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Development of Biological Risk Assessment Protocols for Evaluating the Risks of In-Water Cleaning of Hull-Fouling Organisms

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Abstract: Herein, we evaluate the scientific basis for managing hull fouling of ships entering Korean ports, diagnose biological risks that may occur when in-water cleaning (IWC) systems remove hull fouling, and present a protocol for evaluating these risks (the Korean Infection Modes and Effects Analysis; K-IMEA). Protocol development included the selection of core elements and scenario design for IWC and the evaluation of regrowth experiments. The K-IMEA index was designed by considering the inoculation pathway of attaching organisms in all processes to ships that enter a port for in-water cleaning. A number of risk indices were defined: R1—Introduction/Establishment of alien species before in-water cleaning; R2—Establishment of alien species escaped during in-water cleaning; R3—Introduction/Establishment of alien species after in-water cleaning; and R4—Establishment of alien species in effluent water. K-IMEA regrowth experiments (R2 and R4) using the in-water cleaning effluent showed that the attachment and regrowth of prokaryotes, microalgae, and macroalgae were successfully detected. In particular, prokaryotes were observed in samples filtered through a 5 μm mesh of the in-water cleaning effluent, even at a low fouling rating (Levels 1–2). These experiments suggest a necessity to consider a secondary treatment method in addition to the primary filtration method for the treatment of in-water cleaning effluents.

Keywords: hull fouling; in-water cleaning; IWC; biological risk assessment; Korea infection modes and effects analysis; K-IMEA; in-water cleaning effluent; antifouling paint-coated plates; AFC plates

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1. Introduction

Ship hull fouling, the accumulation of marine organisms and debris on the underwater surfaces of ships, is a long-standing challenge faced by the maritime industry. Fouling can significantly impact ship performance, leading to increased fuel consumption, reduced maneuverability, and higher maintenance costs [1,2]. Moreover, fouling organisms can act as vectors for introducing and spreading invasive aquatic species (IAS), posing risks to marine ecosystems and coastal communities [3–7]. Although many studies on the translocation of organisms attached to ships' hulls have reported various damage cases, international conventions aimed at mitigating the spread of IAS through ships have been limited to ballast water management [4,6,8,9].

The International Maritime Organization (IMO) first discussed approaches to reducing the transfer of invasive species caused by hull fouling in 2006, and in 2011 adopted the *Guidelines for the Control and Management of Ships' Biofouling to Minimize the Transfer of Invasive Aquatic Species* (hereafter referred to as the 2011 Biofouling Guidelines; IMO 2011 [10]) to provide a globally consistent approach to managing ship biofouling and to facilitate international efforts to reduce the risk of introducing IAS from ocean-going ships. In 2023, IMO approved the 2023 Biofouling Guidelines, which revised the 2011 version, and decided to pursue completion of the guidelines for in-water cleaning (IWC), "the development of guidelines on matters relating to in-water cleaning", by 2025. In addition, apart from the IMO's Biofouling Guidelines, New Zealand and the US state of California have implemented mandatory biofouling regulations to protect their environment. In California, a final set of regulations titled "Biofouling Management to Minimize the Transfer of Non-indigenous Species from Vessels Arriving at California Ports" was announced and adopted on 1 October 2017 [11]. This regulation pertains to ships with a gross tonnage of 300 or more arriving at Californian ports. In New Zealand, the "Craft Risk Management Standard for Biofouling on Vessels Arriving to New Zealand" (CRMS-BIOFOUL) was approved on 15 May 2014 and mandatorily implemented on 15 May 2018, applying to all classes of ships, including recreational and human-powered vessels [12]. The CRMS-BIOFOUL regulation requires ship operators to take preventive measures to manage biofouling and maintain a "clean hull" prior to arrival in New Zealand. New Zealand's Ministry for Primary Industries (MPI) also published a report titled *In-water cleaning of ships: Biosecurity and Chemical Contamination Risks* in 2013, based on a multi-year survey of hull-fouling organisms [13]. This report introduced the Infection Modes and Effect Analysis (IMEA) evaluation technique to assess the relative biological risks associated with different in-water cleaning scenarios. However, with the exception of one study conducted in New Zealand, there exist very few studies on biological risk assessment for in-water cleaning of hull-fouling organisms. Furthermore, the New Zealand assessment methodology was developed in consideration of in-water cleaning by divers [13], which limits its direct application to approaches using the type of in-water cleaning robots with capture and post-treatment capabilities that are currently being developed and tested.

The Republic of Korea is surrounded by the sea on three sides and has a temperate climate with four seasons. It is a reasonable expectation that this environmental configuration could be readily vulnerable to marine ecosystem disturbances caused by the introduction of IAS. Furthermore, the Ministry of Oceans and Fisheries (MOF) reported that the number of ships that arrived at Korean ports reached 160,000 in 2018, many of which are expected to have carried out in-water cleaning as part of maintenance or repair. Unfortunately, little research has been carried out regarding the management procedures of ship biofouling in the Republic of Korea.

Based on the resolution of the 2011 Biofouling Guidelines, we, therefore, conducted a two-year study to develop a procedure for hull fouling management that allows decision makers to make case-by-case assessments of in-water cleaning applications. The procedure included the entire process of hull fouling management for all ships entering the ports in Korea. The purpose of this paper is to evaluate the risk of hull-fouling organisms on ships in major ports in the Republic of Korea on a scientific basis, define the risk of biofouling caused by activities before and after entry into the port, and present an evaluation protocol that takes into account the risk of harm to the native ecosystem and environment. The results of this study were proposed as IMO PPR10/INF. 18 on 17 February 2023, titled "Research outcomes on core elements to establish a biological risk assessment protocol for in-water cleaning".

2. Materials and Methods

2.1. Development of Biological Risk Assessment Protocols for In-Water Cleaning of Hull-Fouling Organisms

The developed protocol for the assessment of the biological risks of hull-fouling organisms was named the "Korean Infection Modes and Effects Analysis" (K-IMEA). To develop this protocol, we selected six core elements that should be considered as the inoculation pathway of hull-fouling organisms in all processes from ship entry to in-water cleaning and departure: hull-cleaning area, visit duration before/after in-water cleaning, fouling rating, debris capture performance of in-water cleaning robot, and use of post-treatment system. A total of 160 in-water cleaning scenarios about hull-fouling organisms were designed based on the selected core elements, and biological risk assessment indices (R1–R4) were developed to calculate the risk of each in-water cleaning scenario. These indices covered the following areas: R1—Introduction/Establishment of alien species before in-water cleaning; R2—Establishment of alien species that have escaped during in-water cleaning; R3—Introduction/Establishment of alien species after in-water cleaning; and R4—Establishment of alien species in the effluent water. Details related to the development of the risk assessment procedure are provided in Section 3.1.

2.2. Experimental K-IMEA Evaluation Data

We carried out experiments to generate practical data for the biological risk assessment of hull-fouling organisms before and after the in-water cleaning of incoming ships. The R2 and R4 regrowth experiments were also conducted on three container ships of different tonnage (ship 1: 232,311 GT, ship 2: 228,283 GT, and ship 3: 142,620 GT) with varying fouling conditions that worked the Republic of Korea and Europe route. Divers used representative quadrats (0.5 m × 0.5 m) placed 10 times along the water line to collect waste samples from in-water cleaning. Fouling organisms present in the quadrats were cleaned off until all visibly attached organisms were removed, using a specially designed brush (nylon bristles, 25 mm length by 1 mm diameter, 30 bristles/cm^2), which was connected to a 35 mm diameter tube. All effluents, including debris generated during the entire in-water cleaning process, were transferred to the shore via a diaphragm pump. Of the homogenized in-water cleaning effluents injected into the 100 L container, 6 L were sampled in sterilized high-density polyethylene (HDPE) bottles and transported to the laboratory. The following sections provide further details about the methodology of the regrowth experiments associated with the individual indices (R2 and R4).

2.2.1. Establishment of Alien Species Escaped during In-Water Cleaning (R2)

To confirm the possible establishment of fouling organisms that escaped during in-water cleaning, hull cleaning by divers was performed on three container ships mainly operating on Europe–Asia routes. The waste generated during the in-water cleaning was pumped to a 100 L container on the shore through a diaphragm pump. The homogenized effluent was then transferred into a 6 L sterile bag and moved to the laboratory. Assuming a debris capture efficiency >90%, the wastewater was diluted with filtered seawater to set final concentrations of 1%, 2%, 5%, and 10%, and acrylic attachment plates available for colonization were installed in each tank. Sampling was performed on days 1, 3, 5, 7, 14, and 21 in each experimental treatment.

2.2.2. Establishment of Alien Species in the Effluent Water (R4)

To confirm the possible settlement of fouling organisms in the effluent after in-water cleaning, hull cleaning by divers was performed on three container ships mainly operating on Europe–Asia routes. The waste generated during the in-water cleaning was pumped into a 200 L container through a diaphragm pump. The homogenized effluent in the container was filtered through a net of either 5 μm or 32 μm mesh, and then transferred into a 6 L sterile bag. After transporting the samples to the laboratory, the effluent was diluted with filtered sterilized seawater to set final concentrations of 1%, 2%, 5%, and 10%, and acrylic attachment plates available for colonization were installed in each tank. Sampling was performed on days 1, 3, 5, 7, 14, and 21 in each experimental treatment.

During the K-IMEA R2 and R4 experiments, organisms adhering to the acrylic plates were separated by stripping both sides with cell scrapers or 0.2 μm Supor filters (for prokaryotes). After homogenizing the separated samples, aliquots were taken for quantitative and qualitative analysis of adhering organisms. Analyses of the regrowth experiments were conducted for four groups of organisms that adhere to ship hulls and may be transported (prokaryotes, microalgae, macroalgae, and macroinvertebrates). Three subsample analyses were carried out for each group, but if the number of organisms attached to the reattachment plate was low, the entire sample was evaluated. To estimate abundance and species composition, prokaryotes were determined based on operational taxonomic units (OTUs) identified via 16S rDNA sequencing, and other organisms (microalgae, macroalgae, and macroinvertebrates) were identified via light microscopy.

3. Results and Discussion

The development of the biological risk assessment protocols was divided into three principal stages: selection of core elements, scenario design for in-water cleaning of hull-fouling organisms, and design of biological risk evaluation indices. We also attempted to establish an observationally validated basis for the indices, such as the possibility of the introduction/establishment of organisms released from the ship hull surface before and after in-water cleaning activity and the potential for the establishment of organisms escaped during cleaning. The main research items and their contents are summarized in Table 1.

Table 1. The research items and associated research contents for the development of biological risk assessment protocols.

Research Items	Associated Research Contents
Development of biological risk assessment protocols	
Selection of core elements	Review the latest information on the international regulation of hull fouling. Review previous research to identify risk parameters for leaks of attached organisms during all processes from ship entry to in-water cleaning and departure.
Scenario design for in-water cleaning of hull-fouling organisms	Develop a matrix of scenarios covering the core elements when removing hull-fouling organisms in the water.
Design of biological risk evaluation index	Design biological risk evaluation index for all processes, from ship entry to in-water cleaning and departure. Consult with marine biologists. Conduct experiments for risk assessment for each biological taxon.
Generating experimental data for biological risk assessment	
Ship hull survey	Investigate hull-fouling organisms and their regrowth capacities with regard to ships berthed in ports.
Establishment of alien species escaped during in-water cleaning (K-IMEA R2 index)	Review previous research data related to determining the efficiency of in-water cleaning systems. Determine the capture efficiency of in-water cleaning systems relative to the number of hull-fouling organisms. Conduct regrowth experiments with hull-fouling taxa.
Establishment of alien species in the effluent water (K-IMEA R4 index)	Check the IMO guidelines regarding the particle size that the in-water cleaning filtration system can remove. Conduct experiments to determine the risks arising from organisms passing through the in-water cleaning filtration system.

3.1. Development of Biological Risk Assessment Protocols for In-Water Cleaning of Hull-Fouling Organisms

3.1.1. Selection of Core Elements

The six core elements selected for the development of the biological risk assessment system for in-water cleaning of hull-fouling organisms are as follows:

Hull cleaning area: Depending on the duration of the ship's stay in the port, in-water cleaning can be performed with an effectiveness of 100%, but there may also be cases where only a portion of the cleaning is carried out. In such cases, the chemical components of the antifouling coating that may be leached or the physical damage that may occur during the cleaning process can lead to the release of macroalgae spores or the regrowth of severed fragments [14]. Moreover, Davidson et al. [15] reported that 40% of hull-fouling organisms remained on a very heavily fouled vessel when it was cleaned with hand-held brushes. The hull cleaning area was, therefore, selected considering the fact that in-water cleaning enhances the release of propagules from the hull and the likelihood of infection from residual material after cleaning.

Ship stay duration in ports and on coasts before and after in-water cleaning: This element was selected because previous research indicates that there is a high chance that organisms attached to the hull can settle in the port if a ship stays in port for more than 21 days [14]. In New Zealand, international ships wishing to remain for >21 days must have no more than a slime layer, with allowance for goose barnacles, on any part of the hull. It is, therefore, expected that for ships staying in excess of this threshold, in-water cleaning carried out outside the port prior to entering will be more effective in protecting marine ecosystems.

Fouling rating: This element was selected because it was predicted that the number of species and biomass of alien organisms would vary depending on the fouling rating (Levels 1–4) of the ship's hull. The rating scale ranges from 1 (biofilm only) to 4 (extensive fouling) [14].

Debris capture: This element was selected as correlating to the potential of organism settlement in the port environment from uncollected debris during the in-water cleaning process. Hopkins et al. [16] reported that an experimentally tested hand-operated brush system captured an average of ~95% of material removed from the hull; however, the capture percentage was less when the fouling level was high. Therefore, the possibility of varying capture efficiency of in-water cleaning systems depending on the fouling rating was also considered.

Post-treatment: This element was selected because it substantially determines the size and amount of organisms finally discharged into the port water [7].

3.1.2. Scenario Design Using the Six Core Elements

To evaluate the biological risks depending on the biomass of organisms attached to the hull, the release of propagules, and capture and post-treatment efficiency, a total of 160 in-water cleaning scenarios were designed based on the selected core elements. A number of example scenarios are shown in Table 2; for full details, see Table S1.

3.1.3. Design of Biological Risk Evaluation Indices

The indices for biological risk evaluation of hull fouling were designed considering the inoculation pathway of attached organisms in all processes from ship entry to in-water cleaning and departure (Table 3).

Table 2. Scenarios of in-water cleaning of hull-fouling organisms. Scenarios 1 to 46 out of 160 are shown.

Code	Hull Cleaning Area	Stay Duration before Cleaning	Stay Duration after Cleaning	Biofouling Rating (1)	Debris Capture (%)	Post Treatment?
1	50%	0–10 days	0–10 days	Level 1	Not applicable	Not applicable
2					>99%	Yes
3					90–99%	Yes
4					>99%	No
5					90–99%	No
6				Level 2	Not applicable	Not applicable
7					>99%	Yes
8					90–99%	Yes
9					>99%	No
10					90–99%	No
11				Level 3	Not applicable	Not applicable
12					>99%	Yes
13					90–99%	Yes
14					>99%	No
15					90–99%	No
16				Level 4	Not applicable	Not applicable
17					>99%	Yes
18					90–99%	Yes
19					>99%	No
20					90–99%	No
21			11–21 days	Level 1	Not applicable	Not applicable
22					>99%	Yes
23					90–99%	Yes
24					>99%	No
25					90–99%	No
26				Level 2	Not applicable	Not applicable
27					>99%	Yes
28					90–99%	Yes
29					>99%	No
30					90–99%	No
31				Level 3	Not applicable	Not applicable
32					>99%	Yes
33					90–99%	Yes
34					>99%	No
35					90–99%	No
36				Level 4	Not applicable	Not applicable
37					>99%	Yes
38					90–99%	Yes
39					>99%	No
40					90–99%	No
41		11–21 days	0–10 days	Level 1	Not applicable	Not applicable
42					>99%	Yes
43					90–99%	Yes
44					>99%	No
45					90–99%	No
46				Level 2	Not applicable	Not applicable

(1) Refer to "PPR9/7 Table 2. Rating scale to assess the extent of fouling on inspection target area".

Table 3. Indices of the Korean Infection Modes and Effects Analysis, K-IMEA [1].

Risk Score	R1 Introduction/Establishmer of Alien Species before In-Water Cleaning	R2 Establishment of Alien Species Escaped during In-Water Cleaning	R3 Introduction/Establishmer of Alien Species after In-Water Cleaning	R4 Establishment of Alien Species in the Effluent Water
1 (Lowest risk)	Highly unlikely	Highly unlikely	Highly unlikely	Highly unlikely
2	Unlikely	Unlikely	Unlikely	Unlikely
3	Slight chance	Slight chance	Slight chance	Slight chance
4	Small chance	Small chance	Small chance	Small chance
5	Occasional	Occasional	Occasional	Occasional
6	Moderate chance	Moderate chance	Moderate chance	Moderate chance
7	Frequent	Frequent	Frequent	Frequent
8	Highly likely	Highly likely	Highly likely	Highly likely
9	Very likely	Very likely	Very likely	Very likely
10 (Highest risk)	Certain	Certain	Certain	Certain

[1] The evaluation indices of K-IMEA are being developed based on the report by Morrisey et al. [13], *In-water cleaning of ships: Biosecurity and chemical contamination risks*, Ministry for Primary Industries.

R1—Introduction/Establishment of alien species before in-water cleaning: Fouling rating and stay duration before in-water cleaning were important parameters in the relevant in-water cleaning scenarios. A higher fouling rating indicates a greater potential for the introduction of alien species into the harbor before cleaning, and prolonged harbor stay durations prior to cleaning increase the likelihood of alien species release.

R2—Establishment of alien species that have escaped during in-water cleaning: Fouling rating and debris capture (%) were important parameters in the relevant scenarios. Debris capture efficiency may vary depending on the cleaning equipment used, with capture system performance generally being superior when cleaning microfouled rather than macrofouled hull surfaces.

R3—Introduction/Establishment of alien species after in-water cleaning: Fouling rating, stay duration after in-water cleaning, and hull cleaning area were important parameters in the relevant scenarios. Hopkins et al. [16] reported that the commonly applied brush-type cleaning method can have up to 100% effectiveness when dealing with a fouling rating of 1–2 (predominantly soft-bodied fouling organisms). However, at a rating of 3–4, indicating the presence of hard-bodied organisms, the removal efficiency may decrease significantly to around 60%. Forrest and Blakemore [17] further suggested that using this method runs a risk of failing to remove microscopic life stages of organisms (e.g., gametophytes of *Undaria*), creating a potential for the influx of alien species from the ship's surface after in-water cleaning.

R4—Establishment of alien species in the effluent water: Fouling rating and efficiency of the post-treatment system were important parameters in the relevant scenarios. It is suggested that the potential for the survival and establishment of organisms in discharged water after passing through post-treatment systems may be subject to regulation, similar to the D-2 regulations for ships' ballast water management.

The newly designed R1–R4 indices of K-IMEA are based on the in-water cleaning scenarios in Table 2. These were used to calculate the risk priority number (RPN) for each scenario by multiplying each component, with a high RPN score indicating a high biological risk (Table 4). Scores calculated for the 30 example scenarios presented in this document are shown in Table 5. Among all 160 in-water cleaning scenarios, the highest RPN value of 8000 was assigned to scenario 76 (50% hull cleaning, a port stay duration time of 11–21 days, fouling Level 4, and no debris capture and post-treatment process), while scenario 82 had the lowest value of 1 (100% hull cleaning, port stay duration of 0–10 days, fouling Level 1, and both debris capture (>99%) and post-treatment processing). After plotting the RPN values distribution in decreasing order, scenarios in the section where RPN decreased exponentially were identified as "high-risk", those in the section where RPN gradually decreased were identified as "medium-risk", and those in the section where RPN stabilized were identified as "low-risk" (Figure 1). High-risk scenarios (RPN 8000–1000) include 44 (27.5%) of 160 in-water cleaning scenarios; most feature high fouling levels and the absence of debris capture or post-treatment. The medium risk scenarios (RPN 1000–100) include 53 scenarios (33.1%); except for cases where the fouling level is very low (Level 1), most include debris capture, post-treatment processes, or both. Scenarios with the highest fouling rating (Level 4) were also evaluated as medium-risk even if debris capture and post-treatment were performed. Lastly, the low-risk scenarios (RPN "1–100) include 63 scenarios (39.4%), most of which have a low fouling level (Levels 1–2) and involve debris capture or post-treatment. Here, "high-risk scenario" refers to a scenario in which in-water cleaning in the port is impossible due to high biological risk, and medium-risk and low-risk refer to scenarios where this is possible. The appropriateness of this assessment protocol was confirmed through consultation with marine biologists specializing in prokaryotes, microalgae, macroalgae, and macroinvertebrates.

Table 4. Examples of K-IMEA calculation.

K-IMEA	Examples: Calculation of Overall Risk Ranking (Risk Priority Number, RPN) for Each In-Water Cleaning Scenario
R1—Introduction/Establishment of alien species before in-water cleaning	0–10 days [a] Lv.1 [b]: 1, +Lv.2: 3, +Lv.3: 4, +Lv.4: 7 11–21 days Lv.1: 3, +Lv.2: 4, +Lv.3: 6, +Lv.4: 8
R2—Establishment of alien species escaped during in-water cleaning	Lv.1 + NA [c]: 4, +>99% [d]: 1, +90–99%: 2 Lv.2 + NA: 8, +>99%: 2, +90–99%: 5 Lv.3 + NA: 9, +>99%: 4, +90–99%: 6 Lv.4 + NA: 10, +>99%: 5, +90–99%: 7
R3—Introduction/Establishment of alien species after in-water cleaning	Hull cleaning area: 50% [g] 0–10 days [e] Lv.1 [f]: 2, +Lv.2: 5, +Lv.3: 6, +Lv.4: 8 11–21 days Lv.1: 4, +Lv.2: 6, +Lv.3: 8, +Lv.4: 10
	Hull cleaning area: 100% [g] 0–10 days [e] Lv.1 [f]: 1, +Lv.2: 1, +Lv.3: 4, +Lv.4: 6 11–21 days Lv.1: 2, +Lv.2: 2, +Lv.3: 6, +Lv.4: 7
R4—Establishment of alien species in the effluent water	Post-treatment (filtration system) Yes [h] + Lv.1–3: 1, Lv.4: 2 Post-treatment No and NA +Lv.1: 3, +Lv.2: 6, +Lv.3: 7, Lv.4: 10

[a] 0–10 days and 11–21 days: stay duration before in-water cleaning; [b] Lv. 0–1, 2, 3, 4, 5: hull-fouling rating before in-water cleaning; [c] NA: not applicable; [d] > 99%: capture efficiency > 99%; [e] 0–10 days: stay duration after in-water cleaning; [f] Lv. 0–1: hull-fouling rating after in-water cleaning; [g] 50% and 100%: refers to the percentage of the hull that is cleaned; and [h] Post-treatment Yes: use of post-treatment system.

3.1.4. Summary of Outcomes of Biological Risk Assessment Protocol Development

If a scenario featured a large number of organisms attached to the hull (Levels 3–4), it was classified as high- or medium-risk depending on whether debris capture and post-treatment systems were applied. This result shows the necessity of a debris capture and post-treatment process to reduce the biological risks from in-water cleaning of hull-fouling organisms in port. However, the importance of these processes became relatively low when the number of organisms attached to the hull was small (Levels 1–2). It is likely that the biological assessment protocol can be further improved once the results of the in-water cleaning scenarios and K-IMEA evaluations of incoming and outgoing ships, results of models of port stay duration and particle diffusion, and experimental results from the R1–R4 experiments are incorporated.

Table 5. Examples of risk priority number (RPN) score calculation for 30 scenarios of in-water cleaning of hull fouling (red: high-risk scenario, blue: medium-risk scenario, and green: low-risk scenario).

Code	Hull Cleaning Area	Stay Duration before Cleaning	Stay Duration after Cleaning	Fouling Rating	Debris Capture (%)	Post Treatment?	R1	R2	R3	R4	RPN
76	50%	11–21 days	11–21 days	Level 4	Not applicable	Not applicable	8	10	10	10	8000
36	50%	0–10 days	11–21 days	Level 4	Not applicable	Not applicable	7	10	10	10	7000
56	50%	11–21 days	0–10 days	Level 4	Not applicable	Not applicable	8	10	8	10	6400
16	50%	0–10 days	0–10 days	Level 4	Not applicable	Not applicable	7	10	8	10	5600
80	50%	11–21 days	11–21 days	Level 4	90–99%	No	8	7	10	10	5600
156	100%	11–21 days	11–21 days	Level 4	Not applicable	Not applicable	8	10	7	10	5600
40	50%	0–10 days	11–21 days	Level 4	90–99%	No	7	7	10	10	4900
116	100%	0–10 days	11–21 days	Level 4	Not applicable	Not applicable	7	10	7	10	4900
136	100%	11–21 days	0–10 days	Level 4	Not applicable	Not applicable	8	10	6	10	4800
60	50%	11–21 days	0–10 days	Level 4	90–99%	No	8	7	8	10	4480
38	50%	0–10 days	11–21 days	Level 4	90–99%	Yes	7	7	10	2	980
46	50%	11–21 days	0–10 days	Level 2	Not applicable	Not applicable	4	8	5	6	960
34	50%	0–10 days	11–21 days	Level 3	>99%	No	4	4	8	7	896
58	50%	11–21 days	0–10 days	Level 4	90–99%	Yes	8	7	8	2	896
26	50%	0–10 days	11–21 days	Level 2	Not applicable	Not applicable	3	8	6	6	864
77	50%	11–21 days	11–21 days	Level 4	>99%	Yes	8	5	10	2	800
18	50%	0–10 days	0–10 days	Level 4	90–99%	Yes	7	7	8	2	784
158	100%	11–21 days	11–21 days	Level 4	90–99%	Yes	8	7	7	2	784
6	50%	0–10 days	0–10 days	Level 2	Not applicable	Not applicable	3	8	5	6	720
70	50%	11–21 days	11–21 days	Level 2	90–99%	No	4	5	6	6	720
12	50%	0–10 days	0–10 days	Level 3	>99%	Yes	4	4	6	1	96
93	100%	0–10 days	0–10 day	Level 3	90–99%	Yes	4	6	4	1	96
112	100%	0–10 days	11–21 days	Level 3	>99%	Yes	4	4	6	1	96
132	100%	11–21 days	0–10 days	Level 3	>99%	Yes	6	4	4	1	96
149	100%	11–21 days	11–21 days	Level 2	>99%	No	4	2	2	6	96
28	50%	0–10 days	11–21 days	Level 2	90–99%	Yes	3	5	6	1	90
90	100%	0–10 days	0–10 days	Level 2	90–99%	No	3	5	1	6	90
8	50%	0–10 days	0–10 days	Level 2	90–99%	Yes	3	5	5	1	75
41	50%	11–21 days	0–10 days	Level 1	Not applicable	Not applicable	3	4	2	3	72
65	50%	11–21 days	11–21 days	Level 1	90–99%	No	3	2	4	3	72

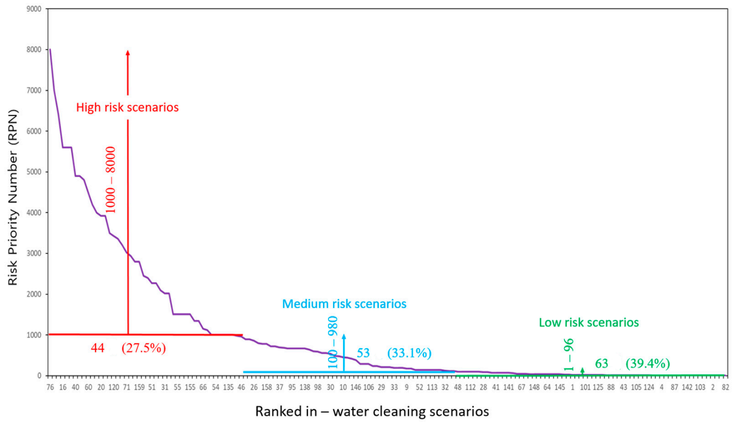

Figure 1. Distribution of risk priority number (RPN) values among in-water cleaning scenarios, shown in decreasing order.

3.2. Experimental K-IMEA Evaluation Data

Regrowth experiments for determining the K-IMEA R2 and R4 were designed and conducted to facilitate the calculation of biological risks for in-water cleaning of hull-fouling organisms in ports using the K-IMEA evaluation index.

3.2.1. Establishment of Alien Species That Escaped during In-Water Cleaning (R2)

Prokaryotes: Samples from ships 1 and 3 (but not ship 2) are currently being analyzed. In the R2 experiment, there were 61 operational taxonomic units (OTUs) of hull-derived prokaryotes attached to the acrylic attachment plate. Among these, *Gammaproteobacteria* (contributing 22 OTUs) was the most diverse and had the highest occurrence rate. *Alphaproteobacteria* and *Flavobacteria* contributed 14 and 13 OTUs, respectively, of which only 6 were classified at the species level. Specifically, the order *Bdellovibrionales* of the class *Deltaproteobacteria*, known as predators of Gram-negative prokaryotes, was identified. Although the characteristics of variation in the number of prokaryote OTUs could not be clarified, the number of OTUs and frequency of occurrence showed a general tendency to increase over time (Figure 2). The number of OTUs on day 21 was 10 or greater under 1%, 2%, and 10% culture, suggesting the continued viability of prokaryotic clusters derived from in-water cleaning of hull fouling.

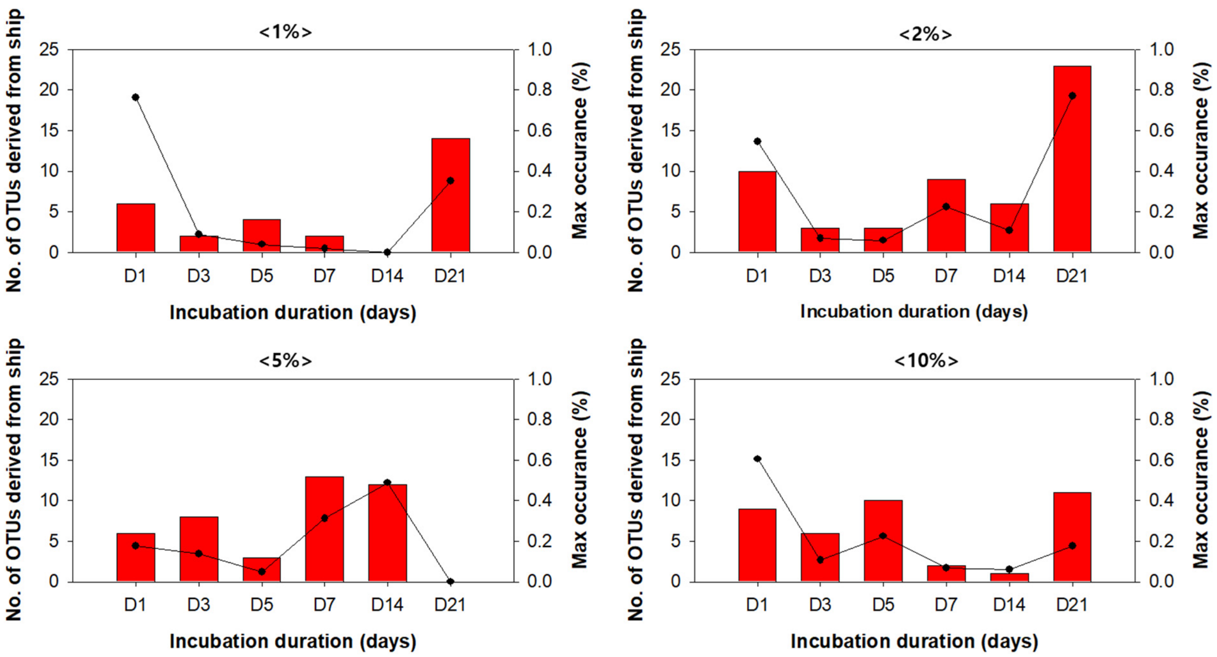

Figure 2. Number (left axis) and maximum observed proportion among total (right axis) of prokaryote OTUs in ship 2 attachment samples based on incubation period. Panel headings (1%, 2%, 5%, and 10%) indicate the percentage of non-captured material, respectively, corresponding to an in-water cleaning system capture efficiency of 99%, 98%, 95%, and 90%.

Microalgae: The viability of attached microalgae was investigated in the effluent samples of ships 1, 2, and 3. In the effluent samples of ship 1, survival of microalgae was not observed at any concentration or incubation time, whereas microalgae survival and reproduction were observed on days 3 and 21 of incubation in the effluent samples of ships 2 and 3, respectively. Regrowth in the sample of ship 2 was observed even in the experimental group, assuming a capture efficiency of 99%, but not in the 99% and 98% groups of ship 3. The dominant species in the samples of ships 2 and 3 was identified as *Halampora* sp., a fine pennate-type diatom less than 20 µm in length, using a scanning electron microscope (SEM) (Figure 3).

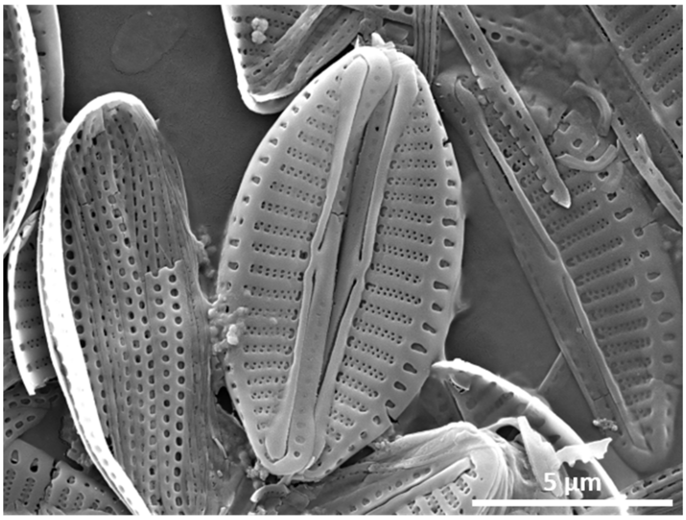

Figure 3. Photograph of dominant microalgae (*Halampora*) in the effluent samples of ships 2 and 3.

Macroalgae: The filamentous green alga *U. compressa* survived in the R2 regrowth experiments of all three ships, with no detection of other macroalgal species. In ships 1 and 3, the species' regrowth was observed at all dilution ratios despite the small amount of organisms attached to the hull (Levels 1–2). This indicates that even if the level of fouling on the ship hull is low, the small size of *U. compressa* may allow it to settle in the surrounding port environment. The thallus of filamentous macroalgae is difficult to eliminate when fragmented, even with a filter of smaller pore size (e.g., 32 μm). In future experiments, it will likely be necessary to evaluate the potential release of filamentous macroalgal species other than those in the *Ulva* genus and to study the quantity of released macroalgal species required for possible regrowth.

Macroinvertebrates: No regrowth of macroinvertebrates was observed in any experimental treatments of any ship until the end of the experiment due to the low level of macroinvertebrate biofouling in all cases. Lists of the macroinvertebrate species recorded on the hulls of ships 1, 2, and 3 can be found in Table S2 in the Supplementary Materials.

3.2.2. Establishment of Alien Species in the Effluent Water (R4)

Prokaryotes: Samples from ships 1 and 3 (but not ship 2) are currently being analyzed. During the R4 experiment, 62 OTUs of hull-derived prokaryotes were found on the attachment plates. Among these, 22 were *Alphaproteobacteria*, 15 were *Flavobacteria*, and 14 were *Gammaproteobacteria*. Only nine of these OTUs were classified at the species level, and most were not classified at all. In contrast to the R2 experiment, variability in hull-derived prokaryotes decreased as the incubation period increased. After 21 days of culture, the number of OTUs had significantly decreased to five or less, and the frequency of occurrence decreased to 0.5% or less. Lists of the prokaryote reattached and regrown in experiment R4 can be found in Table S3 in the Supplementary Materials.

Microalgae: No regrowth was observed in any experimental treatments of samples filtered through 5 μm and 32 μm nets. This indicates that filtration with a mesh size of less than 32 μm could reduce the possibility of settlement of alien microalgae in the port environment.

Macroalgae: In the samples filtered through a 32 μm net, regrowth of *U. compressa* was observed in the samples from ships 1 and 2, but not ship 3. No regrowth was observed in any sample filtered through a 5 μm net. In contrast to the R2 experiment, *U. compressa* could thus be removed through filtration using a 32 μm mesh, and the effectiveness of removal was substantially enhanced using filtration with a mesh size of 5 μm.

Macroinvertebrates: No regrowth of macroinvertebrates was observed in any sample from ships 1, 2, or 3 until the end of the experiment due to the low level of macroinvertebrate biofouling in all ships. Lists of the macroinvertebrate species recorded on the hulls of ships 1, 2, and 3 can be found in Table S2 in the Supplementary Materials.

3.2.3. Summary of K-IMEA Experiments

The fouling rating of all three sampled ships was between Levels 1 and 2. In the experiment to test the settlement potential of organisms not captured during the in-water cleaning process (R2), regrowth of prokaryotes was observed even under 99% debris capture conditions in all sampled ships, while microalgae and macroalgae regrew at 99% debris capture efficiency in some but not all ships. No regrowth of macroinvertebrates was observed. No organism groups except prokaryotes showed regrowth in the effluent of in-water cleaning (R4) when filtered through a 5 µm net. Microalgae and macroinvertebrates did not regrow even when filtered through a 32 µm net, and regrowth in the macroalgae group occurred only in the experimental groups with lower dilutions (5%, 10%). The research results of the R2 experiment indicate that debris capture is necessary, even for the in-water cleaning of low fouling levels. Furthermore, the findings of the R4 experiments demonstrate a significant reduction in the possibility of alien organisms settling in a port environment. Consequently, the implementation of a post-treatment system equipped with a debris capture and filtration system for washing water appears to be essential. Tamburri et al. [7] also highlighted the necessity of a post-treatment system such as reverse osmosis and chemical aggregation to mitigate biological risks associated with aquatic invasive species and chemical risks stemming from the leaching of active substances in antifouling paints [18–21]. However, further ships will have to be sampled to ensure the objectivity of the K-IMEA indices. The results of the K-IMEA experiments are summarized in Table 6.

Table 6. Summary of the results of the K-IMEA experiments.

Organisms	**Index: R2** **Establishment of alien species that escaped during in-water cleaning**
Prokaryotes	- In all dilution treatments, the number of OTUs and occurrence frequency of ship hull-derived prokaryotes tended to increase as the incubation period increased. - This suggests that the released prokaryotic community is viable in a port environment even at a debris capture efficiency of 99% during the in-water cleaning process.
Microalgae	- Microalgae regrowth in the samples of ship 2 was observed even under a debris capture efficiency of 99%, but not in those of ship 3 (no regrowth under 99% and 98%). This confirms that the debris capture efficiency of the in-water cleaning systems affects the introduction/establishment of algae attached to the hull.
Macroalgae	- Regrowth of *Ulva compressa* was observed in all experimental groups despite a low fouling rating in all ships, indicating the possibility of macroalgae regrowth even under low fouling conditions. The occurrence of regrowth under high debris capture efficiency (99%) suggests that macroalgae and especially *U. compressa* are likely to establish in a port environment.
Macroinvertebrates	No regrowth was observed.
Organisms	**Index: R4** **Establishment of alien species in the effluent water**
Prokaryotes	- Prokaryotes attached to debris generated by in-water cleaning were still observed in the effluent after filtration with a 5 µm net, suggesting that prokaryotes of smaller size may be more likely to be present in the effluent. - A longer incubation period correlated with lower viability, but prokaryotes continued to survive until the end of the experiment.
Microalgae	- No regrowth was observed during the incubation period under filtering conditions using either 5 µm or 32 µm nets. - Microalgae were more efficiently removed using filtration than were prokaryotes. - It was confirmed that post-treatment systems such as filtration significantly affect the potential of the port establishment of hull-attached microalgae.
Macroalgae	- No regrowth was observed in the 5 µm net-filtered samples, but some (5–10%) occurred in the 32 µm net-filtered samples. - This suggests the necessity of a post-treatment system with a mesh diameter smaller than 32 µm.
Macroinvertebrates	- No regrowth was observed.

4. Conclusions

Development continues on the K-IMEA biological risk assessment protocol, which is intended to evaluate the impact of hull-fouling organisms on the marine environment that may occur due to in-water cleaning of ships before entering a port. We experimentally confirmed that the proposed K-IMEA indices and testing methods are appropriate for evaluating biological risks under these conditions. We suggest that the protocol can provide a scientific basis for managing hull fouling of ships entering a port, and can be used to diagnose biological risks that may occur when hull fouling is cleaned using in-water cleaning robots. Regrowth experiments were conducted to evaluate the K-IMEA R2 and R4 indices, using samples from three container ships with low fouling ratings (Levels 1–2) working the Republic of Korea and Europe route. No regrowth of macroinvertebrates was observed, but regrowth of prokaryotes, microalgae, and macroalgae was confirmed. In particular, prokaryotes that passed through a 5 μm mesh continued to attach and regrow. It is noteworthy that organisms were observed in cleaning effluent samples filtered through a 5 μm mesh even at the sampled low fouling rating. The experimental results suggest that it is necessary to consider secondary treatment methods along with primary filtration for the treatment of in-water cleaning effluents.

Herein, we present only the experimental results for the K-IMEA R2 and R4 indices based on samples from three ships. Next-generation sequencing analysis results from the R1 and R3 experiments that are currently in progress, and additional experiments for all four indices, should allow the establishment of a more accurate and reliable biological risk assessment framework for in-water cleaning of hull-fouling organisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse12020234/s1, Table S1: Complete scenarios of in-water cleaning of hull-fouling organisms; Table S2: Lists of the macroinvertebrate species observed on the hulls of ships 1, 2, and 3; Table S3: Results of phylotype analyses of bacteria reattached and regrown in the experiment R4. Values represent maximum occurrence over the incubation period.

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