

Original Research Articles

Study on the 96 h-LC₅₀ of five novel non-oxidizing circulating water treatment agents to *Crassostrea gigas* and *Mytilus edulis*

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This study evaluated the acute toxicity and attachment inhibition effects of five novel nonoxidizing circulating water treatment agents on key marine fouling organisms, namely *Crassostrea gigas* eyed larvae and *Mytilus edulis* spat, aiming to screen ecofriendly and efficient antifouling agents for nuclear power cooling systems. A 96 h semistatic acute toxicity assay was adopted combined with byssal secretion parameter determination, and data were analyzed using probit analysis, independent samples t-tests, and ANOVA. The test organisms, *C. gigas* eyed larvae (shell length $\approx$ 200 μ m, actively swimming) and *M. edulis* spat (shell length 1–2 cm, intact shell, healthy), were acclimated under controlled conditions (25 ± 0.5 °C, salinity 20 ± 1 , DO ≥ 6 mg/L, 12L:12D photoperiod). Five agents containing different alkylamine and polyoxyethylene ether components were tested at gradient concentrations (0–1.92 mg/L for *C. gigas* larvae and 0–18 mg/L for *M. edulis* spat), with 50% test solution renewal every 24 h. The measured indicators included 96 h LC₅₀, safe concentrations, attachment inhibition rates, and *M. edulis* byssal secretion parameters (number, total length, diameter of byssal threads, and shedding frequency). All agents showed significant dose dependent toxicity ($P < 0.05$). For *C. gigas* larvae, the toxicity was ranked as Agent 4 > Agent 2 > Agent 5 > Agent 3 > Agent 1, with 96 h LC₅₀ ranging from 0.13 to 1.00 mg/L, while for *M. edulis* spat, the toxicity was ranked as Agent 3 > Agent 2 > Agent 5 > Agent 1 > Agent 4, with 96 h LC₅₀ ranging from 7.65 to 9.74 mg/L. Agent 3 significantly reduced the number, length, and diameter of *M. edulis* byssal threads and increased shedding frequency ($P < 0.05$) through a dual inhibition mechanism by interfering with byssal protein synthesis and substrate adhesion. Overall, Agent 3 exhibited balanced antifouling efficacy and environmental safety, and is recommended at 15 mg/L for biofouling control in nuclear power cooling systems to avoid the toxic byproducts of traditional oxidizing agents.

1. INTRODUCTION

Marine fouling organism adhesion is a persistent, globally prevalent challenge for coastal industrial facilities, with notable implications for the safe and stable operation of nuclear power plant cooling water circulation systems.¹ These organisms, encompassing mollusks, cnidarians, and other taxa, secrete complex adhesive substances composed of proteins, polysaccharides, and lipids to attach to the inner walls of water intake pipelines.² Over time, this attachment

forms dense biological coatings that reduce the effective flow cross-sectional area of pipelines and lower heat exchange efficiency. On-site investigation data from coastal nuclear power plants show that the attachment of *Crassostrea gigas* and *Mytilus edulis* can reduce the cross-sectional area of cooling water pipelines by 30–50% and decrease the heat-exchange efficiency of nuclear power units by 15–25%.³ In individual cases, unchecked fouling can lead to partial or complete pipeline blockage and trigger unplanned shutdowns of nuclear power units, with each shutdown causing significant economic losses (data from

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on-site nuclear power plant statistics). Such incidents result in measurable economic losses and potential operational risks, making fouling control an important requirement for the nuclear power industry. It should be emphasized that *C. gigas* and *M. edulis* are ecologically important species in natural coastal ecosystems; this study addresses their abnormal colonization on artificial substrates of nuclear power cooling systems, rather than questioning their inherent ecological value.⁴

Currently, traditional oxidizing antifouling agents, such as chlorine-based disinfectants and ozone, are widely used for their rapid bactericidal activity and low cost. However, these agents tend to react with organic matter in seawater to produce toxic byproducts, including trihalomethanes and bromate, which pose significant threats to marine ecological systems.⁵ With the increasingly stringent environmental protection regulations globally, the application of oxidizing antifouling agents has been subject to growing restrictions, which creates an urgent demand for the development of high-efficiency, low-toxicity, and eco-friendly non-oxidizing circulating water treatment agents as a sustainable alternative.⁶

The novel non-oxidizing circulating water treatment agent developed represents a promising technical breakthrough for addressing fouling organism adhesion in nuclear power cold source systems. Unlike conventional single-target agents, this new formulation integrates multiple functional components, including alkylamines and polyoxyethylene ethers, designed to target different stages of fouling organism attachment and metabolism. However, before large-scale engineering applications, comprehensive performance evaluations are indispensable. Key parameters requiring clarification include its inhibitory efficacy against a broad spectrum of common fouling organisms and its long-term stability under real-world operating conditions.

Among the diverse array of marine fouling organisms inhabiting coastal waters of China, *C. gigas* (Pacific oyster) and *M. edulis* (blue mussel) are recognized as the most problematic species for nuclear power cooling systems.⁷ On-site investigation data of coastal nuclear power plants in this region have confirmed that the massive attachment of these two mollusks is the primary cause of pipeline blockage and heat exchange efficiency reduction in local nuclear power cooling water systems. Both species are highly prolific, with their larvae readily entering pipelines and forming dense, calcified colonies that significantly exacerbate fouling.⁸ As representatives of mollusks, they share a unique adhesion mechanism relying on byssus secretion, making them ideal test organisms for comprehensively assessing the agent's practical application value in controlling mollusk-related fouling—the core fouling type threatening local nuclear power cold source systems.⁹

In this study, a systematic, multi-faceted approach was adopted to investigate the performance and mechanism of five novel non-oxidizing circulating water treatment agents, with *C. gigas* eyed larvae and *M. edulis* spat—key fouling organisms at control-susceptible critical life stages—selected as test subjects. The research included determining the 96-hour semi-static acute toxicity median

lethal concentration (LC₅₀) to assess the agents' toxicity thresholds, and compiling Material Safety Data Sheets (MSDS) from toxicological test results to provide scientific support for environmental risk assessment. The findings are expected to deliver critical technical parameters for the engineering application of these agents, enrich the theoretical system for marine fouling organism control, and offer practical guidance for fouling management in nuclear power plants and other coastal industrial facilities, thereby contributing to the sustainable development of the coastal industrial sector.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL MATERIALS AND TREATMENTS

C. gigas eye-spot larvae and *M. edulis* spat were sourced from the same commercial hatchery in Yantai, Shandong Province, China. Prior to experiments, both organisms were acclimated for 72 h in the Marine and Biological Engineering College of Yancheng Institute of Technology under the following controlled conditions: water temperature of 25 ± 0.5 °C, salinity of 20 ± 1 , dissolved oxygen (DO) ≥ 6 mg/L, and a photoperiod of 12 h light: 12 h dark (12L:12D). The 72 h acclimation period was set in accordance with GB/T 16882-2022 (Guidelines for marine aquatic biological toxicity test) and international standard protocols for bivalve acute toxicity tests, which is sufficient for test organisms to recover from transportation stress and fully adapt to laboratory physicochemical conditions. During acclimation, they were fed with *Chlorella vulgaris* at a concentration of 5×10^6 cells/mL twice daily at 8:00 and 20:00 (12 h interval). Mortality during acclimation was $<3\%$ for both species, meeting the validity requirement of test guidelines. Dead individuals were removed daily, and only healthy and active individuals were selected for subsequent toxicity tests to eliminate interference from stressed or dead individuals. For subsequent experiments, *C. gigas* eyed larvae (10 days after fertilization, shell length ≈ 200 μ m) with uniform body size and active swimming activity were selected, whereas *M. edulis* juvenile spat (30 days after settlement, shell length 1–2 cm) were selected based on good health status and intact shell morphology. These two stages represent the critical attachment stages of the respective species, the most sensitive life stages to antifouling agents, and the key control targets in nuclear power cooling systems. Although their absolute developmental stages differ, they are functionally equivalent (critical attachment stage), ensuring the comparability of toxicity and inhibition test results.

Five novel non-oxidizing circulating water treatment agents (Agent 1 to Agent 5) were provided by Shanghai Nuclear Engineering Research and Design Institute Co., Ltd., China. All agents were stored in sealed containers at 20 ± 2 °C to avoid contamination and degradation, and fresh stock solutions were freshly prepared with artificial seawater immediately prior to experiments.

Artificial seawater was formulated using analytical-grade Red Sea Coral Pro Salt (Model: RS-CPS01) and deionized water, aerated continuously for 24 h to remove dis-

solved gases, and adjusted to a pH of 8.3 ± 0.1 . Prior to experiments, key physicochemical parameters were measured using a multi-parameter water quality analyzer (YSI ProPlus, YSI Incorporated, Yellow Springs, OH, USA) and a dissolved oxygen meter (HQ40d, Hach Company, Loveland, CO, USA): salinity 20 ± 1 , DO 6.8 ± 0.2 mg/L, chemical oxygen demand (COD) 1.12 ± 0.05 mg/L, nitrate nitrogen 0.158 ± 0.005 mg/L, nitrite nitrogen 0.007 ± 0.001 mg/L, ammonia nitrogen 0.023 ± 0.002 mg/L, inorganic nitrogen 0.178 ± 0.008 mg/L, and reactive phosphate 0.010 ± 0.001 mg/L, all conforming to China's GB 17378-2007 (Marine Monitoring Specifications) and GB 3097-1997 (Sea Water Quality Standard).

2.2. EXPERIMENTAL METHODS

2.2.1. DETERMINATION OF MEDIAN LETHAL CONCENTRATION

A 96 h semi-static acute toxicity assay was performed in a light incubator (MGC-3000, Shanghai Yiheng Scientific Instruments Co., Ltd., China) to determine the 96 h-LC₅₀ values of each agent against *C. gigas* larvae and *M. edulis* spat. Test conditions were consistent across all groups: temperature maintained at $25 \pm 0.5^\circ\text{C}$, light intensity of 1200 lx, which is consistent with the natural shallow coastal light environment (1000–1500 lx) of the test organisms and meets the standard requirements for marine bivalve toxicity tests, photoperiod of 12L:12D, light intensity of 1200 lx, no feeding during the experiment, and 50% test solution renewal every 24 h using the siphon method: the upper 50% of the old test solution was slowly removed without disturbing test organisms, followed by addition of an equal volume of freshly prepared test solution at the target concentration. After each renewal, the actual agent concentration was recalibrated by high-performance liquid chromatography (HPLC), with a deviation $< 5\%$ from the set concentration, ensuring stable exposure concentrations throughout the test. Key water quality parameters, including dissolved oxygen (DO), pH, salinity, and temperature, were dynamically monitored at 0, 24, 48, 72, and 96 h in all groups. The results showed that DO remained ≥ 6.0 mg/L, pH at 8.2 ± 0.2 , salinity at 20 ± 1 , and temperature at $25 \pm 0.5^\circ\text{C}$ throughout the test period, with no significant fluctuations observed. These strict monitoring and water renewal measures ensured stable water quality and agent concentration, eliminating interference from environmental changes on toxicity test results.

Test solutions were prepared using an analytical balance (ME204E, Mettler Toledo International Inc., Zurich, Switzerland). For *C. gigas* eyed larvae, 6 to 10 concentration gradients ranging from 0 to 1.92 mg/L were established for each agent according to preliminary experiments, with key points including 0, 0.06, 0.18, 0.48, 0.96, 1.34, 1.68, and 1.92 mg/L; each group had 3 replicates with 30 larvae per replicate, placed in 500 mL glass beakers containing 300 mL test solution. For *M. edulis* spat, concentration gradients for each agent ranged from 0–18 mg/L, with key points including 0, 6, 9, 12, 14, 16, and 18 mg/L; each group had 3 repli-

cates with 30 spat per replicate, placed in 1 L glass beakers containing 800 mL test solution.

The number of dead individuals was recorded at 24, 48, 72, and 96 h. *C. gigas* larvae were considered dead when no ciliary movement or body contraction was observed under an inverted microscope (IX73, Olympus Corporation, Tokyo, Japan); *M. edulis* spat were considered dead if no response was observed to gentle prodding with forceps and gill movement was completely absent.

2.2.2. DETERMINATION OF MUSSEL BYSSAL THREAD SECRETION PARAMETERS

The optimal agent, selected based on 96 h-LC₅₀ values and attachment inhibition rates, was assayed at its effective concentration to evaluate its effects on byssal thread secretion in *M. edulis* spat. All operations were performed in an ultra-clean workbench (SW-CJ-1FD, Suzhou Antai Air Technology Co., Ltd., China). A control group (artificial seawater without the test agent) and a treatment group (artificial seawater with the optimal agent at an effective concentration) were established, with 10 replicates per group and 1 spat per replicate (housed in 200 mL glass beakers containing 150 mL of test solution).

At 3, 6, 12, and 24 h after exposure, the following parameters were measured: the number of byssal threads (counted under a stereomicroscope), the total length of byssal threads (measured with a digital vernier caliper, accuracy: 0.01 mm), the diameter of byssal threads (measured using an inverted microscope equipped with ImageJ 1.8.0 software; three measurement points were uniformly taken at the middle position of each thread, and the average value was used as the final diameter to avoid variability from base–top thickness differences), and shedding frequency (each spat was gently prodded 3 times at the shell edge with uniform force and 1 min intervals; shedding frequency was recorded as the number of detachments from the substrate in the 3 attempts).

2.3. STATISTICAL ANALYSIS

All data were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD) and analyzed using IBM SPSS Statistics 27 software (IBM Corporation, Armonk, NY, USA). The 96 h-LC₅₀ values and their corresponding 95% confidence intervals (CIs) were calculated via probit analysis. Safe concentration (SC) was calculated in accordance with GB/T 27622-2011 and OECD 206 guidelines, using the formula: $\text{SC} = 96 \text{ h-LC}_{50} \times 0.1$. The safe concentration represents the acute safe concentration for marine aquatic organisms, indicating the maximum concentration at which no acute toxic effect occurs. These data exhibit normal distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Differences in attachment inhibition rates and byssal thread secretion parameters were analyzed using independent-samples t-tests or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Differences were considered statistically significant at $P < 0.05$, and dif-

Table 1. 96 h-LC₅₀ and Safe Concentrations of Five Agents on Eyed Larvae of *C. gigas*

Agent No.	96 h-LC ₅₀ (mg/L)	Safe Concentration (mg/L)	95% Confidence Interval (mg/L)	Regression Equation	χ ² Test Value (p-value)
1	1.00 e	0.10	0.00-2.136	Y = -0.1 + 2.82x (r ² = 0.803)	1.439
2	0.39 b	0.04	0.12-1.41	Y = 0.81 + 1.96x (r ² = 0.947)	0.538
3	0.81 d	0.08	0.34-1.41	Y = 0.33 + 4.62x (r ² = 0.858)	0.836
4	0.13 a	0.01	0.00-0.30	Y = 1.53 + 1.73x (r ² = 0.843)	0.879
5	0.51 c	0.05	0.04-2.03	Y = 0.32 + 1.42x (r ² = 0.728)	2.093

Different lowercase letters in the same column indicate significant inter-agent differences at $P < 0.05$

ferent lowercase letters were used to indicate significant inter-group differences for 96 h-LC₅₀ values.

3. RESULTS

3.1. TOXIC EFFECTS ON EYED LARVAE OF *C. GIGAS*

All five novel non-oxidizing circulating water treatment agents exhibited a significant dose-response relationship in eyed larvae of *C. gigas*, i.e., larval mortality rate increased with increasing agent concentration ($P < 0.05$). Among them, Agent 1 had the highest 96 h-LC₅₀ value of 1.00 mg/L (e) and a safe concentration of 0.10 mg/L, showing the optimal safety for *C. gigas* larvae; Agent 4 was the most toxic, with a 96 h-LC₅₀ of only 0.13 mg/L (a) and a safe concentration as low as 0.01 mg/L; the 96 h-LC₅₀ values of Agents 3, 2, and 5 were 0.81 mg/L (d), 0.39 mg/L (b), and 0.51 mg/L (c), respectively, with corresponding safe concentrations of 0.08 mg/L, 0.04 mg/L, and 0.05 mg/L (Table 1). The toxicity of the tested agents to *C. gigas* larvae was ranked in descending order as follows: Agent 4 (a) > Agent 2 (b) > Agent 5 (c) > Agent 3 (d) > Agent 1 (e).

3.2. TOXIC EFFECTS ON SPAT OF *M. EDULIS*

3.2.1. 96-HOUR MEDIAN LETHAL CONCENTRATION (96 H-LC₅₀)

The five agents showed significant differences in toxicity to the spat of *M. edulis*. Agent 4 had the highest 96 h-LC₅₀ value (9.74 mg/L, e) and a safe concentration of 0.97 mg/L, demonstrating the optimal safety; Agent 3 was the most toxic, with a 96 h-LC₅₀ of 7.65 mg/L (a) and a safe concentration of 0.77 mg/L; the 96 h-LC₅₀ values of Agents 1, 5, and 2 were 9.21 mg/L (d), 8.84 mg/L (c), and 8.35 mg/L (b), respectively, with corresponding safe concentrations of 0.92 mg/L, 0.88 mg/L, and 0.84 mg/L, respectively (Table 2). The toxicity order of the agents to *M. edulis* spat was: Agent 3 (a) > Agent 2 (b) > Agent 5 (c) > Agent 1 (d) > Agent 4 (e) (different lowercase letters indicate significant differences at $P < 0.05$).

Represented by Agent 3, the optimal agent exerted significant effects on the byssal secretion parameters of *M. edulis* spat at effective concentrations ($P < 0.05$). Compared with the control group, the number, total length, and diameter of byssal threads of spat in the experimental group were significantly reduced from 6 hours onwards, and the differences continued to expand with the extension of time; the shedding frequency was significantly increased. After 12 h of exposure, the shedding frequency in the experimental group was significantly higher than in the control group, and at 24 h post-exposure, individuals in the experimental group failed to reattach to the substrate after shedding, indicating a persistent inhibitory effect of Agent 3 on their adhesive capacity.

3.3. COMPREHENSIVE EVALUATION

Based on the comprehensive analysis of 96 h-LC₅₀, safe concentrations, and biological effects, Agent 3 performed the best: it exhibited significant toxic effects on both *C. gigas* larvae and *M. edulis* spat, and effectively inhibited the attachment of these two key fouling organisms through multiple mechanisms. Notably, the higher toxicity of Agent 3 to *M. edulis* spat is a desirable antifouling characteristic rather than an adverse effect, as it directly enhances lethality toward the most problematic fouling mollusk in nuclear power cooling systems. This target-specific toxicity was balanced against its favorable environmental safety profile (safe concentration = 0.77 mg/L) and its unique dual-inhibition mechanism, which interferes with byssal protein synthesis and substrate adhesion. The screening criteria prioritized a comprehensive trade-off: strong antifouling efficacy (lethality + attachment inhibition) against target fouling organisms, moderate environmental safety for non-target species, broad-spectrum activity, and absence of toxic byproducts. Although Agent 5 exhibited moderate toxicity to *M. edulis* spat (c), it showed high toxicity to *C. gigas* eyed larvae (c), thus lacking broad-spectrum antifouling efficacy. In contrast, Agents 1 and 4 presented high environmental safety (e for both test organisms) but exerted limited inhibitory effects on the attachment-related physiological processes of the two fouling organisms; Agent 2 displayed

Table 2. 96 h-LC₅₀ and Safe Concentrations of Five Agents on Spat of *M. edulis*

Agent No.	96 h-LC ₅₀ (mg/L)	Safe Concentration (mg/L)	95% Confidence Interval (mg/L)	Regression Equation	χ ² Test Value (p-value)
1	9.21 d	0.92	6.57-15.56	Y = -4.37 + 4.39x (r ² = 0.898)	1.068
2	8.35 b	0.84	6.62-10.31	Y = -8.95 + 9.65x (r ² = 0.808)	1.307
3	7.65 a	0.77	5.45-9.16	Y = -9.5 + 10.44x (r ² = 0.741)	1.373
4	9.74 e	0.97	5.69-12.31	Y = -7.41 + 7.42x (r ² = 0.811)	1.002
5	8.84 c	0.88	4.98-11.27	Y = -8.68 + 9.13x (r ² = 0.853)	0.693

Different lowercase letters in the same column indicate significant inter-agent differences at $P < 0.05$. Effects on Byssal Secretion of *M. edulis*

moderate toxicity to both organisms (b for both) without obvious advantages.

Considering the actual needs of fouling organism control in nuclear power cold-source systems, Agent 3 is recommended as the preferred choice for engineering applications, with a suggested dosage of 15 mg/L. This is the initial dosing concentration for open circulating water systems, which will be diluted to 0.5–1.0 mg/L in actual operation, ensuring effective control of major molluscan fouling organisms while maintaining favorable environmental safety. This concentration not only ensures the effective control of major molluscan fouling organisms, including oysters and mussels, but also maintains a favorable environmental safety profile, thereby precluding severe ecological risks to non-target marine organisms.

4. DISCUSSION

4.1. TOXICITY DIFFERENCES OF FIVE AGENTS TO *C. GIGAS* LARVAE

The acute toxicity test results indicated that all five non-oxidizing circulating water treatment agents exhibited significant, dose-dependent lethal effects on *C. gigas* eye-spot larvae, consistent with the toxicological characteristics of alkylamine- and polyoxyethylene ether-containing compounds reported in previous studies.¹⁰ Among them, Agent 4 showed the highest toxicity (a), whereas Agent 1 exhibited the lowest toxicity (e), resulting in an approximate 8-fold difference in the 96 h-LC₅₀ values between these two agents. This difference is closely related to the composition and proportion of their active ingredients. Agent 4 contains hydrogenated tallow amine polyoxyethylene ether, whose hydrogenated hydrophobic chain has a higher membrane penetration ability compared to the unsaturated chain in tallow amine polyoxyethylene ether, enabling it to more easily disrupt the cell membrane structure of oyster larvae and induce acute toxicity.¹¹ In contrast, Agent 1 has a lower content of N-oleyl-1,3-propanediamine than Agent 3-5, and the balanced ratio of its three active components may potentially mitigate the mutual synergistic toxicity among them.¹²

Oyster attachment relies on the secretion of cysteine-rich protein complexes by the byssal gland, and cysteine directly affects the structural stability and adhesive activity of byssal proteins.¹³ The agents may interfere with the synthesis of cysteine in oyster larvae, thereby inhibiting the synthesis and secretion of byssal proteins—this is proposed as the core mechanism underlying the inhibitory effect of the tested agents on the attachment of *C. gigas* eyed larvae. The safety concentration of Agent 1 was the highest among the five agents, indicating its potential as an environmentally friendly antifouling candidate. However, its attachment-inhibition effect was not the most pronounced in subsequent tests, which limits its practical application. Agent 3, with a moderate toxicity (d) and a safety concentration of 0.08 mg/L, has balanced toxicity and effectiveness, making it a more suitable choice for engineering applications. This is consistent with the principle that ideal antifouling agents should have moderate toxicity to target organisms while minimizing environmental risks.¹⁴

4.2. TOXICITY AND BYSSAL SECRETION INHIBITION EFFECTS ON *M. EDULIS* SPAT

Unlike the toxicity order to *C. gigas* larvae, the toxicity of the five agents to *M. edulis* spat was ranked as Agent 3 (a) > Agent 2 (b) > Agent 5 (c) > Agent 1 (d) > Agent 4 (e), with Agent 4 showing the lowest toxicity. This species-specific variation in toxicity is a reasonable theoretical inference based on the published literature and is mainly attributed to structural differences in the body surface and detoxification metabolic systems between the two mollusks. *M. edulis* spat has a thicker shell and more developed hepatopancreas, which can enhance the metabolism and detoxification of foreign substances, resulting in higher LC₅₀ values compared to *C. gigas* larvae.¹⁵ Additionally, the byssal gland of mussels has a more complex structure than that of oysters, and the synthesis of byssal proteins involves more metabolic pathways, which may also affect the sensitivity to the agents.¹⁶

The analysis of byssal secretion parameters showed that Agent 3 significantly reduced the number, total length, and diameter of byssal threads in *M. edulis* spat and increased shedding frequency, consistent with the reported inhibitory

effects of alkylamine compounds on mussel adhesion.¹⁷ Mussel byssal proteins, particularly the key components, are rich in 3,4-dihydroxyphenylalanine (DOPA), which is essential for the underwater adhesive performance of byssal threads.¹⁸ The agents may interfere with the synthesis or activity of these key byssal proteins, directly weakening the adhesive strength of byssal threads. Meanwhile, the core component of Agent 3, N-oleyl-1,3-propanediamine, can form a hydrophobic protective film on the substrate surface, hindering the hydrophilic interaction between byssal proteins and the substrate, thus forming a so-called “dual inhibition” mechanism, which involves the internal interference of byssal protein synthesis and the external obstruction of the adhesive interaction between byssal threads and the substrate.¹⁹

High concentrations of the agents may damage mussel spat cells, but the low mortality rate at effective inhibitory concentrations suggests that the agents primarily act on adhesion-related processes rather than causing fatal damage, which is conducive to reducing environmental risks.²⁰

4.3. COMPREHENSIVE EVALUATION AND ENGINEERING APPLICATION PROSPECTS

The comprehensive evaluation based on LC₅₀, safety concentration, and biological effects showed that Agent 3 is the optimal choice for fouling control. It not only exerts significant toxic effects on both *C. gigas* eyed larvae (d) and *M. edulis* spat (a) but also inhibits their attachment through multiple physiological and physical mechanisms, which confer a distinct advantage over conventional single-target antifouling agents.²¹ Agents 1 and 4, despite their high safety, have limited effectiveness in inhibiting adhesion, while Agents 2 and 5 lack broad-spectrum activity, making them less suitable for large-scale application in nuclear power plant cooling systems.

The suggested dosage of 15 mg/L for Agent 3 is based on a comprehensive assessment of its effective inhibition concentration and environmental safety. This 15 mg/L is the initial dosing concentration in the make-up water, which will be rapidly diluted to an actual effective concentration of 0.5–1.0 mg/L in the open circulating water environment. This diluted concentration is below the 96 h-LC₅₀ of *M. edulis* spat (7.65 mg/L) and within the safe concentration range, thus precluding acute toxic effects on non-target marine organisms. This concentration is approximately 187.5-fold and 19.5-fold higher than the safety concentrations of *C. gigas* eyed larvae and *M. edulis* spa, respectively, ensuring long-term effective antifouling control while precluding acute toxic effects on non-target marine organisms.²² Compared with traditional oxidizing antifouling agents, Agent 3 does not produce toxic byproducts such as trihalomethanes, which is in line with the development trend of green antifouling technology.²³

However, this study also has certain limitations. First, the present study was conducted under controlled laboratory conditions, and the actual operating conditions of nuclear power plant cooling water systems may potentially alter the antifouling efficacy of Agent 3.²⁴ Second, the antifouling evaluation was only performed on two key mollus-

can fouling organisms, and the inhibitory effects of Agent 3 on other common marine fouling organisms remain to be further investigated and verified.²⁵ Future research will focus on conducting targeted acute toxicity and attachment inhibition experiments of Agent 3 on typical fouling organisms of different taxa, including cnidarians (*Hydroides ezoensis*), crustaceans (*Balanus amphitrite*), and macroalgae (*Ulva pertusa*), to systematically verify its broad-spectrum antifouling performance. In parallel, standardized field trials will be conducted at a coastal nuclear power plant using three concentration gradients of Agent 3, with continuous monitoring of fouling organism attachment density, species composition, and key water quality indicators at multiple time points to optimize the dosing concentration and application frequency. For long-term environmental risk assessment, a suite of non-target marine organisms will be selected to conduct chronic toxicity tests, reproductive toxicity tests, and embryo-larval development assays, to derive chronic safe concentrations and establish a systematic ecological risk assessment framework for Agent 3 in marine environments, thereby ensuring the sustainability of its engineering application.

In short, Agent 3 exhibits excellent, comprehensive performance across toxicity, attachment inhibition, and broad-spectrum activity, and its mechanism of action is clear. It provides a new technical solution for controlling fouling organisms in nuclear power plant cooling water systems. It has broad application prospects in coastal industrial facilities such as power plants and ports.

5. CONCLUSION

This study systematically assessed the acute toxic and attachment-inhibiting effects of five novel non-oxidizing circulating water treatment agents on *C. gigas* eyed larvae and *M. edulis* spat using 96 h semi-static acute toxicity assays and measurements of byssal secretion parameters. All agents showed significant dose-dependent lethality with distinct species-specific toxicity: Agent 4 (a) was the most toxic to *C. gigas* larvae, while Agent 3 (a) was the most toxic to *M. edulis* spat, a difference attributed to structural and detoxification system variations between the two organisms. Agent 3 exhibited the optimal overall performance, exerting significant toxic effects on both test organisms and inhibiting their attachment via a “dual inhibition” mechanism, while balancing antifouling efficacy and environmental safety. Thus, Agent 3 is recommended as the preferred choice for fouling control in nuclear power cold source systems with a suggested dosage of 15 mg/L, which avoids toxic byproducts of traditional oxidizing agents and provides a reliable technical reference for the green and sustainable operation of coastal industrial facilities. This study enriches the technical framework for marine fouling organism control in nuclear power cold source systems; future research should focus on three key aspects: first, conduct field trials in actual nuclear power cooling water systems to optimize the application parameters (dosage, frequency) of Agent 3; second, expand the test scope to typical non-mollusk fouling organisms including cnidarians (*Hydroides*

ezoensis), crustaceans (*Balanus amphitrite*), and algae (*Ulva pertusa*) to verify the broad-spectrum antifouling efficacy of Agent 3; third, carry out long-term environmental monitoring to assess the ecological risks of Agent 3 to non-target marine organisms.

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AUTHORS' CONTRIBUTION

Conceptualization: Zhengqiang Miao (Lead). Supervision: Zhengqiang Miao (Lead). Writing – original draft: Aijia Lin (Lead). Writing – review & editing: Aijia Lin (Equal), Yiyun Zhang (Equal). Data curation: Shouxia Zhao (Equal), Ning Gao (Equal), Ziyang He (Equal). Validation: Shouxia Zhao (Equal), Ziyang He (Equal). Formal Analysis: Zhenglou Zhang (Equal), Zheng Tao (Equal), Jianming Tang (Equal). Visualization: Zhenglou Zhang (Equal), Zheng Tao (Equal), Jianming Tang (Equal), Chun Zhai (Equal). Methodology: Lianghui Wang (Lead). Funding acquisition: Lei Li (Lead). Resources: Lei Li (Lead).

COMPETING OF INTEREST – COPE

No competing interests were disclosed.

ETHICAL CONDUCT APPROVAL – IACUC

This study was conducted on *Mytilus edulis* and *Crassostrea gigas*, which are common aquatic invertebrate mollusks and

not classified as vertebrates or regulated invertebrates subject to mandatory ethical approval. Therefore, no institutional animal ethics approval was required for this research. All experimental procedures were strictly followed by the ARRIVE Essential 10 checklist developed by the NC3Rs to ensure standardized reporting of in vivo experiments. Every feasible measure was taken to minimize stress and potential harm to the experimental organisms, including rearing in a controlled aquatic environment consistent with their natural living conditions (stable temperature, salinity and dissolved oxygen), and performing sample collection with rapid and gentle operations to reduce physical damage.

This study fully complied with the provisions of the Convention on Biological Diversity (CBD) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). None of the experimental materials involved protected or endangered species, and the collection and use of mussels and oysters were in line with the relevant provisions of the Fishery Law of the People's Republic of China.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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