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Toward reliable viability assessment for resilient life stages of invertebrates in ballast water

Elena Gorokhova, Pia Haecky, Rehab El-Shehawy, Nick Blackburn, Sandra Luecke-Johansson and Elina Viinamäki

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Preface

In this report, results from the project "Toward reliable viability assessment for resilient life stages of invertebrates in ballast water" are presented.

The project is one of nine research projects carried out within the research initiative Management of invasive alien species.

With this initiative, the Swedish Environmental Protection Agency and the Swedish Agency for Marine and Water Management, in cooperation with Formas and the Swedish Transport Administration, wanted to invest in research on the eradication and management of invasive alien species. The aim was to generate new knowledge of managing invasive alien species and specifically to find new or improved methods for eradicating and controlling the incursion and dispersal of such species in nature. Specifically, the call targeted invasive non-native terrestrial plants and invasive non-native aquatic animals.

The project has been financed with funds from the Swedish Environmental Protection Agency's environmental research grant and Formas.

The report was written by Elena Gorokhova, Rehab El-Shehawy, Sandra Luecke-Johansson, and Elina Viinamäki from Stockholm university, as well as Pia Haecky and Nick Blackburn from MicroWISE.

The report has been reviewed for scientific quality by Matthias Obst (Gothenburg university) as well as for practical relevance by Fredrik Lindgren, (the Swedish Agency for Marine and Water Management).

The authors are responsible for the content of the report.

Stockholm, July 2025

Marie Uhrwing
Head of the Sustainability Department

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1. Summary

The discharge of ballast water serves as a critical vector for the global spread of aquatic invasive species, presenting ecological and economic challenges. The International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWMC) was established to mitigate this risk by regulating ballast water treatment. Regulation D-2 under the BWMC sets strict limits on the concentration of viable organisms in ballast water at discharge. While compliance with these standards is essential, it presents significant technical challenges, particularly for the viability assessment of non-motile life stages such as invertebrate eggs, benthic larvae, and dormant stages.

This high-risk, high-gain project aimed to develop a reliable and universally applicable method for viability analysis across diverse species and egg morphologies. However, despite extensive efforts, the project revealed that achieving such a universal method with the proposed approach is unfeasible. The complexity of the diverse integuments and low metabolic activity of non-motile life stages posed insurmountable challenges for the techniques explored.

While a universally suitable method was not achieved, the project provided valuable insights into the methodological barriers and highlighted critical areas for further exploration. Documenting these challenges is an essential contribution to the field, as it helps refine future approaches and directs efforts toward more feasible solutions.

This report captures the lessons learned, the limitations of current viability assessment methods, and the potential for targeted methodologies that focus on specific life stages or species. By doing so, it advances the understanding of ballast water management challenges and informs the development of compliance tools. These findings, while not delivering a universal solution, represent a significant step toward improving regulatory practices and mitigating the ecological risks of ballast water discharge.

2. Sammanfattning

Utsläpp av barlastvatten är en av de största orsakerna till spridningen av invasiva arter i akvatiska miljöer, vilket medför betydande ekologiska och ekonomiska utmaningar. För att minska denna risk har Internationella konventionen för kontroll och hantering av fartygs barlastvatten och sediment (BWMC) införts, med regler för behandling av barlastvatten. Regel D-2 inom BWMC fastställer strikta gränser för koncentrationen av levande organismer i barlastvatten vid utsläpp. Att uppfylla dessa krav är dock tekniskt krävande, särskilt när det gäller att bedöma livsdugligheten hos icke-rörliga livsstadier som ägg och larver från ryggradslösa djur.

Detta högriskprojekt hade som mål att utveckla en pålitlig och universellt tillämpbar metod för livsduglighetsanalys för en mängd olika arter och äggmorfologier. Trots omfattande arbete visade det sig dock att en sådan metod är nästintill omöjlig att utveckla med den föreslagna ansatsen. Den stora variationen bland planktonarter, deras skyddande skal och låga metaboliska aktivitet hos icke-rörliga livsstadier skapade hinder som inte kunde övervinnas med de metoder vi testade.

Även om vi inte lyckades utveckla en universell lösning har projektet gett viktiga insikter i de metodologiska utmaningarna och pekat på områden där ytterligare forskning behövs. Att dokumentera dessa erfarenheter är ett värdefullt bidrag till fältet, då det kan vägleda framtida arbete och hjälpa till att identifiera mer realistiska lösningar.

Denna rapport sammanfattar våra lärdomar, begränsningarna med dagens metoder och möjligheten att utveckla mer specifika strategier för särskilda arter eller livsstadier. Även om målet om en universell metod inte uppnåddes, representerar resultaten ett viktigt steg framåt för att förbättra regelverk och minska de ekologiska riskerna kopplade till utsläpp av barlastvatten.

3. Introduction

The introduction of aquatic invasive species through human activities, particularly via ballast water discharge, is one of the most pressing challenges facing global aquatic ecosystems (Bailey, 2015). These species, transported across the world's oceans and freshwater systems, disrupt native biodiversity, alter food webs, and impose significant economic costs by impacting fisheries, aquaculture, and infrastructure (Crespo et al., 2017). Recognized as a critical environmental threat, this issue has prompted international actions by the International Maritime Organization (IMO), who seeks to mitigate these risks by regulating the discharge of viable organisms in ballast water. However, achieving the current standards set by IMO remains a daunting task due to the complex biological characteristics of organisms present in the ballast water, particularly the non-motile life stages of invertebrates, which exhibit remarkable resilience to traditional viability assessment methods and treatment protocols (Bradie et al., 2018).

Invertebrates are among the primary organisms transported via ballast water, which carries biological materials discharged by ships. These materials often include bacteria, algae, and various animals, such as eggs, cysts, and larvae of invertebrates. When introduced into new environments, these organisms can cause significant ecological problems. Notably, six out of the ten most unwanted invasive species identified by the IMO are invertebrates. Most invertebrates share similar life cycles, typically involving planktonic stages such as eggs, larvae, or dormant forms, with 85% of benthic species having a pelagic larval stage. These planktonic stages are small enough to pass through ballast water intake pumps and are evolutionarily adapted to survive harsh conditions (**Figure 1**).

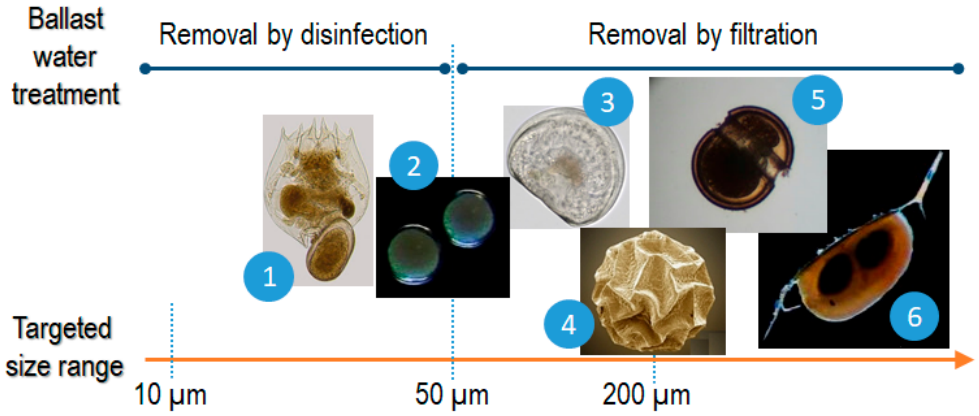

Figure 1. Examples of invertebrate non-adult stages commonly found in ballast tanks and present in both 10-50 µm and >50 µm fractions. 1: rotifer eggs (attached to the female), 2: copepod eggs, 3: bivalve veliger, 4: shrimp cyst, 5: fishhook water flea resting egg, and 6: water flea ephippia. In the typical treatment, a first step of filtration generally removes the larger particles. However, the removal efficiency is often compromised by high turbidity and many organisms that are >50 µm pass through the filters. Note complex integument structures that both increase resilience and hamper viability analysis in these life stages.

3.1 Ballast water and its environmental challenges

The discharge of ballast water is a critical operation for stabilizing ships during voyages. However, this process inadvertently transfers aquatic organisms across ecosystems, facilitating species introductions that can disrupt local biodiversity, economies, and public health. Recognizing these threats, the IMO adopted the Ballast Water Management Convention (BWMC) in 2004, which aims to mitigate the ecological risks of ballast water by imposing stringent standards for its treatment and discharge, particularly through Regulation D-2. This regulation establishes specific thresholds for viable organisms in discharged ballast water (Drake et al., 2014), restricting it to no more than 10 viable organisms per cubic meter for those ≥50 µm in size and 10 viable organisms per millilitre for those in the 10–50 µm range (**Figure 1**). However, implementing these standards in the port state control (PSC) is fraught with challenges, particularly concerning the viability assessment (First et al., 2022).

3.2 Port State Control

Port State Control (PSC) procedures for ballast water management involve verifying that ships comply with international regulations, specifically Regulation D-2 of the BWM Convention, which sets limits on the concentration of viable organisms in discharged ballast water. This verification relies on rapid sample analysis that must be completed within a few hours. To enable this, certified protocols and Compliance Monitoring Devices (CMDs), such as BallastWISE (Microwise, Denmark), Ballast-Check 2 (Turner Designs, USA), and B-QUA ATP (LuminUltra, Canada), etc., for detection and quantification of viable organisms. However, technical challenges remain for viability assessment under field conditions and across diverse organisms. Based on the evaluation of commonly used CMDs, BallastWISE is one of the most promising ballast water management systems for PSC (Romero-Martínez et al. 2024). Unlike other methods relying on biomass proxies, it provides both image and fluorescence-based output, which is particularly suitable for assessing staining of specific objects, such as eggs.

3.3 BallastWISE instrument and detection principles

The BallastWISE system represents a significant advancement in compliance testing for the D-2 regulation integrating Motility and Fluorescence Assay (MFA) to estimate live organisms in the 10–50 µm and >50 µm size fractions through image analysis. The system detects motility to identify active organisms and employs fluorescence analysis for autotrophic organisms or those tagged with specific fluorescent probes. Its adaptability could be extended to identifying viable eggs, benthic larvae, and dormant stages, making it a versatile tool for ballast water management in high-throughput testing methods required by PSC.

3.3.1 Instrument properties and analysis principle

The BallastWISE system operates using the MFA method (Blackburn et al., 2022; Holmstrup et al., 2020). Its optical system comprises a camera, lens, light-emitting diode (LED) lights, and an optical chamber (**Figure 2**). This setup enables detection and imaging of organisms by leveraging autofluorescence (for chlorophyll-containing plankton) and targeted fluorescence staining.

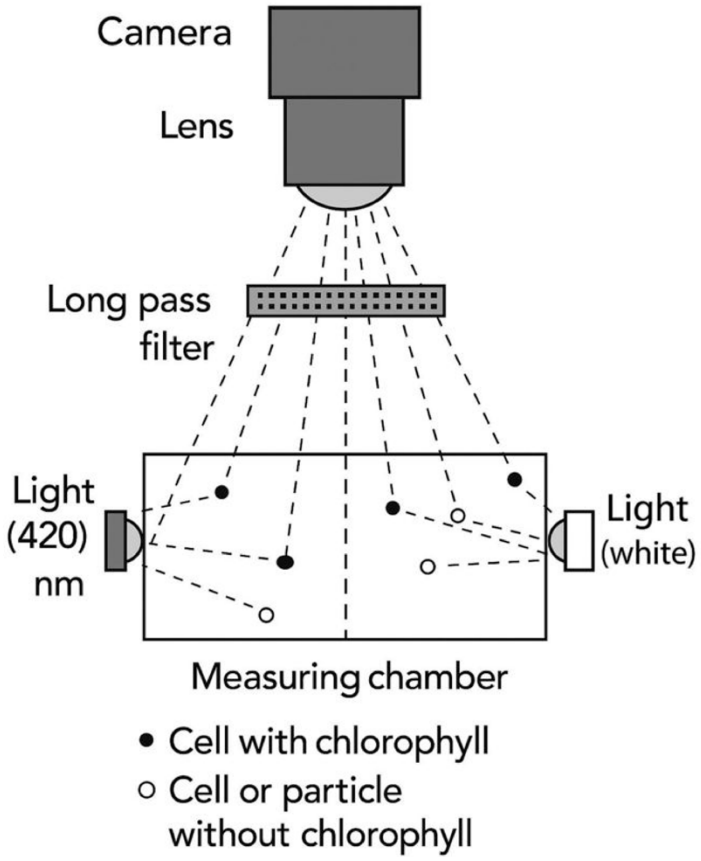

Figure 2. Image analysis and motion detection of individual organisms is used to identify: The number of moving organisms by video analysis. The number of organisms that show active fluorescence (excitation: 420 nm), high-pass filter (590 nm). The size of each organism is measured and processed by dedicated software.

Chlorophyll a within individual cells is detected by switching the light source to a 420 nm wavelength, which stimulates fluorescence. A 590 nm high-pass filter is positioned in front of the camera to ensure only fluorescence emissions are captured by the sensor, allowing fluorescence density at the pixel scale to be imaged. The concentration of viable plankton is then calculated through direct particle counts, based on a comparison of autofluorescence intensity and size (Blackburn et al., 2022).

3.4 Current methods for viability assessment

In the context of egg viability analysis, **viability** refers to the ability of an egg/cyst to successfully develop into a viable embryo, indicating that it possesses the physiological integrity and metabolic activity for normal development. Current assessment methods for the viability of ballast water organisms are broadly categorized into *detailed analyses* and *indicative analyses*, each with specific applications and limitations.

Detailed analyses, such as microscopy, involve directly observing organisms for signs of life, such as motility or cellular integrity. Some key techniques include:

- **Microscopic observation of motility:** Effective for motile stages but unsuitable for non-motile eggs and cysts.
- **Staining with fluorophores:** Viability dyes like fluorescein diacetate (FDA) and 5-chloro-methylfluorescein diacetate (CMFDA) assess membrane integrity but struggle with impermeable integuments.
- **Cell digestion assays:** Evaluate viability indirectly by analyzing degradation products but lack specificity for non-motile stages.

While precise, these methods are time-consuming and resource-intensive, requiring expertise and equipment, limiting their utility for routine compliance monitoring in port state inspections.

Indicative analyses provide rapid assessments of viability. These methods are designed for field applications but often sacrifice accuracy for speed. Common techniques include:

- **Fluorometry:** Measures photosynthetic activity or fluorescence intensity to infer viability, primarily effective for phytoplankton.
- **ATP-based assays:** Detect adenosine triphosphate as a proxy for cellular energy and biomass. These assays are quick but less reliable for dormant or non-metabolically active stages.
- **Flow cytometry:** Automates the detection of stained organisms but is expensive and prone to misclassifications, particularly for non-spherical or aggregated (e.g., an egg sack) organisms.

Despite their advantages, indicative methods frequently fail to address the unique challenges posed by non-motile life stages, leading to false negatives or underestimation of viable populations.

3.5 Non-motile life stages: characteristics and assessment complexities

Non-motile life stages, such as resting eggs, cysts, and dormant larvae, are crucial for the survival and dispersal of aquatic invertebrates. These stages are a widespread phenomenon across phylogenetic groups and geographic zones, being ubiquitous in both planktonic and benthic communities. Evolutionarily, especially in benthic organisms, these stages have emerged as a means to colonize new environments and adapt to unfavorable environmental conditions such as extreme temperatures, salinity fluctuations, and anoxia.

Resting stages are found in nearly all major groups of planktonic and benthic invertebrates, including but not limited to:

- **Rotifers**: Their mictic and amictic eggs are encased in complex protective envelopes.
- **Copepods**: Many species produce quiescent or diapause eggs with zooplankton intricate surface ornamentation, facilitating their survival in sediments.
- **Branchiopoda**: Resting eggs, often housed within ephippia or cysts, exhibit remarkable resistance to desiccation and temperature extremes.
- **Bivalves**: Larvae such as veligers have shells combining organic and inorganic layers that shield against environmental stressors.

Geographically, these stages are found in a range of environments—from polar regions, where they survive freezing conditions, to tropical waters, where they withstand seasonal desiccation. Resting stages also persist in sediments for extended periods, often spanning decades, creating seed banks that allow populations to reemerge when conditions become favorable. This universal occurrence and resilience complicate their assessment and ballast water control, as non-motile stages remain undetected as viable (Holmstrup et al., 2020).

The hardiness and low metabolic activity of resting stages pose further challenges for viability assessment rendering traditional viability indicators, such as enzymatic activity or ATP measurements, ineffective. Moreover, the impermeability of their protective integuments prevents staining agents from penetrating their shells. Consequently, these traits demand highly specialized methods in the context of ballast water compliance monitoring.

3.6 Research gaps and project objectives

The absence of methods for assessing the viability of non-motile life stages underscores a critical research gap in ballast water management. The high diversity of planktonic communities, including holo- (true planktonic forms) and meroplankton (benthic species present in the water column during larval development), necessitates approaches tailored to the specific characteristics of non-motile stages.

The **VIABLEGG project** was initiated to address these gaps by:

1. Exploring existing and developing novel staining protocols for penetrating protective integuments.
2. Adapting automated detection systems, such as BallastWISE, to incorporate non-motile stages.
3. Conducting laboratory and field validations.

This report provides an overview of the project's findings, documenting the challenges encountered and the insights gained. By focusing on the methodological barriers to compliance testing, the project contributes to advancing ballast water management practices.

4. Material and methods

We conducted a set of experimental studies to address the challenges of assessing the viability of non-motile life stages. The main focus was on the testing and validation of staining protocols tailored to diverse invertebrate taxa with varying integument morphologies. The best combinations of chemical, enzymatic, and fluorescence-based protocols established in the laboratory were employed in the field validation using treated and natural plankton samples (**Table 1**). The goal was to integrate these protocols into high-throughput automated workflows using BallastWISE as a test system to provide practical solutions for PSC. Therefore, the key components of this work included

- targeted species selection,
- development of staining protocols,
- controlled exposure experiments to generate eggs/embryos with different viability status (i.e., control vs killed), and
- field validation.

Each of these components is detailed below.

4.1 Species selection and collection

Representative invertebrate species were selected based on their relevance to ballast water transport and their integument morphologies (Guerrero-Jiménez et al., 2020), challenging staining efficiency (**Table 2**). These taxa encompass a range of integument types, from highly resistant diapausing eggs (Branchiopoda ephippia and cysts) to permeable and metabolically active forms (e.g., amphipod embryos), reflecting the spectrum of non-motile stages transported in ballast water.

Rotifer eggs are highly variable in their integument composition and morphology: amictic eggs that develop parthenogenetically, mictic eggs that become males if unfertilized or resting eggs if fertilized (Serra et al., 2019). These eggs may have thin or thick shells, with resting eggs featuring multi-layered structures for survival under extreme conditions. The eggs are encased in integuments where the outer layers are rich in sclerotized proteins and glycosaminoglycans, forming a barrier that resists enzymatic degradation and chemical penetration. Some species produce gelatinous housings around their eggs, further enhancing their resilience. The inner lipid-rich layers contribute to impermeability, making standard staining agents ineffective without pretreatment. These properties were expected to require pretreatment protocols, such as enzymatic or chemical softening, to enable dye penetration.

Copepod eggs have specialized biochemical compositions. Subitaneous eggs are covered by smooth, thin membranes composed predominantly of chitin and proteins, allowing rapid development but offering limited resistance to environmental stressors. In contrast, diapausing eggs possess thick, multi-layered integuments enriched with structural proteins, lipids, and polysaccharides, providing high durability against temperature fluctuations, salinity changes, and chemical

stressors (Couch et al., 2001). These structures make diapausing eggs resistant to staining. Additionally, some diapausing eggs feature spines or ornamentations that may further hinder stain absorption. These biochemical complexities underscore the need for tailored staining protocols.

Cladoceran eggs, including those of Onychopoda like *Cercopagis* and *Bythotrephes*, also exhibit integument adaptations that influence staining. Subitaneous eggs have thin, glycoprotein- and lipoprotein-rich envelopes that are easily penetrable by stains, whereas diapausing eggs are encased in a multi-layered, chitinous ephippium enriched with trehalose, which provides resilience against various stressors (Dodson and Frey, 2001), and, together with the hydrophobic and glycoprotein matrix, hinders dye penetration.

The integument of **veliger larvae** of bivalves, has complex biochemical features (Wanninger and Wollesen, 2019). The epithelium is a single layer of epidermal cells, ciliated cells, and mucocytes, producing a matrix of proteins and glycoproteins supporting mucus production for particle capture and locomotion, and together with the microvillus border and mucopolysaccharide coatings may limit permeability to stains. The larval shell, composed primarily of flexible proteins rather than calcium carbonate, provides a semi-permeable barrier, influencing stain absorption. These features suggest the need for protocols optimized for protein-rich, mucus-coated surfaces. Notably, swimming increases veliger exposure to the staining medium when the staining process occurs in a liquid environment where the veligers are free-moving with semi-open valves.

Amphipod eggs developing in the marsupium are protected by a specialized integument consisting of two main layers with distinct biochemical and structural features. The outer chorion, a thin, transparent, proteinaceous envelope, provides initial protection but is shed as the embryo matures. The inner vitelline membrane, a multi-layered structure composed of glycoproteins and lipids, persists throughout development, supporting gas and nutrient transfer from the marsupial fluid (Browne et al., 2005). These semi-permeable, transparent layers facilitate staining without the need for pretreatments.

Sampling of these organisms within the project involved different gears. Plankton nets with various mesh sizes (25 to 300 µm) were used for collecting rotifers, copepods, cladocerans, and veligers from water columns in marine (Himmerfjärden Bay, Brunnsviken, and Askö area) and freshwater environments (Lake Mälaren). Field sampling sites were chosen based on high species abundance, often informed by local monitoring data. Amphipod embryos were typically obtained by dissecting gravid females collected from benthic habitats using bottom sled.

Table 1. Summary of methods, target species, rationale, and tests conducted in the project. Target taxa were chosen based on egg morphology, ecological relevance, and presence in ballast water.

Category	Aspect	Details	Rationale/Tests conducted
Test species selection and collection	Target taxa	Rotatoria *(Brachionus spp.),* Calanoida *(Acartia tonsa),* Harpacticoida *(Nitocra spinipes*), Bivalvia *(Mytilus spp.)*, Amphipoda *(Pontoporeia femorata, Hyalella azteca*), Branchiopoda (*Daphnia magna, Artemia salina, Cercopagis pengo*i).	Egg size and morphology, ubiquitous presence in ballast water, representativeness as holo- and meroplankton, potential invasiveness of this or other phylogenetically related taxa based on the literature data
	Collection methods	Cultures, plankton nets, bottom sled for field collection from high-abundance areas	Field sampling in Lake Mälaren and the Baltic Sea (Northern Baltic Proper or Western Gotland Basin) for natural assemblages and specific target taxa (when available)
Dye selection and protocols for staining and integument permeability treatments	Review on viability staining in invertebrates	Fluorescent and non-fluorescent dyes employed for viability analysis of invertebrates with a particular focus on egg/embryo	Providing an overview of potentially suitable dyes on the market and existing protocols
	Softening integuments	Chemical treatments (e.g., acids), chitinolytic and proteolytic enzymes). Physical treatments for permeability optimization	Tested chemical and enzymatic treatments; validated via FTIR for hydration and permeability changes
	Vital/mortal dye selection	17 dyes selected by the literature review	Screened multiple dyes for specificity, sensitivity, and compatibility with high-throughput systems
	Compatibility tests	Fluorescence microscopy and image analysis for staining efficacy	Applied staining protocols across different species; optimized protocols for various integument types
Exposure protocols to obtain specimens with different viability status	Simulated disinfection	UV treatment and thermal exposure to induce mortality	Calibrated staining protocols using UV(+TiO2) and heat chock, with positive controls for mortality
	Osmotic shock	Literature-based modeling of mid-ocean ballast water exchanges	Conducted controlled osmotic shock experiments to assess viability changes
Field validation	Treated water sampling	General application of treated plankton samples for method evaluation	Ballast water treated with approved disinfection systems, including natural plankton samples
	Method comparison	Existing studies on BallastWISE and microscopy-based direct methods	Intercalibrated protocols with BallastWISE and microscopy; analyzed discrepancies in sensitivity and specificity

Table 2. Overview of integument morphologies, compositions, and staining challenges for selected taxa

Taxa	Integument layers and composition	Key features and challenges
Rotifers	Glycoproteins and sclerotized proteins, glycosaminoglycans (outer layer)	Cuticle and gelatinous housings around their eggs provide resistance to physical damage and enzymatic degradation
	Chitin-like polymers (middle layer)	Impermeable and nontransparent
Copepods	Chitin-based egg sacs attached to the female	Eggs remain attached to adults, simplifying identification. Permeability can vary depending on sac thickness
	Chitin and proteins with ornamentations (outer shell)	Robust structure with surface complexity (spines/ridges)
	Proteinaceous matrix (middle layer)	Quiescent states reduce metabolic activity
	Lipoproteins and polysaccharides (inner layer)	Challenges in consistent dye penetration
Branchiopods	Silica-like materials and chitin (outer envelope)	Extreme resistance to physical and chemical stressors
	Hydrophobic compounds and proteins (intermediate layers)	Designed for long-term survival in sediment
	Lipid-rich membranes (inner layer)	Requires combined mechanical and chemical treatments for permeability
Veligers (Bivalves)	Calcified outer shell (calcium carbonate matrix)	Outer shell provides strong protection against environmental stressors
	Organic matrix in the mantle layer	Combination of calcified and organic layers challenges staining protocols
	Single layer of epithelial cells	Often produce mucus, complicating dye penetration
Amphipod Embryos	Proteinaceous outer chorion	Highly metabolically active and responsive
	Lipid and polysaccharide-rich vitelline membrane	No challenges expected for viability assessment

4.2 Methods for identifying viable organisms

Viability analysis is based on techniques that do not rely on dye penetration as well as staining-based methods. Both types of methods were relevant for our work, and here, we give a brief description of the state-of-the-art in this field.

4.2.1 Non-staining methods

Non-staining methods for viability assessment rely on various biological, biochemical, or physical indicators to evaluate the viability. These methods can be broadly categorized into several types:

Direct observation of motility. One of the simplest methods for assessing viability (Holmstrup et al., 2020) is that the absence of motion can indicate non-viability. This method is particularly useful for larger, motile organisms such as zooplankton and some larval stages of invertebrates, where motility is easily observable. The advantages of this method include its simplicity and rapidity, requiring minimal equipment and providing immediate qualitative insights into the health of organisms. However, it is not applicable to non-motile life stages, such as eggs and dormant cysts.

Use in the VIABLEGG

The BallastWISE instrument employs motility as its primary criterion for assessing the viability of zooplankton in ballast water. The instrument captures real-time data to identify movement patterns.

Morphological assessments rely on microscopic examination of physical features to infer viability. Healthy eggs typically display intact, well-defined structures, and specific features such as membrane integrity and cellular organization are often associated with viability (Zirbel et al., 2007). Although this method is valuable for identifying abnormalities or deformities that can suggest compromised viability, its applicability is limited due to its subjective nature and inability to provide definitive, quantitative data on whether an organism is alive. Morphological integrity does not always correlate with functional viability, as non-viable eggs may retain outward structural appearance. Consequently, while morphological observations can supplement other methods, they are insufficient as standalone indicators of viability and should be paired with more direct or quantitative techniques for accurate assessment.

Use in the VIABLEGG

Morphological assessment was utilized as an independent method to analyze viability in amphipod embryos. This approach focused on identifying morphological aberrations during embryonic development, such as abnormal segmentation, deformities in appendages, or compromised membrane integrity, which are all indicative of lethal outcomes (Sundelin and Eriksson, 1998). The embryos were extracted from marsupium and classified as either normal (=viable) or malformed (non-viable) using a stereomicroscope. By screening for structural malformations in conjunction with staining methods, we validated the efficiency of staining protocols.

Hatching success. Hatching experiments involve monitoring the ability of eggs or cysts to develop and hatch into motile, juvenile stages under controlled conditions, providing a definitive indication of viability (Briski et al., 2013; Gorokhova, 2010). Direct correlation to staining rate (or color intensity) provides a measure of staining efficiency. However, it may take several days or weeks to observe hatching. Moreover, not all species/egg types can be hatched effectively due to specific environmental or dormancy requirements, and this method may underestimate the viability of diapausing eggs and resting stages that have obligatory dormancy.

Use in the VIABLEGG

Hatching experiments were employed as an independent method to assess viability in rotifers, brine shrimp, and copepods, as a means to evaluate staining efficiency. Eggs were incubated in batches under controlled conditions, including temperature, salinity, and light regime **(Table 3)**, and monitored daily for hatching using a stereo microscope. Staining on the extra batch was performed prior to incubation, and hatching success was compared to staining outcomes to calculate staining efficiency. Using this method, we ensured that % of stained organisms corresponded to % of those with successful hatching.

Metabolic activity measurement. Metabolic activity measurement is used for assessing health status through biochemical indicators, particularly by measuring metabolites such as adenosine triphosphate (ATP) levels. ATP is a key energy carrier within living cells, and its presence indicates that cells are functioning. Bioluminescent assays using commercially available kits with luciferase enzymes can quantify even low ATP levels, providing a rapid means of assessing viability (Van Slooten et al., 2015). However, not all eggs may have measurable ATP levels (Casas-Monroy et al., 2023). Other biochemical assessments may involve measuring specific enzymatic activities or the presence of metabolic byproducts to determine metabolic activity. However, these methods may not correlate directly with viability, as some enzymes can remain active in stressed or non-viable cells, complicating the interpretation of results (Kumar and Ghosh, 2019).

Use in the VIABLEGG

The bioluminescence assay for ATP measurements of the luciferase-catalyzed oxidation of luciferin, proportional to ATP concentration, was utilized alongside staining and hatching experiments **(Table 3)** to assess the metabolic activity of rotifer and copepod eggs. We also tested this method on daphnia ephippia, amphipod embryos, and the blue mussel veligers.

Samples were prepared by lysing eggs (rotifers and copepods: 10 eggs/sample, cladocerans: 1 ephippium or diapausing egg/sample, amphipod and veligers: 1 embryo or veliger/sample) to release intracellular ATP. Using the commercially available B-QUA kit (LuminUltra®, Canada), ATP concentrations (pg ATP mL–1) were measured following the protocol for marine phytoplankton (Amyot et al., 1992; Peperzak et al., 2024). The reaction included a bioluminescent reagent containing luciferase, luciferin, and cofactors like magnesium ions added to the lysate, to initiate the light-emitting reaction. The emitted light is measured using a luminometer, and its intensity is compared against a calibration curve created with ATP standards to determine ATP concentrations. RLU values (Relative Luminescence Units) were used for the between-group comparisons, i. e., when contrasting treated and untreated samples (Romero-Martínez et al., 2024; Van Slooten et al., 2015). By correlating ATP levels with the hatching rate or, alternatively, staining results obtained for the individuals from the same batch, we compared staining efficiency with this metabolic indicator.

Table 3. Incubation conditions and durations for hatching of rotifers *Brachionus plicatilis,* calanoids *Acartia tonsa,* and harpacticoids *Nitocra spinipes.*

Parameter	*Brachionus plicatilis*	*Acartia tonsa*	*Nitocra spinipes*
Temperature	29–30°C	15–17°C	20–22°C
Salinity	29-30 ppt	25 ppt	7 ppt
Light	continuous light, 900 lux	16:8 hours, 700 lux	continuous light, 200 lux
Oxygenation	Gentle aeration to prevent settling	Gentle aeration to maintain suspension	No aeration
pH	7.8	8.0–8.2	7.5–8.0
Medium	Artificial seawater	Filtered and sterilized seawater	Filtered and sterilized brackish water
Incubation duration	24-72 hours	48–192 hours	48–120 hours

4.2.2 Staining-based methods

These techniques, which are widely used in viability assessments, typically utilise dyes that penetrate cells differently depending on their viability status; they are referred to as *vital* and *mortal* stains. The existing approaches for cell viability evaluation are largely based on two properties: membrane integrity and metabolic activity. Although a variety of viability stains and methods are available, there is no systematic investigation of how these methods perform for larger (≥50 µm) micro- and mesozooplankton (Zetsche and Meysman, 2012). For copepod egg viability analysis, vital fluorescent probe Hoeschst 33342 to stain nuclei and to identify anomalies in embryogenesis were employed (Ianora et al., 1989; Poulet et al., 1995) as well as different fluorescent dyes, e.g., FDA, propidium iodide, SYTOX, 7-amino-actinomycin D (7-AAD), and TUNEL (terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling) assay (Buttino et al., 2004, 2003; Romano et al., 2003).

Use in the VIABLEGG

The staining procedure for all stains and test organisms was conducted following manufacturer instructions, with stain concentrations optimized through pilot studies to ensure effective penetration and reliable results. Test organisms, including eggs, embryos, and larval stages, were sourced primarily from laboratory cultures to maintain consistency and control over experimental conditions. When laboratory cultures were unavailable, specimens were collected from the field. The procedure involved optimizing exposure time and concentrations of staining solutions tailored to each stain and organism type, with adjustments made for species-specific integument characteristics.

VITAL STAINS

Vital stains are compounds that can enter live cells through selective mechanisms that maintain membrane integrity without compromising viability, highlighting active cellular components. Small, nonpolar dyes may passively diffuse across the lipid bilayer, while others rely on specific transport proteins or ion channels. Temporary permeabilization agents may be used to facilitate entry. Once inside, dyes bind to intracellular targets or undergo enzymatic modifications, enabling visualization of live cells. Vital stains used in the project (**Table 4**) included:

- **Fluorescein Diacetate (FDA)**: A non-fluorescent substrate converted to fluorescein by intracellular esterases. This method is effective for assessing the viability of various aquatic organisms, particularly in phytoplankton studies.
- **Carboxyfluorescein Diacetate (CFDA)**: Similar to FDA, this compound is a non-fluorescent substrate that can be converted into a fluorescent form by intracellular esterases. CFDA is particularly useful for evaluating the metabolic activity of cells. Its derivative, 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester (5-CFDA-AM), is a neutral compound that can easily pass through cell membranes and is retained in live cells better than FDA.
- **SYTO 9:** A cell-permeant nucleic acid stain that emits green fluorescence when bound to DNA in live cells. SYTO 9 can penetrate the membranes of all cells, regardless of membrane integrity, allowing it to stain both live and dead cells when used alone. It is often used in conjunction with mortal stains to enhance the overall assessment of cell viability.
- **Hoeschst 33342:** This vital fluorescent probe stains nuclei and has been utilized in studies to identify early anomalies in embryogenesis in copepods. Its ability to penetrate live cells makes it valuable for monitoring cell health.
- **DAPI** (4’,6-Diamidino-2-Phenylindole): A fluorescent stain that binds to adenine-thymine-rich regions of DNA, emitting blue fluorescence when bound. It is commonly used to stain nuclei in fixed or live cells, including eggs and embryos, i.e., not strictly vital.
- **PicoGreen:** A non-toxic fluorescent dye that binds to double-stranded DNA, emitting green fluorescence. It is used to quantify DNA in live cells and assess viability in embryos and eggs.
- **MitoTracker:** A cell-permeant dye that accumulates in active mitochondria, providing insights into cellular metabolic activity; useful for assessing metabolic activity in eggs and larvae.
- **Neutral red**: A non-fluorescent vital stain commonly used in cell biology to assess lysosomal activity. It is a weakly basic dye that permeates living cells and accumulates in acidic organelles, such as lysosomes, due to protonation. This accumulation results in a distinct red coloration, serving as a basis for the Neutral Red Uptake (NRU) assay, which measures the capacity of cells to retain the dye as an indicator of membrane integrity and metabolic activity (Repetto et al., 2008).

MORTAL STAINS

Mortal stains are designed to penetrate the membranes of dead or compromised cells that are no longer able to maintain selective permeability. In live cells, intact membranes act as a barrier, preventing the stain from penetrating the cytoplasm, but when cells are dead or dying due to physical damage, chemical exposure, or metabolic failure, their membranes become permeable for the dye molecules to diffuse passively into the cell. Once inside, the stains bind to specific intracellular targets, such as denatured proteins or nucleic acids, often accumulating in regions of cellular damage. Mortal stains used in this project (**Table 4**) included:

- **TO-PRO-1 Iodide** enters cells with compromised membranes, emitting bright green fluorescence when bound to nucleic acids. Gorokhova (2010) demonstrated that TO-PRO-1 could effectively distinguish nonviable eggs in copepods without chitinase pretreatment.
- **Propidium Iodide (PI)** enters cells with damaged membranes, where it binds to nucleic acids and emits red fluorescence. PI is often used in combination with vital stains like SYTO 9 to provide a clearer assessment of cell viability.
- **SYTOX Green** is a membrane-impermeant nucleic acid dye that fluoresces only when bound to DNA in dead or damaged cells; commonly used for algal viability assessments.
- **7-Aminoactinomycin D (7-AAD)** is membrane-impermeant, entering only dead or dying cells, where it emits red fluorescence when bound to nucleic acids; useful in conjunction with other stains for assessing apoptosis.
- **Trypan Blue** is a classic dye for dead cells that absorb it and turn blue. However, it has low efficiency for organisms with thick protective layers, such as certain invertebrate eggs.
- **Aniline Blue** is a commonly used stain that binds to non-viable cells by interacting with denatured proteins and damaged cell membranes. Its simplicity, applicability for light microscopy, and clear contrast in stained samples make it a valuable tool for viability assessments, particularly for organisms with permeable integuments.
- **TUNEL Assay** (Terminal-deoxynucleotidyl-transferase-mediated dUTP Nick End Labeling): This method fluorescently labels fragmented DNA in apoptotic cells and detects non-viable cells by indicating DNA breaks. It is used to assess apoptosis in various cell types, including algal cells. This method was not evaluated at the full scale due to the long and sophisticated protocols requiring specialized equipment and, thus, the low probability of it being applicable in field conditions.

Table 4. List of viability dyes/methods and dye types (vital/mortal) tested in this study.

Stain	Type	Properties	Use	Advantages	Limitations
FDA	Vital	Non-fluorescent substrate converted to fluorescein by esterases	Commonly used in algae viability assessment	Simple and effective for unicellular organisms	Requires intact cellular esterase activity
CFDA	Vital	Similar to FDA; converted into fluorescent CFD by esterases in viable cells	Evaluates metabolic activity in various aquatic organisms	Better retention in cells compared to FDA	Staining efficiency varies; susceptible to interference from auto-fluorescence
SYTO 9	Vital	Cell-permeant nucleic acid stain emitting green fluorescence when bound to live cell DNA	Used in conjunction with mortal stains, offering only a general assessment of nucleic acid presence	Has been earlier applied to copepod eggs	Cannot distinguish between live cells and those with damaged membranes; must be paired with a mortal stain
Hoeschst 33342	Vital	Stains nuclei; penetrates live cells and emits blue fluorescence	Used to identify early anomalies in embryo	Valuable for monitoring embryo viability.	Can be affected by auto-fluorescence
DAPI	Vital	Fluorescent stain that binds to adenine-thymine-rich regions of DNA, emitting blue fluorescence	Commonly used to stain nuclei in fixed or live cells, including eggs and embryos	High sensitivity and specificity for DNA	Requires UV excitation, which can be challenging for live samples
PicoGreen	Vital	Non-toxic fluorescent dye that binds to dsDNA, emitting green fluorescence	Used for quantifying DNA in live cells	Sensitive and suitable for quantifying low DNA levels	Requires careful calibration and controls
MitoTracker	Vital	Cell-permeant dye that accumulates in active mitochondria, indicates metabolic activity	Useful for assessing metabolic activity in eggs and larvae	Provides information about overall cell health	Selectively stains viable cells with active metabolism
Neutral red	Vital	Non-fluorescent dye that permeates living cells and accumulates in acidic organelles, such as lysosomes	Measures the capacity of cells to retain the dye as an indicator of membrane integrity and metabolic activity	Suitable for use with various cell types, including animal and microbial cells	Reliance on lysosomal activity, which may not accurately reflect viability in non-metabolically active or developing stages

Table 4. Cont.

Stain	Type	Properties	Use	Advantages	Limitations
TO-PRO-1 Iodide	Mortal	Membrane-impermeant nucleic acid dye entering only late apoptotic and necrotic cells, emits green fluorescence	Effective for assessing cell viability in various aquatic organisms	High specificity for non-viable cells	Not all cell types may take it up uniformly; may require optimization for different species
Propidium Iodide	Mortal	Fluorescent dye that enters only cells with damaged membranes, binding to nucleic acids and emitting red fluorescence	Used in combination with vital stains like SYTO 9 to assess cell viability	Some differentiation between live and dead cells	Can give false positives in cells with temporarily damaged membranes
SYTOX Green	Mortal	Membrane-impermeant dye that fluoresces only when bound to DNA in dead or damaged cells	Commonly used for algal viability assessments	Effective in detecting non-viable cells	May not be suitable for all cell types; specificity can vary.
7-AAD	Mortal	Fluorescent dye that emits red fluorescence when bound to nucleic acids in non-viable cells; membrane-impermeant	Used in flow cytometry and fluorescence microscopy	Reliable indicator of cell health; effective in conjunction with other stains for apoptosis	May lead to false positives; in some cell types
Trypan Blue	Mortal	Dye exclusion method where viable cells remain unstained while dead cells absorb the dye and turn blue	Commonly used in cell viability assays	Simple and quick visual delineation between live and dead cells.	May not be effective for organisms with thick protective layers
Aniline Blue	Mortal	Binds to denatured proteins and damaged cell membranes	Structural integrity is the target, as viable cells exclude the dye.	Provides quick visual confirmation of non-viability under a microscope	Less effective in organisms with complex integuments
TUNEL Assay	Mortal	Labels fragmented DNA in apoptotic cells; detects non-viable cells by indicating DNA breaks	Used to assess apoptosis in various cell types	Useful for analyzing mechanisms of cell death	Requires more complex protocols; may not differentiate between apoptosis and necrosis effectively

MICROSCOPY-BASED LIVE/DEAD EVALUATION BASED ON STAINING

In microscopy-based assays, simultaneous staining with more than one fluorescent dye, such as SYTO 9 and PI or TO-PRO-1, can be used to observe cellular structures and morphological changes. However, microscopy-based assays have their limitations, e.g., analyzing samples under a microscope can be labor-intensive and time-consuming and only a limited number of specimens per sample are evaluated, which may limit the statistical power of the results. The interpretation of results can also be subjective, as it relies on the visual assessment of fluorescence intensity and organism morphology. Therefore, automatic or semi-automatic image analysis can help to decrease the subjectivity of the interpretations.

Use in the VIABLEGG

After incubation, the stained samples were visually observed and counted using an epifluorescence Nikon Eclipse-Ti inverted microscope system with a standard long-pass blue filter (530-600 nm) at 20× magnification. Image Analysis was conducted using ImageJ software (version 1.53c, National Institutes of Health, USA) with custom macros developed for the study. Images were pre-processed by converting them to 8-bit grayscale and applying a background subtraction algorithm (rolling ball radius: 50 pixels) to normalize intensity. Automatic thresholding using Otsu's method and a watershed algorithm separated organisms from the background and delineated touching organisms. The *Analyze Particles* function identified regions of interest (ROIs) based on size (100–10,000 μm²) and circularity (0.2–1.0). For color analysis, the blue channel of the original RGB image was analyzed to quantify staining. For example, for Aniline Blue, mean blue intensity was measured for each ROI, and organisms were classified as live or dead using a threshold value of 150 (on a scale of 0–255). Morphological parameters such as area, perimeter, aspect ratio, and roundness were also measured for each organism. Results were exported to CSV files for further statistical analysis.

4.3 Sample preparation to improve penetrability of integument

The poor penetrability of chitinous and proteinaceous structures in integument poses a significant challenge for the application of staining techniques. In addition to preventing stains from reaching the internal structures of the egg, the eggshells often exhibit autofluorescence, complicating the detection and interpretation of staining results. For example, as illustrated in **Figure 3,** the integument not only obstructs stain penetration (*CDFA* panels) but also generates background fluorescence (*No stain* panels), presenting the dual challenge of overcoming both physical and optical interference for effective staining applications.

Increasing the penetrability of chitinous and proteinaceous structures requires a sample preparation tailored to the structural composition of the integument. Key challenge is the balance between effective disruption of chitin or protein layers and the preservation of specimen viability. Therefore, the treatment must be optimized to ensure compatibility with dyes while minimizing damage to the structural integrity of the specimen.

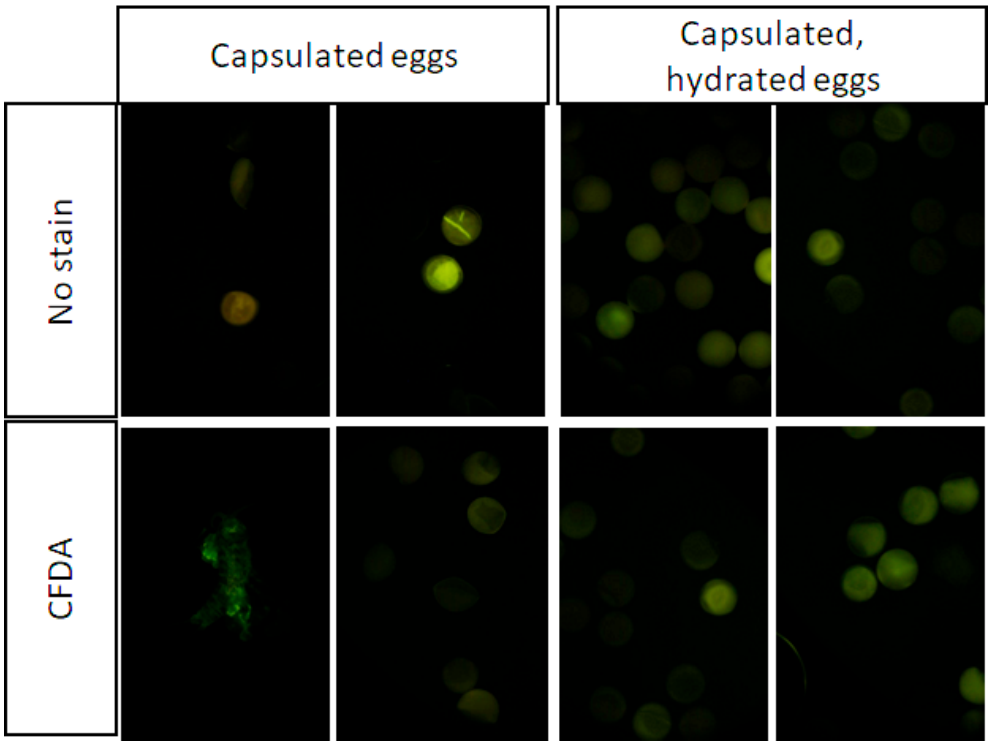

Figure 3. Fluorescence microscopy images showing capsulated *Artemia* eggs (left panels) and capsulated, hydrated eggs after a 48-hour hydration period (right panels). The upper panels display the eggs without staining, where autofluorescence of the eggshell is evident. The lower panels show eggs stained with CFDA. The results highlight the autofluorescence of the eggshell and the limited penetration of the stain into the egg interior.

In the VIABLEGG project

several methods (sections 4.3.1 and 4.3.2) were tested to improve the penetration of stains into chitinous and proteinaceous structures of rotifer and copepod eggs, bivalve veligers, shrimp cysts, and cladoceran ephippia. The treatment efficacy was evaluated by (1) staining and (2) confirmatory hatching experiments as described above, and (3) structural and chemical changes in the integuments by Fourier Transform Infrared Spectroscopy (FTIR) analysis **(Figure 4)**.

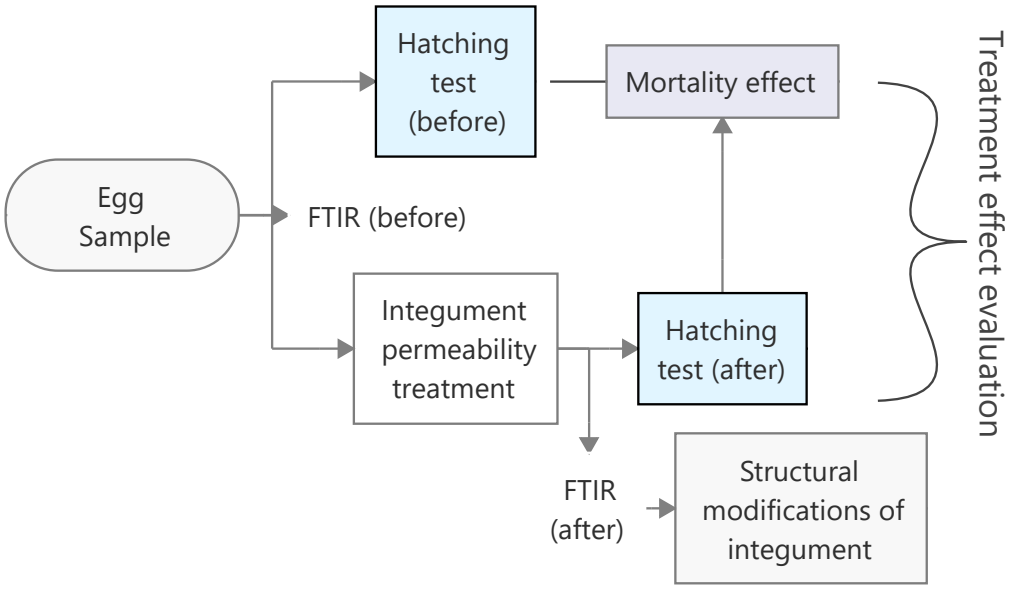

Figure 4. The integration of hatching experiments to establish the percentage of viable eggs as the ultimate measure of mortality, permeability enhancement (chemical and/or physical treatments) outcomes, and FTIR analysis showing the bulk molecular modifications in the sample following the treatment. This general workflow was applied to evaluate treatment efficacy.

4.3.1 Chemical and photochemical treatments

Chemical treatments employed to enhance the integument permeability in eggs, ephippia, and veligers included the following methods:

Enzymatic digestion involved the use of chitinase (1–5 mg/mL) to break down chitin in copepod eggs and trypsin (0.5–1 mg/mL) to degrade protein layers, with incubation times ranging from 30 minutes to 2 hours at 25°C. Before staining, eggs were incubated for 24 hours in 250 µ l of chitinase enzyme dissolved in 50 mmol l^{-1} citrate buffer, pH 6, at 25°C, to permeabilize the chitinous wall. After rinsing several times in PBS, embryos were incubated for 2 hours in 0.1% Triton X-100 at room temperature and rinsed in PBS (buffer) containing 1% BSA (albumin).

LED-assisted bleach decapsulation is a method used in aquaculture to remove the outer shell (chorion) of *Artemia* cysts using a combination of LED light and bleach treatment. We followed the method that involves hydrating *(Artemia)* cysts in water, exposing them to a blue LED light, and then treating them with a sodium hypochlorite solution, combined with sodium hydroxide to maintain an alkaline pH. The reaction is subsequently stopped using sodium thiosulfate, which neutralizes the bleach and halts the decapsulation process (Delbos et al., 2019).

Alkaline treatments used sodium hydroxide (NaOH) at concentrations of 0.1–1% (w/v) for 5–30 minutes to deacetylate chitin and soften tough ephippial layers in daphnia eggs, followed by neutralization or thorough rinsing.

Acid treatments, including hydrochloric acid (0.01–0.1 M) or acetic acid (0.5–2% v/v), were applied for 2–10 minutes to dissolve calcium carbonate structures in bivalve veliger shells, with post-treatment neutralization or rinsing.

Organic solvent treatments used acetone (20–50%) for 10–30 minutes to remove lipid layers in rotifer eggs.

4.3.2 Physical treatments

The following physical methods were employed to enhance the integument permeability by mechanically or thermally disrupting structural barriers:

Sonication. Ultrasonic waves were applied to create microcavities that disrupted outer integuments without causing significant damage to the specimens. Samples were treated in an ultrasonic bath at low frequencies of 10–20 kHz for durations of 10-15 seconds, depending on the egg size and integument thickness.

Microwave irradiation. Short bursts of microwave radiation were used to enhance the effectiveness of chemical treatments by softening integuments and accelerating molecular interactions. Samples were exposed to microwave bursts of 5–15 seconds at low power settings (10–20% of maximum power), with careful monitoring to avoid overheating.

Electroporation. This method utilizes short, high-voltage electrical pulses to create transient pores in the lipid bilayer of membranes, to temporarily increase the penetration of vital dyes that would otherwise be excluded. Test organisms (rotifer and copepods eggs, diapausing eggs and ephippia) were suspended in a conductive saline buffer and subjected to controlled electrical pulses in an electroporation chamber. Typical parameters included voltages of 200–600 V/cm, pulse durations of 1–10 milliseconds, and 1–3 pulses (generally lower values for rotifers and copepods and higher for Branchiopoda). Post-electroporation, organisms were immediately subjected to staining and then allowed to recover in fresh medium to re-establish membrane integrity before examination.

Combining physical and chemical approaches. Physical treatments such as sonication and microwave irradiation were often paired with chemical methods, such as enzymatic or alkaline treatments, to enhance penetration synergistically. For example, sonication was combined with chitinase digestion for copepod eggs, significantly improving stain access to internal structures.

4.3.3 Assessing structural and chemical changes in the integument

The integrity of egg and embryo integuments was evaluated using Fourier Transform Infrared Spectroscopy (FTIR) to assess structural and chemical changes induced by the treatments. FTIR provides a molecular-level analysis of integument components, enabling the detection of alterations in chitin, protein, and lipid structures (Ishigaki et al., 2016). Key functional groups were monitored, such as the amide I (~1650 cm^{-1}) and amide II (~1550 cm^{-1}) peaks for chitin-protein matrices, which indicate deacetylation or protein degradation from enzymatic or alkaline treatments. FTIR spectra of chitosan (degraded chitin) show a disappearance of the band at 2532 cm^{-1} (Beil et al., 2012). Lipid layers were analyzed through CH_2 stretching peaks at ~2850–2920 cm^{-1}, with reductions suggesting lipid removal by, e.g., organic solvents. Protein denaturation or hydrolysis caused by treatments was reflected in variations in amide band intensities and positions. Also, a decrease in the OH stretching peak around 3460 cm^{-1} after a treatment, as observed in FTIR spectra, typically indicates significant structural and chemical changes in the material. This reduction may result from the loss of hydroxyl groups due to dehydration or chemical reactions, such as conversion to carbonyl groups, polymer degradation, structural breakdown, or altered crystallinity, particularly in materials like chitin (Lewandowska, 2012). Oxidation processes and dehydration of bound water can further contribute to the observed decrease.

4.4 Evaluation of stain performance in BallastWISE

Stained samples of live and dead zooplankton species, including copepods (*Nitocra spinipes*) and cladocerans (*Daphnia magna*), were analyzed using the BallastWISE system. The analysis employed excitation light at 420 nm and 500 nm with a 590 nm high-pass filter to evaluate the fluorescence response of different stains. The stains demonstrating the most consistent performance during laboratory evaluations under light and fluorescence microscopy (see Section 5.2) were CFDA, TO-PRO-1 Iodide, and Aniline Blue.

Sample preparation. The test specimens used in these experiments originated from cultures, ensuring a reliable source of healthy copepods (*Nitocra spinipes*) and cladocerans (*Daphnia magna*), with both species exhibiting natural population dynamics, including egg-bearing females. A set of mixed samples (copepods and cladoceras, 50:50) was also prepared.

Live specimens for the tests were carefully selected from the culture beakers. Only egg-bearing females displaying visible motility and normal morphological appearance were included to ensure the viability and representativeness of the test samples. Each individual was picked manually and gently transferred to sample containers to minimize handling stress.

Samples were assembled with a total of 25–100 individuals per sample. Efforts were made to maintain a consistent size distribution across all test samples to ensure comparability between replicates and stains. This preparation process minimized variability and ensured that the samples accurately reflected the zooplankton population characteristics under study.

Staining procedure. To evaluate stain performance, all samples were divided into two groups: live and dead individuals. For the "dead" group, heat-killing was applied using a water bath set to 55°C for 30 minutes. This treatment was previously validated to ensure 0% hatching success in both *Nitocra spinipes* and *Daphnia magna*, confirming the effectiveness of this approach for inducing mortality. Following the heat-killing process, both the treated (dead) and non-treated control (live) samples were stained in parallel using the same staining protocols employed during laboratory evaluations (see Section 5.2). Additionally, "no stain" controls were prepared for both live and dead groups. These controls were essential for assessing background fluorescence and autofluorescence levels, ensuring that the fluorescence signals observed in stained samples were stain-specific. The sample were shipped to Denmark with a carrier mail, where they were analyzed within a few days after the staining.

Focus on whole-body staining. In the context of BallastWISE testing, the decision to evaluate whole-body fluorescence intensity, despite the primary focus on eggs, was driven by practical considerations. In both copepods and cladocerans, the eggs are physically attached to the adult females, either contained within egg sacs in copepods or housed in the brood chamber in daphnids. These close physical associations made it impractical to isolate the eggs for fluorescence measurements without disrupting their structural integrity. As a more feasible and efficient approach, whole-body fluorescence intensity was analyzed to reflect the staining of the entire organism, including the eggs. Image-based evaluations were used to confirm that the eggs within the egg sacs or brood chambers exhibited similar

staining patterns to the rest of the body (**Figure 5**). This dual approach ensured that the fluorescence data captured the viability status of the eggs while avoiding the technical challenges and potential biases introduced by attempting to analyze eggs separately from the adult organisms in a practical and minimally invasive manner.

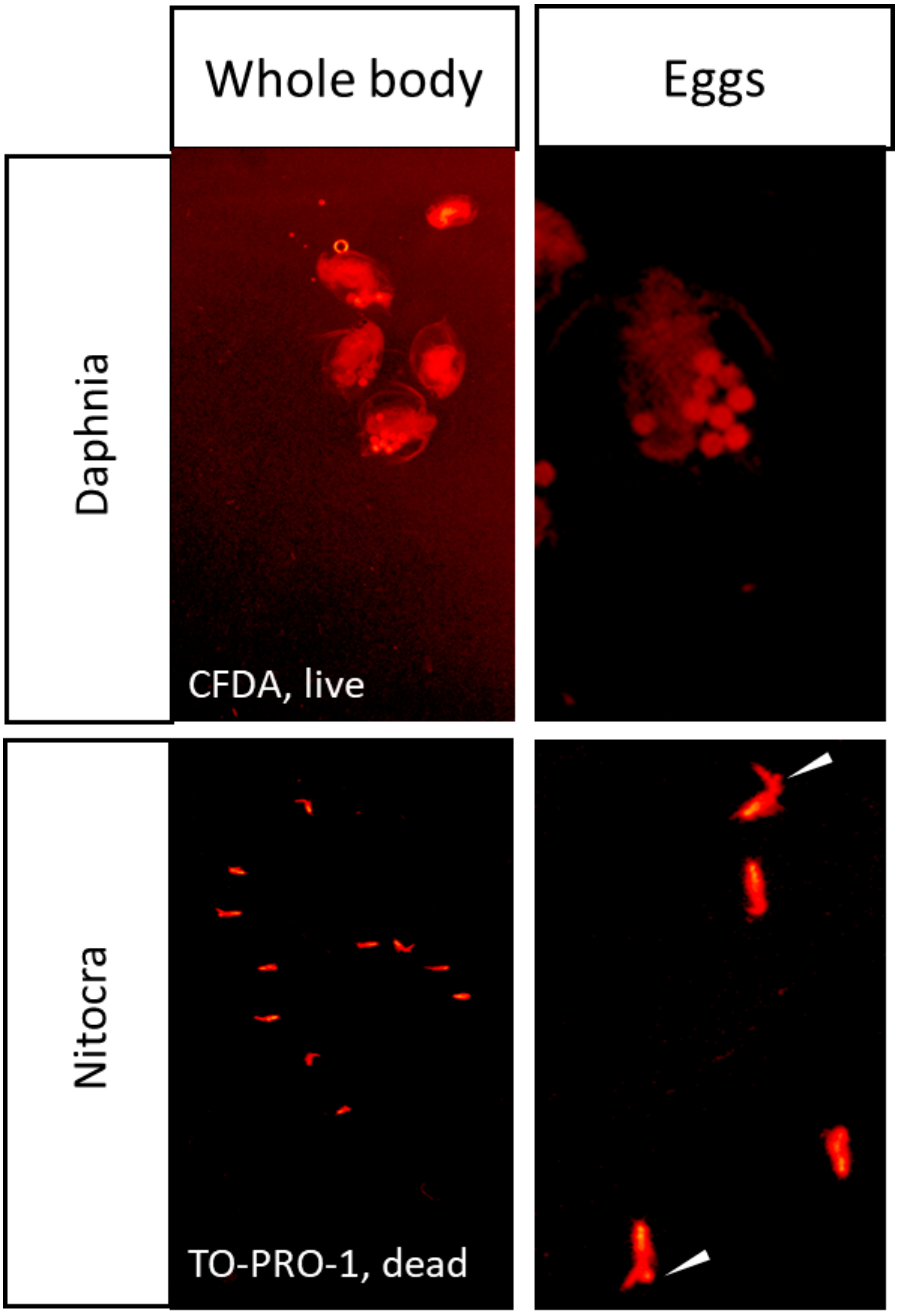

Figure 5. Fluorescence staining of whole bodies and eggs for *Daphnia magna* (live, stained with CFDA) and *Nitocra spinipes* (dead, stained with TO-PRO-1 Iodide) The left panels show whole-body fluorescence and the right panels provide close-up views of the eggs located in the brood chamber of *Daphnia magna* (top right) and the egg sac of *Nitocra spinipes* (bottom right) from the same individuals depicted in the whole-body images on the left. The stain used and the viability status of the organisms are indicated. Arrowheads highlight stained egg sacs in the copepod females. In both species, the staining is uniform for the whole body and the egg material.

4.5 Data analysis

The efficiency of staining (based on image analysis output) and metabolic activity (measured as ATP levels) as indicators of viability was evaluated by comparing these metrics to either hatching success (for *Artemia*, rotifers and copepods) or the presence of embryo aberrations (for amphipods). For each replicate, the percentage of hatched eggs (or normal embryos) was calculated, and the results were correlated with staining outcomes. Similarly, the ATP levels were compared between the egg batches designated as dead and live (i.e., control vs killed by exposure to different disinfection treatments; **Table 1**). Concordance between hatching/aberration rate and viability determined by staining or ATP measurements was used to assess the accuracy of these test methods.

The effects of chemical and physical treatments on the permeability and survivorship of the test organisms were evaluated using generalized linear models (GLMs) or Mann-Whitney U tests, with a significance threshold of $p < 0.05$. The data were Box-Cox transformed if necessary to test treatment-specific impacts on staining efficiency and organism mortality due to the damage caused by the treatment while accounting for treatment type, duration, and organism type.

5. Results

5.1 Summary of main findings

This study evaluated the feasibility of viability assessment methods for non-motile invertebrate life stages using staining-based techniques. Despite substantial species- and stage-specific variability, several stains showed promising results under laboratory conditions. Among them, CFDA (vital stain) and TO-PRO-1 Iodide (mortal stain) demonstrated high diagnostic accuracy (above 90%) in subitaneous copepod eggs and amphipod embryos (**Table 5**). Neutral Red was effective for swimming veligers due to their active uptake of the staining medium via feeding and developed lysosomal systems. Also, Anilin Blue showed promise for non-diapausing copepod and cladoceran eggs, though it was not tested in other taxa. In contrast, diapausing stages generally showed poor staining efficiency due to impermeable integuments and treatment-related viability loss (**Table 6**).

Table 5. Summary of viability stain performance based on the experimental results. The table highlights the type, mechanism, effective taxa, strengths, and limitations of the stains tested for assessing the viability of non-motile life stages of invertebrates, focusing on egg sacs, embryos, and veligers. Only stains with efficiency showing potential for further investigations are included.

Stain	Type	Target/Mechanism	Effective taxa	Strengths	Limitations
Aniline Blue	Mortal	Binds to denatured proteins/damaged membranes	Copepod eggs, rotifer eggs	Quick and simple; selective staining of egg sacs	False positives in live specimens (dye ingestion); uneven staining
TO-PRO-1 Iodide	Mortal	Penetrates damaged membranes, binds to nucleic acids	Copepod eggs, amphipod embryos	Bright, consistent fluorescence; high sensitivity for copepod eggs	Requires optimization; autofluorescence interference, false positives (15%) in amphipod embryo
CFDA	Vital	Esterase activity converts to fluorescent product	Shrimp cysts, copepod eggs, amphipod embryos	Effective for metabolically active eggs	Poor penetration in impermeable integuments; some autofluorescence
Neutral red	Vital	Accumulates in lysosomes	Bivalve veligers	Reliable for certain taxa only	Ineffective for eggs with no lysosomal activity
MitoTracker	Vita	Accumulates in mitochondria	Copepod eggs (subitaneous), last embryonic stages	Reliable only for well-developed eggs	Poor penetration in impermeable integuments

When applied in the BallastWISE system, stain performance decreased substantially, with diagnostic accuracy dropping below 60% for most tests. This discrepancy was partly due to autofluorescence interference, suboptimal filter settings, and reduced stain uptake in intact or poorly permeabilized specimens. Nevertheless, even in the BallastWISE tests, the fluorescence intensity of CFDA and TO-PRO-1 was ~2-fold and 3-fold higher for viable and dead eggs, respectively, demonstrating the system's utility for egg viability screening.

No universal protocol emerged due to the inherent diversity in egg integuments and physiological states. Nonetheless, the findings provide a practical framework for selecting viable stain–species combinations and demonstrate the need for tailored protocols when assessing viability in ballast water monitoring.

Table 6. Summary of viability stain performance for different egg types based on the experimental results. The table highlights the taxa, types of stages, and the challenges revealed by our findings. Only stains with efficiency showing potential for further investigations are included.

Species	Egg Type	Stain	Success	Challenges
Rotifers	Mictic	CFDA	Poor	Integument impermeability; high mortality with chitinase and NaOH treatments
	Amictic		None	Integument impermeability; high mortality with chitinase and NaOH treatments
		MitoTracker	Good	Ineffective in staining other egg types lacking a substantial mitochondrial activity
Copepods	Subitaneous	CFDA	Good	False-positive staining in heat-killed embryos; some autofluorescence
		TO-PRO-1	Good	Autofluorescence from chitinous exoskeleton
	Diapausing	CFDA	None	Integument impermeability; high mortality with chitinase and NaOH treatments
Amphipods	Embryos	CFDA	Yes	False-positive staining in heat-killed embryos
		TO-PRO-1	Yes	None
Bivalves	Veligers	Neutral Red	Yes	Ineffective in staining other egg types lacking a developed lysosomal system
Artemia	Decapsulated cysts	CFDA	Limited	High autofluorescence from decapsulated cysts interfered with stain fluorescence detection

5.2 Enhancing integument permeability

The impermeability of egg integuments—particularly in diapausing stages—was a major barrier to effective staining. To address this, we tested a range of chemical, enzymatic, and physical treatments to improve dye penetration without compromising viability. Treatment efficacy was validated through staining efficiency, hatching success, and structural changes assessed via FTIR spectroscopy. The results revealed a clear trade-off: treatments that significantly enhanced permeability often resulted in reduced hatching success, highlighting their detrimental effects on organism viability. For instance, electroporation improved stain uptake for rotifers but reduced hatching success by 25%, while FTIR confirmed alterations in the chitinous and proteinaceous layers of their integuments. Conversely, no significant permeability enhancements were observed for tough integuments such as ephippia or diapausing eggs, and FTIR indicated minimal structural modifications, reflecting their resilience. The integration of FTIR with hatching experiments demonstrated the importance of understanding treatment-induced integument changes to balance permeability improvements with the preservation of viability, ensuring the practicality of staining protocols for viability assessments.

5.2.1 FTIR analysis of integument modifications

FTIR spectroscopy proved valuable in interpreting the structural and biochemical changes induced by sample preparation treatments on the integuments. As an example, we show how FTIR spectra of *Cercopagis pengoi* eggs treated with chitinase (1 mg/ml) and NaOH (0.3%) were affected by the treatment (before vs after). These spectra allowed the identification of specific alterations in key functional groups associated with the chitinous and proteinaceous components of the eggshells (**Figure 6**).

In the upper panel, the spectra demonstrate the impact of chitinase treatment. A reduction in the intensity of the amide I (~1650 cm^{-1}) and amide II (~1550 cm^{-1}) peaks suggests partial degradation of the chitin-protein matrix. Additionally, the subtle changes in the OH stretching region (~3460 cm^{1}) may indicate minor disruptions in the hydrogen bonding network, reflecting localised changes to the chitin structure.

The lower panel illustrates the effect of NaOH treatment. Significant reductions in the amide I and amide II peaks indicate extensive protein degradation and chitin deacetylation. Moreover, the sharp decline in the OH stretching peak (~3460 cm^{-1}) points to substantial loss of hydroxyl groups, likely due to deacetylation and dehydration. These changes were more pronounced compared to the chitinase treatment, consistent with the stronger impact of alkaline treatments on integument integrity (see section 5.1.2). Overall, the FTIR spectra highlight the treatment-specific changes to the integuments, offering insights into the mechanisms of structural modification.

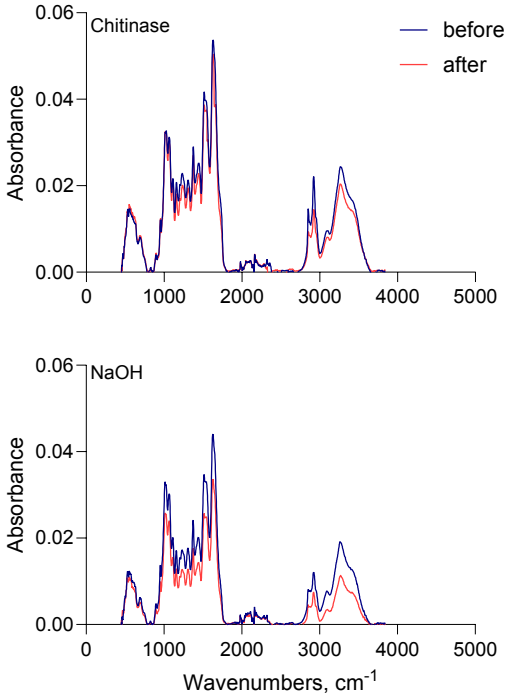

Figure 6. FTIR spectra illustrating the effects of chemical treatments on the integument of *Cercopagis pengoi* diapausing eggs. The upper panel shows the impact of chitinase treatment, with reductions in the amide I (~1650 cm^{-1}) and amide II (~1550 cm^{-1}) peaks, indicating partial degradation of the chitin-protein matrix. The lower panel depicts the effects of NaOH treatment, with more pronounced reductions in amide peaks and a sharp decline in the OH stretching peak (~3460 cm^{-1}), reflecting significant deacetylation and loss of hydroxyl groups.

5.2.2 Chemical treatments

Chemical and enzymatic treatments, including enzymatic digestion, alkaline solutions, acid demineralization, organic solvent treatments and LED-assisted decapsulation, were tested to enhance stain penetration through chitinous and proteinaceous integuments. Among the most effective were chitinase and NaOH, both of which significantly altered the structure of egg envelopes, as confirmed by FTIR and hatching tests (**Table 7**).

Chitinase (1–5 mg/mL) improved dye uptake in rotifer and copepod eggs, but was often lethal, especially when combined with detergents. FTIR spectra revealed reduced amide I and II peaks, indicating chitin-protein matrix degradation.

NaOH (0.1–1%) was effective in softening tough layers, such as *Daphnia* ephippia, but also resulted in elevated mortality. It caused pronounced reductions in hydroxyl groups and protein peaks, confirming structural alterations.

Acid and trypsin treatments enhanced permeability only marginally, with uptake rates below 15% for mortal dyes and 8% for vital dyes. Given their low efficiency and added risk of structural damage, these treatments were discontinued.

LED-assisted decapsulation of *Artemia* cysts successfully removed the chorion, but introduced high autofluorescence (**Figure 7**), likely resulting from residual chemical reactions or structural modifications during the bleaching process, complicated the interpretation of stain fluorescence. Staining with CFDA and TO-PRO-1 showed some positive results, but viability (hatching) remained below 3%, implicating autofluorescence as the main source of the signal (**Figure 8**).

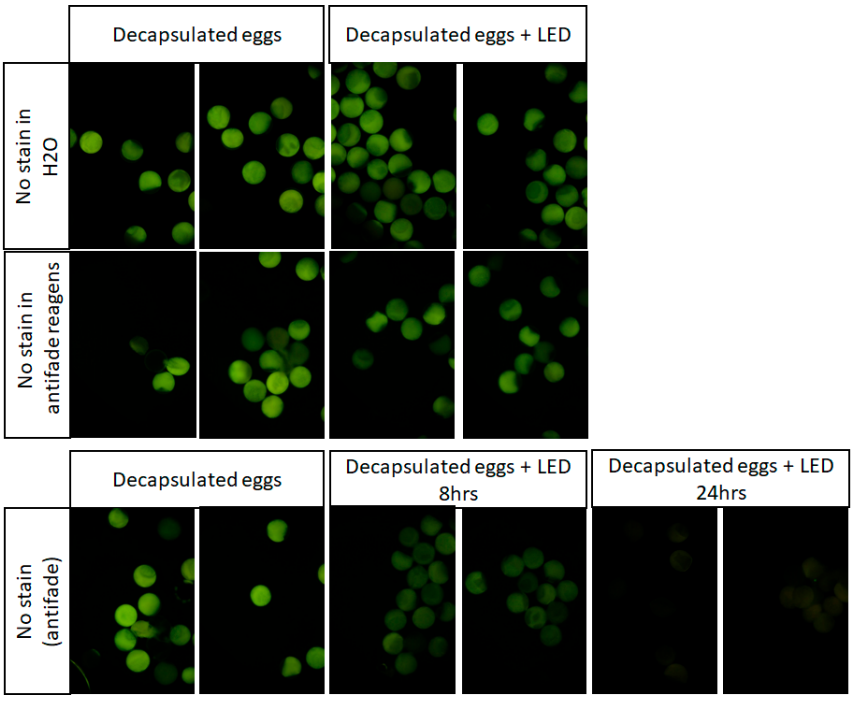

Figure 7. LED-assisted decapsulation of *Artemia* cysts showing successful removal of the chorion after the treatment. High autofluorescence observed in decapsulated cysts under fluorescence microscopy, which impeded the accurate assessment of stain uptake.

Upper and middle rows: decapsulated cysts without and with LED exposure in water (upper row) and antifade reagents (middle row); the antifade was added as a part of the common procedure working with fluorescence imaging. *Bottom row:* decreasing autofluorescence over the course of 24 h under LED exposure. In no case a staining was applied.

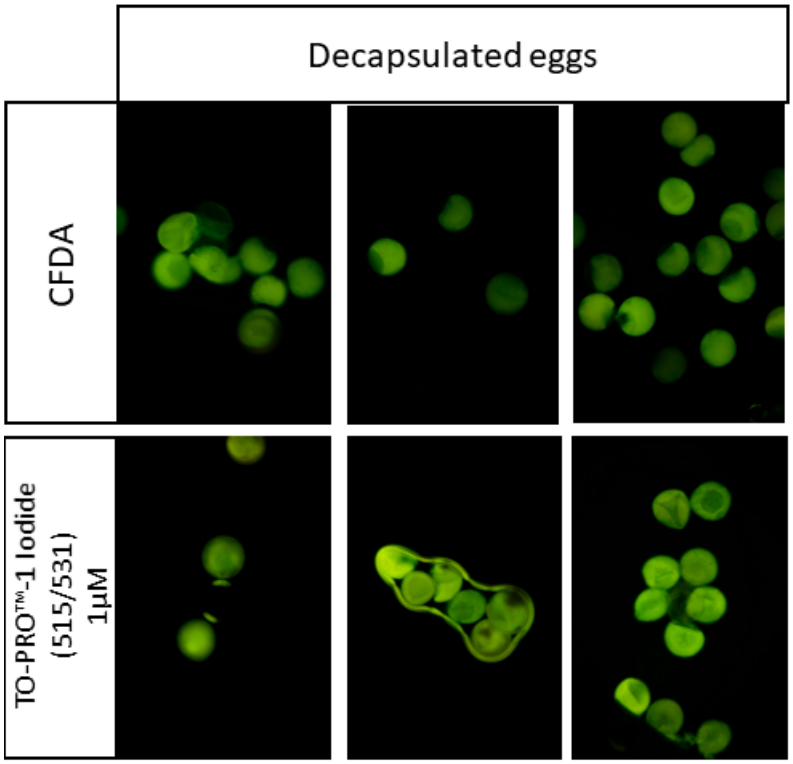

Figure 8. Decapsulated *Artemia* eggs stained with CDFA (vital stain; upper panels) and TO-PRO-1 Iodide (mortal stain, lower panels). Both stains produced apparently positive results, although the viability of these eggs in the hatching experiments was less than 3%. The fluorescence was mostly due to the autofluorescence of the shells.

A generalized linear model (GLM) with a binomial error distribution was used to analyze the effects of treatment type (i.e., the specific agent applied to improve the stain permeability), species, and exposure duration on the egg mortality determined by hatching. The model revealed significant main effects of both treatment type ($p < 0.001$) and exposure duration ($p < 0.001$) on mortality, as well as a significant interaction between species and treatment type ($p = 0.015$). More, specifically, mortality was significantly higher for subitaneous eggs compared to diapausing eggs in *Acartia tonsa* ($p < 0.001$), reflecting the greater susceptibility of subitaneous eggs. Also, NaOH consistently resulted in significantly higher mortality rates across all species, emphasizing its stronger negative impact (GLM; $p < 0.001$; **Table 7**).

Acid treatments (HCl at 0.01–0.1 M) primarily targeted calcium carbonate components in the veliger shells but failed to disrupt integuments in rotifers lacking calcified layers. Similarly, trypsin (0.5–1 mg/mL) had minimal impact on chitinous or proteinaceous barriers in the rotifer eggs, as its proteolytic activity did not penetrate the tough outer layers effectively (**Table 8**).

Table 7. Egg/ephippia mortality (%) and the corresponding 50% lethality (LC_{50}, the median lethal dose) in different species following the integument permeating treatments with chitinase (1 or 5 mg/mL) and NaOH (0.1%).

Species/egg morphology	Treatment	LC_{50} min	10 min	20 min	30 min	60 min
*Daphnia magna** (ephippia)	Chitinase (5 mg/mL)	**27.6**	10	35	67	95
Daphnia magna (ephippia)	Chitinase (5 mg/mL)	**34.3**	0	15	27	44
Acartia tonsa (subitaneous egg)	Chitinase (1 mg/mL)	**30.4**	5	16	24	31
Acartia tonsa (diapausing egg)	Chitinase (1 mg/mL)	**131.6**	4	21	44	68
Nitocra spinipes (egg sac)	Chitinase (1 mg/mL)	**134.9/NC**	5	8	13	27
Brachionus plicatilis (mictic egg)	Chitinase (1 mg/mL)	**NC**	2	4	3	5
*Daphnia magna** (ephippia)	NaOH	**23.0**	13	35	73	92
Acartia tonsa (subitaneous egg)	NaOH	**29.3**	12	23	57	81
Nitocra spinipes (egg sac)	NaOH	**20.0**	20	45	75	98
Brachionus plicatilis (mictic egg)	NaOH	**100.9/NC**	10	7	25	33
Artemia salina (cysts)	NaOH	**NC**	5	0	13	19

*: applied after sonication; NC: non-complete dose-response, LC_{50} calculations are not possible.

Table 8. Acid and trypsin treatments enhanced dye permeability only marginally, with uptake rates below 15% for mortal dye and 8% for vital dye. Given their low performance relative to other tested methods, they were not pursued further in the experimental workflow.

Species	Treatment	Aniline Blue uptake (%)	CFDA uptake (%)
mictic egg	HCl (0.1 M)	10	5
amictic egg	HCl (0.1 M)	15	8
veligers	HCl (0.2 M)	12	6
mictic egg	Trypsin (2 mg/mL)	8	3
amictic egg	Trypsin (2 mg/mL)	10	5
veligers	Trypsin (1 mg/mL)	9	4

Overall, chitinase and NaOH had the highest impact on permeability, but their toxic effects often outweighed the benefits. Most other chemical treatments either failed to improve dye uptake or caused excessive damage. Thus, chemical treatments, while effective in enhancing integument permeability, carry significant risks to specimen viability. Future work should focus on refining these methods to balance improved stain penetration with reduced mortality rates.

5.2.3 Physical and combined treatments

To minimize chemical damage while improving permeability, several physical methods, including microwave irradiation, sonication, and electroporation, and their combinations with chemical treatments were explored. The results revealed significant variability in effectiveness and impact on viability, with distinct taxon-specific responses.

Electroporation yielded the most promising results, significantly enhancing CFDA permeability for rotifer eggs (up to 55%) while causing a moderate increase in mortality (25%). However, electroporation showed minimal effect on *Nitocra spinipes* (5% increase in permeability) and no measurable effect on *Daphnia magna* ephippia. Mortality rates for these taxa were lower, 20% for *Nitocra spinipes* eggs and 10% for *Daphnia magna* ephippia, reflecting their resilience to electroporation and good recovery.

Microwave treatment showed poor performance, with minimal improvement in permeability and consistently high mortality rates. For rotifers, microwave treatment resulted in only 20% permeability (measured as CDFA uptake) but caused ~70% mortality. Similarly, for *Nitocra spinipes* egg sac and *Daphnia magna* ephippia, microwave treatment did not enhance permeability and caused mortality rates of 85% and 10%, respectively. Due to these strong negative effects, microwave treatment was discarded from further evaluations.

Sonication produced inconsistent results, with permeability enhancements for rotifers varying widely (34% ± 20%) and associated with a high mortality rate. For *Nitocra spinipes* egg sac and *Daphnia magna* ephippia, sonication failed to enhance permeability (0% uptake) and elevated mortality rates to 56% and 10%, respectively. When applied in combination with the chitinase **(Table 5)**, sonication significantly elevated the mortality response to the enzyme (GLM: $p < 0.05$). These findings suggest that although sonication may primarily affect the organism itself rather than its integument, it limits its utility for permeability enhancement.

The GLM confirmed significant effects of treatment type ($p < 0.001$), taxon ($p < 0.01$), and their interaction ($p < 0.05$) on permeability and mortality. More specifically, electroporation significantly improved permeability for rotifer eggs stained with CFDA compared to other treatments ($p < 0.001$), while microwave and sonication treatments were ineffective across all taxa. Mortality analysis showed electroporation had a lower impact on rotifers compared to microwave and sonication ($p < 0.01$), but no significant differences were observed among treatments for *Nitocra spinipes* eggs and *Daphnia magna* ephippia.

In conclusion, our efforts to enhance stain penetration through chemical and physical treatments provided valuable insights but failed to identify an effective method. While electroporation demonstrated some potential for enhancing permeability in rotifers, none of the tested physical treatments was effective for tough integuments such as ephippia and diapausing eggs.

5.3 Staining efficiency assessment

To evaluate stain performance, microscopy-based observations were conducted on control (viable), treated (non-viable), and mixed samples across a range of invertebrate eggs and embryos. Since no pretreatment method was identified to simultaneously enhance integument penetrability and preserve organism viability, no such method was applied during the staining efficiency assessment. Instead, staining efficiency was evaluated using a selection of representative species. The focus shifted to organisms with thin-shelled eggs to minimise the impact of the integument.

Fluorescence intensity, staining specificity, and diagnostic accuracy were used as performance indicators, with hatching success and ATP levels serving as reference methods. A total of 17 stains, comprising 8 vital and 9 mortal dyes, were tested (**Table 4**), and several stains demonstrated potential for species- and stage-specific applications, but no single dye proved universally effective. Below, we summarize key results for the most informative stains.

5.3.1 Vital stains

CFDA showed strong fluorescence in viable subitaneous copepod eggs (**Figure 9**) and amphipod embryos (**Figure 10**), correlating with high hatching rates and ATP levels (**Figure 11**, **Figure 12**). In the copepods *Nitocra spinipes*, a high CFDA concentration (10 µM) effectively stained viable egg sacs, while a low concentration failed to do so. Staining of live females was also significantly more intense than in dead controls (**Figure 9**), suggesting reliable discrimination under these conditions. However, a weak signal (<15%) was also observed in dead egg sacs, possibly due to esterase activity persisting after death or autofluorescence. No recovery was observed for the dead controls (i.e., heat-killed females with egg sacs, 45 °C for 60 min, no movements before staining) within 24 hours post-treatment, and their eggs were classified as 100% dead by the hatching experiments (**Figure 11**). A weak autofluorescence was observed in the non-stained females, with no evidence of the autofluorescence associated with the egg sacs (**Figure 9**).

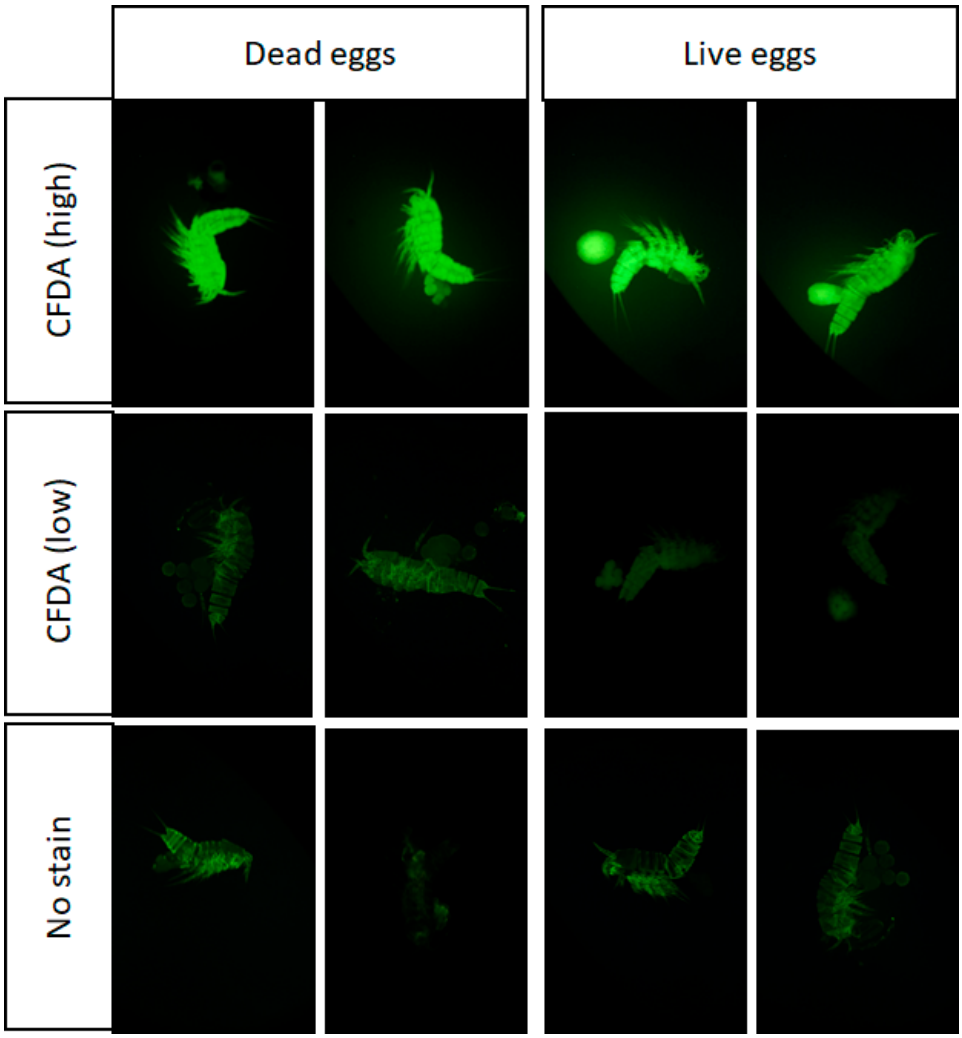

Figure 9. Harpacticoid copepod *Nitocra spinipes* (females with egg sacs) stained with CFDA at high (10 µm; upper panel) and low (1:1000 dilution; middle panel) dye concentrations. No-stain reference samples are shown in the lower panel; some autofluorescence is visible.

The high concentration of CFDA resulted in effective staining of viable egg sacs (top-right panels). Dead eggs were obtained by exposing females with egg sacs to high temperatures (45 °C for 60 minutes; top-left panels). Note that the low CFDA concentration produced fluorescence levels comparable to those of non-stained specimens.

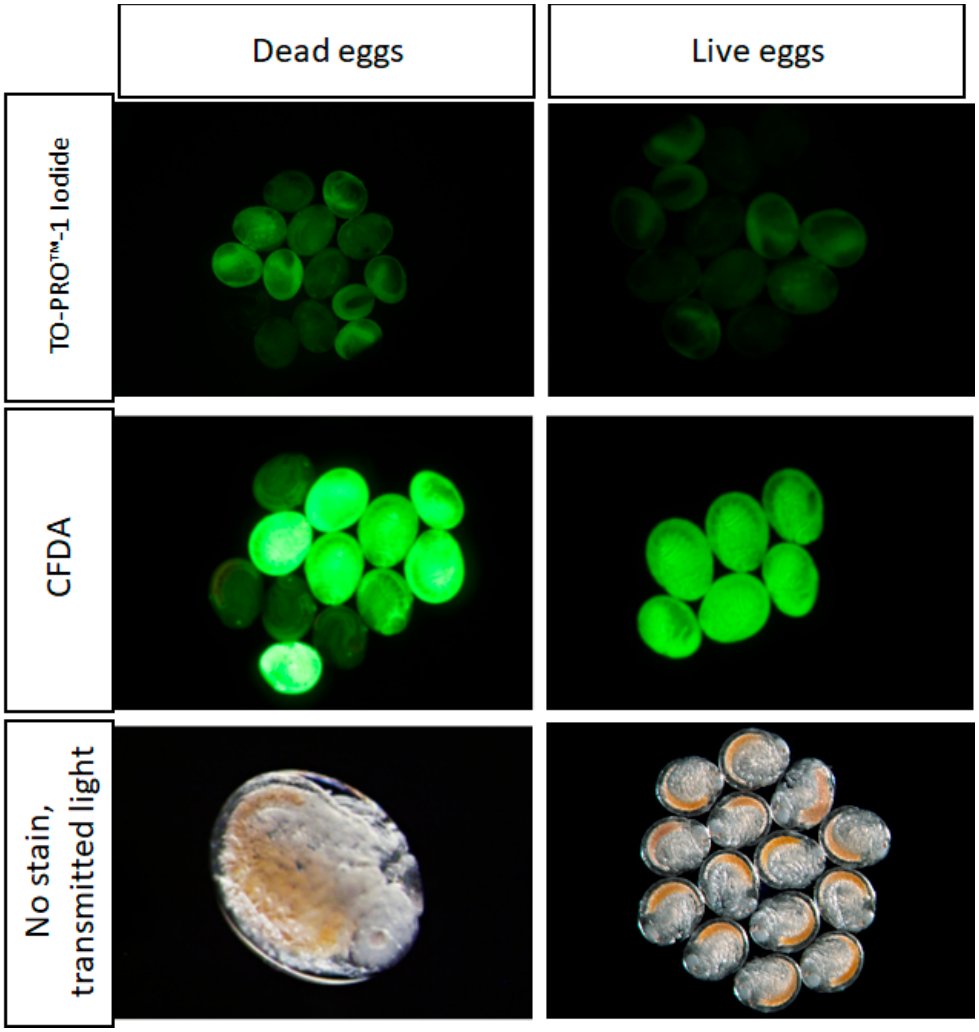

Figure 10. Embryos of the amphipod *Monoporeia affinis* extracted from the brood pouch were stained with TO-PRO-1 Iodide (top panel) and CFDA (middle panel). The bottom panel displays unstained embryos observed under transmitted light, with a heat-killed necrotic embryo appearing on the left and healthy viable embryos on the right.

The dead eggs were generated by submerging at 55–60 °C for 30 minutes; after that, the eggs stayed overnight at 5 °C before staining. Less than 1% of the eggs subjected to this procedure did not develop necrosis over the next 3 days **(Figure 9**), and none recovered after a week, suggesting that they were not viable at the time of the staining.

In the amphipods *Monoporeia affinis*, the dye penetrated well due to a very thin outer membrane (**Table 2**), but resulted in >50% false positives, as dead embryos still retained metabolic activity sufficient for fluorescence activation (**Figure 10**). The heat-killed embryos had sufficient esterase activity for several hours after the heat treatment, resulting in positive staining even though none of these embryos survived, and necrotic changes were apparent the next day. No false negatives were observed for the viable embryos (**Figure 10**; image on the right, middle panel) and no autofluorescence. Thus, the viability status indicated by CFDA was inconsistent across non-viable embryos but consistent across viable ones.

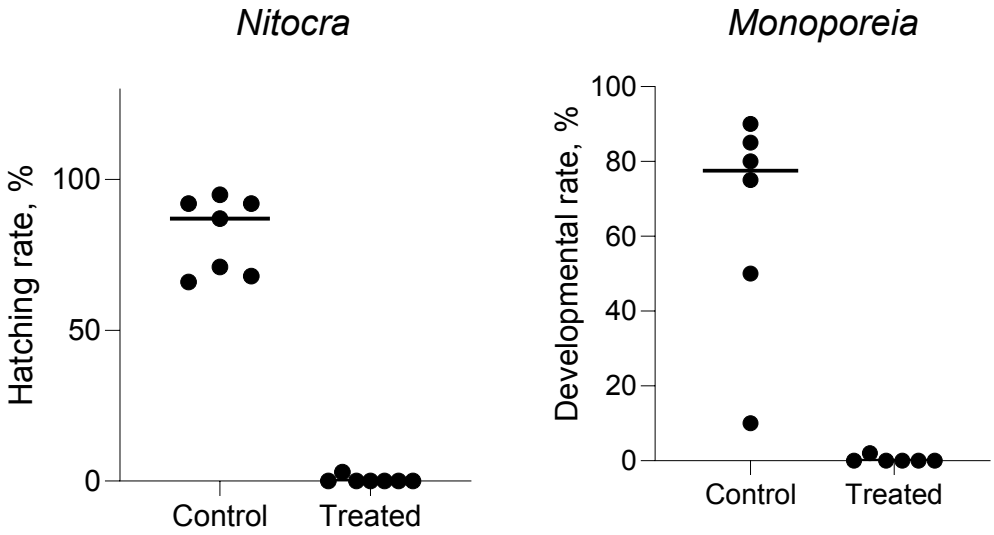

Figure 11. Comparison of hatching rate (%) and developmental progression in untreated (Control) and heat-killed (Treated) *Nitocra spinipes* and *Monoporeia affinis*. For each species, the CDFA and TO-PRO-1 staining was conducted **(Figures 7 and 8)** using the specimens from the same batch. The Treated groups exhibited significantly lower hatching success (****: $p < 0.0001$) and virtually no development over three days.

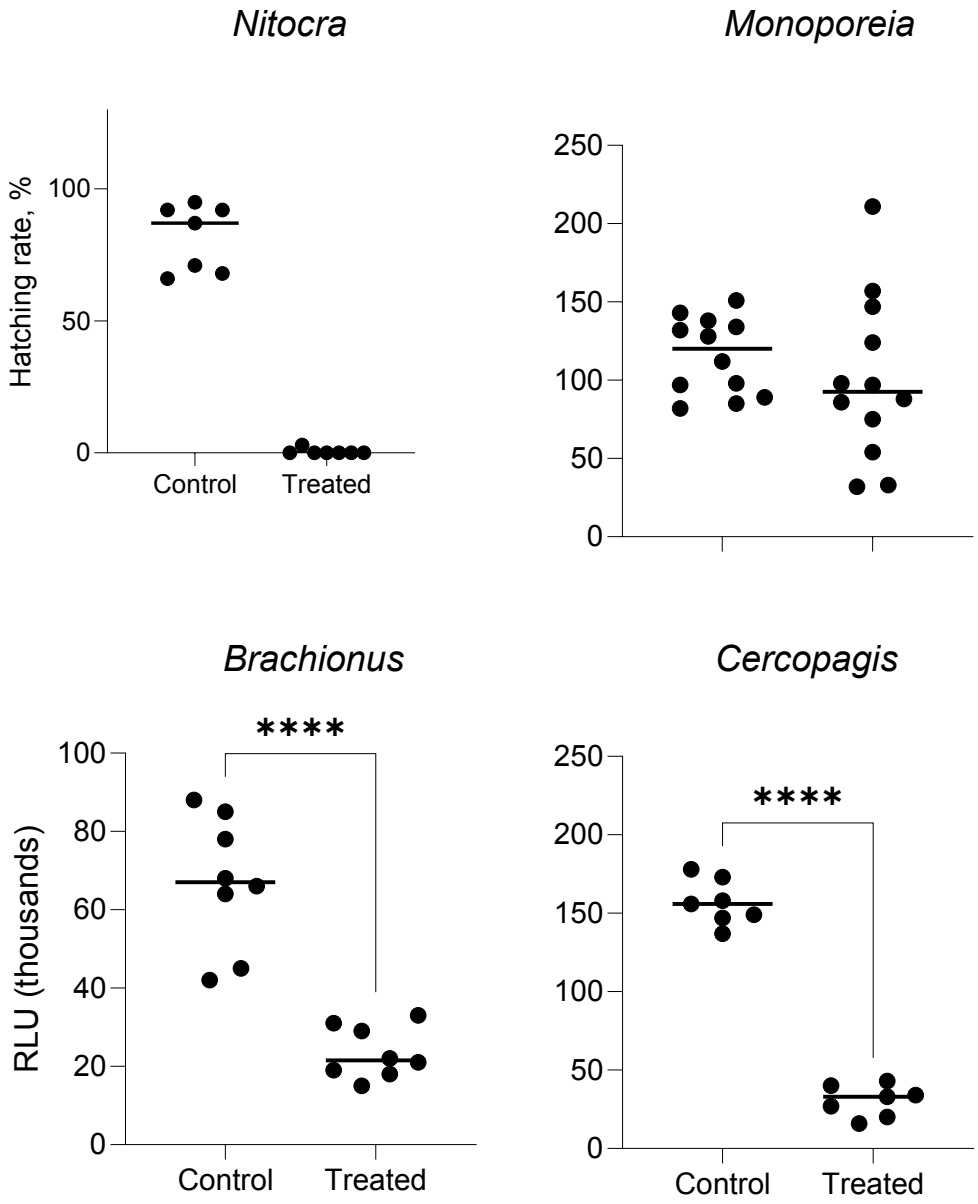

Figure 12. ATP levels (RLU/egg) were measured across different species under two conditions: Control (no treatment applied) and Treated (exposed to 55 °C for 30 minutes or UV light for 12 hours to induce egg mortality). The effect of treatment was assessed using an unpaired t-test, with the significance of differences denoted by asterisks (**: $p < 0.01$; ****: $p < 0.0001$); the horizontal line indicates the group mean.

Hence, CFDA proved effective in staining viable subitaneous copepod eggs and amphipod embryos, with consistent identification of living specimens. However, heat-killed amphipod embryos and copepod eggs exhibited false-positive staining (50% and up to 15%, respectively) due to residual esterase activity and potential autofluorescence. Therefore, while CFDA is a useful tool for viability screening, it is not entirely dependable for distinguishing live from dead specimens, particularly immediately after a disinfection treatment. Thus, these results partially support using CFDA as a vital stain for copepod eggs but not the adults, which

stained clearly while being non-viable. The latter is a valuable observation for zooplankton viability analysis, even though it was not included in the study questions.

Neutral red showed excellent performance in bivalve veligers of *Mytilus* spp. and *Macoma balthica* collected in the Baltic Sea, with >96% of viable individuals stained after 3 h exposure (**Figure 13**). The distinct pink color in live (and swimming) veligers, but not in dead ones was easily observable in light microscope. However, the stain was ineffective in eggs from other taxa, likely due to the absence of lysosomes and impermeable chorion barriers. Its long staining time also limits field use. For example, copepods eggs showed inconsistent staining, with only 10% exhibiting faint dye retention and no clear correlation with hatching rate (data not shown).

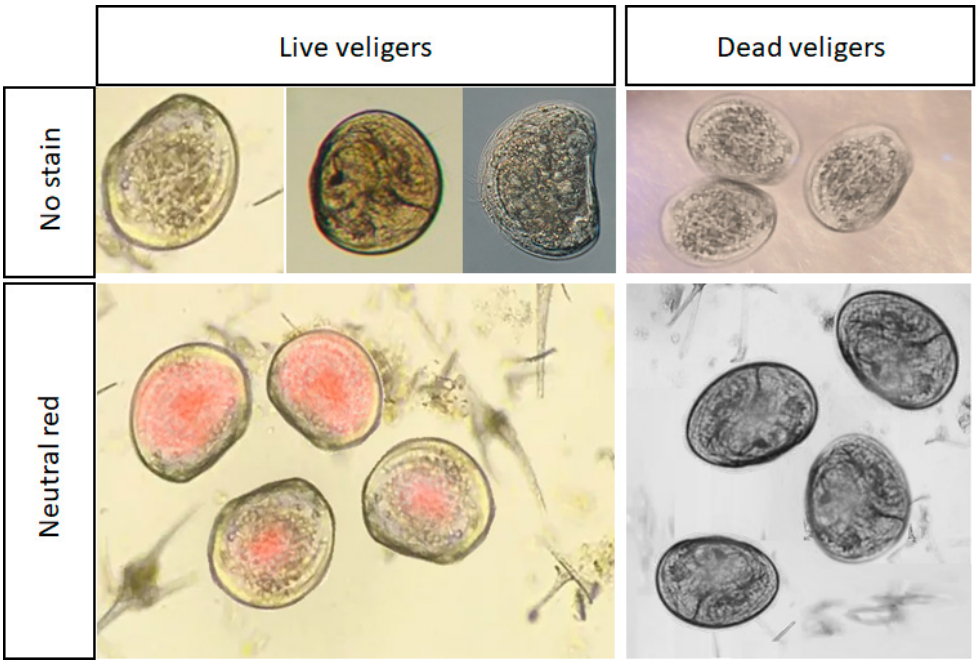

Figure 13. Staining veligers (larval stages of Bivalvia) with Neutral red. Positive staining, albeit with varying intensity was always obtained for live (actively swimming) veligers, whereas no staining was seen for the heat-killed (assigned as dead) specimens. The dye penetration was facilitated in swimming veligers due to the direct contact of the body interiors with the staining medium via semi-open valves.

The efficiency of Neutral red in the veligers can be attributed to their functional lysosomal activity (Repetto et al., 2008), which facilitates dye accumulation in acidic organelles. In contrast, eggs lack a lysosomal system, which likely impedes the dye's ability to accumulate. Additionally, the presence of a protective eggshell or chorion might have acted as a barrier, preventing dye penetration into the egg contents, but this was not possible to verify due to the poor/absent lysosomal activity. These results suggest that Neutral red is suitable for viability assessment in veligers and possibly other feeding and swimming benthic larvae that occur as plankton. However, it is not effective for non-motile, metabolically quiescent stages such as eggs.

Other vital stains such as SYTO 9, MitoTracker, PicoGreen, and DAPI showed low or no staining efficiency in eggs with developed integuments. SYTO 9 did not

distinguish viable from non-viable copepod eggs or amphipod embryos, while MitoTracker (which provides a measure of mitochondrial mass and activity) occasionally produced false-positive staining but failed overall to indicate viability (**Figure 14**). The MitoTracker staining observed in the heat-killed eggs was most likely due to the interaction of the vital stain with the damaged or compromised eggshell rather than actual biological activity within the egg. Such nonspecific binding or adherence of the stain, may create an artifact that appears as fluorescence. No penetration was detected for Hoechst 33342, DAPI, or PicoGreen, even in permeabilized eggs.

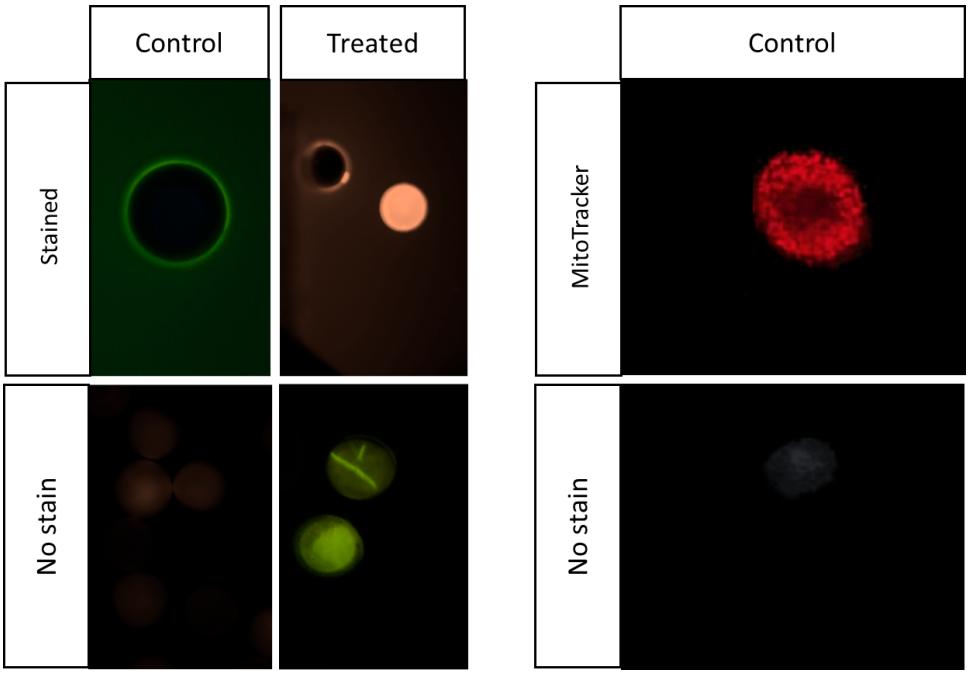

Figure 14. Unsuccessful staining of eggs with intact integuments. Controls are samples not subjected to any treatment. Treated are heat-killed samples.

The aura-like staining is a result of an insufficient penetration of a fluorescent stain (e.g., PicoGreen, Hoechst 33342 and SYTO 9). The reddish coloration for the treated stained egg stained with MitoTracker (a vital stain) is a false-positive result. The proper staining for MitoTracker was observed only for about 30% of the fast-developing copepod eggs (upper left panel).

In summary, CFDA and Neutral red were the most promising vital stains, effective in specific taxa and developmental stages. However, both have limitations: CFDA may give false positives in recently killed specimens, while Neutral red is unsuitable for non-motile stages.

5.3.2 Mortal stains

TO-PRO-1 Iodide showed high fluorescence intensity in heat-killed copepod eggs and amphipod embryos (mean 120.5 ± 5.2 vs. 15.3 ± 2.1 in live eggs; $p < 0.001$; **Figure 15**). It stained >60% of dead eggs with minimal signal in live controls, indicating good specificity. Staining was most effective at 1 µM concentration. While some background autofluorescence was present in chitinous layers **(Figure 14)**, it did

not interfere with microscopy-based evaluation. However, TO-PRO-1 was ineffective in dormant stages, such as ephippia and resting copepod eggs.

Both dead *Nitocra spinipes* females and their egg sacs were stained (**Figure 15**), with the highest intensity for non-viable eggs and a lower female body staining and minimal live (Control) organisms staining. The ability of TO-PRO-1 to effectively penetrate the eggshells of dead eggs likely reflects alterations in membrane integrity upon cell death, allowing the dye to bind nucleic acids. Notably, the fluorescence signal in dead eggs was consistent across treatments, with no significant differences observed between chemically and UV-induced egg death ($p = 0.45$, ANOVA; data not shown)

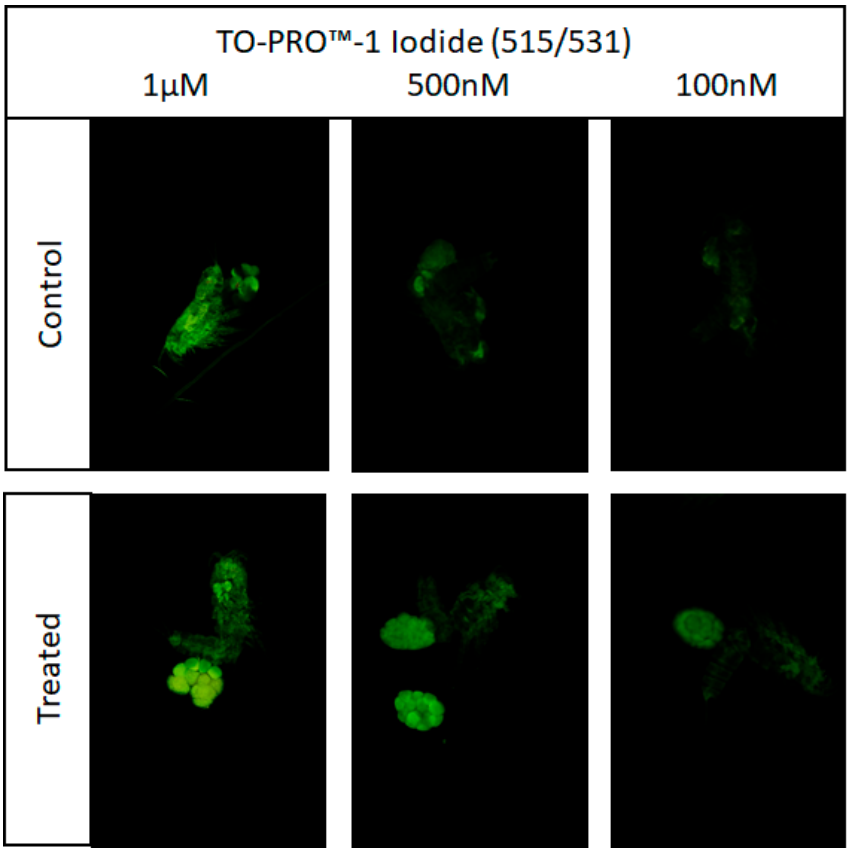

Figure 15. Harpacticoid copepods *Nitocra spinipes* (females with egg sacs) stained with TO-PRO-1 Iodide at different concentrations (shown on top). Control animals are live females from the culture; Treated are the heat-killed animals exposed for 3 min at 90 °C in the water bath. The optimal test concentration was 1 µm as indicated by the bright, consistent fluorescence, while live controls showed minimal staining across all concentrations tested.

The results suggest that TO-PRO-1 is a reliable marker for identifying non-viable eggs (**Table 9**). However, its inability to stain eggs with well-developed integuments, such as ephippia and dormant copepod eggs, underscores its limitations. Moreover, due to the excitation at ~500 nm, some autofluorescence of the copepod chitinous exoskeleton was always present (**Figure 16**), although it did not present a problem for the microscopy-based analysis, because its intensity was comparable to that in the live (Control) samples.

Table 9. Staining efficiencies for TO-PRO-1 Iodide using test samples containing heat-killed copepods (Dead; 100%), controls (Live; 100%), and a mixed sample consisting of 50% live and 50% dead specimens. The staining outcome was determined as % of individuals displaying a definitive bright green fluorescence.

	%	Stained	Not stained
Dead	100	94	5.9
Mixture	50/50	55	45
Live	100	25	75

Aniline Blue, applied via transmitted light microscopy, stained dead rotifer and copepod egg sacs with strong contrast and minimal (<10%) false positives (**Figure 17**). The stain exhibited some selective affinity for egg sacs, but penetration into the eggs required extended staining times to achieve consistent and visible coloration. Moreover, the bodies of the heat-killed copepods displayed heterogeneous staining, with some regions staining more intensely than others, likely due to variability in tissue permeability. Also, live individuals showed some internal coloration in the mouth and gut area due to ingestion of Aniline Blue crystals during the staining period, but these locations did not interfere with egg viability assessment.

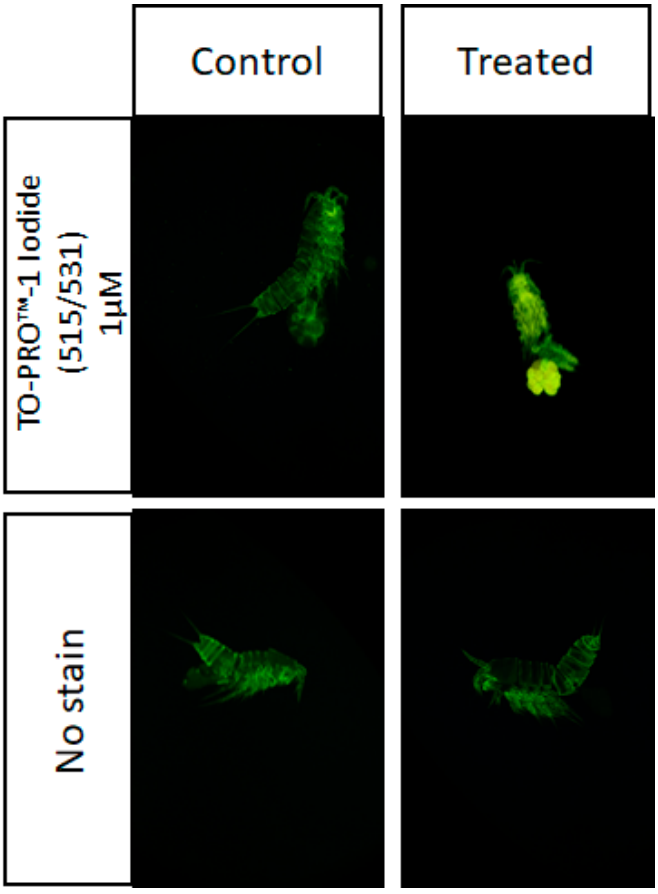

Figure 16. TO-PRO-1 Iodide staining of the Controls (live females with egg sacs) was significantly lower than that of the heat-killed females and statistically indistinguishable from the autofluorescence in the non-stained individuals regardless of their viability status. The egg sac autofluorescence was generally lower than the body fluorescence of the female.

The intensity of Aniline Blue staining in egg sacs was quantified using image analysis to compare heat-killed (treated) and live (control) eggs of *Nitocra spinipes*. Mean stain intensity values (arbitrary units, AU) were significantly higher in the egg sacs of heat-killed individuals compared to live controls (mean ± SD: 150 ± 20 AU vs. 30 ± 10 AU, respectively; $p < 0.001$, t-test). This indicates a strong affinity of Aniline Blue for egg sac structures in non-viable eggs, likely due to increased permeability or altered structural integrity following the heat treatment. In live individuals, the low mean intensity was consistent with negligible staining, though some variation was observed due to light conditions in the field of view and staining in the gut region close to the egg sacs.

For rotifer eggs, mean stain intensity values were also significantly higher in the heat-killed samples compared to live controls (45 ± 13 AU vs. 21 ± 8 AU, respectively; $p < 0.05$, t-test). This indicates a weaker affinity of Aniline Blue for this type of egg compared to the *Nitocra* egg sacs, possibly due to lower permeability or protein content of the rotifer eggs.

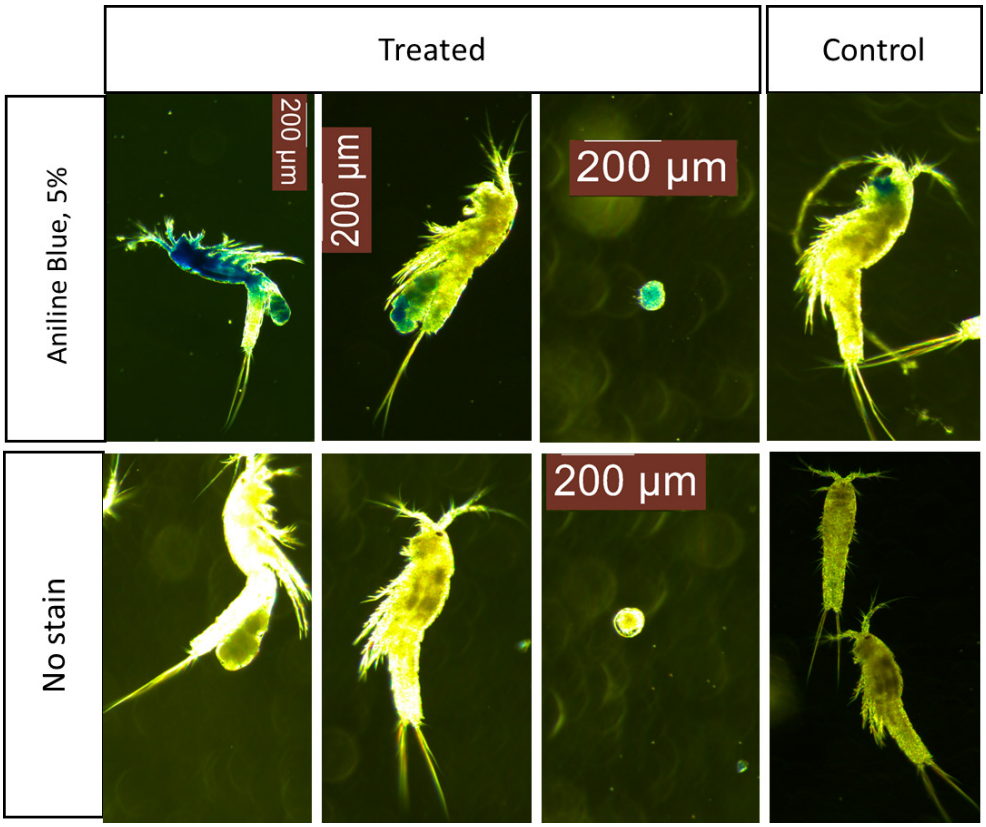

Figure 17. Staining of heat-killed (treated) and live (control) *Nitocra spinipes* and rotifer *Brachionus plicatilis* with 5% Aniline Blue. Egg sacs in heat-killed individuals stained prominently blue, demonstrating the selective affinity of Aniline Blue for egg sac structures. In contrast, control or non-stained individuals showed no visible staining, except for the mouth region (top right panel) suggesting ingestion of the Aniline Blue crystals during the staining procedure. Single eggs (rotifers; the right panel for the Treated group) also showed a clear staining.

These findings highlight both the potential and limitations of Aniline Blue staining for eggs, particularly in distinguishing viable eggs. Under fluorescence mode, however, signal intensity was similar in the treated and control samples, suggesting that fluorescence-based detection is unsuitable for Aniline Blue. Further optimization of staining protocols might improve staining homogeneity and accuracy.

Other mortal stains tested, including Propidium Iodide, SYTOX Green, Trypan Blue, and 7-AAD, failed to penetrate egg integuments, even in heat-killed specimens. Their molecular properties (e.g., size, charge, hydrophilicity) likely prevented diffusion through dense, cross-linked structures like ephippia or diapausing eggshells. Additionally, the TUNEL assay, although useful in detecting apoptotic cells, was not applicable to eggs due to the large size of reagents and complex protocols. No signal was observed in the few tests conducted, and the method was excluded from full-scale evaluation.

In conclusion, TO-PRO-1 and Aniline Blue were the most effective mortal stains, suitable for identifying dead individuals in taxa with permeable integuments. Their use, however, remains limited in stages with highly developed protective structures.

5.4 Comparison with BallastWISE system

To assess the applicability of fluorescence-based viability assessment under Port State Control (PSC) conditions, the best-performing stains (CFDA, TO-PRO-1 Iodide, and Aniline Blue) were evaluated using the BallastWISE system. While BallastWISE captured the general fluorescence trends observed in microscopy for CFDA and TO-PRO-1 Iodide, its diagnostic accuracy was limited by current filter settings and background autofluorescence, highlighting areas for targeted technical improvements.

5.4.1 Instrument modifications implemented for the VIABLEGG project

A series of modifications were introduced to the BallastWISE system to enable viability assessment of non-motile invertebrates. Originally designed for detecting autofluorescent plankton, the instrument uses a 420 nm excitation source and a 590 nm high-pass emission filter (**Figure 2**). This setup was suboptimal for detecting CFDA and TO-PRO-1 Iodide, which emit in the green spectrum (510–540 nm), i.e., largely outside the detection range of the system's default filter. The system's grayscale camera further limited the interpretation of colour-specific features that could otherwise help detect Anilin Blue staining under standard microscopy.

To improve compatibility, a second excitation channel at 500 nm was implemented during the project. This allowed better alignment with the excitation spectra of CFDA and TO-PRO-1. However, the existing emission filter (≥590 nm) could not be replaced within the test setup, which meant that green fluorescence (i.e., where these stains emit the most) was only partially captured. As a result, some of the emitted signal was visible due to spectral bleed-through, but much of it was attenuated or shifted, reducing contrast and diagnostic clarity.

Despite this constraint, the system detected general differences in fluorescence intensity between live (CFDA) and dead (TO-PRO-1) specimens, indicating that

the core staining principles are compatible with automated imaging. The fact that these patterns were still discernible under suboptimal filter conditions is highly promising and shows the instrument's potential for adaptation.

In the context of Port State Control (PSC), where sample volumes often exceed 50 mL and results are needed rapidly, BallastWISE offers clear advantages in terms of real-time analysis. To fully realize its potential, two further modifications are recommended: (1) Integration of alternative or interchangeable emission filters, particularly in the 480–550 nm range, to support stains with green fluorescence, and (2) Optimization of sample throughput and flow paths to handle larger volumes efficiently while maintaining resolution and object recognition. While it is not possible to modify or remove the 590-nm filter during analysis, any additional high-pass filter could be installed and used. With these improvements, BallastWISE could be effectively adapted for high-throughput viability screening in PSC settings.

5.4.2 Stain performance in the BallastWISE evaluations

The performance of viability dyes, CFDA (vital stain) and TO-PRO-1 Iodide (mortal stain), varied considerably between laboratory microscopy and BallastWISE evaluations, underlining both the potential and the current limitations in using automated systems for viability assessments of non-motile stages. Both dyes showed a potential for applications in PSC; however, the BallastWISE system's optical setup, sample characteristics, and fluorescence background affected both the signal intensity and separation between live and dead specimens.

Vital stain: CFDA showed strong discrimination between live and dead specimens in the microscopy-based evaluations, with over 90% staining efficiency and ~12-fold higher fluorescence in live organisms, aligned with its enzymatic activation. However, in BallastWISE, this contrast was reduced to a 1.6-fold difference, with high variability caused by sample condition, integument barriers, and suboptimal detection.

Similarly, TO-PRO-1 Iodide showed a 10-fold difference in fluorescence under microscopy, but only a 3-fold difference in BallastWISE, again with overlapping signal ranges. Aniline Blue, although effective under visible light microscopy, could not be evaluated properly in BallastWISE due to spectral mismatch. Additionally, background autofluorescence, especially in treated or structurally complex eggs, further complicated automated detection. These results underscore the importance of optimized filter settings, stain-specific protocols, and possibly hybrid methods to ensure accurate viability assessments in regulatory contexts.

The fluorescence responses of stained samples analyzed using the BallastWISE system demonstrated considerable variability (**Figure 19**). This variability indicates challenges in reliably distinguishing between live and dead organisms (**Figure 20**), limiting the definitive conclusions about stain efficacy. Below is an assessment of the results for each stain.

No-Stain control. Fluorescence intensities in no-stain controls were similar for live (27 ± 0.2) and dead (28.5 ± 16.5) samples, highlighting background autofluorescence as a serious issue. The presence of significant autofluorescence in the test organisms, with particularly variable values in the dead specimens, complicates the interpretation of fluorescence intensity attributed to stains. Therefore,

autofluorescence must be accounted for in all analyses to ensure stain-specific signals are correctly interpreted.

CFDA. Live organisms exhibited higher relative fluorescence intensity (59.6 ± 40.7) compared to dead organisms (25.5 ± 7.5). However, the wide variability in fluorescence intensities among live samples complicates the differentiation between live and dead groups. Therefore, while CFDA fluorescence mechanism theoretically aligns with its role as a vital stain, the inconsistent intensity values across samples suggest challenges in reliably using this stain for live organism detection in this setup. Factors such as stain uptake efficiency, organism handling, and environmental conditions likely contribute to the observed variability.

TO-PRO-1 Iodide. Dead organisms had >4-fold higher fluorescence intensity (36 ± 20.6) compared to live organisms (8.5 ± 8.5). However, there was substantial overlap between live and dead samples, reducing confidence in TO-PRO-1's ability to consistently differentiate viability states. Therefore, although TO-PRO-1 theoretically targets nucleic acids in dead organisms, its performance in this experiment was inconsistent, likely due to variability in sample staining or imaging conditions. Improvements in staining protocols and/or optical system adjustments may improve the selectivity.

Challenges in the BallastWISE setup

The BallastWISE system employs a 590 nm high-pass filter, which alters the detection of fluorescence signals for stains such as CFDA and TO-PRO-1 Iodide, whose emission maxima are in the green spectrum (520 and 533 nm, respectively). This filter blocks most green-light emissions, allowing red light to dominate in the captured images. As a result, stains that appear bright green in laboratory fluorescence microscopy tests are detected as red signals by BallastWISE, reflecting the spectral cutoff imposed by the filter rather than the stains' true emission properties (**Figure 18**). This shift complicates direct comparisons between BallastWISE and laboratory results and highlights the need for filter optimization to better match the emission spectra of these stains for improved fluorescence-based assessments.

Aniline Blue. The fluorescence intensities for both live and dead specimens were similar to the autofluorescence levels observed in the no-stain controls, although live specimens appeared to have a stronger signal. The mean fluorescence for Aniline Blue-stained dead organisms (4.5 ± 4.5) and live organisms (11.5 ± 8.2) was comparable to the background fluorescence in no-stain samples (27 ± 0.0 for live and 28.5 ± 16.5 for dead specimens.)

Challenges in the BallastWISE setup

In the laboratory, Aniline Blue was evaluated using light microscopy under transmitted light, which relies on visible color changes corresponding to the stain's absorbance spectrum. This approach does not involve fluorescence and was effective in the manual evaluation of stained specimens. The stain's specifications, however, indicate that it can also be used for fluorescence-based detection with UV excitation.

The BallastWISE instrument is equipped with a black-and-white camera, making the transmitted light approach impractical for Aniline Blue. Black-and-white imaging cannot differentiate the color variations required to interpret the stain's absorbance spectra. As a result, fluorescence-based detection was tested instead, utilizing the system's existing fluorescence excitation and detection settings.

The incompatibility of Aniline Blue with the 590 nm high-pass filter was evident, as fluorescence signals were weak and did not align with expected staining outcomes. A lower cutoff filter (e.g., 480 nm) is necessary to evaluate Aniline Blue's true potential as a mortal stain.

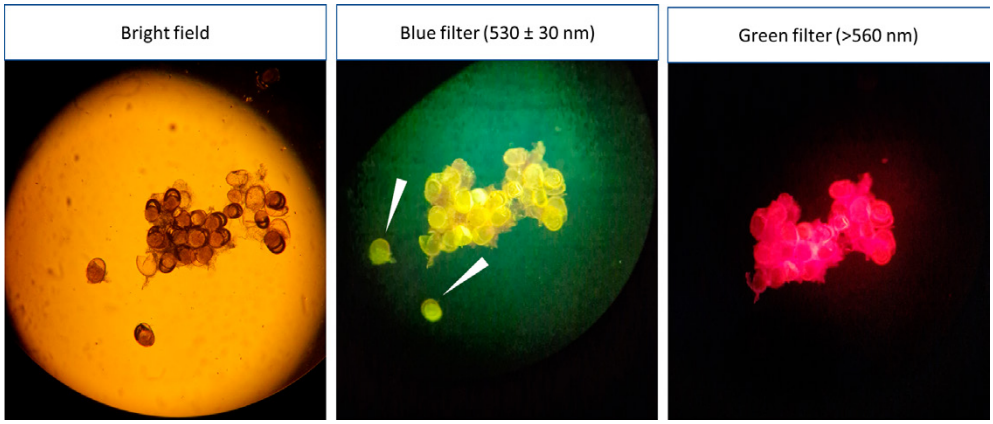

Figure 18. Rotifer eggs stained with TO-PRO-1 and visualized under a microscope using different filters to highlight staining patterns. The left panel shows the eggs under bright field illumination, where their overall structure and arrangement are visible. The middle panel displays the eggs using a blue filter (530 ± 30 nm), highlighting the fluorescence emitted by TO-PRO-1 (emission peak 533 nm) in permeabilized cells. The right panel uses a green filter (>560 nm), capturing the dominant red fluorescence signal emitted by TO-PRO-1 in the red part of the spectra. These images demonstrate the filter-dependent visualization of TO-PRO-1 staining. The arrowheads in the middle panel indicate two eggs that are mostly visible as green but absent under the green filter because the red part of the emitted light spectrum is not sufficient to be visible (the right panel).

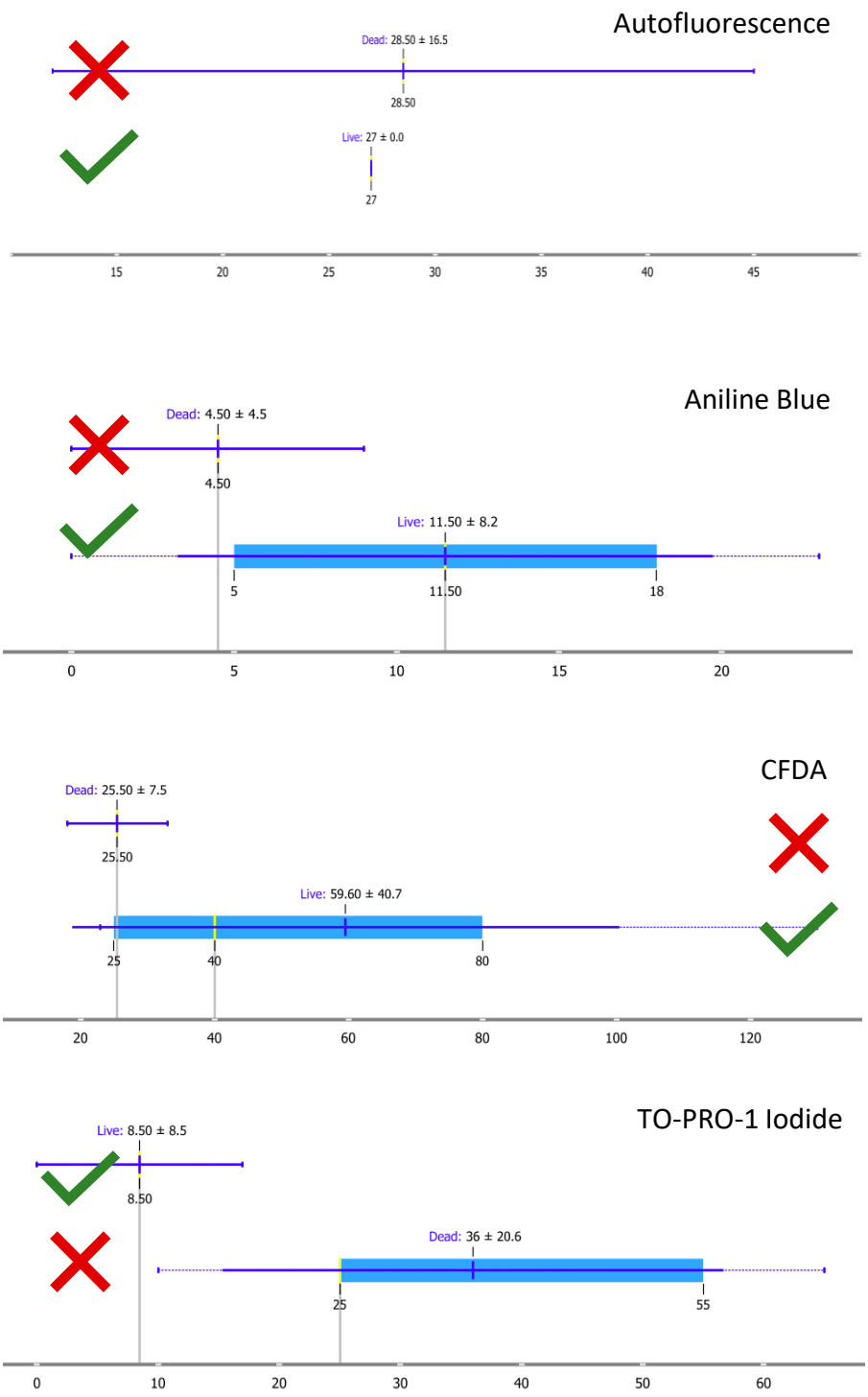

Figure 19. Relative fluorescence intensity (x-axis) for the test stains (Aniline Blue, CFDA, and TO-PRO-1 Iodide) applied to the copepod and cladoceran samples and for the non-stained samples (i.e., autofluorescence). In the plots, the red cross (X) indicates data points for dead specimens, while the green checkmark (✓) represents live specimens. The data are shown as box-and-whiskers plots with 25% and 75% variability, mean and median values indicated by vertical lines, and min and max values indicated by the horizontal lines.

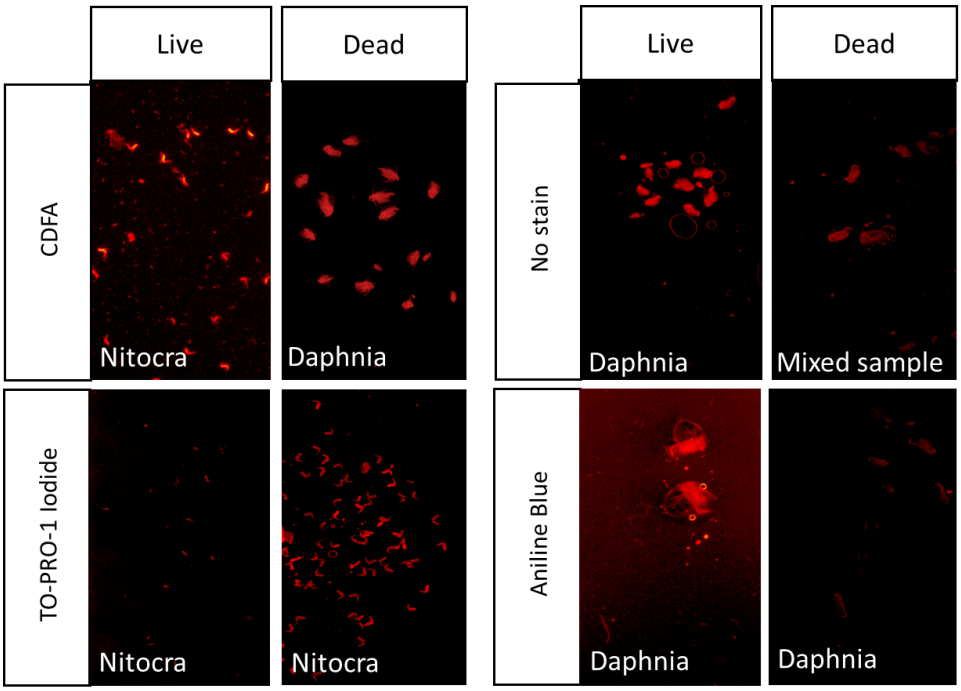

Figure 20. Images of live and dead zooplankton (*Nitocra spinipes*, *Daphnia magna* and their mixture) specimens stained with CFDA, TO-PRO-1 Iodide, or Aniline Blue, and corresponding no-stain controls, acquired using the BallastWISE system. Each live/dead pair was imaged under the same excitation wavelength (420 or 500 nm). The CFDA and TO-PRO-1 Iodide stains produced distinguishable fluorescence patterns between live and dead specimens, whereas Aniline Blue fluorescence and no-stain controls showed overlapping signals, reflecting challenges with background autofluorescence and filter incompatibility. For this selection, the most contrasting live/dead images were used. The actual variability in the staining intensity is shown in **Figure 18**.

6. Conclusions and recommendations

The VIABLEGG project has made important progress in addressing a complex and pressing issue in ballast water management: the viability assessment of non-motile invertebrate life stages. Through a comprehensive evaluation of staining methods, sample preparation techniques, and analytical tools, the project has generated a unique dataset and practical knowledge that serve as a solid foundation for future method development and policy refinement.

We established a solid experimental foundation, tested a broad range of staining methods, and produced critical insights into the biological and technical constraints that hinder the development of universal viability assessment tools. The systematic evaluation of stains and sample preparation protocols, as well as the integration of microscopy, metabolic indicators, and high-throughput systems such as BallastWISE, represent important methodological advances.

However, the project also confirmed that achieving a universally applicable and reliable viability assay for hard-shelled, non-motile stages is not currently feasible. While CFDA and TO-PRO-1 Iodide performed well for some taxa, no stain provided reliable results across all tested life stages. The wide variability in integument structure and permeability, combined with the low metabolic activity of many resting stages, poses significant obstacles to both staining and detection. Even the most promising stains, such as CFDA and TO-PRO-1 Iodide, showed inconsistent performance across taxa and were ineffective for encysted or diapausing stages. The need for extensive pre-treatment to improve stain penetration often compromised organism viability or introduced artefacts.

These findings underscore the complexity of the problem and the limitations of current tools, but also provide a clearer direction for future work. Crucially, they confirm that the development of effective vital staining methods is essential for reliably detecting viable non-motile stages—something that current morphological or ATP-based proxies cannot achieve. Rather than seeking a universal solution, research and regulatory efforts should focus on species- or group-specific viability assays based on vital stains, developed with realistic expectations of their applicability in port state control contexts and regulations.

6.1 Necessity of vital stains for compliance with Regulation D-2 requirements

Regulation D-2 under the BWMC requires accurate quantification of viable organisms in ballast water to ensure compliance and mitigate biological invasions. The use of mortal stains to identify non-viable organisms, while useful for evaluating, e.g., treatment efficacy, does not provide sufficient evidence to meet the regulatory requirement of determining **viable organism concentrations.** Theoretically, the difference between the total count of organisms before staining and the count

of non-viable specimens after applying a mortal stain could imply the number of viable organisms. However, this approach faces several practical and regulatory challenges (Outinen et al., 2024) that undermine the feasibility of using solely mortal stains for compliance testing:

- *Inadequate evidence of compliance*: Regulation D-2 explicitly focuses on the quantification of viable organisms. Identifying non-viable specimens does not provide direct information about the viable population, leaving compliance unverifiable.
- *Regulatory and operational constraints*: The interpretation of non-viable counts risks creating ambiguity in compliance assessments. Regulatory bodies require clear evidence of viable organism counts to ensure enforcement is consistent and defensible.
- *Practical limitations*: Approaches relying on total counts and subsequent staining are operationally complex, requiring precise equipment and handling. Such complexity is impractical for field testing, where methods must be rapid, simple, and robust.

Given these limitations, the development of direct methods for quantifying only viable organisms remains essential.

6.2 Specific recommendations

The VIABLEGG project has clarified the scale of the problem and offered practical advances for selected taxa, even if a universal method remains out of reach. These results should inform both the refinement of existing protocols and the design of future solutions within the broader context of ballast water management. Moreover, promising methods should be tested across different laboratories and environmental conditions to ensure reproducibility and robustness.

6.2.1 For research

Prioritize taxa-specific method development: Given the significant structural and biochemical diversity among non-motile life stages, especially in resting eggs and encysted forms, future efforts should move away from the pursuit of universal methods. Instead, viability assessment tools should be developed for specific taxa or functional groups that are particularly relevant for ballast water monitoring and management. Additionally, methods like the osmotic imbalance technique (Zirbel et al., 2007) allow for staining nuclei in formalin-preserved copepod eggs using semi-permeant dyes like DAPI and PicoGreen without destroying the chorion, which can provide a testable approach in the search for the stain carriers and other methods facilitating eggshell penetration.

Improve and diversify staining protocols: Research should focus on optimizing combinations of pre-treatment and staining protocols that enable reliable viability detection without compromising sample integrity. This includes refining enzymatic and chemical treatments to enhance permeability in a controlled and reproducible manner and reducing background autofluorescence that interferes with stain signal detection. Using multiple stains simultaneously as well as new stain-delivery approaches, such as nanoparticles (Shin et al., 2022), a more com-

prehensive assessment of cell diversity in terms of viability within a sample can be gained. For example, using both TO-PRO-1 and CFDA can provide complementary information about cell viability based on membrane integrity and metabolic activity (Gorokhova, 2010; Gorokhova et al., 2012).

Explore alternative viability indicators: In addition to staining, other biomarkers such as metabolic enzyme activity or stress protein induction could offer more sensitive and stage-relevant indicators of viability, provided that rapid assays are available for PSC surveys. These should be explored with the aim of integration into practical, field-adaptable formats.

Advance automated and semi-automated analysis: High-throughput image analysis tools, such as those embedded in systems like BallastWISE, should be further developed to quantify staining responses objectively. Algorithms tailored to recognize specific taxa and viability-associated fluorescence patterns could reduce operator bias and improve standardization.

6.2.1 For port control of ballast water

Integrate viability stains into compliance monitoring where possible: Although viability stains such as CFDA and TO-PRO-1 Iodide do not provide reliable diagnostics across all taxa and egg morphologies – particularly for diapausing or encapsulated stages – they represent a valuable addition to the suite of indicative analysis methods currently used in port state control (as discussed in Section 3.4). When applied to taxa where stain performance has been validated, these methods can support rapid, operationally feasible assessments of viability and help identify high-risk samples that warrant further investigation. Their use should be encouraged as a practical compromise between speed, accessibility, and diagnostic value, with clear guidance on interpretation and limitations.

Develop practical workflows combining stains and imaging: Semi-automated protocols combining quick staining, microscopy or imaging, and classification tools can offer a compromise between feasibility and accuracy. Pilot implementations at selected ports could help define operational protocols and logistical needs.

Train inspectors in taxon-specific recognition and interpretation: The effectiveness of any stain-based method depends on the ability to recognize target life stages and interpret viability signals. Training and guidance materials should be developed for inspectors to enhance consistency and confidence in results.

Set realistic thresholds and expectations using a risk-based, taxon-informed approach: Given the current limitations in assessing viability across all taxa, regulatory frameworks should recognize that full diagnostic certainty is not always possible. Instead, ballast water inspections should:

- Apply **species-specific interpretation criteria**, using validated stains only where performance is reliable.
- Implement **flag-and-follow-up protocols**, where uncertain results trigger secondary checks rather than automatic enforcement.
- Define acceptable uncertainty ranges, acknowledging which taxa cannot yet be reliably assessed.

- Use **biological risk profiles** to guide inspection intensity—for example, prioritizing water from high-risk regions.
- Allow flexibility in compliance protocols, updating them as new methods are validated.

6.2.1 For regulatory bodies

Support method development through targeted funding and coordination: Regulatory agencies should actively support method development focused on high-risk taxa and encourage collaborative efforts between researchers, industry, and technology developers. Encourage the adoption of high-throughput systems like BallastWISE in port state inspections and ensure these systems are equipped with flexible configurations to accommodate diverse stains to selectively detect viable taxa and life stages.

Update guidance documents and protocols: Current regulations should be revised to reflect the known limitations for non-motile stages and to include recommendations for using complementary methods such as vital staining**.** Regulation D-2 should account for the limitations of current viability assessment methods, particularly for non-motile life stages. Current regulations, such as those under the BWMC, rely on size-based thresholds for viable organisms. However, as demonstrated by the VIABLEGG project, the viability of eggs with the same appearance and within the same size class can differ drastically.

Consider interim criteria for compliance: In recognition of methodological constraints, interim assessment protocols or decision frameworks could be adopted for non-motile stages, using a weight-of-evidence approach that combines staining, morphological, and metabolic indicators.

Promote international harmonization of methods: To ensure consistency in compliance and facilitate data comparability across ports and regions, international coordination is essential. Organizations such as HELCOM, IMO, and ICES should be engaged to:

- **Establish shared protocols and a centralized database** of validated methods for the use of viability stains and pretreatment methods, tailored to key taxa.
- Develop **intercalibration exercises** and **joint training programs** between national inspection authorities to ensure reproducibility and alignment.

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9. Publications and data availability

9.1 Peer-reviewed publications

Blackburn, N., Haecky, P., Jurgensone, I., Griniene, E., Brugel, S., Andersson, A., Carstensen, J., 2022. The use of an automated organism tracking microscope in mesocosm experiments. *Limnol. Oceanogr. Methods* 20.

Gorokhova E., El-Shehawy, R, Luecke-Johansson, S., Viinamäki, E. (2024) Viability assessment of pelagic eggs stained with fluorescent dyes: toward improved analysis of live organisms in ballast water. Preprint.

9.2 Master theses

Sara Sundin, 2023. Inventering av infärgningsmetoder för LIVE/DEAD-analys av ägg och larver hos ryggradslösa djur: Hur kan vi upptäcka livsdugliga organismer i barlastvatten? MSc Thesis, Stockholms universitet, 55 pp.

9.3 Scientific conference presentations

Haecky P., Blackburn N. (2024) Compliance monitoring of ballast water treatment systems. IMAREST Marine Biosecurity Symposium 2024. https://www.imarest.org/resource/mp-ballast-water-and-biofouling-at-the-2024-biosecurity-symposium.html

Haecky P. (2024) Ballast Water Management Convention Implementation. European Maritime Safety Agency (EMSA), SEA EUROPE Workshop, Lisbon, March 2024

Blackburn N. (2020) The use of an automated organism tracking microscope in a mesocosm experiment. EcoChange Conference 2020, Umeå University

Gorokhova E., Sundin S. (2022) Assessing invertebrate egg viability in ballast water: Challenges and more challenges, 24th Ballast Water Management Conference, 16th March 2022 - 17th March 2022, Rotterdam - The Netherlands. Poster presentation.

9.4 Stakeholder presentations

Gorokhova E. Informal Consultation Session of the Expert Group, Finnish Transport and Communications agency. Online meeting - February 7-8, 2023. Presentation of VIABLEGG and discussion on viability testing.

Gorokhova E. Assessing invertebrate egg viability in ballast water (presentation); JEG: OSPAR/HELCOM Joint Expert Group on Non-Indigenous Species, February 2022.

Gorokhova E. Consultations to Transportstyrelse, Sjö- och luftfart, Sektionen för internationell samordning. IMO/MEPC 82 & ISWG-GHG 17 (2024-09-11), Typgodkännande BWTS vid MEPC 82 och Baltic SIREN (2024-08-11).

9.5 Open access data

All data generated during the project are openly available on Zenodo (zenodo.org) and can be accessed using the following DOI: 10.5281/zenodo.15762932.

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Toward reliable viability assessment for resilient life stages of invertebrates in ballast water

This report presents results from the VIABLEGG project, which made important progress in identifying viable eggs and resting stages of invertebrates in ballast water.

The findings show that some methods work well for certain species, offering practical value for inspections at ports. At the same time, the project provides new insight into why some life stages are difficult to assess and what biological and technical challenges must be addressed. The report offers concrete suggestions for future research, practical application, and the need for international coordination. These results lay a valuable foundation for efforts to prevent the spread of invasive species through global shipping.

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