by issues encountered during the testing phase before the 2023 test, which required improvements to the initial protocol.

Our findings indicate that these adjustments have had a notable impact on both the development of the organisms under study and the accuracy of the testing outcomes. Importantly, these modifications have brought our procedures more in line with the standards outlined in ISO 14669:1999 [33], enhancing the reliability and consistency of our experimental results.

Feeding Protocol Adjustment

In the initial test conducted in 2019, the copepod culture was fed with a suspension of *I. galbana* (Haptophyta), while parallel cultures received a mixture of *Nannochloris* sp. and *Tetraselmis suecica* (Chlorophyta) along with *I. galbana*. Our observations revealed significant differences in development between the cultures. Specifically, those fed exclusively with *I. galbana* showed superior growth and vitality compared to those fed with the algae mixture. This result aligns with the guidelines of ISO 14669:1999 [33], which recommends *I. galbana* as a food source. Consequently, we included *I. galbana* as a food source for copepods cultures. In the 2021 test, cultures initially fed with *I. galbana* experienced high mortality rates; therefore, we incorporated *R. baltica* (also mentioned by ISO) as a substitute for *I. galbana*. This adjustment aims to provide the cultures with an optimal diet with low mortality rates, thereby enhancing the reliability and reproducibility of our experimental results.

Test Chamber Specification Update

Evaluation of organisms in the original square wide-mouth bottles proved challenging, primarily due to limited visibility and higher risks of contamination, evaporation, and

volatilization. To address these issues, we introduced an alternative test chamber: borosilicate glass Petri dishes (height 20 mm; diameter 100 mm). These dishes provide superior visibility for organism evaluation and mitigate contamination risks. The modified protocol allows for the use of either the traditional square bottles or the new Petri dishes, offering greater flexibility and improving overall testing conditions.

The modifications to the feeding regimen and test chamber specifications collectively enhance the accuracy and reliability of our marine organism testing protocols. By aligning these protocols more closely with ISO 14669: 1999 [33] standards, we ensure that our testing conditions are optimal for organism development and sensitivity, ultimately leading to more reliable and reproducible test results.

In 2021, borosilicate glass Petri dishes (20 mm in height and 100 mm in diameter) were replaced with 50 mL cell sterile cell culture flasks made of polystyrene, closed with screw caps, for 24 and 48 h. This modification was made to improve culture handling and sterility, thereby enhancing the consistency and reliability of our experimental outcomes.

Future studies should continue to refine these protocols, exploring additional adjustments that may further improve testing outcomes.

It was concluded that these modifications should be added to the initial protocol.

4.3. *A. tonsa* Findings and Other Existing Research

Our study's consistent maintenance of environmental parameters aligns with the rigorous standards required in the *A. tonsa* bioassay, thereby validating our findings. The laboratory was actively engaged in simultaneously testing, developing specific research methodologies for culturing and studying *Skeletonema costatum* (Mediophyceae) and golden grey mullet *Chelon auratus* (Risso, 1810) as reference species for ecotoxicity tests in the Black Sea [21]. By including multiple trophic levels, from primary producers like phytoplankton (such as *Skeletonema costatum*) to higher-level consumers like the golden grey mullet *Chelon auratus*, researchers can better evaluate the ecological risks and predict the overall impact of contaminants on biodiversity and ecosystem health in environments like the Black Sea.

Copepod growth and reproductive success, including egg production rates and egg viability or hatching success, are often seen as a response to food availability (typically measured in terms of carbon, C), with higher rates corresponding to an increased food supply [70,71].

The findings of this study align with and extend previous research on the dietary preferences and nutritional needs of *A. tonsa*. Prior studies have highlighted the importance of diet quality on the physiological performance of copepods, indicating that nutritional content directly influences survival, growth, and reproductive success [70–74]. Our results demonstrated that *A. tonsa* fed with *R. baltica* exhibited a significantly higher survival rate compared to those fed with *I. galbana* and a mixed algal diet. These findings are consistent with another study, which reported that diets rich in essential fatty acids, such as those found in *R. baltica*, enhance copepod survival [75]. The high survival rate observed in this study underscores the superior nutritional quality of *R. baltica*. While previous studies have established the general importance of algal quality in copepod diets, our study provides specific evidence for the benefits of *R. baltica*. For instance, the research by Arendt et al. [76] showed that copepods fed with algae rich in essential fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), exhibited improved physiological performance [77]. *R. baltica* is known for its high EPA content, which likely contributed to the positive outcomes observed in our study. Additionally, several studies [78–80] demonstrated the crucial role of carotenoids in enhancing copepod reproductive success, *R. baltica* containing high levels of carotenoids [81].

Research involving the cultivation of copepods, such as *A. tonsa*, faces several challenges that must be addressed. These challenges include the significant resource and labor demands for maintaining cultures, the increased risk of contamination, and the potential decline in genetic diversity over time [82–85]. Furthermore, copepods in artificial culture environments may undergo stress, leading to altered behavior and physiology, which

can affect the validity of experimental results [66,82,84,86,87]. These considerations are essential when designing studies that utilize cultured copepod populations. Additionally, purchasing eggs can be expensive, subject to supply chain issues, and may lack genetic diversity and environmental context. The health status of commercially sourced eggs can also vary. Balancing these factors against the study's specific objectives is crucial for making informed decisions.

Future research on the use of copepods, such as *A. tonsa*, in toxicity testing is important for advancing our understanding of environmental pollutants and their impacts on marine ecosystems, particularly in the Black Sea. One promising direction involves developing standardized protocols for using copepods in toxicity assays to ensure reproducibility and comparability of results across various studies and laboratories [88]. However, it is necessary to compare, evaluate, and improve these protocols, as toxicology is an evolving experimental science that undergoes continuous reassessment and methodological changes [89], as in the present study. This includes refining methods for culturing and maintaining copepod populations under controlled conditions and improving techniques for assessing their physiological and behavioral responses to toxicants.

Additionally, studies should explore the ecological relevance of toxicity testing with copepods by incorporating multiple stressors that more accurately reflect natural conditions, such as temperature changes, salinity fluctuations, and food availability [58,77,78,83,90–92]. Understanding these interactions is essential for predicting the real-world impacts of pollutants on copepod populations and, consequently, on the marine food web, with a particular focus on the unique dynamics of the Black Sea environment.

Given their vital role in marine ecosystems, copepods are key components in the transfer of energy and nutrients through the food web [32,67,68,93]. They serve as primary consumers of phytoplankton and as prey for higher trophic levels, including commercially important fish species [94–98]. Therefore, understanding the effects of pollutants on copepods is essential for assessing the health of marine ecosystems [99–101] and the potential risks to biodiversity and fisheries [94,102].

Future research should also integrate advanced molecular techniques, such as genomics and metabolomics, into standardized protocols. These techniques can uncover specific biochemical and genetic pathways affected by pollutants, providing deeper insights into the mechanisms of toxicity.

5. Conclusions

This study successfully established a validated protocol for testing chemical effluents using *Acartia tonsa* as a model organism, specifically employing the reference toxicant 3,5-dichlorophenol. By standardizing procedures for culturing, maintaining, and testing *A. tonsa*, the research provides a scientifically robust method for assessing the ecotoxicity of pollutants. This approach contributes to a deeper understanding of the impacts of pollutants on marine ecosystems, which is crucial for informing conservation and management strategies, particularly in sensitive environments like the Black Sea.

The standardized protocol ensures reliable and comparable results, enhancing the sensitivity and accuracy of ecotoxicity assessments. Future research should focus on refining these protocols to account for specific environmental conditions and expanding their application to a wider range of pollutants.

Utilizing the data generated from *A. tonsa* ecotoxicity tests can inform environmental policies, promote sustainable product innovation, and guide conservation efforts. By broadening the use of *A. tonsa* in toxicity testing, a more comprehensive understanding of the ecological impacts of various pollutants can be achieved.

In light of these findings, the following recommendations are proposed to enhance the effectiveness and scope of ecotoxicity testing using *A. tonsa*:

- continue refining and validating *A. tonsa* testing protocols to ensure they account for specific environmental conditions, enhancing sensitivity and accuracy in ecotoxicity assessments;

- expand the use of *A. tonsa* in toxicity testing to include a wider range of pollutants, providing a more comprehensive understanding of their ecological impacts;
- utilize the data generated from *A. tonsa* ecotoxicity tests to inform environmental policies, promote sustainable product innovation, and guide conservation efforts;
- conduct comparative studies with other marine organisms to improve the ecological relevance and robustness of toxicity assessments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14219861/s1, Figure S1. *Acartia tonsa* eggs (original photo—OlympusSZX10 stereomicroscope, 4xzoom), Figure S2. Inoculation of *A. tonsa* eggs (original photo), Figure S3. Cultures of *A. tonsa* transferred to dilution water (original photo), Figure S4. *Chryptomonas baltica* (original photo), Figure S5. *C. baltica* microalgae cultures (original photo), Figure S6. Analysis of *C. baltica* under the microscope (left) using the hemocytometer (right) (original photos), Figure S7. Copepods transfer into the test chamber (original photo), Figure S8. Monitoring of parameters for copepod culture (original photo), Figure S9. Light intensity check before the start of the experiment (original photo), Table S1. Walnes medium composition (per 1 L), Table S2. Dilution series of the test substances, Table S3. Water quality parameters, Table S4. Nutrient concentrations and maximum admissible levels, Table S5. Concentrations of heavy metals and maximum admissible levels, Table S6. Concentrations of organochlorine pesticides and maximum admissible levels, Table S7. Concentrations of polycyclic aromatic hydrocarbons (PAHs) and maximum admissible levels, Table S8. Water quality measurements before and after the experiment.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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