Hull biofouling

An ecological and ecotoxicological approach for common suitable management and policy strategies

Mar Santos Simón

International PhD Thesis

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" The ocean keeps refusing
to stop kissing the shoreline "

- Sarah Kay

And just like the ocean, tenacious musician and sonorous on its creations, science seeks answers that need to be sung.

And just like the ocean, persistent on its motion, nature, stubborn as it is, unavoidably comes across, even to the most unforeseen hideouts.

And just like the ocean, bound and determined on its sole purpose of eternal caresses, you, my greatest certainty, infallibly guide me through the heavy sea to the gentle shore.

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Key definitions

Antifouling system = any method, coating, paint, treatment, surface or device that aims to control or prevent the attachment of unwanted organisms on artificial substrates. The antifouling system(s) of choice, together with a planning (that can be more or less elaborated or fulfilled) and the practice itself, make up an **antifouling strategy**.

Biofouling = unwanted settlement and growth of organisms on artificial hard surfaces exposed to aquatic environments.

Coating = an external layer that covers a surface. It can be a paint, but not only.

> *Traditional coating* = an antifouling coating containing biocides that leach to the environment. Conventionally, biocides were embedded in insoluble matrixes, but to optimize their efficiency, the matrix types have evolved. In this thesis we refer to an ablative coating (learn more in the General Introduction)
>
> *Alternative coating* = an antifouling coating qualified as non-toxic that substitutes traditional biocides by natural compounds or based on different action mechanisms, such as physical ones. In this theses, we refer to a foul-release coating (learn more in the General Introduction).

Maintenance practice = an adopted behaviour to keep the surface of interest (including coating status) in good condition, with minimum fouling levels.

Niche area = areas on a vessel particularly susceptible to biofouling colonization due to different hydrodynamic forces, inaccessibility to proper coating and maintenance, exposure to wear and damage of coatings, etc. They are considered a hotspot for biofouling accumulation.

Paint = a particular type of coating, usually a coloured liquid that may content other additives or active compounds.

Recreational boat = refers to boats with a hull length of 2.5 – 24 m that are intended for leisure or sport use.

List of abbreviations

ABFMR = Australian Biofouling Management Requirements

AFMA = Australian Fisheries Management Authority

AFM = Antifouling measures

AFS = International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention, 2001)

APP = Antifouling paint particles

BC = Biocide-based coating

BFMP = Biofouling Management Plan (BFMP)

BFRB = Biofouling Record Book (BFRB)

BMCC = Basque Microalgae Culture Collection

BPD = Biocidal Products Directive (BDP; Directive 98/8/EC)

BWM = International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Covention, 2004)

C = Control

CBD = Convention on Biological Diversity (CBD, 1992)

COP = Conference of the Parties

CRMS = Craft Risk Management Standard

CSLC = California State Lands Commission

DAFF = Department of Agriculture, Fisheries and Forestry (Australia)

DEPA = Danish Environmental Protection Agency

EEZ = Exclusive Economic Zone

EPA = Environmental Protection Agency

ECHA = European Chemicals Agency

EIA = Environmental Impact Assessment

FR = Foul-release coating

GHG = Greenhouse gas

GI = Growth inhibition

IMO = International Maritime Organization

IWCC = In-water cleaning and capture

M = Maintenance

MARPOL = International Convention for the Prevention of Pollution from Ships (MARPOL, 1973)

MARS = Maritime and Aircraft Reporting System

MEPC = Marine Environment Protection Committee

MPI = Ministry of Primary Industries (New Zealand)

MSAS = Member State Audit Scheme

MSFD = Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC)

NCV PAR = Non-commercial vessel pre-arrival report

NIS = Non-indigenous species

NM = No maintenance (unmaintained)

NMA = Norwegian Maritime Authority

OECD =Organization for Economic Cooperation & Development

PAR = Pre-arrival report

PVC = Polyvinyl chloride

REACH = Registration, Evaluation, Authorisation and Restriction of Chemicals (Regulation (EC) No 1907/2006)

RIS = Regulation Impact Assessment

TBT = Tributyltin

UNCLOS = United Nations Convention on the Law of the Sea (UNCLOS, 1982)

UNEP = United Nations Environment Programme

WCN = World Charter for Nature (WCN, 1982)

WFD = Water Framework Directive (WFD, Directive 2000/60/EC)

General abstract

Underwater hard-substrates are fairly diverse and so are the communities that colonize them. Any hard substrate exposed to or submerged in aquatic environments is susceptible to be colonized by organisms in a succession process that culminates with mature, three-dimensionally complex communities. The specific case of unwanted settlement and growth of organisms on artificial hard substrates is referred to as **biofouling**, and is considered a cross-sectorial issue for many blue economy industries, with costs estimated in billions annually. In fact, **controlling its development is essential** to ensure the correct functioning and operability of submerged or exposed structures. In mobile elements, such as vessels, fouling development can increase in hydrodynamic resistance, fuel consumption and risk of structural damage, resulting in higher emissions and operational costs. Furthermore, biofouling can pose important biosecurity risks due to translocation of organisms, acting as a vector of introduction and spread of non-indigenous species (NIS), which is globally recognized as a major threat to biodiversity. Still, today there is no international legally binding framework on biofouling, despite it being a bottomless pit of costs and risks. Generally, it is in everyone's interest to control and minimize the development of these communities and **antifouling** (AF) measures are usually applied, coatings being the most common ones, offering a plethora of choices. Biocide-based (BC) coatings have long been used and still are the most widely applied; however, environmental concerns have been raised in relation to their environmental impacts and, in fact, a number of products have been withdrawn from the market. Recent advances in antifouling technology have paved the way to alternative non-toxic coatings, which are becoming valuable options with the potential to replace conventional toxic ones, among which foul-release (FR) coatings are the main product of choice. The objective of this project is to study the effectiveness, implications and performance of selected antifouling coatings (BC, FR), and to integrate these outcomes and find their fit into the current regulatory scenario, focusing on leisure boats.

The work is **divided into three main blocks**, according to the methodological approach. The first one is focused on laboratory experiments and includes **chemical characterization of AF coating lixiviates** and the **assessment of their toxicological profiles** from a multi-taxa and multi-level approach, selecting target and non-target species. These involved planktonic (*Isochrysis galbana* and *Tetraselmis* sp.) and benthic (*Cylindrotheca* sp.) microphytes and a model zooplankton species (the copepod *Acartia tonsa*) as main test organisms. According to the test species a battery of selected biomarkers at different levels was designed. The second block is focused on **in-field testing of the selected AF coatings**, which was divided into two sampling seasons; this task was carried out with a field experiment in realistic conditions: two recreational marinas in the

Mediterranean Sea, known to host abundant biofouling communities and numerous NIS. Biofouling communities were grown on surfaces with different AF treatments and their species identified to the species to the lowest taxonomic level possible, in order to assess community structure variables and presence/abundance of NIS. The last block covers a bibliographic **review of the evolution of marine environmental legislation**, analyses the current **existing regulatory instruments** and **integrates the experimental outcomes as learnings and proposals**.

By and large, this work revealed that, as one could expect, lixiviate metal compositions differ greatly between coating types, based on their chemical formulations. Environmental factors such as temperature and salinity were found to influence the release of metals from the coatings, suggesting potential implications for antifouling usage under different environmental conditions and future climate scenarios. Toxicity of BC coatings was particularly high, inhibiting growth of microphyte cultures and causing mortality of the model copepod at relatively low lixiviate concentrations, while FR lixiviates did not cause such effects. Furthermore, changes in photosynthetic activity and pigment content were observed in all the microalgae species under BC treatments. Narrower lixiviate concentration ranges allowed to detect sub-lethal effects at different levels of biological responses, including enzymatic activities and sorption of metals in microphytes, and fecundity endpoints and target-gene expression patterns for *A. tonsa*. In short, exposure to coatings, particularly BC treatments, affect target and non-target organisms and their responses and magnitude vary across species. Phenomena of sensitivity and tolerance were observed and backed-up by additional toxicity screening assays on other target species. fieldwork experiment revealed that a traditional BC coating is effective in the short term and can reduce the coverage of biofouling; however, longer idle-periods reduce their performance without reaching half of its service life. The communities differed greatly between treatments and unveiled potential tolerance of certain species to the tested BC coating (a finding adds upon laboratory outcomes), coinciding with the initial submersion period and high boating season. FR recruited considerable fouling biomass, but proved easy to clean and even self-detach organisms. Overall, a holistic approach provided a broad understanding of the toxicity profiles and performance of the selected coatings and helped to identify associated environmental risks.

A revision of the evolution of international marine environmental protection and analysis of the existing biofouling regulatory instruments proved useful to identify gaps and challenges on the matter. The experimental outcomes of laboratory and field work could be used as learnings and proposals for biofouling management, addressing some of the identified priorities.

Resumen general

Los substratos duros presentes en los ambientes marinos son notablemente diversos, como también lo son los organismos que los colonizan. Cualquier substrato duro expuesto a ambientes acuáticos, bien total o parcialmente, es susceptible a ser colonizado por organismos en un proceso de sucesión que culmina con comunidades maduras y tridimensionalmente complejas. El caso específico del asentamiento y crecimiento no deseado de organismos sobre substratos duros artificiales se conoce como incrustación biológica o **bioincrustación** y se considera un problema intersectorial que afecta a muchas industrias de la economía azul, con costos estimados en miles de millones anuales. **Controlar su desarrollo es esencial** para asegurar el correcto funcionamiento y operatividad de las estructuras sumergidas o expuestas a dichos ambientes. En elementos móviles como los buques, por ejemplo, el desarrollo de bioincrustaciones puede aumentar la resistencia hidrodinámica, el consumo de combustible y el riesgo de daño estructural, lo que resulta en mayores emisiones y costos operativos. Además, las bioincrustaciones pueden suponer importantes riesgos para la bioseguridad local debido a la translocación de organismos, actuando como vector de introducción y propagación de especies alóctonas (EAs), globalmente reconocido como una de las principales amenazas para la biodiversidad. Sin embargo, hoy en día no existe un marco legal internacional vinculante sobre el control de las bioincrustaciones, a pesar de que se trata de un pozo sin fondo de costos y riesgos. En general, es de interés para todos controlar y minimizar el desarrollo de estas comunidades y, para ello, se suelen aplicar **medidas antiincrustantes** (del inglés antifouling; AF), siendo los recubrimientos los más comunes, ofreciendo una gran variedad de opciones. Los recubrimientos basados en biocidas (BC) se han utilizado tradicionalmente y, a día de hoy, siguen siendo los más utilizados; no osbtante, debido a sus impactos ambientales, su empleo ha provocado una creciente preocupación, llegando a retirarse varios productos del mercado e impulsando el desarrollo de alternativas sostenibles. De hecho, los avances recientes en la tecnología antiincrustante han allanado el camino a recubrimientos alternativos no tóxicos, que se están convirtiendo en opciones valiosas con el potencial de reemplazar a los tóxicos convencionales. Entre los recubrimientos alternativos, aquellos llamados liberadores de incrustaciones (del inglés, foul-release; FR) son el principal producto de elección. El objetivo de esta tesis es estudiar la efectividad, las implicaciones y el rendimiento de recubrimientos antiincrustantes seleccionados (BC, FR), e integrar los resultados experimentales como aprendizajes y propuestas, buscando su encaje en el escenario regulatorio actual, centrándose en las embarcaciones de recreo.

El trabajo se divide en **tres bloques principales** según el enfoque metodológico. El primero se centra en experimentos de laboratorio e incluye la **caracterización química de los lixiviados de**

los recubrimientos AF y la **evaluación de sus perfiles toxicológicos** desde un enfoque multinivel y multi-taxón, seleccionando especies diana y no diana. Éstas incluyeron micrófitos planctónicos (*Isochrysis galbana* y *Tetraselmis* sp.) y bentónicos (*Cylindrotheca* sp.) y una especie de crustáceo modelo de zooplancton (el copépodo *Acartia tonsa*) como principales organismos de estudio. De acuerdo a dichas especies, se diseñó una batería de biomarcadores a diferentes niveles. El segundo bloque se centra en las **pruebas de campo de los recubrimientos AF seleccionados**, que se dividieron en dos temporadas de muestreo. Esta tarea se llevó a cabo por medio de un experimento de campo en condiciones realistas: dos puertos deportivos en el mar Mediterráneo, conocidos por albergar abundantes comunidades de bioincrustantes y numerosas EAs. Se sumergieron unidades experimentales con superficies tratadas con diferentes AF para el reclutamiento de comunidades bioincrustantes y se identificaron las especies presentes hasta el nivel taxonómico más bajo posible, con el fin de evaluar variables de estructura comunitaria y presencia/abundancia de EAs. El último bloque abarca una **revisión bibliográfica** de la evolución de la **legislación ambiental marina**, analiza los **instrumentos regulatorios existentes** en la actualidad e **integra los resultados experimentales** como aprendizajes y propuestas.

En general, este trabajo reveló que las composiciones de metales lixiviados difieren en gran medida entre los tipos de recubrimiento, como cabe esperar en función de sus formulaciones químicas. Se observó que los factores ambientales como la temperatura y la salinidad influyen en la liberación de metales de los recubrimientos, lo que implica importantes consideraciones para el uso de antiincrustantes en diferentes condiciones ambientales y escenarios climáticos futuros. La toxicidad de los recubrimientos BC fue particularmente alta, inhibiendo el crecimiento de cultivos de micrófitos y causando la mortalidad del copépodo modelo a concentraciones de lixiviado relativamente bajas, mientras que los lixiviados FR no causaron tales efectos. Además, se observaron cambios en la actividad fotosintética y el contenido de pigmentos en todas las especies de microalgas bajo tratamientos BC. Rangos de concentración de lixiviado más acotados permitieron detectar efectos sub-letales en diferentes niveles de respuestas biológicas, incluidas las actividades enzimáticas y la sorción de metales en micrófitos, y parámetros de fecundidad y los patrones de expresión de genes diana en *A. tonsa*. En resumen, la exposición a los recubrimientos, en particular los tratamientos con BC, afecta a los organismos diana y no diana, aunque sus respuestas y magnitud de éstas varían según la especie. De hecho, se observaron fenómenos de sensibilidad y tolerancia, respaldados por ensayos adicionales de toxicidad en otras especies diana. El experimento de campo reveló que el recubrimiento BC tradicional es eficaz a corto plazo y puede reducir la cobertura de bioincrustaciones; sin embargo, durante períodos de inactividad más prolongados reduce su rendimiento sin alcanzar la mitad de su vida útil. Las

comunidades diferían mucho entre los tratamientos y, en determinadas especies, se detectó cierta tolerancia al recubrimiento BC seleccionado (un hallazgo que se suma a los resultados de laboratorio), coincidiendo con el período de inmersión inicial y la temporada alta de navegación. El FR reclutó una biomasa de incrustaciones considerable, pero resultó fácil de limpiar, llegando incluso a desprenderse parte de ésta por sí sola. De la parte experimental, se puede concluir que el enfoque holístico de este trabajo proporcionó una amplia comprensión tanto de los perfiles de toxicidad y como del rendimiento de los recubrimientos seleccionados y ayudó a identificar los riesgos ambientales asociados.

Finalmente, la profunda revisión de la evolución de la protección ambiental marina internacional y el análisis de los instrumentos regulatorios de bioincrustaciones existentes resultaron útiles para identificar lagunas y desafíos en la materia. Los resultados experimentales del trabajo de laboratorio y de campo se utilizaron como aprendizajes y propuestas para la gestión de las bioincrustaciones, abordando algunas de las prioridades identificadas.

Riassunto generale

Negli ambienti marini esiste una grande varietà di substrati duri e gli organismi che li colonizzano sono altrettanto diversi. Qualsiasi substrato duro esposto agli ambienti acquatici, sia total o parzialmente, è suscettibile di essere colonizzato da organismi in un processo di successione che culmina in comunità matura e tridimensionalmente complessa. Il caso specifico dell'insediamento e della crescita indesiderata di organismi su substrati duri artificiali è noto come incrostazione biologica (**biofouling**), considerato un problema trasversale per molte industrie dell'economia blu, con costi stimati in miliardi all'anno. **Controllare questo sviluppo è fondamentale** per garantire il corretto funzionamento e l'operatività delle strutture sommerse o esposte a questi ambienti. Sugli elementi in movimento, come le navi, lo sviluppo del biofouling può aumentare la resistenza idrodinamica, il consumo di carburante e il rischio di danni strutturali, con il conseguente aumento delle emissioni e dei costi operativi. Inoltre, il biofouling può comportare rischi significativi per la biosicurezza locale a causa della traslocazione di organismi, agendo come vettore per l'introduzione e la diffusione di specie aliene (dall'inglese non-indigenous species; NIS), riconosciute a livello globale come una delle principali minacce alla biodiversità. Tuttavia, oggi non esiste un quadro giuridico internazionale vincolante sul controllo del biofouling, nonostante sia un abisso senza fondo di costi e rischi. In generale, è nell'interesse di tutti controllare e minimizzare lo sviluppo di queste comunità e, a tal fine, vengono solitamente applicate **misure antivegetative** (AF), di cui i rivestimenti sono i più comuni e offrono un'ampia varietà di opzioni. I rivestimenti a base biocida (BC) sono stati tradizionalmente utilizzati e, oggi, continuano ad essere i più applicati. Tuttavia, a causa del suo impatto ambientale, il suo utilizzo ha suscitato crescente preoccupazione, portando al ritiro di numerosi prodotti dal mercato e promuovendo lo sviluppo di alternative sostenibili. In effetti, i recenti progressi nella tecnologia antivegetativa hanno aperto la strada a rivestimenti alternativi non tossici, che stanno diventando opzioni preziose con il potenziale per sostituire quelli tossici convenzionali. Tra i rivestimenti alternativi, quelli denominati foul-release (FR) rappresentano il principale prodotto di scelta. L'obiettivo di questa tesi è studiare l'efficacia, le implicazioni e le prestazioni di rivestimenti antivegetativi selezionati (BC, FR) e integrare i risultati sperimentali come apprendimento e proposte, cercando il loro inserimento nell'attuale scenario normativo, concentrandosi sulle imbarcazioni da diporto.

Il lavoro è suddiviso in **tre blocchi principali**, secondo l'approccio metodologico. Il primo si concentra su esperimenti di laboratorio e comprende la ca**ratterizzazione chimica dei percolati provenienti dai rivestimenti AF** e la **valutazione dei loro profili tossicologici** da un approccio multilivello e multitassonimico, selezionando specie bersaglio e non bersaglio. Questi includevano

microfiti planctonici (*Isochrysis galbana* e *Tetraselmis* sp.) e bentonici (*Cylindrotheca* sp.) e una specie di crostaceo zooplanctonico modello (il copepode *Acartia tonsa*) come principali organismi di studio. A seconda delle specie è stata progettata una batteria di biomarcatori a diversi livelli. Il secondo blocco si concentra sulle **prove sul campo dei rivestimenti AF selezionati**, che sono state divise in due stagioni di campionamento. Questo compito è stato portato avanti attraverso un esperimento sul campo in condizioni realistiche: due porti turistici nel Mar Mediterraneo, noti per ospitare abbondanti comunità di biofouling e numerose NIS. Le unità sperimentali sono state sommerse con superfici trattate con diversi AF per il reclutamento di comunità di biofouling e le specie presenti sono state identificate fino al livello tassonomico più basso possibile, al fine di valutare variabili di struttura della comunità e presenza/abbondanza di NIS. L'ultimo blocco prevede una **rassegna bibliografica dell'evoluzione della legislazione ambientale marina**, analizza gli **strumenti normativi attualmente esistenti** e **integra i risultati sperimentali** come apprendimenti e proposte.

Nel complesso, questo lavoro ha rivelato che le composizioni dei metalli lisciviati differiscono notevolmente tra i tipi di rivestimento, come ci si aspetterebbe in base alle loro formulazioni chimiche. È stato osservato che fattori ambientali come la temperatura e la salinità influenzano il rilascio di metalli dai rivestimenti, il che rivela importanti considerazioni per l'uso degli AF in diverse condizioni ambientali e scenari climatici futuri. La tossicità dei rivestimenti BC era particolarmente elevata, inibendo la crescita delle colture di microfite e causando la mortalità del modello di copepode a concentrazioni di percolato relativamente basse, mentre i percolati FR non hanno causato tali effetti. Inoltre, sono stati osservati cambiamenti nell'attività fotosintetica e nel contenuto di pigmenti in tutte le specie di microalghe sottoposte a trattamenti BC. Intervalli di concentrazione dei percolati più ristretti hanno permesso di osservare effetti subletali a diversi livelli di risposte biologiche, comprese le attività enzimatiche e l'assorbimento dei metalli nelle microfite, e parametri di fecondità e modelli di espressione dei geni bersaglio in *A. tonsa*. In sintesi, l'esposizione ai rivestimenti, in particolare ai trattamenti BC, colpisce gli organismi bersaglio e non bersaglio, sebbene le loro risposte e la loro entità varino a seconda della specie. Sono stati infatti osservati fenomeni di sensibilità e tolleranza, supportati da ulteriori test di tossicità in altre specie bersaglio. L'esperimento sul campo ha rivelato che il tradizionale rivestimento BC è efficace a breve termine e può ridurre la copertura del biofouling; tuttavia, durante periodi di inattività prolungati si riducono le sue prestazioni senza raggiungere la metà della sua vita utile. Le comunità differivano notevolmente tra i trattamenti e, in alcune specie, è stata rilevata una certa tolleranza al rivestimento BC selezionato (un risultato che si aggiunge ai risultati di laboratorio), in coincidenza con il periodo di immersione iniziale e l'alta stagione di navigazione. La FR ha

reclutato livelli importanti di biomassa, ma risultava facile da pulire, anche staccandosene da sola pezzi di essa. Dalla parte sperimentale, si può concludere che l'approccio olistico di questo lavoro ha fornito un'ampia comprensione sia dei profili di tossicità che di prestazione dei rivestimenti selezionati e ha contribuito a identificare i rischi ambientali associati.

Infine, l'esame approfondito dell'evoluzione della protezione dell'ambiente marino a livello internazionale e l'analisi degli strumenti normativi esistenti sul biofouling sono stati utili per identificare lacune e sfide in materia. I risultati sperimentali del laboratorio e del lavoro sul campo sono stati utilizzati come apprendimenti e proposte per la gestione del biofouling, affrontando alcune delle priorità identificate.

Laburpen orokorra

Itsas inguruneetan substratu gogorrak ugariak dira eta horiek kolonizatzen dituzten organismoak anitzak. Ingurune urtarren eraginpean, guztiz edo partzialki, dagoen edozein substratu gogor organismoz koloniza daiteke, eta seguida prosezu bat jarraituz, komunitate heldu eta komplexuetan bilakatzen dira. Substratu gogor artifizialetan nahi ez den organismoen finkapen eta hazkundeari **inkrustazio biologikoa** deritzo (ingelesetik, biofouling), ekonomia urdineko industria askori eragiten dien arazotzat hartzen da, urtero milaka milioiko kostuak izanik. Haren garapena kontrolatzea ezinbestekoa da ingurune urtarren eraginpean dauden egituren funtzionamendu egokia eta eraginkortasuna bermatzeko. Higikorrak diren elementuetan, itsasontziak esate baterako, inkrustazio biologikoen garapenak erresistentzia hidrodinamikoa, erregai-kontsumoa eta egitura-kalteak izateko arriskua areagotu ditzakete, ondorioz, isuriak eta funtzionamendu-kostuak areagotuz. Gainera, inkrustazio biologikoek arrisku handiak dakartzate tokiko biosegurtasunerako, organismoen lekualdatzearen ondorioz, espezie arrotzak (EA) sartzeko eta hedatzeko bektore gisa jokatzen baitute, biodibertsitatearen mehatxu nagusietako bat bezala mundu osoan aintzatetsita egonik. Hala ere, inkrustazio biologikoen kontrolari buruz gaur egun ez dago nazioarteko lege-esparru loteslerik, kostu eta arriskuek hondorik gabeko putzua izan arren. Oro har, guztion interesekoa da komunitate horien garapena kontrolatzea eta gutxitzea eta, horretarako, **anti-inkrustazio neurriak** (ingelesetik, antifouling; AF) aplikatzen dira normalean, ohikoenak gainestaldurak izanik, eta aukera ugari eskainiz. Tradizionalki, biozidak (BC) dauzkaten gainestaldurak erabili izan dira eta, oraindik ere gehien erabiltzen diren mota dira. Hala ere, ingurumen-inpaktuak direla eta, horien erabilerak gero eta kezka handiagoak sustatzen ditu, hainbat produktu merkatutik kentzera eta alternatiba jasangarrien garapena bultzatuz. Izan ere, anti-inkrustazio teknologien azken aurrerapenek estaldura ez-toxiko alternatiboetarako bidea ireki dute, ohiko aukera toxikoak ordezkatzeko aukera baliotsu bihurtzen ari direnak. Estaldura alternatiboen artean aukeratutako produktu nagusiak *foul-release* (FR) izenekoak dira, inkrustazioen askapena errazten dutenak. Tesi honen helburua anti-inkrustazio gaiestaldura hautatuen (BC, FR) eraginkortasuna, inplikazioak eta errendimendua aztertzea da, eta emaitza esperimentalak ikaskuntza eta proposamen gisa integratzea, haien egokitzea bilatuz egungo araudian, aisialdiko itsasontzietan arreta jarriz.

Tesi hau **hiru bloke nagusitan** banatuta dago, ikuspegi metodologikoan oinarrituta. Lehenengoa, laborategiko esperimentuetan zentratzen da eta **AF gainestalduren lixibiatuen karakterizazio kimikoa** eta haien **profil toxikologikoen ebaluazioa** barne hartzen ditu, maila eta taxoi anitzeko ikuspegitik, xede eta xede ez diren espezieak hautatuz. Horien artean, mikrofito espezie ezberdinak, planktonikoak (*Isochrysis galbana* eta *Tetraselmis* sp.) eta bentonikoak (C*ylindrotheca*

sp.), eta zooplankton espezie-modelo bat (*Acartia tonsa* kopepodoa) aztergai hautatu ziren. Espezie horien arabera, maila ezberdinetako biomarkatzaileen bateriak diseinatu ziren. Bigarren blokea **hautatutako AF gainestalduren eraginkortasuna aztertzen ditu baldintza errealetan,** bi laginketa-denboralditan banatutako zelaiko lanetan. Zeregin hori zelai-esperimentu baten bidez gauzatu zen Mediterraneo itsasoko bi kirol-portutan, inkrustazio biologiko komunitate ugari eta EA askotarikoak ostatzeagatik ezagunak. Unitate esperimentalak AF gainestaldura ezberdinekin tratatu eta urperatu ziren, inkrustazio biologikoen komunitateak haz zitezen eta ondoren espezie horiek ahalik eta maila taxonomiko baxuenera identifikatu ziren, komunitatearen egituraren eta EAen presentzia/ugaritasuna ebaluatzeko. Azken blokeak, alde batetik **itsas ingurumeneko legediaren bilakaeraren berrikuspen bibliografikoa** jasotzen du eta, bestetik, **gaur egun dauden arau-tresnak aztertzen ditu** eta **emaitza esperimentalak** ikaskuntza eta proposamen gisa **integratzen ditu**.

Oro har, lan honek agerian utzi zuen lixibiatuen metalen konposizioak oso desberdinak direla gainestaldura moten arabera, haien formulazio kimikoen arabera espero zitekeen bezala. Ingurumen-faktoreek, tenperatura eta gazitasuna zehazki, gainestalduren metalen askapenean eragiten zutela ikusi da, anti-inkrustazioen erabilpena egokitzeko ingurumen baldintzetara eta etorkizuneko klima agertokira kontuan hartu beharreko emaitza izanik. BC estalduren toxikotasuna bereziki altua izan zen, mikrofitoen hazkuntza galaraziz *eta A. tonsa*ren hilkortasuna eraginez lixibatuen kontzentrazio nahiko baxuetan; aldiz, FR lixibatuek ez zuten halako efekturik eragin. Gainera, jarduera fotosintetikoan eta pigmentu-edukian aldaketak ikusi ziren mikroalga espezie guztietan BC tratamendupetan. Lixibatuen kontzentrazio-tarte estuagoek erantzun biologikoen maila ezberdinetan efektu subhilgarriak detektatzeko aukera eman zuten, besteak beste, aktibitate entzimatikoak eta mikrofitoetako metalen xurgapena, eta kopepodoaren ugalkortasun parametroen aldaketak eta itu-geneen adierazpen ereduak. Laburbilduz, estalduren esposizioak, bereziki BC tratamenduak, xede eta xede ez diren organismoetan eragina izan zuen, nahiz eta haien erantzunak eta magnitudea espeziearen arabera aldatu. Izan ere, sentsibilitate- eta tolerantzia-fenomenoak ikusi ziren, xede-espezie batzuen toxikotasun-proba gehigarriek berretsita. Zelaiko esperimentuak agerian utzi zuen BC gainestaldura tradizionala eraginkorra dela epe laburrean eta inkrustazioen estaldura maila murrizten zuela. Hala ere, gelditasun epe luzeagoetan eraginkortasuna murrizten zen, bere bizitza erabilgarriaren erdira iritsi gabe. Komunitateak asko desberdintzen ziren tratamenduen artean eta, zenbait espezietan, aukeratutako BC gainestaldurarekiko nolabaiteko tolerantzia antzeman zen (laborategiko emaitzei gehitzen zaien aurkikuntza), hasierako murgiltze aldiarekin eta nabigazio garaiko denboraldiarekin bat eginez. FRak eskala handiko biomasa metatu zuen, baina erraz garbitzen

zen, batzuetan biomasa bere kabuz askatuz ere. Atal esperimentaletik ondoriozta daiteke lan honen ikuspegi holistikoak aukeratutako gainestalduen toxikotasun- eta errendimendu-profilen ulermen zabala eman zuela eta lotutako ingurumen-arriskuak identifikatzen lagundu zuela.

Azkenik, berrikuspen sakon bat egin zen nazioarteko itsas ingurumenaren babesaren eboluziari buruz eta baita inkrustazio biologikoen kontrola arautzeko dauden tresnen azterketei buruz ere. Bildutako informazioa, baliagarria izan zen gaiaren hutsuneak eta erronkak identifikatzeko. Laborategiko eta zelai-lanen emaitza esperimentalak inkrustazio biologikoak kudeatzeko ikaskuntza eta proposamen gisa erabili ziren, identifikatutako lehentasunei helduz.

General Introduction

Introduction and state of the art
Hypothesis and objectives

Hard substrate communities: the specific case of biofouling

Underwater hard-substrates are rather diverse and so are the communities that colonize them. Hard-substrates include non-living natural biogenic (shells, organogenic substrates such as coral remains, bioclasts, etc.) and abiogenic materials (rocks and stones from various origins, sedimentary hardgrounds), as well as anthropogenic surfaces (docks, boulders, piles, ships, pipelines, buoys or plastic debris, to mention some) (Taylor & Wilson, 2003). Living organisms too, such as algae, plants and animals, can act as surfaces for others to settle. Any hard substrate exposed or submerged to aquatic environments is susceptible to be colonized by organisms that compose the so called hard-substrate communities, in a succession process that goes from a biochemical conditioning and an initial biofilm cover to more mature, three-dimensionally complex communities (Railkin, 2003) (Figure 1 and 2). While epibiosis, intended as the growth of organisms on other organisms, is a natural phenomenon, the specific case of unwanted settlement and growth of organisms on artificial hard substrates partially or totally exposed to aquatic environments is referred to as biofouling (Lewis, 1998).

Despite the diversity of hard-substrate communities, generally, the succession process follows a basic pattern composed of 4 stages, regardless the type of substratum and the geographical region (Wahl, 1989). The stages are described in detail below but, in short, the process starts with the submersion of a surface into an aquatic body, right after which macromolecules are adsorbed to it and the conditioning process of the bare surface begins. This conditioning triggers the bacterial colonization and the formation of an initial biofilm. The first two stages are relatively fast and soon new unicellular eukaryotic forms arrive, such as protozoans, fungi and microalgae, particularly diatoms, increasing the complexity and diversity of the initial microfouling film. As colonization progresses, multicellular eukaryonts, from spores to meroplanktonic larvae, settle and develop, leading to the formation of a mature community of macrofoulers (Evans, 1981; Wahl, 1989; Railkin, 2003).

Stages in biofouling development

As summarized by Wahl (1989) (Figure 1), fouling development starts with the biochemical conditioning of the surface, which is characterized by the adsorption of dissolved chemical compounds (e.g. polysaccharides, glycoproteins, proteoglycans, etc.) in a purely physical, spontaneous and random process. Interactions between macromolecules and the surface occur seconds after the contact with seawater (or other aqueous media) and the dynamic equilibrium is usually reached after a few hours. The biochemical conditioning gives way to the bacterial colonization, consisting of a reversible phase (adsorption) and a non-reversible attachment phase

(adhesion). Similarly to the biochemical conditioning, the first phase is governed by physical forces: Brownian motion, electrostatic interactions, gravity, Van-der-Waal forces etc. (Wahl, 1989), and cell movement has often been comparted to that from colloid particles (Marshall, 1972; Characklis 1984). Previous forces determine the approach and weak bonds of the bacteria to the macromolecular film and the establishment of covalent bonds between bacterial glycocalix and the existing film finally leads to the adhesion phase (Wahl, 1989). Bacterial colonization start approximately an hour after immersion and the community changes continuously by the effect of disturbance, competition, predation, succession, etc.

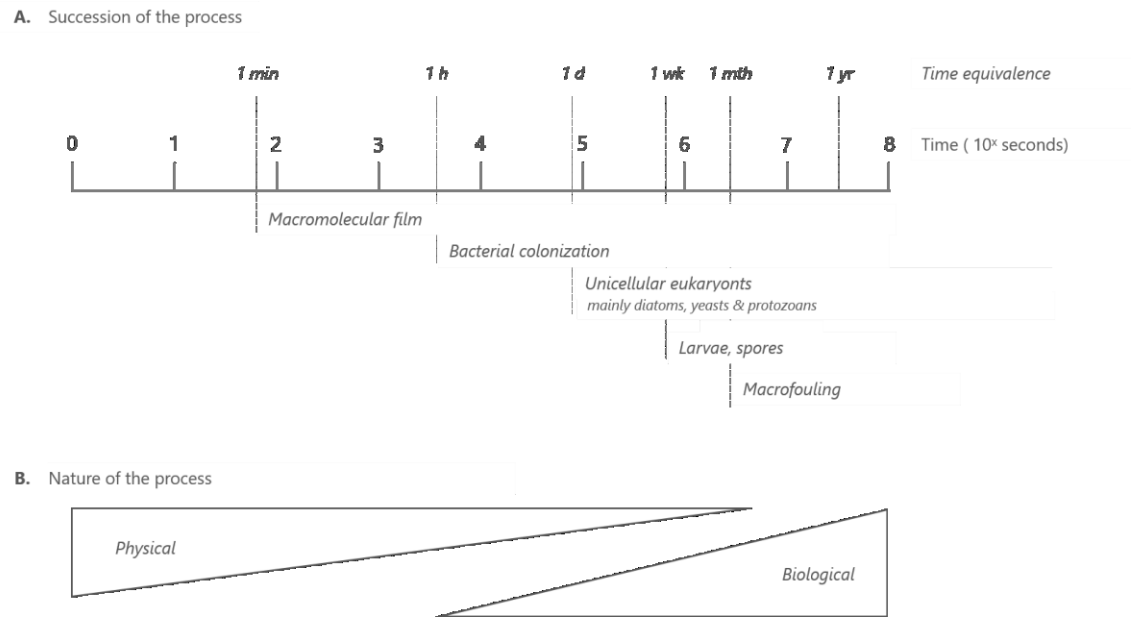


Figure 1. Schematic representation of the succession process through time. The nearly adsorption of macromolecules occurs immediately after the submersion, and is followed by prokaryotic colonization. Diatoms and protozoa typically settle from the second day onward. Larvae and algal spores follow with a lag of one to several weeks (according to latitude, season, etc.). (Slightly modified from Wahl, 1989).

The formation of the bacterial microfilm is followed by the arrival of unicellular eukaryotes (Figure 1), mainly yeasts, protozoans and diatoms, being the latter quantitatively dominant. This phase is also characterized by a succession process, in which initial pioneers and later recruits can be distinguished. The presence of different life forms contribute to greater diversity and complex interactions within the microfouling community. Extracellular secretions, such as those from diatoms, enhance attachment and build-up of organisms.

The last and longest stage in the fouling development begins with the settlement of meroplanktonic larvae and propagules of algal species (Figure 1). It can start as early as days after biochemical conditioning and as late as weeks after (Wahl, 1989) and it overlaps with the

continuous recruitment and evolution of the microfouling community. Initially, fast-growing foulers arrive and dominate the macrofouling community (e.g. bryozoans, serpulids), followed by the settlement of slow-growing invertebrates later on in the succession process (Railkin, 2003). The community will evolve towards a climax (mature) stage characterized by stable abundances and number of species, as well as high structural complexity and three-dimensionality that can sustain heterogeneous assemblages (Bradshaw *et al.*, 2003; Railkin, 2003; Tempesti *et al.*, 2022). Sessile forms often dominate hard-substrate communities in terms of abundances and biomass, and act as biocontructors creating microenvironments, such as crevices and cavities that host vagile forms (Railkin, 2003). Furthermore, their constructions provide new substrate for other sessile organisms to settle in the advanced stages of the colonization process. This last phase extends in time and, last for several years before a climax is reached, which will not necessarily happen in all cases.

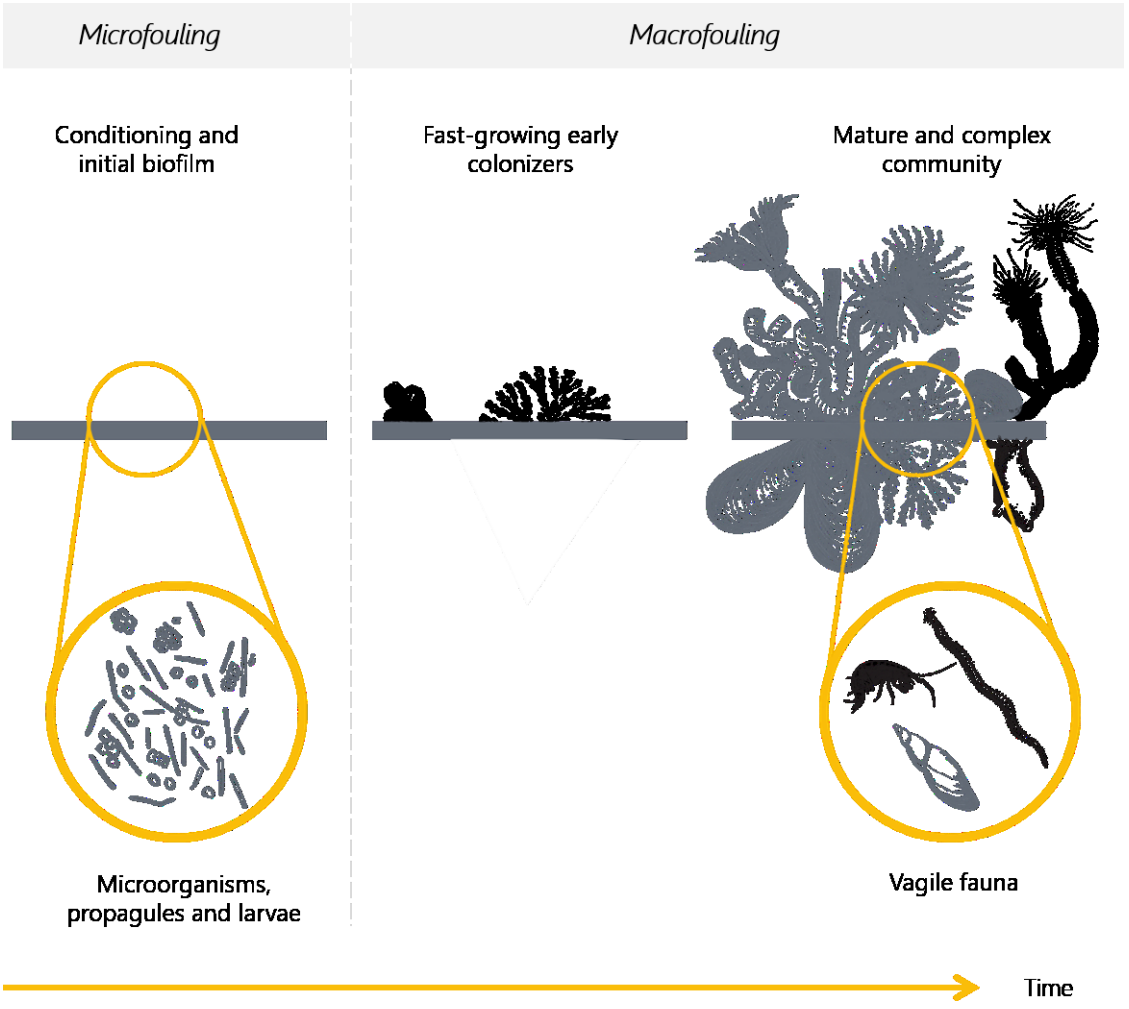


Figure 2. Simplified succession process of biofouling from the initial surface conditioning and biofilm formation towards a climax community.

Overall, the succession phenomenon has well defined stages, however, the development of these communities depends on five underlying processes that determine the dynamics of the assemablages: 1) transport, 2) settlement, 3) attachment, 4) development and 5) growth. Simultaneously, other environmental variables such us temperature, light, depth, currents, nutrient concentration, etc. dictate fouling dynamics and contribute to its complexity.

Implications of biofouling

The development of fouling communities is a fast, dynamic and cumulative process that can pose several problems for many human activities in sectors such as aquaculture, extractive industry, renewable energy production and its transportation, monitoring systems, maritime defence, maritime transport, tourism and other forms of navigation. In other words, marine biofouling is a cross-sectorial issue with implications for key blue economy sectors. Controlling its development is essential to ensure the correct functioning and operability of submerged or exposed elements, including cooling systems, piping, sensors, gear assemblies and bare surfaces, to mention some. Furthermore, reducing fouling levels can be crucial for productivity in aquaculture, as it can otherwise reduce water circulation in caging systems or reduce fitness of shellfish due to competition (de Nys & Guenther, 2009; Bannister *et al*., 2019).

Because of the mobility and relevance in many sectors, it is of particular interest the case of vessels. Shipping and boating include vessels belonging to the sectors of maritime transport and defence, fishing, as well as tourism, from passenger transportation to recreational activities. Due to the drag in fouled surfaces, vessel operability and efficient navigation can be seriously impacted by the increase in hydrodynamic resistance, fuel consumption and structural damage, which ultimately can lead to higher emissions and operational costs (Shultz, 2007; Shultz *et al*., 2011). Furthermore, biofouling can pose important biosecurity risks (Davidson *et al*., 2016; Georgiades *et al*., 2020) and, together with ballast water, has been identified as a major human-mediated vector of introduction and spread of non-indigenous species (NIS) (Hewitt *et al*., 2009; Ros *et al*., 2023).

Climate change is expected to exacerbate the growth and development of biofouling communities, while also impacting the distribution range of many species (Khosravi *et al*., 2019). Generally, alterations in ocean chemistry, salinity, temperatures, productivity and circulation patterns, among others, could potentially alter fouling dynamics and shift the composition of these communities, with potential additional economic and environmental effects derived from the battle against biofouling. Temperature in particular is known to be an important factor affecting fouling dynamics, due to its role in the growth, metabolism and reproduction of

organisms. Future climate projections may result in an increase of certain fouling groups at higher latitudes, particularly in the northern hemisphere where the greatest warming is expected (Poloczanska & Butler, 2009).

Economic implications of biofouling for shipping and boating

Maritime transport is undoubtedly a strategic pillar for socioeconomic activities. The shipping industry accounts for approximately 90% of the EU's external cargo trade (EC, 2024a) and more than 80% of the world's merchandise is seaborne mediated (COM, 2009; UNCTAD, 2017), which has almost doubled over the past two decades (UNCTAD, 2019) (Figure 3). Besides, the recreational boating sector contributes a total of €20 billion to EU revenue, with great growth prospects for the sector in the upcoming years (EC, 2024b).

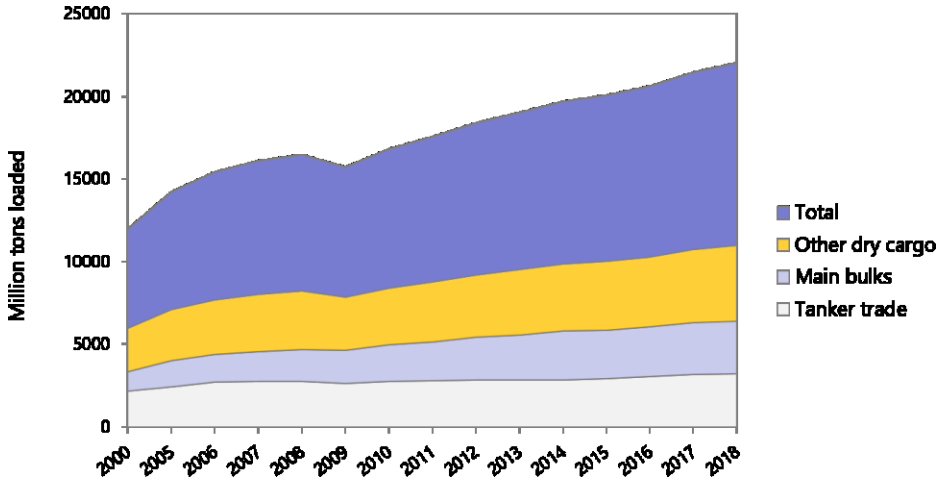


Figure 3. Development in international maritime trade (million tons loaded), selected years. Compiled by the UNCTAD secretariat based on data supplied by reporting countries, as posted on government and port industry websites, and data provided by specialist sources. Modified from: UNCTAD, 2019.

The build-up of organisms in vessel hulls increases the roughness of the surfaces and, consequently, the frictional drag, leading to greater hydrodynamic resistance to the ship's movement. In order to keep up with the operational parameters, fouled ships need to increase power, that is, fuel consumption, to overcome the loss of speed or, otherwise, face the loss of speed at constant power and increase operational times (Abbott *et al.*, 2000). Slime films alone can impact powering penalties by 21%, while heavy calcareous biofouling increases the penalty up to 86% (Schultz, 2007). Figure 4 shows the expected increases in costs of propulsive fuel in relation to fouling level and, in fact, an increase in 20% of fouling rating, in monetary terms, can be translated into an additional cost of $1M (Schultz *et al.*, 2011).

Although these estimations are based on a number of assumptions regarding vessel and hull type, powering characteristics etc., the model by Schultz *et al* (2011) suggests that operating with fouling has important additional costs that could be otherwise spent differently on management, maintenance, acquisition, research and innovation, for example.

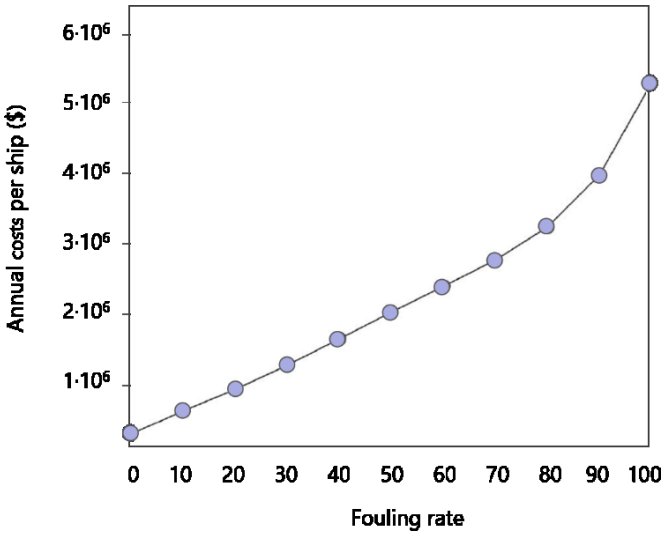


Figure 4. The annual costs (per ship) for a range of hull fouling levels (fouling rates). The cleaning and coating costs are assumed to be the same as the Navy's present practices including qualified ablative AF coatings and regular interim and full hull cleanings (Schultz et al., 2011).

Considering the size and number of vessels of the shipping industry, operating with none or minimized levels of fouling can greatly reduce the total operational costs.

Environmental implications of vessel biofouling

Following up with powering penalties and fuel consumption, the dichotomy of overcoming the loss of speed increasing power or maintaining power losing speed and increasing operational times has, in both cases, a direct consequence: an increase in greenhouse gases (GHGs) emissions (Figure 5). Ships are powered with low-grade remnants of crude oil, such as heavy fuel oil, that can emit air pollutants when combusted (e.g. NO, SO_2, black carbon) (Eyring *et al.*, 2010) and contribute to atmospheric SO_4^{-2}, NO_3^-, and organic aerosols.

Therefore, in order to comply with the requirements of the IMO (International Maritime Organization), namely a) fuel-sulphur limit under an amendment to Annex VI of the International Convention for the Prevention of Pollution from Ships (i.e. MARPOL) and b) carbon intensity requirements (IMO, 2023a), biofouling levels must be kept at its minimum. In other words,

managing biofouling is pivotal to reduce global emissions by vessels and to shift towards a greener shipping industry that aligns with the current environmental targets.

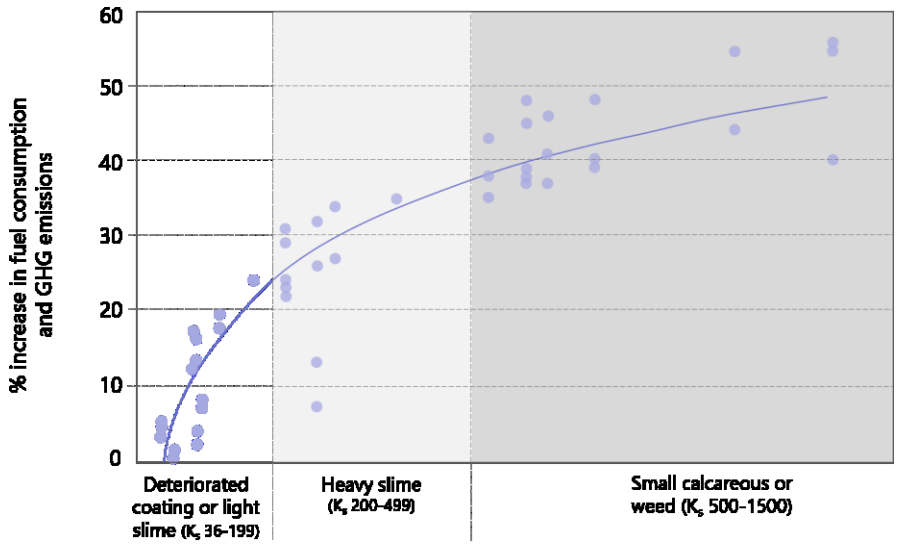


Figure 5. Increase of fuel consumption and GHG emissions in relation to hull biofouling condition (modified from GEF-UNDP-IMO GloFouling Partnerships Project & GIA for Marine Biosafety, 2022).

Yet, fuel consumption is not the only consequence associated with vessel biofouling. Human-mediated introduction of marine non-indigenous species is a historical phenomenon and, currently, is seen as an important driver of change (Ojaveer *et al.*, 2018) with considerable implications in the structure and functioning of marine ecosystems (Costello *et al.*, 2010) and their biodiversity.

Non-indigenous species (NIS) are defined as species, or lower taxa, occurring outside their past or present natural distribution range and outside their natural dispersion potential (Olenin *et al.*, 2010). The term includes any part, gamete or propagule of such species with the potential to survive and thrive. Their arrival to a non-native area could be intentional or unintentional and the vectors that may mediate it are varied and, generally, allow to overcome environmental barriers (Occhipinti-Ambrogi & Galil, 2004; Occhipinti-Ambrogi, 2021). It is important to remark that the natural shifts in distribution ranges (passive or active dispersal of individuals or propagules following natural processes such as marine currents or zoochory, including climate-related shift of species) are not considered introductions, but naturally spreading species, and, therefore, are not NIS (Olenin *et al.*, 2010; Essl *et al.*, 2019).

Anthropogenic pathways and vectors for the introduction and spread of NIS outside their natural range are very diverse (Ruiz & Carlton, 2003) and vessels have long been identified as important

ones (Chilton, 1910; Allen, 1953; Skerman, 1960), through ballast water and biofouling. While commercial shipping is a major vector for primary introductions, biofouling on recreational boats largely contributes to secondary spread of NIS (Murray *et al.*, 2014), offering frequent opportunities for transfers and high connectivity between locations, including areas of conservation and special interest (Ulman *et al.*, 2019; Ashton *et al.*, 2022). Furthermore, unlike other vectors of introduction such as ballast water (Bailey, 2015; Drake *et al.*, 2021) and aquaculture (Grosholz *et al.*, 2015; Marchini *et al.*, 2015), biofouling is still largely unregulated at global scale, although some virtuous exceptions exist (California Legislature, 2007; MPI, 2014; DAFF, 2015).

Still, over 6 million boats are kept in European waters while 10,000 marinas provide over 1 million berths both inland and in coastal areas (EBI, 2023). Europe's two largest markets for recreational boating are located in France and Spain, which, along with Italy, account for 80% of total demand in the Mediterranean region (Ramieri *et al.*, 2022). Undoubtedly, recreational boating, yachting and other forms of maritime tourism constitute important sector (Cappato *et al.*, 2011), pivotal in the case of the Mediterranean, where many efforts of sustainable development are focused on (Ramieri *et al.*, 2022). However, despite the role of these vessels in the secondary spread of NIS and their relevance within the tourism sector, as regards biofouling, most of the measures rely on self-management and good practices (MEPC, 2011; MEPC, 2023b). Yet, the level of awareness of recreational boaters regarding their involvement in the NIS spreading is very poor at Mediterranean scale (Ferrario *et al.*, 2016; Martinez-Laiz *et al.*, 2019), a fact that does not favour the application of good practices and evidences that little is being done to curb this problem.

Some of the most notorious species introductions via hull biofouling include the freshwater mussel *Dreissena polymorpha* (Pallas, 1771), the marine '*spaghetti*' bryozoan *Amathia verticillata* (delle Chiaje, 1822), the marine serpulid *Ficopomatus enigmaticus* (Fauvel, 1923) or the colonial ascidian *Didemnum vexillum* Kott, 2002; all of which impacted local environments or activities (Winston, 1995; Padilla, 1997; Schwindt *et al.*, 2001; Fletcher *et al.*, 2013).

Controlling biofouling: solutions to a *sticky* problem

Antifouling coatings: History and current situation

For many sectors, controlling biofouling is key to the optimal performance of the involved systems and machinery. The development of preventive measures is closely related to that from the maritime transport and dates back to the classical era. In fact, evidences suggest that early Phoenicians (1500-300 BC) used pitch and copper to sheath the hulls of the ships, as well as wax and asphalt (Laidlaw, 1952; Almeida *et al.*, 2007). Similarly, Greeks used tar, wax and lead

sheathing as early as 3rd century BC, and Plutarch described the issue of fouling as it follows already by AD 99, although attributing it to a single fish species:

> '*It is probable that it lightly glides, and as long as it is clean, easily cuts the waves; but when it is thoroughly soaked, when weeds, ooze and filth stick upon its sides, the stroke of the ship is more obtuse and weak; and the water coming up upon this clammy matter, doth not so easily part from it; and this is why they usually calk their ships. Now it is likely that the echeneis in this case, sticking upon the clammy matter, is not thought an accidental consequent to this cause, but the very cause itself.*' (Plutarch [translated] 1909; Davidson *et al.*, 2016). Curiously, later on Linnaeus referred to it when describing a species of remora, *Echeneis remora* (Linnaeus, 1758) and, in fact, *Echeneis* is rooted in Greek for 'to hold a ship' (Davidson *et al.*, 2016).

Many fleets throughout the history (Archimedes, the Romans, Spanish and French fleets, etc) have repeatedly used lead sheathing afterwards (Laidlaw, 1952). Although some early forms of coatings were used already in 412 BC, being it the first mixture applied to ships to improve sailing, it was not until the 17th century that the first coating has been recorded as a patented solution specifically for fouling prevention, in 1625 (Laidlaw, 1952).

Still now, antifouling measures mostly include the use of coatings that protect exposed surfaces in order to inhibit or minimize the recruitment of organisms. The nature and action mechanism of those can vary and, currently, there are multiple options (Figure 6).

Biocide-based coatings

Traditionally, coatings have toxicants embedded into a matrix that gradually releases the active compounds from the coating's surface layer into the environment. The matrix type, that is, the carrier agent of the antifouling agent, determines the action mechanism and release rate of the active compounds (Valkirs *et al.*, 2003; Miller *et al.*, 2020; Tian *et al.*, 2021).

Conventional AF coatings are constituted by insoluble matrixes that do not degrade and from which active compounds diffuse into the water (Cao *et al.*, 2011; Wezenbeek *et al*, 2018). Chlorinated rubber, polyvinyl chloride and rosin are common insoluble binders within which high concentrations of biocides such as Cu_2O and booster biocides are physically dispersed in the hard matrix (Takahashi, 2009). Because of the intrinsic insolubility of the matrix, the release rate of the active compounds tends to decrease exponentially with time and have, consequently, relatively shorter lifetimes. However, this solution has higher mechanical strength and stability to oxidation and photodegradation (Cao *et al.*, 2011). To lengthen the lifespan of the coatings, soluble matrixes

were developed, which erode by the dissolution of their binding compound, simultaneously releasing the active compound, exposing with time the active compounds contained deeper in the coating layer (Cao *et al.*, 2011).

Oppositely, ablative coatings erode by hydrolysis of the matrix, usually a mixture of soluble/hydration binder (e.g. vinyl chloride-vinyl acetate copolymer, vinyl chloride-vinyl isobutyl ether copolymer and rosin) (Takahashi, 2009). These coatings release the biocide as the matrix hydrolyses. Other hydrolysable matrixes are those from self-polishing coatings, which contain hydrolysable units that lead to a progressive degradation of immersed binder and a greater control of the erosion and leaching rates (Takahashi, 2009; Wezenbeek *et al*, 2018), resulting in longer lifetime.

In all cases, for effective prevention of fouling, a defined and constant concentration of the active compound is desirable at the surface of paint, i.e. effective concentration. Currently, copper represents the most common active compound in combination with booster co-biocides (Jones and Bolam, 2007; Ytreberg *et al.*, 2010; Tian *et al*, 2021). Although the application of BC coatings has been a common practice and still is, it has raised environmental concerns due to the release of toxic chemicals to the environment and their associated implications for the marine life (Amara *et al.*, 2018; de Campos *et al.*, 2021).

Biocide-free coatings

Recent advances in antifouling technology have given rise to alternative solutions that substitute toxic active compounds by either natural non-toxic compounds or coatings with different action mechanisms, rather than the chemical approach (Figure 6) (Wezenbeek *et al*, 2018). Foul-release coatings (FR) are among the most common alternatives to traditional biocide-based coatings (BC) and act by reducing the strength of attachment of foulers to the surface of interest, due to the low surface energy (Magin *et al.*, 2010; Tian *et al.*, 2021). Most FR coatings are based on silicone elastomers, fluoropolymers or a combination of both (Wezenbeek *et al*, 2018) and have proved to be a valuable alternative (Lagerström *et al.*, 2022).

Similarly to BC self-polishing coatings, non-toxic self-polishing coatings rely on a continued hydrolysis reaction, leading to a constant renewal of the active surface and causing the detachment of the organisms from the upper layer. Natural substitutes obtained from or inspired by chemical defences of certain sessile organisms, such as sponges, corals, seaweed and other algae, have been proved to exhibit antifouling properties and successfully applied in some cases (Xie *et al.*, 2019; Kyei *et al.*, 2020). Additionally, other molecules, e.g. oxidoreductases, transferases,

hydrolases, lyases, or medetomidine that interacts with octopamine receptor of barnacle's larvae, have already reached the market (Dahlström *et al.*, 2000; Cao *et al.*, 2011; Gizer *et al.*, 2023).

Hard coatings, in turn, are generally made of epoxies, polyesters, vinylesters or ceramic-epoxy compounds, being in many cases silicon (Si) a major ingredient, and sometimes reinforced with glass flakes. However, their surface properties alone do not control fouling development and are intended to be used in conjunction with routine cleaning (Wezenbeek *et al*, 2018). Still, they have an increased life service and easier maintenance due to their resistance.

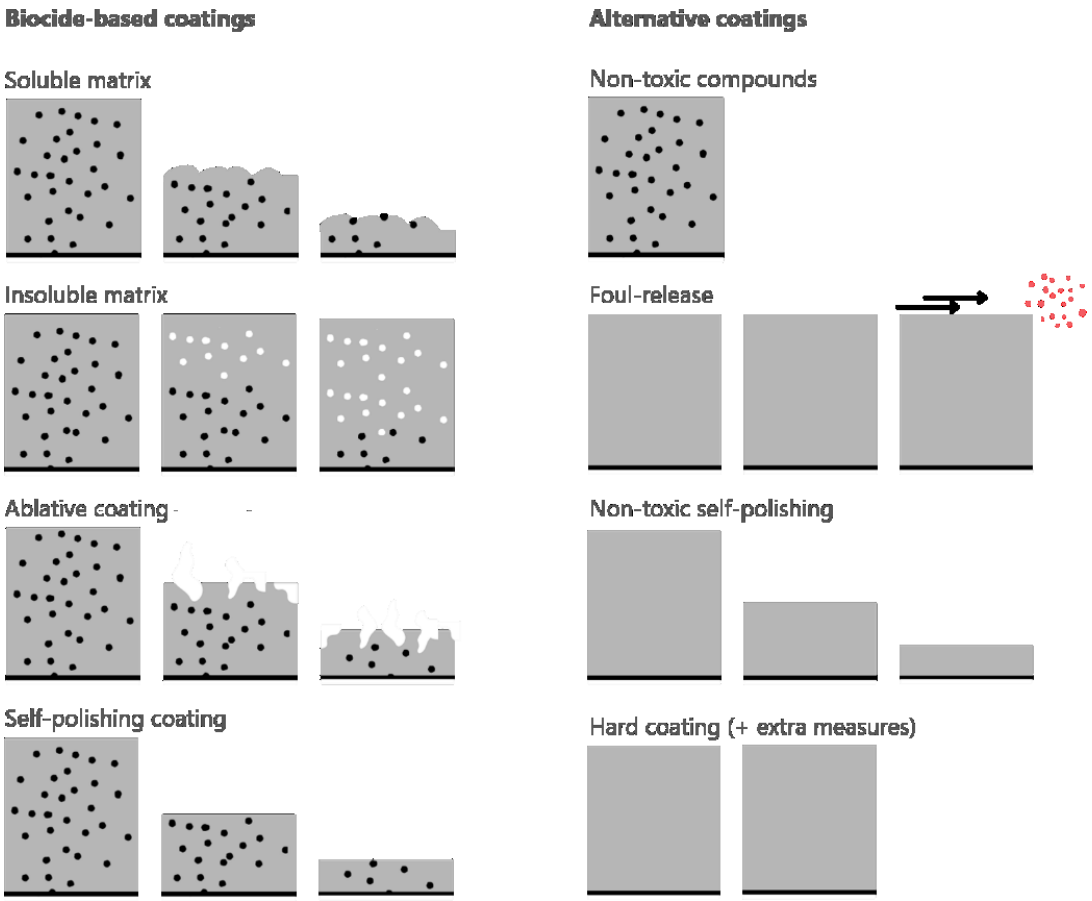


Figure 6. Schematic representation of the main coating typologies and their action mechanism, mostly given by the characteristics of the embedding matrix.

Although there are other bioinspired options, like micro-structured surfaces that lower contact points for adhesion mimicking shark skin, crab shells or even artery's endothelium, there is uncertainty on their long-term performance (Magin *et al.*, 2010; Xie *et al.*, 2019) and efficiency alone (Gizer *et al.*, 2023), besides being uncommon due to the difficulty of application in real environments. Other bioinspired coatings or *wrappings*, such as fibre films mimicking the spiky hairs of certain organisms, have reached the market, have easier application and are being used in different sectors.

Beyond coatings: other antifouling solutions

Aside from the coatings, other AF measures can be used to prevent or control the attachment of unwanted organisms, alone or as additional preventive and maintenance practices (Figure 7). Normally, the application of any coating requires maintenance, although depending on the action mechanism, the measures that are suggested to follow may vary.

Physical measures

The most common measures have a physical basis, and they usually rely on the mechanical removal of fouling organisms from colonized surfaces. The devices that can be employed for this purpose go from simple sponges, scrapers and brushes, to cleaning robots and high-pressure water. Cleaning can be done in dry-dock conditions, although underwater cleaning making use of hand tools or remotely controlled devices is also a common practice. The choice of device in dry-docking often is power washer, using high-pressure water. Despite its effectiveness, it has some major drawbacks: 1) impact the integrity of the coatings, 2) usage of large volumes of water, leading to big amounts of contaminated run-off, and 3) production of backsplash, aerosols and spray, carrying and redistributing paint-derived contaminants (Leighton, 2013), posing human-health and biosecurity risks.

In-water cleaning is the alternative to dry-docking and usually relies mechanical removal by divers, using hand tools, hydraulically powered devices or remotely controlled cleaning devices. Best Management Practices (BMPs) developed by the California Professional Divers Association (2011) include cleaning frequently enough to use the gentlest cleaning tool and least amount of effort to remove fouling species, ensuring minimal coating damage (Culver *et al*., 2012). Similarly to dry-docking, underwater cleaning also has its drawbacks, since it can pose important environmental risks if derived wastes are not collected and treated appropriately (Scianni & Georgiades, 2019; Kim *et al* 2023). In particular, NIS propagules can be facilitated by such operations and successfully colonize new areas once removed from the hull (Hopkins & Forrest, 2008; Kim *et al*., 2023), alongside with and enhanced biocide leaching and release during execution (Scianni & Georgiades, 2019). Thus, although there is a wide variety of instruments and approaches for hull cleaning (Kim *et al*., 2023) there is need for effective waste management methods and plans, including in-water cleaning and capture (IWCC) devices, that minimize the impacts derived from maintenance practices.

A recent alternative to reactive cleaning and maintenance, both dry-docking and standard in-water practices, is proactive cleaning, also known as grooming, which consists in a frequent, habitual and gentle mechanical cleaning, with options of doing so in-transit. It has proven to be

an effective option that could eliminate the need of other cleaning methods while reducing operational costs and minimizing coating damage (Hearin, 2016; Ralston *et al.*, 2022).

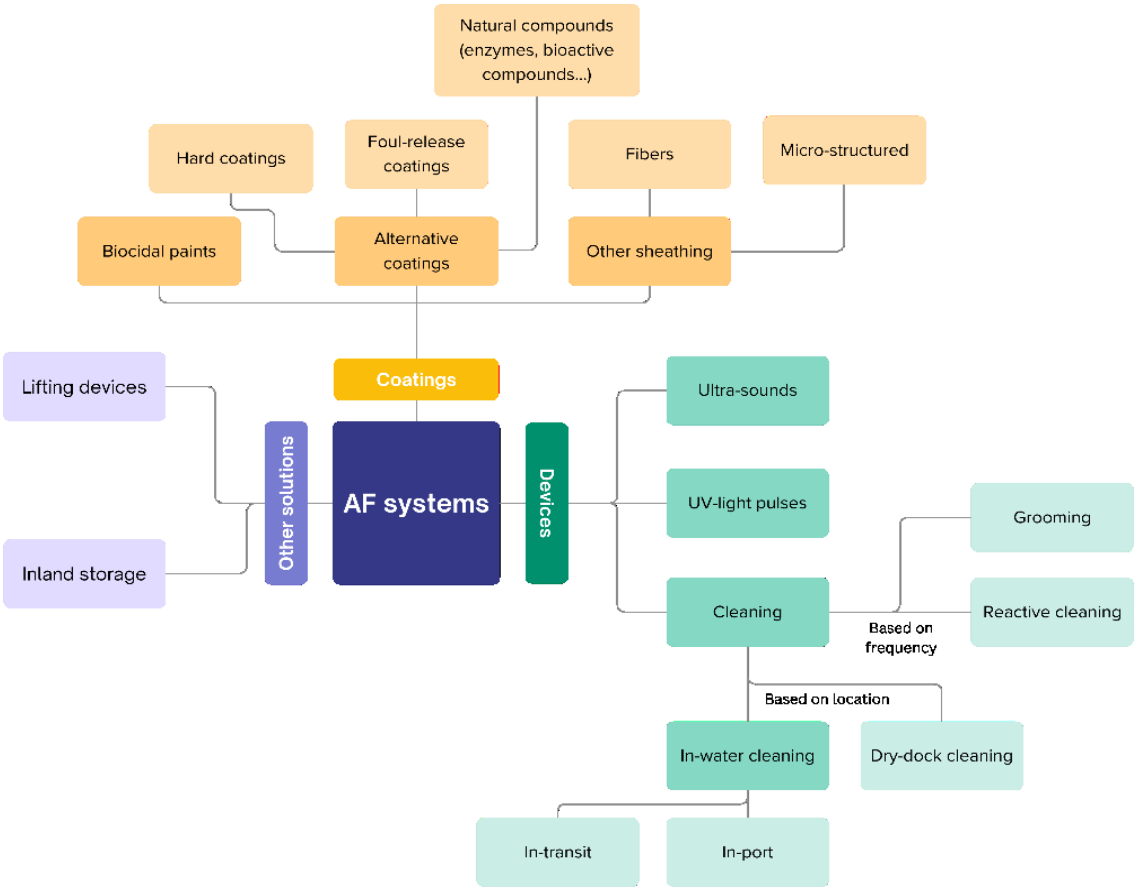


Figure 7. Schematic summary of the current fouling prevention measures

Because some mechanical measures may affect the integrity and performance of the coating, other alternative physical measures can be employed, such as ultrasounds and UV. Ultrasound technology can be applied to restrain fouling development by means of cavitation, vibration, heating etc. associated to the ultrasonic frequencies. Numerous studies have addressed the effectiveness of acoustic methods (Legg *et al.*, 2015; Park & Lee, 2018; Gizer *et al.*, 2023), both audible and ultrasonic, and new advancements in technology has allowed real application of these measures. Ultraviolet-light (UV) has been applied successfully in various industries, like food production water management and biomedicine, to avoid contamination, and recently it has been considered to be a potential measure in fouling control, after its use in ballast water treatments (Tsolaki & Diamadopoulos, 2010), with some studies on its application (Ryan *et al.*, 2020; Richard *et al.*, 2021). Lastly, desiccation by means of lifting devices requires a re-structuration of mooring sites, allowing the lifting of vessels, in other to dry-off the surfaces and prevent new recruitments during long idle times.

Cultural measures

Although cultural measures act mainly on habits and routines of ship and boat owners, other interested parties, such as marina managers, port and local authorities etc., may play a significant role in implementation and progress towards efficient measures. They are considered to be benign measures and conform the basis of any fouling management plan (Figure 8). Culver *et al*. (2012) listed a series of measures that make up these cultural measures and Table 1 lists them, including further updates, according to the final executor.

Table 1. Summary of cultural measures (updated and completed from Culver *et al*., 2012).

Boat owner	
Suitable strategy selection	Location, boat type, usage (frequency, speed)
Scheduling	
- Coating application	Most effective just before the peak of recruitment. It may require additional measures
- Hull cleaning	Adjusted to type and age of coating, seasonality, travel plans, geographical area and harbour location
Recording	Follow up of the applied measures and associated information
Marina manager	
Facilities and services	Cleaning services, waste management plans and services, qualified personnel. Improvement of infrastructures and offer of alternative options
Dissemination plans	Promotion of good practices, spread of latest information among clients, outreach events, etc.
Authorities	
Access to information	Updated and clear information that include fact sheets, protocols, best practices, summarized regulatory information and requirements, appointed office and contact people
Outreach programs	Stakeholder meetings, round tables, social media campaigns, specific events
Implementation plans	Clear legal information, stated responsibilities, engagement activities, dedicated staff to ensure compliance.

Cultural measures act from a preventive approach, opposed to other tactics that require direct intervention (Figure 8), and can be considered a fundamental part of the implementation phase of any regulatory measure. An example of this is the development and application of the *Craft Risk Management Standard for Biofouling on Vessels Arriving to New Zealand* ("CRMS-BIOFOUL"; MPI, 2014), with an initial voluntary compliance that evolved into mandatory enforcement in 2018. This time window allowed an important outreach campaign from the Ministry of Primary Industries to the stakeholders, including stakeholder meetings, the development of guidance documents and fact sheets, and organized media and social media campaigns (Scianni *et al*.,

2021). Currently, the official website of the MPI for Biofouling management includes clear information, tools and appointed contacts that assist stakeholders with the entry requirements (MPI, 2024a; MPI, 2024b).

In summary, the current scenario of antifouling measures (AFM) is composed by a great diversity of measures (Figure 6 and Figure 7), most of which have already reached the market. Generally, they can be classified into four main classes of *tactics* or approaches, three of which have been addressed above: 1) cultural, 2) physical, 3) biological based on the release of 'biological control agents' (out of our scope) and 4) chemical, as in Culver *et al*., 2012 (Figure 8, Table 2).

Table 2. Summary of the most common antifouling measures by tactic category.

Type	Action mechanism	Examples
Chemical approaches		
Biocides	Release of toxicants	Copper & zinc oxides
Photoactive release	Light generated H_2O_2	
Natural active compounds	Deterrence, repellency or inhibition	Hydrolases, lyases, or medetomidine
Physical approaches		
Foul release	Reduced attachment strength	Silicones, fluoroploymers
UV light pulses	DNA damage, prevention of biofilm formation	UV light-emitting diodes in a protective coating
Ultrasounds	Ultrasonic waves create micro-local temperature increase, biofilm prevention	Transducers
Cleaning devices	Mechanical removal of foulers	
Cultural approaches		
Timing maintenance	Planning and scheduling	
Recording maintenance	Logbook recording maintenance practices	
Maintenance facilities	Improving and increasing maintenance facilities	Cleaning services and waste management
Dissemination strategies	Implementing and promoting good practices	Outreach & info events, newsletters, etc

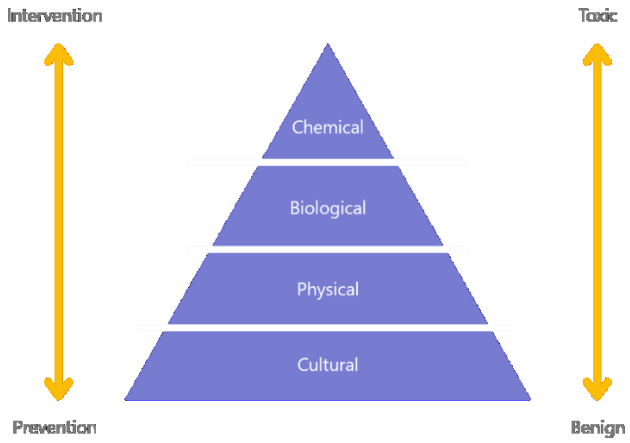


Figure 8. Classification of antifouling tactics and the required level of intervention (left) and associated impact in terms of toxicity (right). Pyramid of Integrated Pest Management from Culver et al., 2012.

Challenges in fouling prevention

Service life

Long-term performance of AF coatings is one of the major challenges to be faced. Biocide-based coatings, BC, and especially copper-based ones, are characterized by a high release rate during the initial service months, making them highly efficient in the short-term. However, their performance drops when release rates get below a critical level, allowing recruitment of species with greater copper tolerance (Dafforn *et al.*, 2011). Furthermore, these coatings show a release rate of biocides greater than necessary after application (Lagerström *et al.*, 2020), raising concerns on the potential environmental effects (Dafforn *et al.*, 2011; Amara *et al.*, 2018). Furthermore, copper release rates vary according to certain environmental parameters such as salinity (Yebra *et al.*, 2004; Lagerström *et al.*, 2020), showing a positive correlation, which may also influence the long-term performance of the coating.

Optimal coating application

Optimal application is key to maximize coating performance. Limited accessibility to some areas may hamper proper coating application and, together with areas of damaged coating, act as *niche areas* (Figure 9). Therefore, niche areas are known to be hotspots for biofouling accumulation, with fouling significantly more prevalent in those areas than any other hull surface (Figure 10, A) (Davidson *et al.*, 2016). Consequently, niche areas tend to raise biosecurity concerns related to a higher risk of species introduction.

Therefore, biofouling development varies across areas within a ship and makes management tricky and heterogeneous. Figure 10 illustrates fouling accumulation profiles under different scenarios (Davidson *et al.*, 2016). Uncoated areas, niche areas and dock block areas (present in

large vessels) tend to accumulate biomass faster. Poorly applied or damaged coating and coatings mismatching vessels' operational profiles accumulate biofouling quicker, leading to reduced operability and functioning, while also increasing bioinvasion risks. Proper coating selection and application maximizes performance and durability, while reactive cleaning maintenance helps to reduce fouling levels temporally, requiring increased frequency with time.

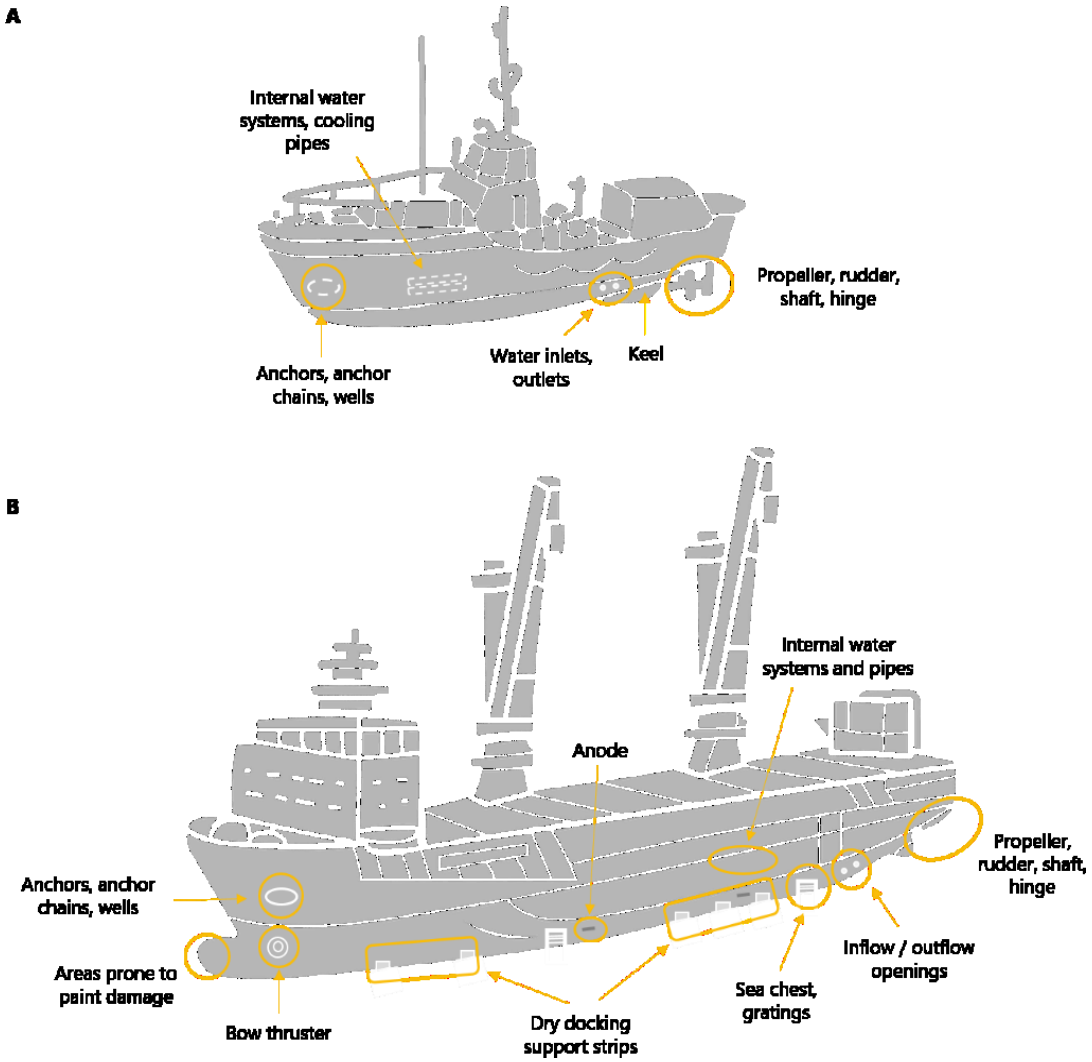


Figure 9. Localization of main niche areas for recreational boats (A) and commercial vessels (B). Adapted from Georgiades, E., & Kluza, D. (2020) and MPI (2024b).

Suitability of maintenance measures

As regards cleaning and maintenance measures, efficient capture of derived wastes and a suitable treatment is still a challenge. Although new technology advancements allow also in-water capture, their applicability in certain sectors is limited. In fact, despite the tide of antifouling measures that

progressively are reaching the market, their social acceptability and convenience, feasibility and applicability are unclear for sectors such as recreational boating.

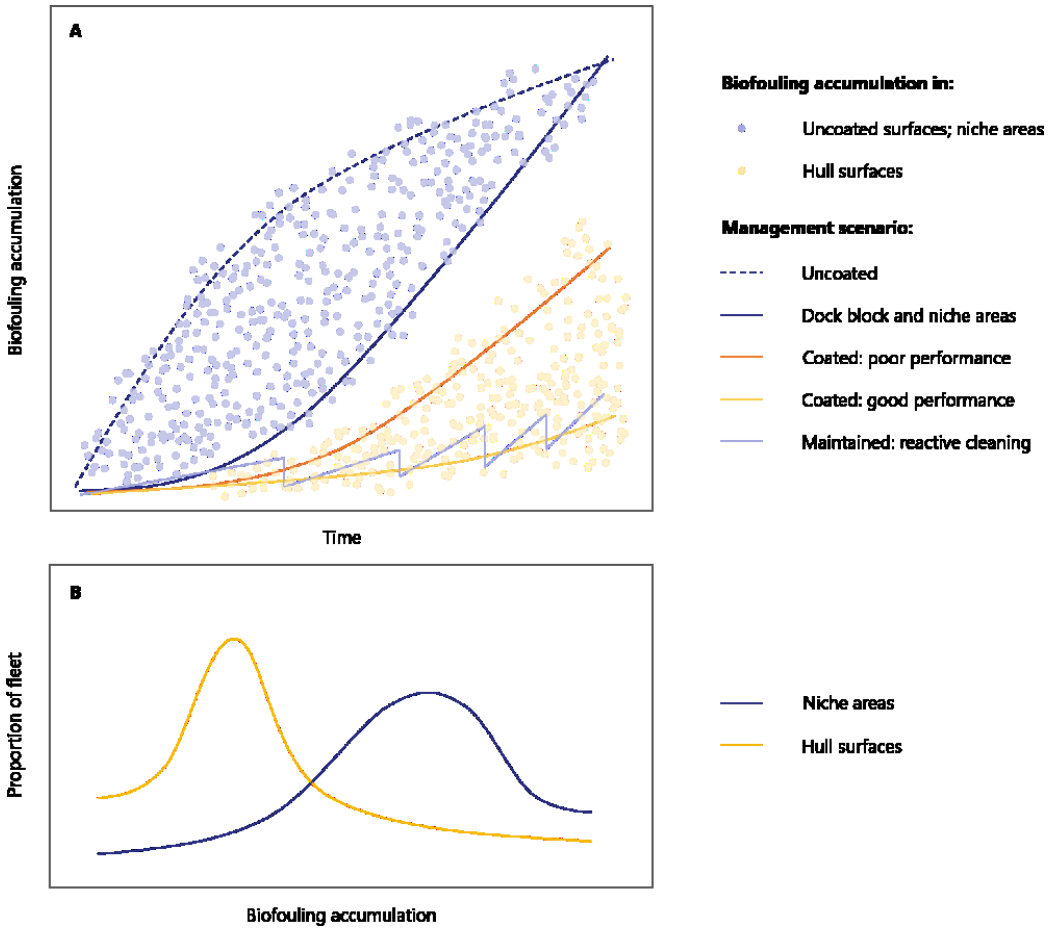


Figure 10. Conceptual diagrams of biofouling accumulation. A) Dots represent a theoretical biofouling accumulation on uncoated surfaces and niche areas (powder blue) and on managed hull surfaces (pale yellow). Lines represent accumulation tendencies based on the boat area and management scenario: uncoated surfaces (dashed blue line), dock block and niche areas (solid blue line), coated with low performance coating or poor application (solid orange line), coated with good performance coating (solid yellow line) and maintained with in-water periodical reactive cleaning (solid powder blue line). (B) Proportions of ships and biofouling accumulation rates in niche areas (solid blue line) and hull surfaces (solid orange line). Adapted from Davidson et al., 2016.

Environmental impacts of antifouling measures

Although antifouling measures are of common use in many industries, there are still uncertainties and knowledge gaps on their potential environmental impacts. Regarding AF paints, the major concern is their indirect effects on the environment and fate of their components. In the 1960s, tributyltin (TBT) based AF paints were developed and widely used due to their high effectiveness. However, TBT showed important negative consequences for many marine organisms, including

imposex in gastropods and consequent reduction in reproductive capability, deformation and abnormalities in oysters, as well as bioaccumulation potential and persistence in sediments (Bryan & Gibbs, 1991; Stroben *et al.*, 1995; Santillo *et al.*, 2002; Price & Readman, 2013). These environmental effects lead to progressive restrictions on its use (Council Directive 76/769/EEC, 1989) and finally to its global ban in 2001 (IMO, 2001; Regulation (EU) 782/2003, 2003), aiming for its effective elimination from ships by 2008, although some studies keep reporting their use and commercialization (Paz-Villarraga, 2022; Uc-Peraza *et al.*, 2022). Following the entry into force of the regulations, copper-based compounds became the major biocides used in the industry (Yebra *et al.*, 2004; Dafforn *et al.*, 2011), usually accompanied by booster co-biocides.

Copper itself is an essential element and its sources in the marine environment are diverse. However, being the main active compound of current biocide-based coatings and due to their wide use, AF paints have been identified as the main source of diffuse copper input to the marine environment (EMTER, 2021). Furthermore, studies such as the one by Lagerström *et al* (2020) determined excessive leaching of copper from AF into the environment. Yet, according to what stated by the Biocidal Products Regulation (Regulation (EU) 528/2012, 2012) (BPR) recommended dose of a biocidal product should be the minimum necessary to achieve the desired effect (Annex VI Art. 77). Moreover, biocidal agents must have fast degradability to reduce their environmental persistence, low bioaccumulation potential and low toxicity to non-target species.

Copper usage, as well as that from other co-biocides, is currently under scrutiny, as they fail to meet those requirements (Readman, 2006; Lagerström *et al*, 2020; de Campos *et al.*, 2021). Booster biocides such as Irgarol (cybutryne) are not readily biodegradable and have been reported to be highly toxic for non-target marine organisms (Yebra *et al.*, 2004), leading to its recent European ban in 2016 (Commission Implementing Decision (EU) 2016/107, 2016). Metals, such as copper, on the other hand, are known to have effects on different biological endpoints: survival and developmental (Lee *et al.*, 2008; Biandolino *et al.*, 2018; Charry *et al.*, 2019); reproductive (Hook & Fisher, 2001; Hussain *et al.*, 2020); as well as other physiological and molecular endpoints (Pinto *et al.*, 2003; de Almeida-Rodrigues *et al.*, 2021). Some studies have reported toxicological effects on non-target species (Katranitsas, 2003; Karlsson *et al.*, 2010; Ytreberg *et al.*, 2010; Oliveira, 2017; Amara *et al.*, 2018) and biocide resistance in certain fouling (target) species (Floer *et al.*, 2004; Piola & Johnston, 2006). In the case of copper, however, due to complexation, their fate and exposure pathways can vary. Its associated environmental risk is also expected to vary in relation to abiotic parameters, being some, such as salinity, directly related with its leaching rates (Singh & Turner, 2009; Lagerström *et al*, 2020). Therefore, depending of the local environmental conditions, the application of certain coatings could pose a higher risk. Other factors, such as

water renewal, boat density, and proximity to shipbuilding and cleaning infrastructures could additionally contribute to biocide accumulation risk (Schiff *et al.*, 2007; Brooks & Waldock, 2009; Ytreberg *et al.*, 2010).

Additionally, combination of active compounds and presence of other paint components are to be considered when studying biological impacts to understand the additive and synergistic effects that contribute to the overall toxicity (Karlsson *et al.*, 2010). Recent studies have pointed out to other forms of antifouling pollution beyond leaching of biocides. In fact, antifouling paint particles (AFPPs) are gaining attention due to their abundance, being even among the most common microplastic forms in certain marine environments, (Kang *et al.*, 2015; Turner *et al.*, 2021) which could act as micro-sources of metal pollution, leaching biocides locally (Soroldoni *et al.*, 2020; Gaylarde *et al.*, 2021; Turner *et al.*, 2021). Hull maintenance particles can also contribute to an increase dislodge of these fragments and their release into the environment. Inadequate management of maintenance-derived wastes can also be, indeed, an important source of chemical and biological pollution (Kim *et al.*, 2023).

Today, there is uncertainty on the efficiency of antifouling coatings in fouling prevention (Culver 2012; Culver *et al.*, 2021) and, thus, effective antifouling strategies usually require combining chemical approaches with other physical and even cultural tactics to maximize their performance (Culver *et al.*, 2012; Wezenbeek *et al.*, 2018; Xie *et al.*, 2019; Kim *et al.*, 2023). New products and technologies have reached the market; however, these recent developments in antifouling technologies have also given raise to associated concerns and new challenges. Hull cleaning is known to release not only biocidal compounds, but also biofouling propagules and viable fragments that, without proper waste management, increase environmental hazards associated to biofouling prevention. Although the diversity of antifouling strategies has evidenced a lack of standards, creating a gap in toxicological information and potential environmental risks (Kim *et al.*, 2023), the application of standard and rigorous assessments for some technologies, such us in-water cleaning and capture (IWCC) devices, is proven challenging but possible and a need within the sector (Tamburri *et al.*, 2020). These assessments help product maturation and optimization, as well as development and implementation of management protocols.

Ecotoxicology and field monitoring

In this context, ecotoxicology and field monitoring become two pivotal tools to 1) assess the impacts and effectiveness of antifouling strategies and 2) to address the lack of standards and toxicological gaps associated to the available range of antifouling technologies.

The ultimate goal of ecotoxicological testing is to predict ecological effects of chemicals and other stressors (Cairns & Pratt, 1989). Bioassays are procedures that aim to understand the effects of certain chemical substances or compounds at different biological levels. They rely on effect-based measurements and are specifically designed to investigate a target effect (biological response). When studying a certain compound, acute toxicity tests are usually the first approach in toxicity screening, narrowing down the concentration range to establish sub-lethal concentrations for further testing, as lethal toxicity alone is not considered an environmentally relevant endpoint (Cairns & Pratt, 1989). Acute toxicity bioassays are designed to be quick, replicable and inexpensive and to provide a useful baseline to develop more specific experimental testing. A battery of approaches often gives a wider appraisal of biological effects of chemical substances, combining multiple response measurements across different taxa (Blaise & Gagné, 2009). For the specific case of antifouling coatings, bioassays can range from initial lethal toxicity screening to assays on settlement, adhesion strength, growth inhibition, behaviour, etc. (Briand *et al.*, 2009; Dahms & Hellio, 2009). Besides, other classical endpoints can also be tested, such as fecundity or development (Lee *et al.*, 2008; Biandolino *et al.*, 2018; Hussain *et al.*, 2020).

Biomarkers constitute the second pillar in ecotoxicology and are defined as measurements at molecular or cellular level that indicate that the organism has been exposed to toxic chemicals (biomarker of exposure) and the magnitude of the organism´s response to the contaminant (biomarker of effect) (McCarthy & Shugart, 1990). In antifouling testing, biomarker groups involved in biotransformation and detoxification, antioxidant activity and indices of oxidative stress, immunotoxicity, alterations at gene expression level (toxicogenomics) and histological alterations, among others, are often applied (Murugadas *et al.*, 2016; Park *et al.*, 2016; George *et al.*, 2017; Yun *et al.*, 2023).

Broad-taxa approach is gaining importance in the field of ecotoxicology, emphasizing the need of *in vivo* toxicity testing based on multiple model organisms and endpoints (Rosner *et al.*, 2024), to overcome the interspecies differences in sensitivities. Tests done with organisms from different trophic levels (Blaise & Gagné, 2009; Oliveira *et al.*, 2017) or belonging to various functional groups (Soroldoni *et al.*, 2020) contribute to understand toxicity pathways, transfer mechanisms and sensitivities.

Effectively combining bioassays and biomarkers constitutes a comprehensive approach to assess biological effects at various levels and even across species. However, laboratory restricted experimentation fails to resemble the complexity of the natural environment. Field studies can help to complete the picture, particularly when studying substances like antifouling, which are

aimed to perform in-situ to prevent the development of fouling communities and, ideally, with a minimum impact in the surrounding environment. Therefore, a comprehensive approach in antifouling testing should be composed of quick screening tools (bioassays), measurements of specific endpoints (multilevel biomarkers) and *in situ* performance testing of the antifouling product. Furthermore, in relation to the nature of antifouling products, it is of especial importance to include a broad-taxa approach for *in vivo* testing, which encompasses both target and non-target species.

Regulatory framework in biofouling management

Although shipping has long been identified as a vector for NIS introduction and spread (Bishop 1951; Elton 1958; Carlton, 1985), international regulatory measures only arrived in 2004, substituting prior guidelines and focusing mostly on ballast water as a vector (*International Convention for the Control and Management of Ships' Ballast Water and Sediments*; IMO, 2004), and did not enter into force globally until September 2017, with September 2024 as deadline for its global implementation.

Regarding biofouling as a vector, international efforts began in 2006, when the issue was formally discussed at the IMO (Scianni *et al.*, 2021). Those efforts lead IMO's Marine Environmental Protection Committee (MEPC) to the approval in 2011 of the voluntary *Guidelines for the Control and Management of Ships' Biofouling to Minimize the Transfer of Invasive Aquatic Species* (MEPC, 2011) and *Guidance for Minimizing the Transfer of Invasive Aquatic Species as Biofouling (Hull Fouling) for Recreational Craft* (MEPC, 2012). These guidelines have been updated (MEPC, 2023b), but they keep they status as guidelines, intended to provide useful recommendations for biofouling management practices.

There are other conventions and directives address and regulate fouling associated issues, without directly tackling the topic, such as the "*Biocidal Products Directive*" (BPD; Directive 98/8/EC and Regulation (EU) No 528/2012), the "*International Convention on the Control of Harmful Anti-fouling Systems on Ships*" (AFS; 2001), the "*International Convention for the Prevention of Pollution from Ships*" (MARPOL).

To date, the only existing regulations and requirements in force that specifically address biofouling are implemented solely in California (California Legislature, 2007; California State Lands Commission, 2017, adopted and in force since 2017), New Zealand (MPI, 2014; in force since 2018) and Australia (DAFF, 2015; amended and in force since 2022). All three fall within the biosecurity

standards of each country/state and focus on vessel biofouling control to minimize the transfer of NIS into their territorial waters.

This scenario evidences unclear fragmented response towards biofouling management and, thus, a lack of standards and regulatory tools in biosecurity requirements. In fact, there is undoubtedly an imbalance across geographical areas and sectors, with biofouling guidelines and regulations mostly applying to the shipping and boating sector. However, other industries affected by biofouling and relevant for the global biosecurity, such as aquaculture, are generally left aside. Additionally, within the same sector, some regulations do not equally apply certification regimes and inspections to all type of structures or ship types (e.g. Regulation (EU) 782/2003, 2003 on the prohibition of organotin compounds on ships).

State of the art

Artificial structures exposed to or submerged in aquatic environments are prone to be colonized in absence of any preventive measure by organisms that compose the so-called biofouling. The development of these communities can lead to reduced functionality and operability of the structures and systems, risks to their structural integrity, and potential biosecurity hazards, which generally are translated into increased operational and maintenance costs as well as ecological costs. Biofouling is a cross-sectorial issue with profound implications for the concerned industries and, to date, there is no long-term preventive method capable of effectively reducing its development, nor a binding legal framework at international level that addresses the matter. Still, biofouling control is in everyone's interest and there is a wide antifouling market offering a plethora of measures.

Traditionally, BC coatings have been primarily applied as main measure on biofouling prevention and, currently, they constitute the typology dominating the market, encompassing most of the products offered by the big manufacturers. Copper has long been used as biocide, even when other compounds dominated the market, such as TBT. After the later one was banned from the market, copper usage experienced a rise again. However, environmental concerns on its effects are increasing, as new scientific evidence is being made available. Recently, in response to these environmental concerns and uncertainties regarding the performance of traditional BC coatings, novel alternative solutions have reached the market and are starting to find their niche within it, even with some of these products being manufactured by major companies in this sector.

Although regulations on biocidal compounds exist, they encompass multiple substance types and usages, while some antifouling systems remain outside their provisions. Additionally, the current regulatory instrument on antifouling systems focuses on prohibiting harmful substances, with only two listed in its Annex since its approval. Biofouling control, from a holistic environmental protection perspective, has not been addressed at jurisdictional level and relies on guidelines and good practices. This scenario has encouraged some exceptions to regulate biofouling and limit the application of other biocides at regional level, but they remain isolated cases.

At the light of today's context, there is a need of standard procedures for antifouling testing, which, because of the complexity and nature of the issue, require of integrative approaches that provide a full picture of the fouling control system, including their effects, performance, durability and suitable usage. Such procedures could ease the evaluation of new antifouling measures and find their fit within the existing guidelines and regulations, as well as their integration in management plans.

Scope

This work focuses on leisure boats, a pivotal sector for blue tourism and an important vector for the introduction and secondary spread of non-indigenous species. Therefore, the selected coatings are commercially available and used by boat owners.

Hypothesis

Current traditional biocide-based coatings have diverse toxicological effects on target and non-target species and fail to meet *in situ* performance goals. Contrarily, alternative non-toxic foul-release coatings do not show adverse toxicological effects on target species, nor in non-target species, and perform, at least, as their traditional counterpart. Existing guidelines on biofouling management are insufficient and fail to assist final users in the selection of suitable antifouling measures.

Objectives

In order to experimentally test the hypothesis, the main objective of present work is to study and understand the toxicity profiles and performance of a selection of commercial antifouling coatings (BC, FR) from a holistic approach, ultimately aiming to develop precise and appropriate management strategies, for which a series of sub-objectives were established:

1. To develop a standard procedure on antifouling coating lixiviate preparation and their chemical characterization by determining metal release and lixiviates' composition under different temperature and salinity scenarios in laboratory controlled conditions.

2. To compare the toxicity profiles of the selected antifouling coatings and to investigate their toxic effects in various target and non-target marine organisms exposed to the respective lixiviates, from a biological multi-level and multi-taxa approach, including **a.** microalgae and **b.** zooplankton.

3. To assess in situ performance of the selected antifouling coatings and to characterize the biofouling communities and their dynamics in response to the different AF strategies, with NIS recruitment in the limelight.

4. To review the evolution of international marine environmental protection and the existing biofouling regulatory instruments to, eventually, integrate the experimental outcomes of antifouling multi-approach testing into the current policy framework, serving as updated recommendations for stakeholders.

01

Chemical characterization

Development of a standard procedure for antifouling testing: chemical characterization of antifouling coating lixiviates and their behaviour under different laboratory simulated environmental scenarios

Congress contribution:

Santos-Simón, M., Barbieri, E., Etxebarria, N., Marchini, A., Ortiz-Zarragoitia, M. (2024). Chemical Characterization and Toxicity Assessment of Antifouling Coatings' Lixiviate: Implications for Biofouling Management. Society of Environmental Toxicology and Chemistry (SETAC), Sevilla, Spain.

Abstract

Antifouling coatings are usually the first and main measure in biofouling control. Traditionally coatings contain active compounds embedded in a matrix (biocides) that are released into the environment to prevent the growth of organisms. Due to concerns on their potential indirect effects in the environment and uncertainty regarding their performance, new solutions have reached the market, being foul-release (FR) coatings the most commonly used alternative. This section 1) describes the selected antifouling products that are used in the current project, a biocide-based coating (BC) and a FR one; 2) establishes a standard procedure for coating lixiviate preparations to be used for toxicity tests and their characterization; and 3) analyses the effects of environmental factors on the chemical leaching from the two types of antifouling coatings. Results show a clearly distinct metal composition of lixiviates based on their specific formulations and different behaviours under the experimental scenarios of modified temperatures and salinities. In fact, these factors affect the release of metals differently and according to the metal identity. Interestingly, Cr concentration is remarkably higher in FR lixiviates, suggesting some leaching from the inner paint coat despite the external silicone coat. Undeniably, incubation conditions can alter the leaching of chemicals from the coatings to lixiviates, affecting their final composition. Efficient and suitable biofouling management plans should needfully consider the chemical behaviour of the AF of choice and the local environmental conditions, while accounting for risk mitigation under a global changing scenario.

Keywords | Antifouling · lixiviate · metal release · risk mitigation · ICP-MS

Resumen

Los recubrimientos antiincrustantes (del inglés, antifouling; AF) suelen ser la primera y principal medida para el control del biofouling. Tradicionalmente, contienen compuestos activos embebidos en una matriz (biocidas) que se liberan al medio ambiente para evitar el crecimiento de organismos. Debido a sus posibles efectos indirectos en el medio ambiente y la incertidumbre sobre su rendimiento, nuevas alternativas han alcanzado el mercado, siendo los recubrimientos antiincrustantes a base de silicona (FR) la alternativa más utilizada. Esta sección 1) describe los productos antiincrustantes seleccionados que se utilizan en el proyecto actual, un recubrimiento basado en biocidas (BC) y uno FR; 2) establece un procedimiento estándar para las preparaciones de lixiviados de los recubrimientos AF, que serán utilizados para pruebas de toxicidad, y para su caracterización; y 3) analiza los efectos de los factores ambientales en la lixiviación química de los dos tipos de recubrimientos antiincrustantes. Los resultados muestran una composición metálica claramente distinta de los lixiviados en función de su formulación y diferentes comportamientos bajo los escenarios experimentales de temperaturas y salinidades modificadas. De hecho, estos factores afectan la liberación de metales de manera diferente y de acuerdo con la identidad del metal. Cabe destacar que la concentración de cromo es notablemente más alta en los lixiviados de FR, lo que sugiere que este metal se libera desde la capa de pintura interna a pesar de la capa exterior de silicona. Indudablemente, las condiciones de incubación pueden alterar la liberación de sustancias químicas de los revestimientos a los lixiviados, lo que afecta a su composición final. Los planes de gestión de las incrustaciones biológicas deben considerar necesariamente el comportamiento químico del AF elegido y las condiciones ambientales locales, al tiempo que tienen en cuenta la mitigación de riesgos en un escenario global cambiante.

Palabras clave | Antiincrustante · lixiviado · liberación de metales · mitigación de riesgos · ICP-MS

Riassunto

I rivestimenti antivegetativi (dal inglese, antifouling; AF) sono solitamente la prima e principale misura per il controllo del biofouling. Tradizionalmente contengono composti attivi (biocidi) incorporati in una matrice e vengono rilasciati nell'ambiente per impedire la crescita di organismi. A causa dei possibili effetti indiretti sull'ambiente e dell'incertezza sulle loro prestazioni, nuove alternative hanno raggiunto il mercato, tra cui i rivestimenti antivegetativi a base siliconica (FR) sono l'alternativa più utilizzata. Questa sezione 1) descrive i prodotti antivegetativi selezionati per il loro studio nel progetto attuale, un rivestimento a base di biocidi (BC) e uno FR; 2) stabilisce una procedura standard per la prepazione di percolati dei rivestimenti AF da utilizzare per i test di tossicità; e 3) analizza gli effetti dei fattori ambientali sulla lisciviazione chimica delle due tipologie di rivestimenti antivegetativi. I risultati mostrano una composizione metallica chiaramente diversa dei percolati a seconda della loro formulazione e comportamenti diversi negli scenari sperimentali di temperature e salinità modificate. Questi fattori, infatti, influenzano il rilascio dei metalli in modo diverso e d'accordo all'identità del metallo. In particolare, la concentrazione di cromo è notevolmente più elevata nei percolati FR, suggerendo che questo metallo viene rilasciato dallo strato di vernice interno nonostante lo strato esterno di silicone. Indubbiamente, le condizioni di incubazione possono alterare il rilascio di sostanze chimiche dai rivestimenti ai percolati, influenzandone la composizione finale. Piani di gestione del biofouling efficienti e appropriati devono necessariamente considerare il comportamento chimico dell'AF scelto e le condizioni ambientali locali, tenendo conto della mitigazione del rischio in uno scenario globale in evoluzione.

Parole chiave | Antivegetativo · percolato · rilascio metallico · mitigazione del rischio · ICP-MS

Laburpena

Inkrustazioen aurkako gainestaldurak (ingelesetatik, antifouling; AF) izan ohi dira inkrustazio biologikoak kontrolatzeko lehen neurria eta nagusiena. Tradizionalki, konposatu aktiboak (biozidak) dituzte matrize batean txertatuta ingurunera askatzen direnak, organismoen hazkuntza saihestuz. Ingurumenean izan ditzaketen zeharkako eraginak eta haien errendimenduaren inguruko ziurgabetasunaren ondorioz, alternatiba berriak iritsi dira merkatura, eta silikonazko gainestaldurak (FR) dira alternatibarik erabiliena. Atal honetan, 1) proiektuan zehar erabiliko diren produktuak deskribatzen dira, biozidetan oinarritutako estaldura bat (BC) eta FR estaldura bat; 2) toxikotasun-probak egiteko erabiltzen diren AF lixibiatuen prestakuntza prozedura estandarizatua ezartzen da eta baita haien karakterizazioentzako metodologia ere; eta 3) ingurumen-faktoreek duten eragina aztertzen da hautatutako bi gainestalduren lixibazio kimikoan. Emaitzek lixibatuen konposizio metalikoa argi eta garbi desberdina dela erakusten dute, haien formulazioaren arabera, eta gainera, portaera kimiko ezberdinak dituzte baldintza esperimentalak aldatzean, zehazki tenperatura eta gazitasuna. Izan ere, faktore hauek metalen askapenean eragiten dute era ezberdinean, metalaren identitatearen arabera. Adierazgarria da kromoaren kontzentrazioa FR lixibatuetan, bereziki handia dena eta, beraz, metal horren askapena iradokitzen duena barneko pintura-geruzatik uretara, kanpo silikona-geruza bat izan arren. Zalantzarik gabe, inkubazio-baldintzek gainestalduren konposatu kimikoen askapena alda dezakete, lixibatuen konposizioan eraginez. Inkrustazio biologikoak kudeatzeko plan eraginkor eta egokiek nahitaez kontuan hartu behar dituzte aukeratutako AFaren portaera kimikoa eta tokiko ingurumen-baldintzak, baita agertoki aldakor batean arriskuak arintzeko neurriak ere.

Gako hitzak | Anti-inkrustazio · lixibatua · metalen askapena · arrisku arintzea · ICP-MS

1. Introduction

Antifouling coatings are usually the first and main measure in the control of biofouling. They traditionally contain active compounds embedded in a matrix that dictates their action mechanism and release rate. The earlier formulations were based on insoluble matrices as main carriers of the active biocides, which dissolve from the matrix into the water (Cao *el at*., 2011; Wezenbeek *el at*, 2018). However, the matrix does not erode and the release of active compounds decreases relatively fast. To lengthen the lifespan of the coatings, soluble matrices were developed based on the dissolution of the binder compound and therefore, the release of the active component (Cao *el at*., 2011). More recently, in order to control pace of biocidal release and further extend their durability, modern chemical antifouling coatings contain different polymers and copolymers with specific chemical characteristics that rely on hydrolysis of the matrix binder (Cao *el at*., 2011). The hydrolysis rate is controlled by the liberation of the active compound and its substitution by water in the free spaces. Ablative coatings erode by hydrolysis of the matrix, usually a mixture of soluble/hydration binder, while self-polishing coatings contain hydrolysable units that have a more progressive degradation and a greater control of the erosion and release rates (Takahashi, 2009; Wezenbeek *el at*, 2018). Currently, copper represents the most common active compound in combination with booster co-biocides (Jones and Bolam, 2007; Ytreberg *el at*., 2010; Tian *el at*, 2021) and, in fact, copper-based antifouling coatings constitute by far the major part of the available products of the main manufacturers. Interviews to leisure boat owners in the Mediterranean by Ulman *el at*., 2019 (Figure 1) show their antifouling coating preferences, supporting BC coatings as the main category of choice.

Although there is not much data available on the usage trends of antifouling coatings for leisure boats, available non-toxic alternatives from the main manufacturers are mostly limited to foul-release silicone based products. FR coating market is indeed, the main leading alternative market to traditional BC based coatings (Lagerström *el at*., 2022). Their use, however, was not particularly extended among leisure boat owners in the Mediterranean according to the unpublished data from interviews by Ulman *el at*., 2019 (Figure 1).

For the purpose of this thesis, two antifouling coatings were selected from the plethora of commercially available products, a traditional BC ablative antifouling coating and a FR coating. This section describes the selected antifouling products that are used in the current project.

Additionally, it covers the chemical aspects related to the selected coatings including leaching experiments and chemical analyses, with the aim of 1) understanding the effects of environmental

conditions on the chemical leaching from the two types of antifouling coatings (BC, FR) and 2) adapting an existing protocol to develop a standardized procedure for antifouling testing.

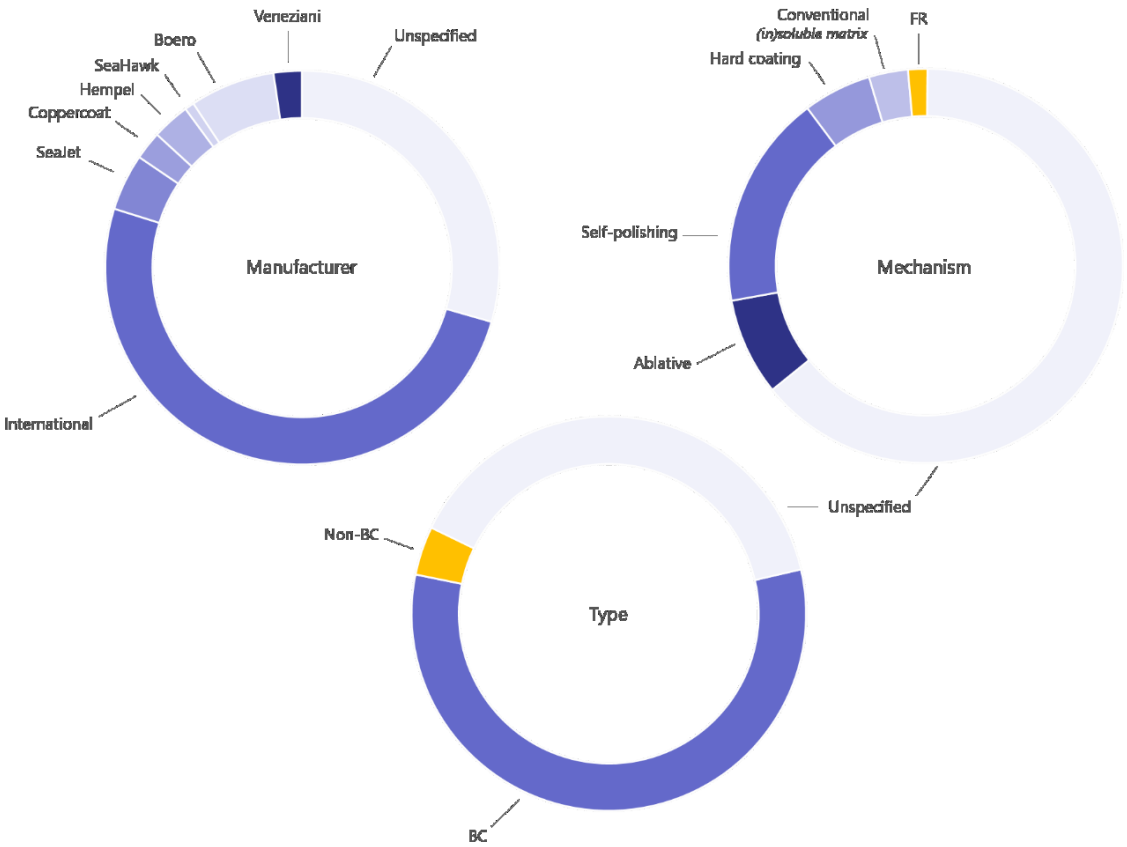


Figure 1. Descriptive graph illustrating the main choices of boat owners surveyed in the Mediterranean during the years 2015-2016. The data was extracted from face-to-face interviews (unpublished data collected by Ulman *el at*. 2019), focusing on Italian and French marinas and selecting only the answers with clear replies on antifouling practices.

2. Materials and methods

Understanding the chemical behaviour of the selected antifouling coatings is a key component for the subsequent sections of this work. Therefore, this chapter describes the selected coatings, the methodological procedures for the characterization of coating lixiviates and an experimental approach to determine the release of metals under different temperature and salinity scenarios.

2.1 Selection of the tested coatings

Two commercially available coatings were selected, belonging to the BC and FR categories. Based on 129 interviews to Mediterranean boaters collected in the years 2015 and 2016 by the University of Pavia team (unpublished data), for the preparation of the work Ulman *el at*. (2019), *International* was selected as the main manufacturer for the BC coating for leisure boats. Being one of the

named coatings (generally simply as Micron) and due to local availability in a specialized store, 'Micron 350w' was the coating of choice for the BC category. Table 1 summarizes the information provided both in the technical data excerpt and safety data sheet, also accessible online.

Table 1. Summary of technical and safety datasheets of the selected biocide-based coating (BC).

Biocide based antifouling coating				
General info	Name	Micron 350 w	Manufacturer	AkzoNobel (International)
	Colour	Dover White	Product code	YBB628
	Type	Biocide-based coating	Application	Professional or consumer
	Performance	Up to 2 years	Use	Fresh and salt water
	Other	Biocide release including in stationary periods. Application possible 12 months before launch.		
Composition	Compound		Identifier (CAS n.)	% of weight
	Dicopper oxide		1317-39-1	≥ 25 – 50 ≤
	Colofonia		8050-09-7	≥ 10 – 25 ≤
	Naphtha (dissolvent, light aromatic fraction)		64742-95-6	≤ 9.5
	Zinc oxide (booster biocide)		1314-13-2	≤ 10
	Xylene		1330-207	≤ 8
	Ethylbenzene		100-41-4	≤ 2
Application	Prior to the application, all fouling and contamination must be thoroughly removed including the leached layer. Thoroughly stir until product is mixed to a uniform consistency before use. To prevent premature failure, ensure the correct amount of paint is applied using the coverage as a guide. Product and ambient temperatures should be minimum 5°C and maximum 35°C; substrate temperature should be 3°C above dew point and maximum 35°C. To adapt it for PVC surfaces a pre-treatment (sanding) of the surface was done to maximize the adherence.			
Complete guide and references	Technical datasheet and safety info available at: https://www.international-yachtpaint.com/en/gb/boat-paint-products/antifouling/micron-350w			

Alternative non-toxic coatings were not particularly extended among boat owners according to the results of the interviews from Ulman *el at*. (unpublished data, Figure 1). Still, among those that did use alternative coatings, some specified the use of FR ones and, in some cases, the application of different coatings for propellers and hull area. Previous studies indicated FR coatings as the main substitute for traditional BC ones (Lagerström *el at*., 2022) and, indeed, available eco-friendly market products from the main manufacturers relay on this mechanism. After a research on the available market products, *Propspeed* kit was the product of choice as the alternative non-toxic FR coating. Table 2 summarizes the information provided both in the technical data excerpt and safety data sheet, accessible also online, of the three components from the kit that conform the coating layers.

Table 2. Summary of technical and safety datasheets of the foul-release coating (FR).

Foul-release antifouling paint				
General info	Name	Propspeed	Manufacturer	Propspeed ®
	Product	Propspeed kit	Product code	PSLKIT-EU
	Type	Foul release coating	Application*	Professional or consumer
	Performance	1-2 years	Use	Fresh and salt water
	Other	Anti-adhesion coating, foul-release system. If protected from sun and mechanical abrasion, coated surfaces can stay out of the water.		
	Kit components	Propclean; Propprep; Etching Primer Base (A) & Hardener (B); Clear Coat (C)		

Composition	Compound	Identifier (CAS number)	% of weight
	2-propanol	67-63-0 (kit component A)	≥ 30 – 60 ≤
	2-Metilpropan-1-ol	78-83-1 (kit component A)	≥ 10 – 30 ≤
	Zinc chromate	13530-65-9 (kit component A)	≥ 5 – 10 ≤
	Xylene	1330-20-7 (kit component A)	≥ 5 – 10 ≤
	Talc	14807-96-6 (kit component A)	≥ 2 – 5 ≤
	2-propanol	67-63-0 (kit component B)	≥ 60 – 100 ≤
	Phosphoric acid	7664-38-2 (kit component B)	≥ 10 – 20 ≤
	Xylene	1330-20-7 (kit component B)	≥ 10 – 30 ≤
	Methyl tris(methylethylketoximine)silane	22984-54-9 (kit component C)	≥ 2 – 8 ≤
	3-Aminopropyltrimethoxysilane	13822-56-5 (kit component C)	≥ 0.1 – 2 ≤
	Titanium(IV) bis(ethylacetoacetato) Diisopropoxide	27858-32-8 (kit component C)	≥ 0.1 – 2 ≤

Application	*Network of professionals for application purposes – professional application recommended. Prior to the application, all fouling and contamination must be thoroughly removed including any other coating layer. The surface must be sanded previously. First, use Propclean to degrease the surface and chemically prepare the surface with Propprep (this step can be avoided for PVC surfaces). Temperature is a key factor in the application process. Thoroughly stir the primer until is mixed to a uniform consistency before adding the catalyser. Add the catalyser (hardener) to the primer: 1 part of hardener for every 4 parts of primer. Immediately apply the mixture to the surface, two layers with adequate drying times. Finally apply the silicone (Clear Coat) after proper mixing with a paintbrush. To prevent premature failure, ensure the correct amount of paint is applied and follow correct time spacing between applications. Product and ambient temperatures should be minimum 5°C and moisture not higher than 85%. Do not apply under direct sun exposure.
Complete guide and references	Technical datasheet, safety info and application guidelines available at: https://www.propspeed.es/documentacion-propspeed (accessed 2021)

2.2 Lixiviate preparation

The materials herewith described are based in the standard procedure of the Smithsonian Environmental Research Center (SERC; Edgewater, MD, United States) for biofouling studies (Marraffini *el at*., 2017; Jimenez *el at*., 2018). Polyvinyl chloride (PVC) panels with a standardized surface of 14x14 cm were sanded and coated, when required, with the selected coatings (BC, FR,

and 'bare': no coating) according to the manufacturers' procedures. Plates were left to dry in the dark and transferred into a glass container with 1L of filtered sea water (0.22 µm mesh size), in such a way that the plate was fixed in the surface, with the coated side facing downwards and completely submerged. Subsequently, the container was placed in an incubator (MaxQ500 Incubator Shaker – Refrigerated/Heated) with an integrated shaker for 24 h at a speed of 150 rpm.

2.3 Experimental design

To test how different scenarios affect the leaching of metals from antifouling coatings, metal release was measured under six experimental conditions (Table 3), modifying temperature or salinity alone, to understand the contribution of individual factors to the metal release. The baseline condition has reference salinity and temperature values of 33.5 PSU and 20 °C and were used subsequently for all the incubation steps of the laboratory experiments of this thesis. The remaining leaching scenarios were determined by modifying the value of a single factor and maintaining the other factor at baseline condition values, resulting in: a) three temperature scenarios at 33.5 PSU, and b) three salinity scenarios at 20ºC. Additionally, uncoated PVC plates were used as a control correction for the plate material, maximizing detection of the measured elements (ensuring beyond detection limit, BDL, concentrations) by incubating them at high temperature and salinity conditions (30 °C and 37.5 PSU).

Table 3. Experimental conditions for the incubation of coated plates for the characterization of metal leaching under different simulated scenarios, corresponding to a range of possible environmental conditions where the antifouling products are used.

	Low temperature	Low salinity	Baseline condition	High temperature	High salinity
Temperature	14 °C	20 °C	20 °C	30 °C	20 °C
Salinity	33.5 PSU	20 PSU	33.5 PSU	33.5 PSU	37.5 PSU

After the incubation (see '*Lixiviate preparation*'), water samples from the lixiviates were taken to measure the metal release from the coated surfaces with inductively coupled plasma mass spectrometry (ICP-MS).

2.4 Chemical characterization of coating lixiviates by ICP-MS

Since samples were mostly prepared using seawater, they were filtered through 0.45 µm filters (PVDF, Milipore) and diluted around ten times with MilliQ water. ICP-MS analyses were carried out inside a class 100 cleanroom using an ICP-MS (NexION 300, Perkin Elmer) in which the most abundant isotopes were measured. Plasma conditions such as nebuliser Ar flow, torch position and instrument lens voltages were optimised daily by aspirating a standard containing Mg, Rh, In,

Pb and U at 1 ng·mL-1. The nebuliser gas flow was optimised to obtain a balance between sensitivity and low level of oxides (below 2.5% for the CeO/Ce ratio). Finally, quantification was performed on a multi-elemental calibration curve obtained from commercial standards of 1000 µg·g-1 (Alfa Aesar, Specpure) using Be, Sc, Ge, In, Ge, and Bi as internal standards.

2.5 Statistical analysis

Statistical analyses were conducted using RStudio (`version 4.2.2`; R Core Team, 2022) and its following packages: `vegan` (Oksanen *el at*., 2022), `car` (Fox and Weisberg, 2019), `rcompanion` (Mangiafico, 2023), `lmPerm` (Wheeler & Torchiano, 2016) and `ggplot2` (Wickham, 2016).

Metal composition of lixiviates was compared using a multivariate approach considering 14 selected elements from the ICP-MS analysis, which are provided as supportive material in the Annex (Table S4; 3. Supportive material). For this purpose a Permutational Analysis of Variance (PERMANOVA) (Anderson, 2001) was carried out, based on Euclidean distances and scaled metal concentration values. Furthermore, dispersion within treatment groups was checked with PERMDISP (Anderson, 2006), running '`betadisper`' function on the distance matrix for the homogeneity of multivariate dispersions. PERMANOVA was performed applying the function '`adonis2`' of the `vegan`, under the reduced model following a sequential addition of terms and 9999 free permutations. Multivariate data visualization was done through a Principal Component Analysis (PCA), using the packages '`corrr`' (Kuhn *el at*., 2020), '`ggcorrplot`' (Kassambara, 2023) and '`FactoMineR`' (Lê *el at*., 2008) with scaled data. The PCA plot included ellipses with 0.95 confidence level.

Univariate analysis were performed to study the individual chemical behaviour of metals of interest (copper (Cu), zinc (Zn), chromium (Cr) and arsenic (As)), selected after an initial data screening and/or in relation to the coatings' formulation. Prior to the test, normality and homogeneity of variances were checked with Levene's (Levene, 1960) and Shapiro tests (Shapiro & Wilk, 1965), respectively. Whenever the assumptions were met, ANOVA (Chambers & Hastie, 1992); otherwise, permutational ANOVA (Wheeler & Torchiano, 2016) was applied. Both multivariate and univariate analyses were done with untransformed data.

3. Results

Metal concentrations obtained from the ICP-MS analysis of lixiviates are summarized in the third section of the Annex (Table S4; 3. Supportive material). Results from multivariate analysis showed clearly distinct composition of coatings, as could be expected from their respective formulations,

and diverse chemical behaviour under the experimental scenarios. The statistical outputs the PERMANOVA analysis can be found in Table 4 and are graphically supported with Figure 2. The conducted analysis revealed the significance of the tested factors, including coating type, selected incubation parameters and their interaction and the PCA graph visualizes these outcomes. Three clear clusters spatially separated can be observed, corresponding to groupings of bare, BC and FR treatments and, within each cluster of coating type, the effects of the incubation parameters. Salinity affected the release of metals from both coating types, BC and FR, visible as small well-defined clusters of salinity modified scenarios within each coating group (Figure 2). This parameter was the main responsible of differences among BC lixiviates, while differences across FR treatments we due to both tested incubation parameters, temperature and salinity. In fact, FR derived lixiviates appeared as four well-delimited clusters in Figure 2.

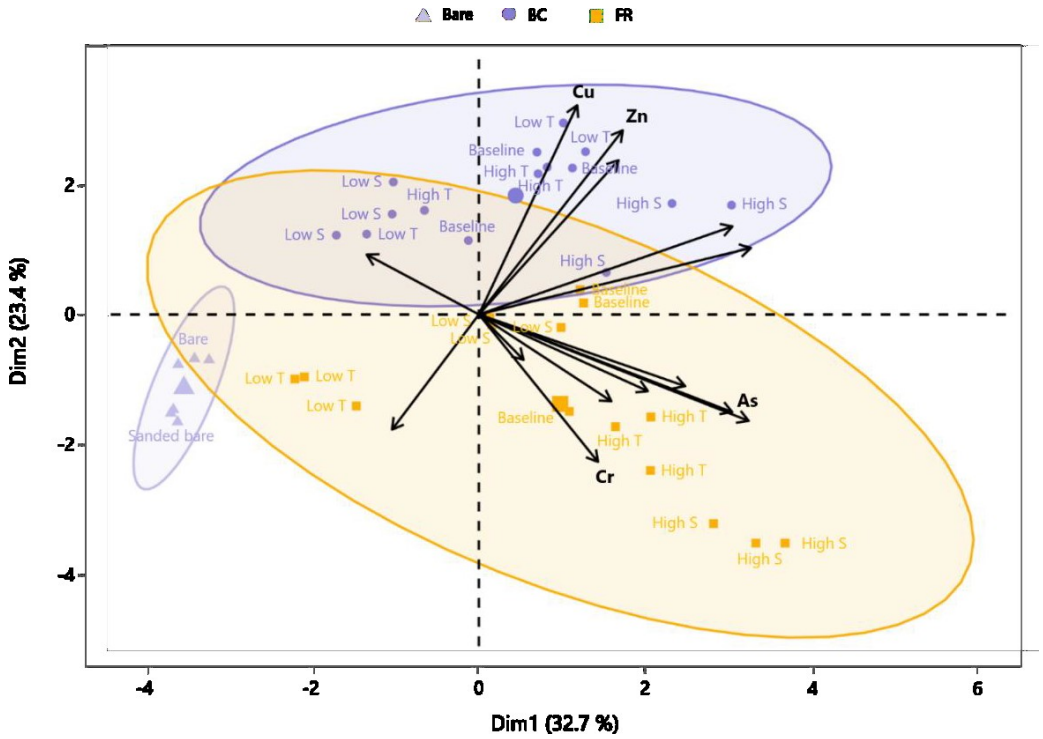


Figure 2. PCA analysis and visualization. Colours and symbols indicate coating treatments: powder blue triangles = bare (control plates); indigo dots = biocide-based (BC) and orange squares = foul-release (FR) treatments. Scores ID indicate the incubation scenarios, i.e. baseline and low or high salinity (S) or temperature (T). Black arrows indicate the variable (metal concentration), with the identity of four metals or interest specified in bold. Percentage of contribution of each component is also noted.

Univariate analysis were carried out to elucidate the chemical behaviour of the selected elements of interest, being these Cu, Zn, Cr and arsenic As (see *Statistical analysis* of the **Materials and methods** for the selection criteria). The concentration of these metals on lixiviates under different scenarios was analysed individually (Table 4) and visualized on Figure 3, additionally supported

with Figure 4. Overall, the results from these analyses support those derived from the multivariate approach.

Table 4. Statistical outputs of the analyses on metal composition. The response variable and the test are specified in the first column. Significant differences are indicated with an asterisk (*).

	Ind. variable	DF	R SumSq	R2	F value	Pr (>F)	
Metal composition	Coating	2	204.83	0.41802	35.2591	1e-04	*
PERMANOVA	Temperature	2	50.80	0.10368	8.7451	1e-04	*
Euclidean distances	Salinity	2	99.37	0.20279	17.1049	1e-04	*
	Coating : Temperature	2	30.76	0.06278	5.2952	1e-04	*
	Coating : Salinity	2	31.62	0.06453	5.4428	1e-04	*
	Residuals	25	72.62	0.14820			
			Homogeneity of multivariate dispersions p-value = 0.614				

	Ind. variable	DF	R SumSq	Mean Sq	F value	Pr (>F)	
Cu composition	Coating	2	19020093	9510046	203.918	3.31e-16	*
ANOVA	Temperature	2	2570056	1285028	27.554	4.77e-07	*
	Salinity	2	2121075	1060537	22.740	2.36e-06	*
	Coating : Temperature	2	20722	10361	0.222	0.802348	
	Coating : Salinity	2	898319	449160	9.631	0.000792	*
	Residuals	25	1165917	46637			

	Ind. variable	DF	R SumSq	Mean Sq	F value	Pr (>F)	
Zn composition	Coating	2	829823	414911	108.023	4.99e-13	*
ANOVA	Temperature	2	58101	29051	7.563	0.0027	*
	Salinity	2	147579	73790	19.211	8.83e-06	*
	Coating : Temperature	2	19409	9705	2.527	0.1001	
	Coating : Salinity	2	4773	2387	0.621	0.5453	
	Residuals	25	96024	3841			

	Ind. variable	DF	R SumSq	Mean Sq	F value	Pr (>F)	
Cr composition	Coating	2	82435	41218	1e+06	< 2e-16	*
Permutational	Temperature	2	5959	2979	1e+06	0.06952	·
ANOVA	Salinity	2	1934	967	1e+06	0.39782	
	Coating : Temperature	2	6032	3016	1e+06	0.07004	
	Coating : Salinity	2	1994	997	1e+06	0.38642	
	Residuals	25	25370	1015			

	Ind. variable	DF	R SumSq	Mean Sq	Iter	Pr (Prob)	
As composition	Coating	2	7058.1	3529.0	1e+06	< 2.2e-16	*
Permutational	Temperature	2	2798.5	1399.3	1e+06	< 2.2e-16	*
ANOVA	Salinity	2	2910.1	1455.1	1e+06	< 2.2e-16	*
	Coating : Temperature	2	3291.1	1645.5	1e+06	< 2.2e-16	*
	Coating : Salinity	2	596.5	298.2	1e+06	0.001209	*
	Residuals	25	780.2	31.2			

Individual metal concentrations of the lixiviates reflect the composition of the coatings (Figure 3), as also reported above in the multivariate analysis (Figure 2). Copper and zinc have significantly higher concentrations in the BC coating lixiviates, and, in spite of having significantly lower concentrations, these were also present in FR lixiviates. Interestingly, chromium concentration is remarkably higher in FR lixiviates, suggesting some leaching from the inner paint layer despite the external silicone coat. Similarly surprising, arsenic concentrations too are higher in FR lixiviates in most cases, except for the low temperature scenario, in which BC lixiviates tends to display higher concentrations. Besides, metal leaching varies across incubation treatments and the main factors shaping it are different for each metal (Figures 3 and 4). Zinc and arsenic leaching is affected by both factors, temperature and salinity, while copper leaching is mostly dictated by the salinity values. Chromium does not show such a clear response, although temperature tends to impact its leaching.

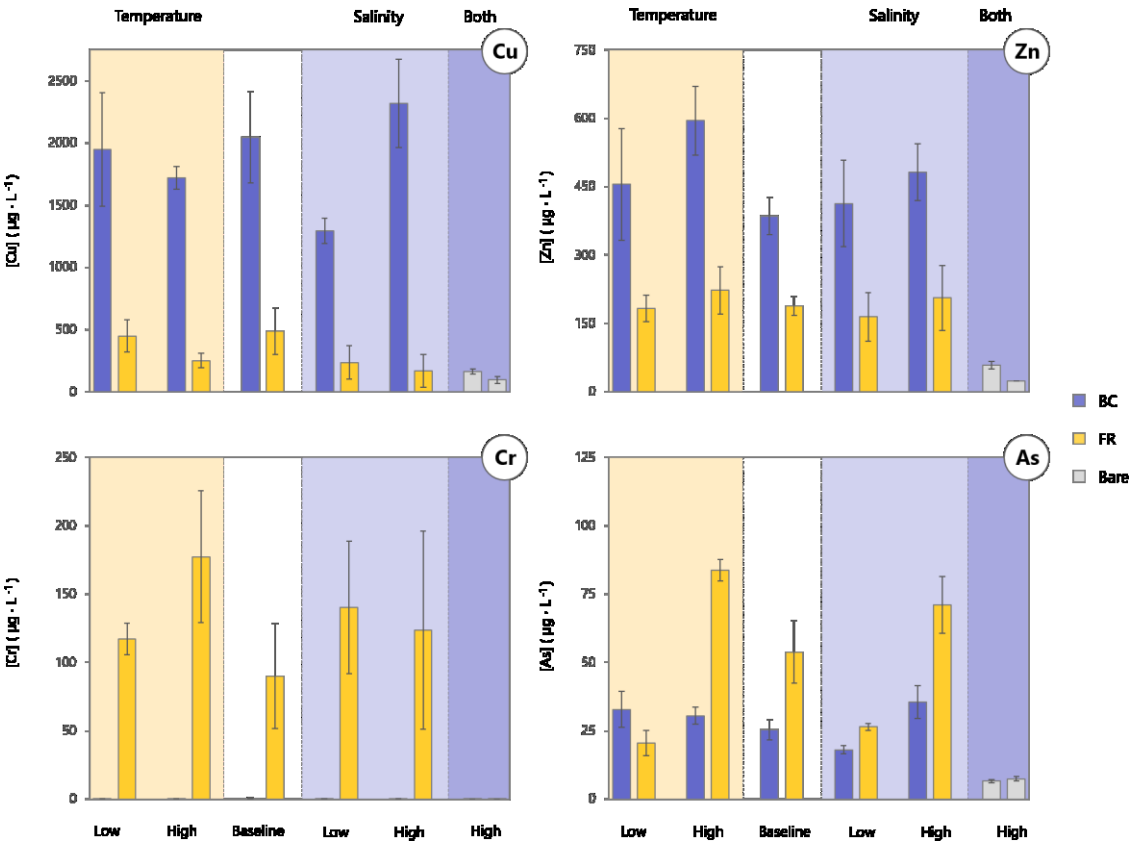


Figure 3. Bar-plot of individual metal concentrations in lixiviates incubated under different scenarios. Bar colours represent coating treatment: violet for biocide-based coatings (BC); amber yellow for foul-release coatings (FR) and grey for bare PVC plates (controls). Background colours indicate incubation conditions: light yellow for modified temperatures; white for baseline conditions; powder blue for modified salinities, and darker blue for high salinities and temperatures of the control incubation (maximized release in bare plates). Error bars represent standard deviation.

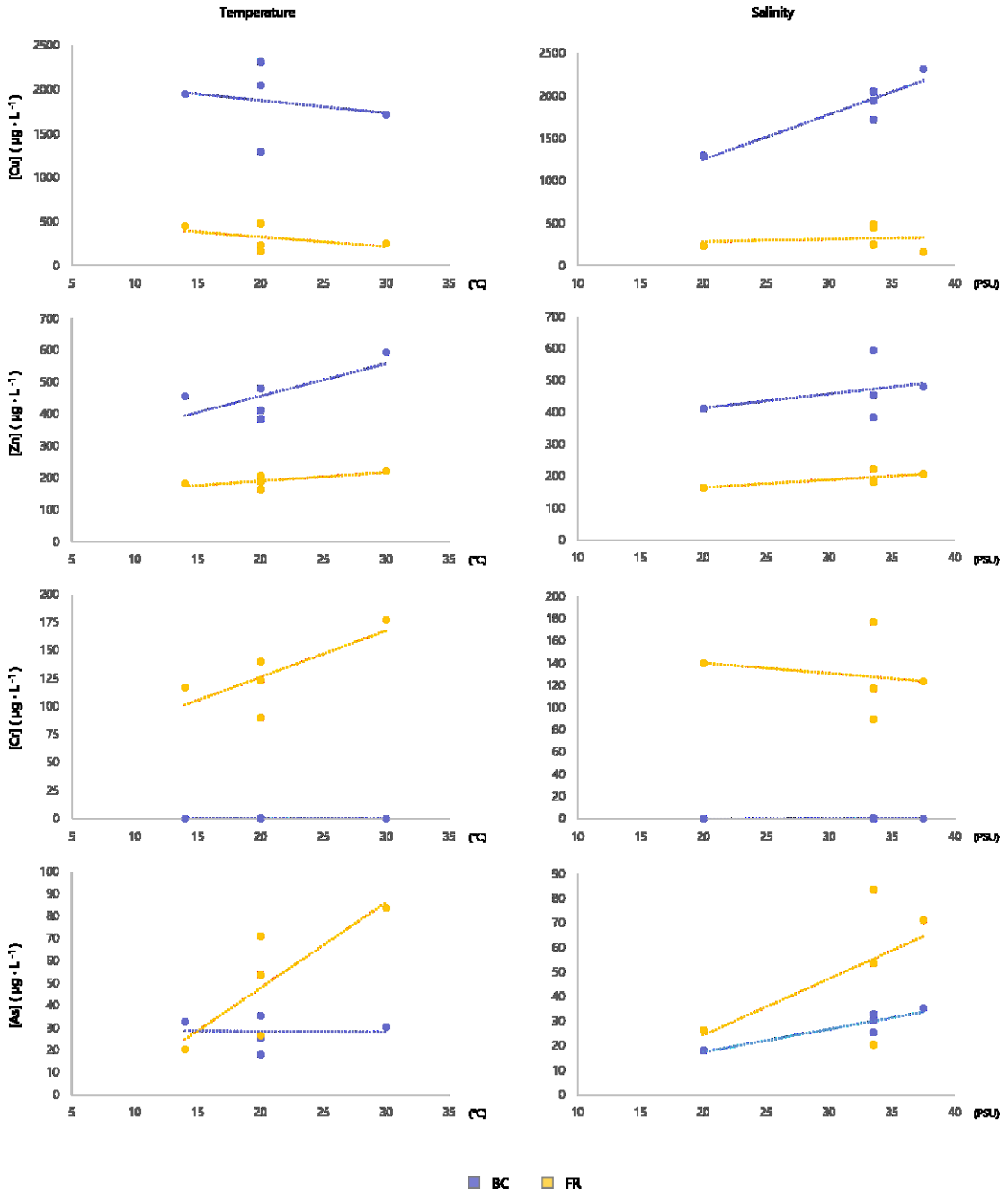


Figure 4. Individual metal concentration values in lixiviates incubated under different scenarios. The factors temperature and salinity have been separated to understand their role in the final measured concentrations and a lineal tendency line has been added to facilitate visualization of trends. Colours represent coating treatment: violet for biocide-based coatings (BC); amber yellow for foul-release coatings (FR).

4. Discussion and Conclusions

Studying chemical behaviour of antifouling coatings is the first step of this project, allowing us to understand how lixiviates are composed and which variables affect their composition. The

selected coatings fall into two types of categories (BC and FR) with different action mechanisms and, therefore, distinct formulations. The traditional BC coating contains mainly copper and zinc (Table 1) and the values of these two metals are, indeed, remarkably higher in BC lixiviates. The selected FR coating contains zinc chromate as a component of the inner paint layer isolated by an external silicone coat (Table 2). Interestingly, the results of the current experiment indicate that, despite the external silicone layer, chromium does leach to the water and is present in significant concentrations under all scenarios. This metal is long known to be toxic under certain oxidation states (Mearns *el at*., 1980; Wong & Trevors, 1988; Aslam & Yousafzai, 2017; de Almeida Rodrigues *el at*., 2022). In fact, chromium, together with lead, mercury, cadmium and arsenic, are regarded as metals of concern (de Almeida Rodrigues *el at*., 2022). Additionally, arsenic is present in both lixiviate types and, in this case too, tends to be higher in FR coating lixiviates. These results deserved special attention, as the selected FR coating is regarded as a non-toxic substitute of traditional BC coatings and, although the action mechanism is different, the release of these metals to the environment shall not be overlooked.

Undeniably, incubation conditions can alter the leaching rates of chemicals from the coatings to lixiviates and, therefore, affect their final composition. Prior studies have linked environmental conditions with metal leaching rates (Yebra *el at*., 2004; Singh & Turner, 2009; Lagerström *el at*., 2020) and our results build up on them. This evidence suggests that environmental factors such as temperature and salinity can affect leaching rates and, thus, could have effects on the efficiency, durability and toxicity of AF coatings. Furthermore, under global changing scenario, the performance of AF coatings could be altered and their associated risks increased. Climate change related alteration of physical and chemical environmental parameters could change contaminants' behaviour affecting their worldwide distribution and toxicity potential and, consequently, reshaping environmental risks and impacts of marine chemical pollutants (Cabral *el at*., 2019).

Hence, efficient and suitable biofouling management plans not only would needfully take into account the chemical behaviour of the AF of choice and the local environmental conditions, but also account for risk mitigation under a global changing scenario.

02

Toxicity in microalgae

Toxicity of vessel antifouling coating lixiviates in target and non-target marine microalgal species: multi-taxa and biological multi-level approach testing

Congress contribution:

Santos-Simón, M., Blanco-Rayón E., Seoane-Parra, S.., Etxebarria, N., Ortiz-Zarragoitia, M. (2023). "Antifouling Coatings: Assessment of the Effects on Phytoplanktonic Non-target Species". Society of Environmental Toxicology and Chemistry (SETAC), Dublin, Ireland.

Abstract

The development of a microfilm is one of the very first steps in the succession process that takes place in bare surfaces exposure to aquatic environments, where microalgae, and especially diatoms, are among the early colonizers. To prevent or minimize this undesired growth in human-made structures antifouling (AF) measures are applied and coatings are the most common ones. This work studied the effects of two commercially available coatings, a traditional biocide-based one (BC) and an alternative non-toxic foul-release (FR) one, from a multi-taxa and multilevel approach. Different microalgal species were selected, including pelagic non-target species and a benthic target one, which were exposed to AF lixiviates to measure various biological endpoints. Toxicity screening assays revealed that exposure to BC lixiviates inhibited growth in all three species and affected the photosynthetic efficiency differently, the diatom being the most sensitive one, while FR did not have any effect on them. Additionally, exposure affected the total pigment content, particularly for BC treatments. Subcellular responses to narrower concentration ranges indicated change tendencies in the selected biomarkers of oxidative stress (the catalase and glutathione S-transferase activities and lipid peroxidation levels), both for BC and FR treatments. Finally, cellular metal sorption did not show clear differences across treatments nor species. Overall, exposure to lixiviates directly affected both target and non-target microalgae species, although the type and magnitude of the responses varies according to the species and treatments. Multi-taxa and multi-level approaches provide a broad overview of the biological responses and have the potential to be a valuable tool in aquatic toxicology.

Keywords | Antifouling · *Isochrysis galbana* · *Tetraselmis* sp · *Cylindrotheca* sp · growth · pigments · cellular metal sorption · oxidative stress.

Resumen

El desarrollo de un microfilm es uno de los primeros procesos que tiene lugar durante la sucesión que se da en superficies expuestas a un ambiente húmedo o acuático. En este proceso, las microalgas, y especialmente las diatomeas, se encuentran entre los primeros colonizadores. Para prevenir o minimizar este crecimiento no deseado en estructuras artificiales, se aplican medidas antiincrustantes (AF por sus siglas en inglés), siendo los recubrimientos los más comunes. Este estudio evaluó los efectos de dos recubrimientos comerciales, uno tradicional basado en biocidas (BC) y uno alternativo no tóxico (FR), desde un enfoque multi-taxón y multinivel. Se seleccionaron diferentes especies de microalgas, incluidas especies pelágicas no diana y una especie bentónica diana, que fueron expuestas lixiviados AF para evaluar las respuestas de parámetros biológicos de interés. Los ensayos de detección de toxicidad revelaron que la exposición a lixiviados BC inhibe el crecimiento en las tres especies y afecta la eficiencia fotosintética de manera diferente, siendo la diatomea la más sensible, mientras que la exposición a FR no desencadena ningún efecto. Además, el contenido total de pigmentos se vio particularmente afectado bajo los tratamientos BC. Las respuestas sub-celulares a rangos de concentración más acotados indicaron tendencias de cambio en los biomarcadores de estrés oxidativo seleccionados (actividades enzimáticas de catalasa y glutatión S-transferasa; niveles de peroxidación lipídica), tanto para tratamientos BC como FR. Finalmente, la sorción celular de metales no mostró diferencias claras entre tratamientos ni especies. En general, la exposición a lixiviados afectó directamente tanto a las especies de microalgas diana como a las no diana, aunque el tipo y la magnitud de las respuestas varían según la especie y los tratamientos. Los enfoques multitaxónicos y multinivel brindan una visión general amplia de las respuestas biológicas y tienen el potencial de ser una herramienta valiosa en toxicología acuática.

Palabras clave | Antiincrustante · *Isochrysis galbana* · *Tetraselmis* sp · *Cylindrotheca* sp · crecimiento · pigmentos · sorción metálica celular · estrés oxidativo.

Riassunto

Lo sviluppo di un microfilm è tra i primi processi che avviene durante la successione biologica in superfici esposte a un ambiente umido o aquatico. In questo processo, le microalghe, e in particolare le diatomee, sono tra i primi colonizzatori. Per prevenire o ridurre al minimo questa crescita indesiderata sulle strutture artificiali, vengono applicate misure antivegetative (AF dalle sigle in inglese), essendo i rivestimenti le più comuni. Questo studio ha valutato gli effetti di due rivestimenti commerciali, uno tradizionale a base di biocidi (BC) e uno alternativo non tossico (FR), da un approccio multi-taxa e multi-livello. Diverse specie di microalghe, comprese specie pelagiche non bersaglio e una specie bentonica bersaglio (una diatomea), sono state selezionate ed esposte a percolati di AF per valutare le risposte di certi parametri biologici. I test di screening della tossicità hanno rivelato che l'esposizione al percolato di BC inibisce la crescita in tutte e tre specie e influenza l'efficienza fotosintetica in modo diverso, risultando la diatomea come la specie più sensibile, mentre l'esposizione a FR non innesca alcun effetto. Inoltre, il contenuto totale di pigmento risultava particolarmente influenzato dai trattamenti BC. Le risposte subcellulari a intervalli di concentrazione più ristretti hanno indicato tendenze di cambiamento nei biomarcatori dello stress ossidativo (attività enzimatiche della catalasi e della glutatione S-transferasi; livelli di perossidazione lipidica), sia per i trattamenti BC che FR. Infine, il sorbimento cellulare dei metalli non ha mostrato chiare differenze tra trattamenti o specie. Nel complesso, l'esposizione al percolato ha un effetto diretto sia sulle specie di microalghe bersaglio che su quelle non bersaglio, sebbene il tipo e l'entità delle risposte varino a seconda della specie e dei trattamenti. Gli approcci multitassonomici e multilivello forniscono un'ampia panoramica delle risposte biologiche e hanno il potenziale per essere uno strumento prezioso nella tossicologia acquatica.

Parole chiave | Antivegetativo · *Isochrysis galbana* · *Tetraselmis* sp · *Cylindrotheca* sp · crescita · pigmenti · sorbimento celulare dei metalli · stress ossidativo.

Laburpena

Mikrofilm baten garapena ingurune heze edo urtar baten eraginpean dauden gainazaletan gertatzen den lehenengoetariko prozesu bat da. Prozesu horretan, mikroalgak, eta bereziki diatomeak, lehen kolonizatzaileen artean daude. Egitura artifizialetan nahi ez den hazkuntza hori saihesteko edo gutxitzeko, anti-inkrustazio (AF, ingeleseko antifouling hitzatik) neurriak aplikatzen dira, ohikoenak gainestaldurak izanik. Ikerketa honek bi gainestaldura komertzialen efektuak aztertu ditu, biozidak (BC) dituen tradizional bat eta ordezko ez-toxiko bat (FR) zehazki, taxoi eta maila anitzeko ikuspegia erabiliz. Mikroalga espezie desberdinak hautatu ziren, xede ez diren espezie pelagikoak eta xede-espezie bentiko bat barne, eta intereseko parametro biologikoen erantzunak aztertu ziren AF lixibatuen eraginpean. Toxikotasun frogak agerian utzi zuten BC lixibatuak hiru espezietan hazkuntza galarazten zuela eta efizientzia fotosintetikoan eragiten zuela modu ezberdinetan, diatomea sentikorrena izanik; FR lixibatuak, ostera, ez zuen eraginik izan. Gainera, pigmentu-edukia, kopuru totala zein konposizioa, bereziki aldatu zen BC tratamendupetan aztertutako hiru espezieetan. Bestalde, kontzentrazio-tarte estuagoetan aztertutako erantzun subzelularrei dagokionez, hautatutako estres oxidatiboko biomarkatzaileetan aldaketa-joerak behatu ziren (katalasaren eta glutation-S-transferasaren jarduera entzimatikoak; lipidoen peroxidazio mailak), bai BC bai FR tratamendupetan. Azkenik, metalen xurgapen zelularra ez zuen desberdintasun argirik erakutsi tratamenduen edo espezieen artean. Orokorrean, lixibatuen esposizioak eragin zuzena izan zuen hautatutako mikroalga espezieetan, xede diren eta ez direnetan, nahiz eta erantzun mota eta haien magnitudea tratamenduarekiko eta espeziearekiko aldatu. Taxoi eta maila anitzeko ikuspuntutik egindako ikerketek erantzun biologikoen ikuspegi zabala orokorra eskaintzen dute eta toxikologia eremuan tresna baliotsu bat izan litezke.

Gako-hitzak | Anti-inkrustazio · *Isochrysis galbana* · *Tetraselmis* sp · *Cylindrotheca* sp · hazkuntza · pigmentuak · metalen xurgapen zelularra · estres oxidatiboa.

1. Introduction

Any bare surface exposed to a wet or aquatic environment is susceptible to be colonized by diverse organisms. As described in the introduction, the growth of such organisms follows a succession process that culminates with the development of a mature community and, when associated specifically to artificial substrates, the unwanted development of those communities is referred to as biofouling or just fouling (Lewis, 1998; Dürr & Thomason, 2009). Right after the submersion, organic macromolecules are adsorbed, initializing the surface conditioning and subsequent bacterial colonization. This phase is relatively fast and followed by the arrival of unicellular eukaryotes, increasing the complexity of the initial microfilm. In fact, microalgae, and especially diatoms, are among the first incomers together with fungi and protozoa, constituting the so-called microfouling (Wahl, 1989; Railkin, 2003; Salta *et al*., 2013). The succession process is complex and many of the underlying mechanisms are not fully understood. Still, it is widely accepted that the presence of certain molecules and organisms, as well as their interactions, determine the settlement of subsequent foulers (Yebra *et al*., 2004; Salta *et al*., 2013; Cacavelos *et al*., 2020). The dynamics of biofilms can mediate not only microbial colonisation but also macrofoulers' recruitment, influencing the subsequent stages of biofouling development (Dobretsov & Qian., 2006; Qian *et al*. 2007). Thus, the characteristics of the microfouling layer can be critical for the successive incomers and determinant for the development of the macrofouling, an interesting aspect for biotechnological purposes, particularly for antifouling technologies (Dobretsov *et al*., 2006).

Biofouling development can be considered a cross-sectorial issue, affecting multiple industries of the blue economy (Schultz *et al*., 2011; Bannister *et al*., 2019; Vinagre *et al*., 2020) and can have important implications on the optimal performance and functioning of certain artificial structures, leading to eventual consequences on their associated costs, such as operability, efficiency, maintenance, etc. (Schultz *et al*., 2011). Therefore, the application of antifouling measures is essential to prevent or minimize the undesired growth of fouling communities in submerged or wetted artificial surfaces and the effects derived from it. Antifouling (AF) coatings are amongst the most common measures and, in particular, biocide-based (BC) coatings are the most frequently applied AF coatings. After the global ban on tributyl-tin (TBT) (IMO 2001; Regulation (EU) No 782/2003) copper (as copper oxide) is, at present, the most commonly used biocide (Jones & Bolam, 2007; Ytreberg *et al*., 2020), typically in combination with booster co-biocides, such as zinc and copper pyrithione (Wallström *et al*., 2011).

In spite of the wide use of BC coatings in many maritime sectors, there is uncertainty regarding their actual efficiency (Culver *et al.*, 2021) and indirect effects in the marine environment (Yebra, 2004; Amara *et al.*, 2018; de Campos *et al.*, 2021). Besides, AF from maritime shipping and leisure boating are considered an important source of metals into water systems (Ytreberg *et al.*, 2022). Not only do some non-target species seem to be affected by the release of this compounds into the aquatic environment (Katranitsas, 2003; Karlsson *et al.*, 2010; Ytreberg *et al.*, 2010; Oliveira, 2017), but some target species can develop resistance to biocides (Floer *et al.*, 2004; Piola & Johnston, 2006; Culver *et al.*, 2021; Santos-Simón, 2024), resulting in a reduced performance of the coating. In this context, in order to dodge the potential problems and risks associated with traditional BC coatings, new alternatives that avoid the use of toxic compounds are being explored. From natural compounds based on chemical defences of marine organisms (Qian *et al.*, 2015; Kyei *et al.*, 2020) to physical alterations of the surface properties and repelling mechanisms, antifouling technologies are evolving towards sustainable alternatives that have already reached the market (Yebra *et al.*, 2004; Wezenbeek *et al*, 2018). Among those, foul-release (FR) technologies based on silicone polymers are becoming more common.

Overall, it can be hypothesized that coatings could potentially affect surrounding organisms through waterborne exposure to leached components. Based on the action mechanism of the coating type, the expected effects would vary, BC coatings being the most concerning ones. Furthermore, it is expected that biological characteristics of the organisms will determine the level of toxicity and their responses. Microalgae are a greatly diverse and are ubiquitous in aquatic environments, playing a key role in ecosystems (Singh & Saxena, 2015). Their rapid response to environmental changes, including anthropogenic-derived ones, makes them suitable as indicators and bioassays with microalgae as test organisms have become a valuable tool in ecotoxicology and have been applied for decades now (Nyholm & Peterson, 1997). The main goal of the current study was to assess the effects of selected AF coatings on different microalgae species: three free-swimming non-target microalgae and a benthic 'target' diatom. Studying various endpoints (from biochemical to organism level responses), this work addresses experimentally the potential indirect effects of two AF coatings belonging to different typologies (BC and FR coatings) from a multi-taxa and biological multi-level approach.

2. Materials and methods

2.1 Culture media

Seawater was filtered by using 0.22 µm pore size nitrocellulose filters (FisherScientific) and sterilized afterwards in a bath at 80 °C for 1 hour. Once cooled, it was kept at 4 ± 1 °C until its use

for algal cultures and/or lixiviate preparation (usage within 48 h). Media was directly added to the experimental flasks, at 1 mL per 1 L ratio, using f/2-silica enriched medium ('easy algae f/2 modified + sicilca' product from Fitoplancton marino).

2.2 Algal cultures

The selected test organisms were *Isochrysis galbana* Parke, 1949; *Tetraselmis chui* Butcher 1959; *Tetrasemis suecica* (Kylin) Butcher 1959 and *Cylindrotheca* sp. L. Rabenhorst, 1859, belonging to three distinct phyla, namely Haptophyta, Chlorophyta and Heterocontophyta, and common in estuaries of the Bay of Biscay (Seoane *et al.*, 2005; Bilbao *et al.*, 2023). The first three species have been largely used in ecotoxicological assays (Liu *et al.*, 2011; Sathasivam *et al.*, 2018) and are commonly used for feeding purposes of diverse species in aquaculture and laboratory cultures. Therefore, their wide use, the amount of information and studies on these species make them suitable for the purpose of the research. As regards the benthic diatom *Cylindrotheca* sp., despite not being particularly common as test species, Araújo *et al.* (2010) already highlighted the importance of microphytobenthos in ecotoxicology and there is some work already done on the genus itself (Satoh *et al.*, 2005; Lozano *et al.*, 2014). Thus, being benthic biofilm forming species, it was selected as a target species. Additionally, their distinct biological traits provides a better understanding on how different species respond to common contaminants and their presence in natural marine habitats contributes to environmentally relevant outcomes.

Axenic stock cultures of *T. chui*, *T. suecica*, and *I. galbana* were purchased from the Marine Science Station of Toralla (ECIMAT, Spain) as non-target planktonic species, and *Cylindrotheca* sp. (strain BMCC 385), a benthic diatom, was obtained from the Basque Microalgae Culture Collection (BMCC). Stocks were grown and kept at 21±1 °C, with a photoperiod of 16:8 under white LED illumination (100 µmol · s^{-1} · m^{-2}) and filtered aeration.

2.3 Antifouling coatings

Two commercially available antifouling coatings were selected, corresponding to different categories: a biocidal coating (BC) and a foul-release coating (FR). The BC coating contained between 25-50 % in weight of dicopper oxide (Cu_2O_2) and less than 10 % in weight of zinc oxide (ZnO) as the active compounds. In addition, the coating contained among the other components colophony, naphtha, xylene and ethylbenzene (see Table 1 of I. Chemical characterization). The FR coating was composed of two layers of coatings: 1) an external layer of silane composites (between 2-8 % in weight of methyl tris(methylethylketoximine)silane and 0.1-2% in weight of 3-Aminopropyltrimethoxysilane), and 2) an inner isolated paint coating that contains zinc chromate,

xylene, talc, propan-2-ol; 2-methylpropan-1-ol and phosphoric acid (see Table 2 of the chapter I. Chemical characterization for more specifications).

2.4 Preparation of the lixiviates

Exposure to AF coatings was done by incubation of coated PVC plates in a known volume of water, following a standardized procedure, as detailed in the chapter I. Chemical characterization. The lixiviate containing leached contaminants from the coated plates was used and test contaminant. To prepare the lixiviate, PVC plates with an standard surface of 14x14 cm were cut, sandblasted and coated using a painting roll, following the manufacturers' coating procedures, simulating a real scenario. Plates were left to dry for 24 h and, afterwards, transferred into a glass container with 1 L of sterilized culture media, with plates fixed in the surface, with the coated side submerged. Subsequently, the container was placed on a shaker at 150 rpm for 24 h. Lastly, samples of the obtained lixiviates were taken for chemical characterization (Table 1).

2.5 Exposure bioassays and sample collection

The current work was divided into two experimental phases: 1) toxicity screening and general physiological stress responses (growth inhibition, photosynthetic efficiency and pigment content) and 2) subcellular responses (biochemical responses and bioaccumulation) with narrower concentration range (Table 1; Figure 1).

Table 1. Summary of the design of the exposure experiments.

Test	**Treatment**				**Replicates**
	Control	FR	BC	Category	
1) Growth inhibition, pigment content & photosynthetic efficiency	-	25 %	5 %	Low	3 per concentration and treatment
	-	50 %	15 %	Mid	
	-	75 %	25 %	High	
2) Metal accumulation in cells & biochemical responses *	60 %	25 %	5 %	Low	4 per concentration and treatment
	60 %	50 %	10 %	Mid	
	60 %	75 %	15 %	High	
Initial cell density					
[*I. galbana*]$_i$ = 10^5 cell · mL^{-1}	[*Cylindrotheca* sp.]$_i$ = 10^5 cell · mL^{-1}				[*Tetraselmis*]$_i$ = 5 ·10^4 cell · mL^{-1}

* Due to stock and supplier issues, *T. chui* was substituted by *T. suecica.*

The number of replicates and lixiviate concentrations used in each phase varied due to the experimental nature of each phase and experience acquired. A total of 3 concentrations per coating typology plus a control treatment were prepared per algal species, with 3 replicates per

treatment during the first experimental phase, and 4 replicates for the second one (Table 1; Figure 1). Because of limitation with algal cultures from the genus *Tetraselmis*, two different species with very similar characteristics were used for each experimental phase and hereby will be referred to as *Tetraselmis* sp (Table 1).

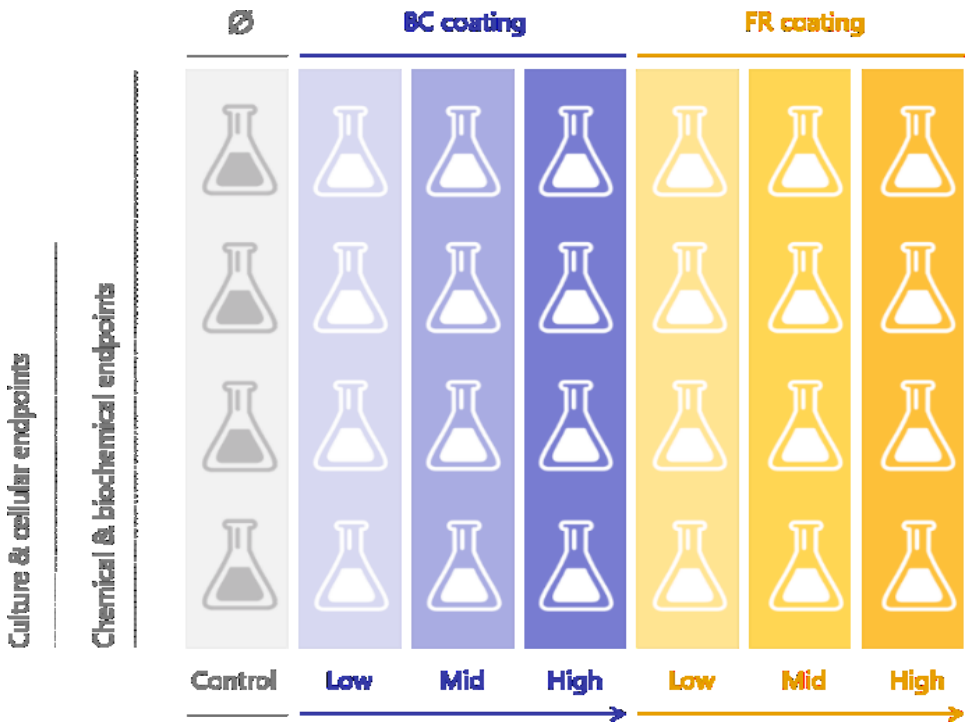


Figure 1. Schematic illustration of the experimental design, replicated accordingly for each microalgae species. The number of replicates per treatment varies according to the endpoints measured, being 3 the number of replicates for the culture and cellular responses and 4 for the subcellular endpoints. Colour represents coating type (BC in violet scale and FR in orange scale) from light to brighter according to increasing concentrations

Exposure media was prepared accordingly for each treatment and concentration in a 250 ml Erlenmeyer glass flask. The inoculation densities varied for each species (10^5 cell · mL $^{-1}$ *for I. galbana*; 5 ·10^4 cell · mL $^{-1}$ for *Tetraselmis* sp. and 105 cell · mL $^{-1}$ for *Cylindrotheca* sp.) (Table 1) and were calculated according to their biovolume (Sun & Liu, 2003). Flasks were kept in an incubator at a constant temperature of 21 ± 1 °C, with a photoperiod of 16:8 h (light/dark) under a white LED (100 µmol · s^{-1} · m^{-2}) light and were manually shook three times per day. The duration of the exposure was 72 h, throughout which daily values of culture growth were measured manually by means of a Neubauer cell-counting chamber. The content of the experimental flasks was centrifuged to collect algal pellets that were then weighted, frozen in liquid nitrogen and stored at -80 °C. Centrifugation speeds varied across algal species, being 2700 rpm for *I. galbana* and *Cylindrotheca* sp., and 3500 rpm for *Tetraselmis* sp.

2.6 Chemical analysis of the lixiviates

Metal concentration was determined for each lixiviate preparation with inductively coupled plasma mass spectrometry (ICP-MS) measured in water samples, as described in the chapter I. Chemical characterization.

2.7 Quantification of daily growth and growth inhibition calculation

The average specific growth rate for a given period under a certain treatment was calculated as the logarithmic increase in biomass from the equation (1); where μ_{i-f} is the average specific growth rate from moment time i to f ; t_i is the initial starting time and t_f the final time; B_i is the biomass concentration at the initial time (t_i) and B_f is the biomass concentration at the final time (t_f) (OECD, 2011).

$$\mu_{i-f} = \frac{lnB_f - lnB_i}{t_f - t_i} \cdot d^{-1} \quad (1)$$

The percentage of growth inhibition (% GI) was calculated using the average growth rate over the whole test duration for the controls (μ_C) and the specific treatments (μ_T), as indicated in the equation (2):

$$\%GI = \frac{\mu_C - \mu_T}{\mu_C} \cdot 100 \quad (2)$$

Finally, GI-50, that is the concentration at which the growth is inhibited in a 50%, was calculated by scatter-plotting the % GI results and using the equation of the line to calculate the concentration at which growth was inhibited in a 50%.

2.8 Determination of photosynthetic efficiency

Photosynthetic efficiency was measured using a Water-PAM™-II (Heinz Walz Gmbh, Effeltrich, Germany) and WinControl-3 software. *In vivo* fluorescence data was calculated as maximal PSII quantum yield (ϕ_M) and used as a proxy of photosynthetic efficiency. The fluorescence yield (i.e. the probability that excitation energy is emitted as fluorescence), was recorded as the variable fluorescence (F_V) using the values of minimum fluorescence yield (F_0) of a dark-acclimated state and the maximum fluorescence yield (F_M) after irradiation with a saturating pulse, normalized to the condition of maximum fluorescence as in equation 3 (Juneau *et al.*, 2002; Schansker, 2020).

$$\Phi_M = F_V / F_M = \frac{F_M - F_0}{F_M} \quad (3)$$

2.9 Quantification of pigment content

Filtration of samples was carried out in dark conditions using 25 mm diameter Cytiva Whatman™Binder-Free Glass Microfiber Filters GF/F (FisherScientific) and 3 mL of sample per treatment and replicate. Filters were covered individually with aluminium, immediately frozen in liquid nitrogen, and stored at -80 °C first and then transferred to -40 °C until their analysis. Pigment extraction was done under low light conditions, grounding each filter using a glass stick in 3 mL of 90 % acetone for 20 minutes at 4 °C. Subsequently, extracts were filtered through syringe filters (Millex, 0.22 µm pore size) into glass vials, and stored at -20 °C until high performance liquid chromatography (HPLC) analysis. The identification and quantification of pigments based on their absorbance spectra and retention times was carried out following the extraction and analytical procedures described by Zapata *et al* (2000), including the modifications by Seoane *et al* (2009) to the solvent A and HPLC equipment.

2.10 Biomarkers of oxidative stress: enzymatic activities and lipid peroxidation (LPO)

Microalgae pellets were homogenized in 0.05 M phosphate buffer (pH 7.0) at a ratio 1 mL per 0.1 g, by ultrasonication (Bandelin SONOPULS HD 3100 ultrasonic homogeniser). Homogenates were centrifuged at 10000 g at 4 °C for 20 min for enzymatic activities and 10 min for LPO quantification. Supernatants were then collected for enzymatic assays, following standard spectrophotometric protocols using High-performance microplate spectrophotometer (BioTek Eon ™).

Total protein content of the samples was determined by the Bradford colorimetric assay (Bradford, 1976; ref. Bio-Rad 500-0205), using commercial bovine serum albumin (Sigma, A2153) as standard. Catalase activity was measured in UV-light specific microplates for unique absorbance readings at 240 nm, following the method of Claiborne (1985) and adapted for microalgae. GST activity was determined spectrophotometrically following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) with unique measurements at 340 nm (Roldán-Prieto *et al*., 2024). Enzymatic activities were normalized to the total protein content of the samples. Lastly, LPO was determined in the homogenates by measuring thiobarbituric acid reactive substances (TBARS), as a by-product of lipid peroxidation, at 535 nm, based on Bird and Draper (1984) and adapted for microalgae (Roldán-Prieto *et al*., 2024).

2.11 *Cellular metal concentration*

To determine metal accumulation in cells after exposure, algal pellets were collected, frozen in liquid nitrogen and stored at -80 °C. Samples were unfrozen and pellets cleaned thoroughly to remove all the extracellular metals that could have remained, by doing three cycles of

resuspension in filtered seawater, centrifugation (3000 rpm) and supernatant removal. Once washed, pellets were lyophilized (24 h at -80 °C), weighed, sonicated and digested in 1 mL HNO_3 to release the cellular content. Metal concentration was finally measured using ICP-MS (see chapter I. Chemical characterization) and normalized to the pellet weight.

2.12 Statistical analysis

The software RStudio (`R version 4.1.2`; R Core Team, 2022) was used to perform the statistical analysis, complemented, when required, with Microsoft Excel. Statistical differences across treatments in the response variables of growth rate and photosynthetic efficiency were checked for each microalgae species individually, using the Kruskal-Wallis test ('`stats`' package; R Core Team, 2022), as the assumptions for a parametric test were not met. Pairwise comparisons were done for each algal species individually, using the function 'pairwise.t.test' and the adjustment method by Bejamini & Hochberg (1995). Tests were done using x confidence level value of 0.95 (default value).

Pigment content and intracellular metal accumulation were analysed using a multivariate approach with Permutational Analysis of Variance (PERMANOVA) (Anderson, 2001), based on Euclidean distances and scaled pigment and metal concentration values. Furthermore, dispersion within treatment groups was checked with PERMDISP (Anderson, 2006), running 'betadisper' function on the distance matrix for the homogeneity of multivariate dispersions. PERMANOVA was performed applying the function 'adonis2' of the `vegan`, under the reduced model following a sequential addition of terms and 9999 free permutations. Pairwise distances among treatments were graphically represented with a Non-metric Multidimensional Scaling (nMDS) plot.

The Integrated Biological Response (IBR) index (Beliaeff & Burgeot, 2002) was used as an integrative statistical tool using a battery of selected biomarkers at different levels to feed the analysis. Using star plots as a multivariate graphic method, plotted areas of standardized biomarker responses were calculated into the IBR index as described by Beliaeff & Burgeot (2002). This analysis was used specifically to integrate the responses of culture growth rate, photosynthetic efficiency, protein content, catalase activity and LPO level. Significant differences were calculated using the t-score. Additionally, PCAs with all the measured endpoints were created for each microalgal species and provided as supportive material (see Figures S1, S2 and S3 of the section 1. Additional results of the Annex).

3. Results

3.1 Chemical analysis of the lixiviates

Table 2 summarized the mean metal concentration of selected metal(oid)s obtained from the chemical analysis of the lixiviates, divided according to the experiment type. Metal concentrations are strictly linked to coating typology.

Table 2. ICP-MS chemical analyses of the coating lixiviates showing mean metal concentration of selected elements in $\mu g \cdot L^{-1}$.

		Coating	[Cu]	[Zn]	[Cr]	[As]
1) Toxicity screening	*I. galbana*	BC	774.204	111.779	1.912	21.852
		FR	87.504	73.950	5.520	31.910
	T. suecica	BC	1168.500	295.944	2.229	28.432
		FR	106.636	126.544	3.207	27.348
	Cylindrotheca sp.	BC	1612.253	1539.007	19.322	273.835
		FR	285.177	345.827	4.367	64.968
2) Subcellular responses	*I. galbana*	C	121.648	<LoD	2.030	28.893
		BC	519.683	183.147	3.336	26.811
		FR	135.129	13.434	10.658	28.081
	T. suecica	C	111.983	13.802	2.307	26.859
		BC	1504.413	373.026	2.010	28.111
		FR	138.870	18.199	7.630	25.551
	Cylindrotheca sp.	C	116.880	27.965	3.005	25.136
		BC	1278.155	337.618	2.832	26.617
		FR	126.433	74.536	15.206	27.837

3.2 Effects on culture growth

Throughout the duration of the exposure, algal cell density was calculated every 24 hours and results are plotted in Figure 2, as growth curves (Figure 2 A-C) and growth rate (Figure 2 D). Overall, growth rates significantly varied across treatments (K-W p-values = 0.004; 0.013 and 0.0065, for *I. galbana*, *Tetraselmis* sp. and *Cylindrotheca*, accordingly), being significantly lower in BC exposed cultures than control rates in all cases. Exposure to FR lixiviate only reduced significantly the growth of *I. galbana* at medium and high concentrations (Figure 2D), still allowing some growth without reaching GI-50 after 72 h. In fact, the cultures exposed to FR coating lixiviates of the three microalgae species exhibited growth after 72 hours, indicating no growth inhibition effect (Figure 2A, B and C). However, even the lowest concentration of the BC coating had a significant impact on the final culture concentration (Figure 2D), with a 50 % of growth inhibition at 8.47 %; 30.17 % and 1.91 % of coating lixiviate, for *I. galbana*, *Tetraselmis* sp. and *Cylindrotheca* sp., respectively (Figure 2A, B and C). The latter one, aside of being the most sensitive species, had slower growth rates (Figure 2D) than the other two algal species.

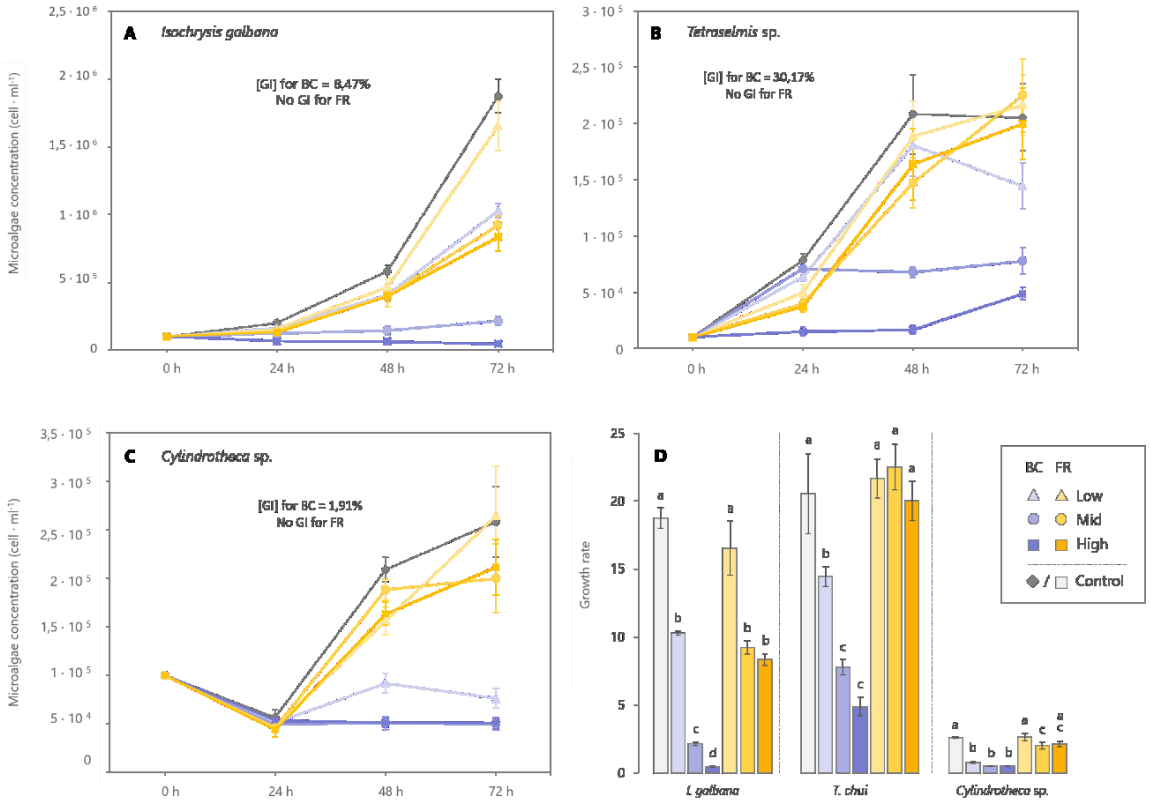


Figure 2. Daily microalgal growth curves as an estimation of cell density (cell · mL $^{-1}$) per treatment for *I. galbana* (A), *T. chui* (B) and *Cylindrotheca* sp. (C). Growth inhibition (GI) concentration is also indicated for each case. Additionally, final growth rates were calculated for each treatment (D) and post-hoc test were done individually for each algal species, equal letters group treatments with no significant differences. Colour indicates coating (violet scale for BC and orange scale for FR; controls are represented in white), and colour intensity and symbol indicate lixiviate concentration (light colour triangle for low lixiviate concentration, mid colour circle for mid lixiviate concentration and intense colour square for high lixiviate concentration). Error bars indicate standard error.

3.3 Effects on photosynthetic efficiency

Fluorescence yield was used as a proxy of photosynthetic efficiency. The response patterns greatly varied across species (Figure 3). Indeed, *Tetraselmis* sp. did not show any statistically significant differences across any treatment (K-W p-value = 0.0896), while BC lixiviate had an impact in the total yield of the other two species (p-values = 0.0006 and 0.0057, for *I. galbana* and *Cylindrotheca*, respectively), reducing it with increasing concentrations of lixiviate for *Cylindrotheca* sp. and just the opposite for *I. galbana*. FR lixiviate had no effect on *Tetraselmis* sp. and *I. galbana*, although it significantly increased the photosynthetic yield of FR exposed *Cylindrotheca* sp. cultures, equally regardless the concentration (Figure 3).

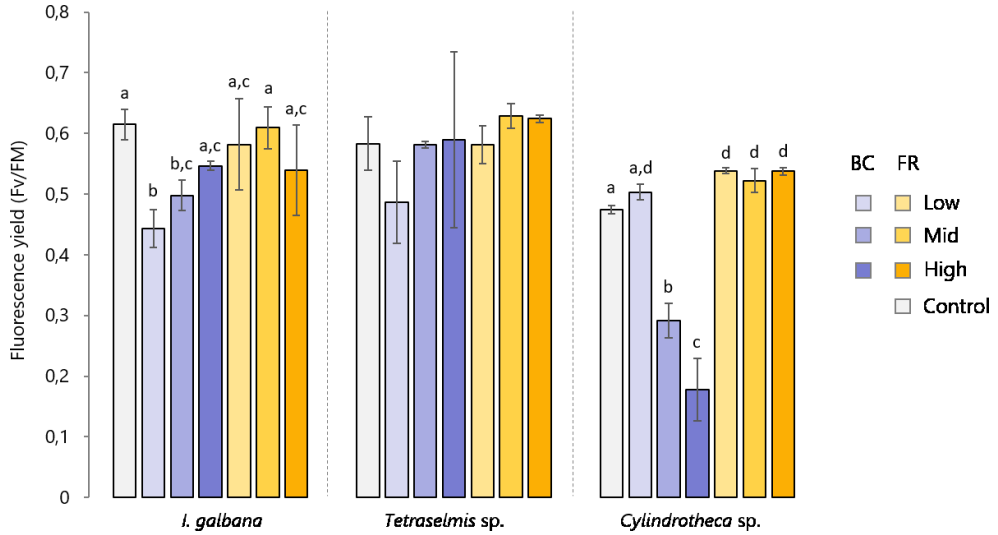


Figure 3. Fluorescence yield per treatment as an estimation of the photosynthetic activity for *I. galbana*, *T. chui* and *Cylindrotheca* sp. Colour indicates coating (violet scale for BC and orange scale for FR; controls are represented in light grey), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration). Post-hoc test were done for each algal species, equal letters group treatments with no significant differences. Error bars represent

3.4 Effects on total pigment content

Pigment composition was species specific and pigment concentrations varied across treatments (Figure 4). Both coating and concentration, as well as their interaction, were significant factors shaping the pigment composition for all three species (Figure 5; Table 3).

Overall, the pigment composition and concentrations varied across treatments (Figure 5; Table 3). Exposure to BC concentrations generally decreased concentration of most of the accessory pigments (Figure 4). Contrarily, chlorophyll-a was the most important and less affected pigment, even with increasing tendencies under exposure to any BC concentration for *I. galbana,* and for *Tetraselmis* sp. at the lowest BC concentration (Figure 4). Interestingly, under medium and high concentrations of BC lixiviate, *Tetraselmis* sp. presented an additional chlorophyll b-like pigment that was not observed under any other treatment nor species. These changes in pigment composition were analyzed with PERMANOVA and integrated in Figure 5, which allows seeing similarities across samples and treatments on a nMDS plot. Exposure to BC coating has a clear effect in the content and composition of the pigments of all three species, forming defined clusters separated from FR and control treatments (Table 3).

Table 3. Statistical outputs of the multivariate analysis of the total pigment content per microalgae species using Euclidean distances

	Ind. variable	DF	R SumSq	R2	F value	Pr (>F)	
Isochrysis galbana	Coating	2	30.165	0.31752	5.9328	0.0047	*
	Concentration	2	14.253	0.15003	2.8033	0.0416	*
	Coat : Concentration	2	17.534	0.18457	3.4487	0.0197	*
	Residuals	13	33.048	0.34788			
			Homogeneity of multivariate dispersions p-value = 0.117				
Tretaselmis sp.	Coating	2	34.716	0.20302	4.9791	0.0062	*
	Concentration	2	41.242	0.24118	5.9151	0.0033	*
	Coat : Concentration	2	49.723	0.29078	7.1316	0.0012	*
	Residuals	13	45.320	0.26503			
			Homogeneity of multivariate dispersions p-value = 0.870				
Cylindrotheca sp.	Coating	2	62.410	0.62410	30.3233	0.0001	*
	Concentration	2	9.690	0.09690	4.7080	0.0101	*
	Coat : Concentration	2	13.493	0.13493	6.5559	0.0032	*
	Residuals	14	14.407	0.14407			
			Homogeneity of multivariate dispersions p-value = 0.138				

Under low BC concentrations, pigments from *I. galbana* and *Tetraselmis* sp. appear clustered together near the controls and FR, suggesting no big differences from them. *Cylindrotheca*, however, was sensitive to any BC concentration. FR treatments, regardless concentration, display a similar pigment composition to control treatments, with the sole exception of two samples of mid-FR concentration in *I. galbana* (Figure 5). Homogeneity of multivariate dispersions was confirmed with PERMDISP analysis, indicating that dispersion within treatment groups does not differ and, therefore, the differences are entirely due to treatments (p-values = 0.117; 0.87 and 0.138 for *I. galbana*, *Tetraselmis* sp. and *Cylindrotheca* sp., respectively).

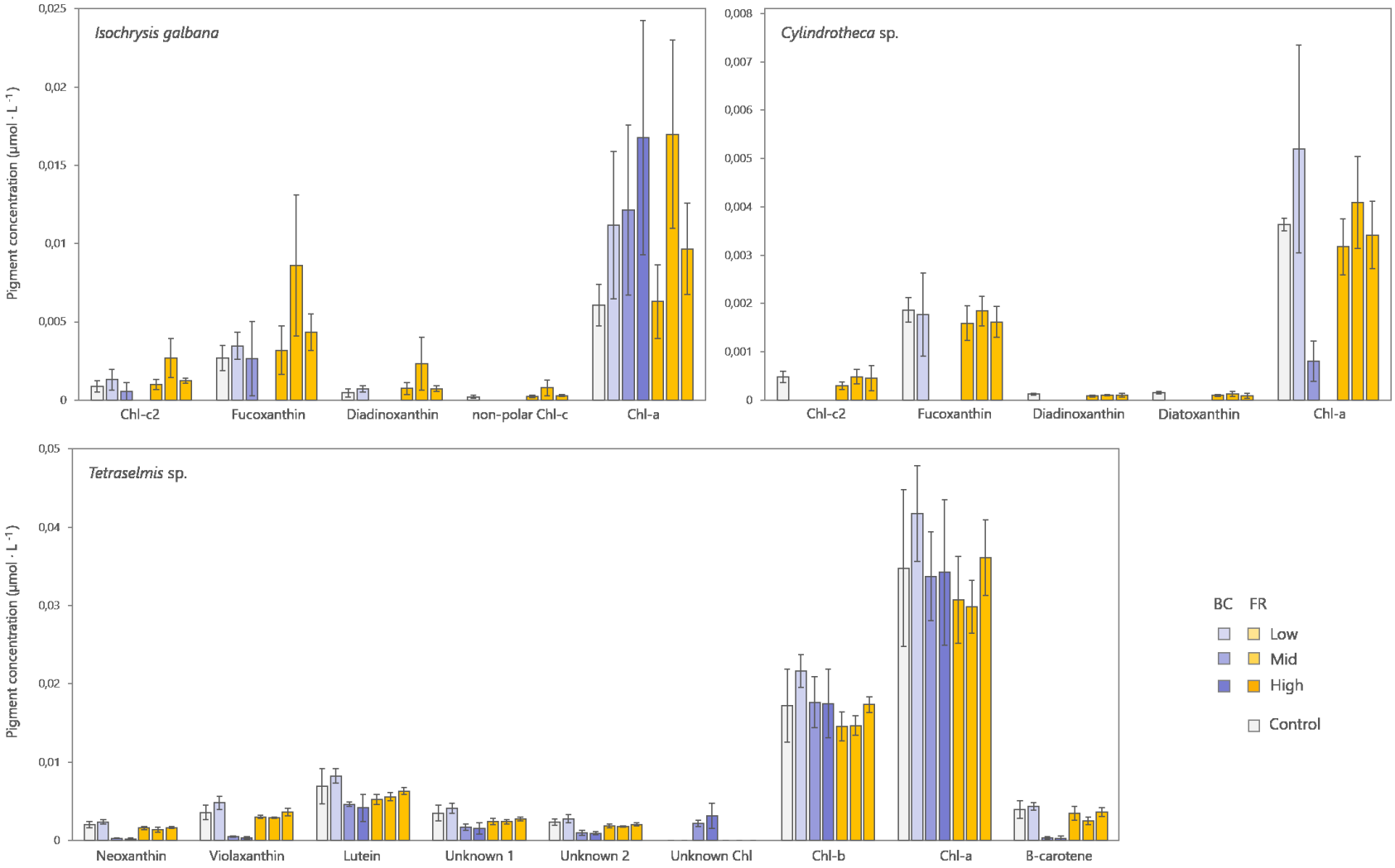


Figure 4. Mean pigment concentration per treatment for each microalgae species, relativized to total growth. Colour indicates coating (violet scale for BC and orange scale for FR; controls are represented in light grey), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration). Error bars represent standard deviation.

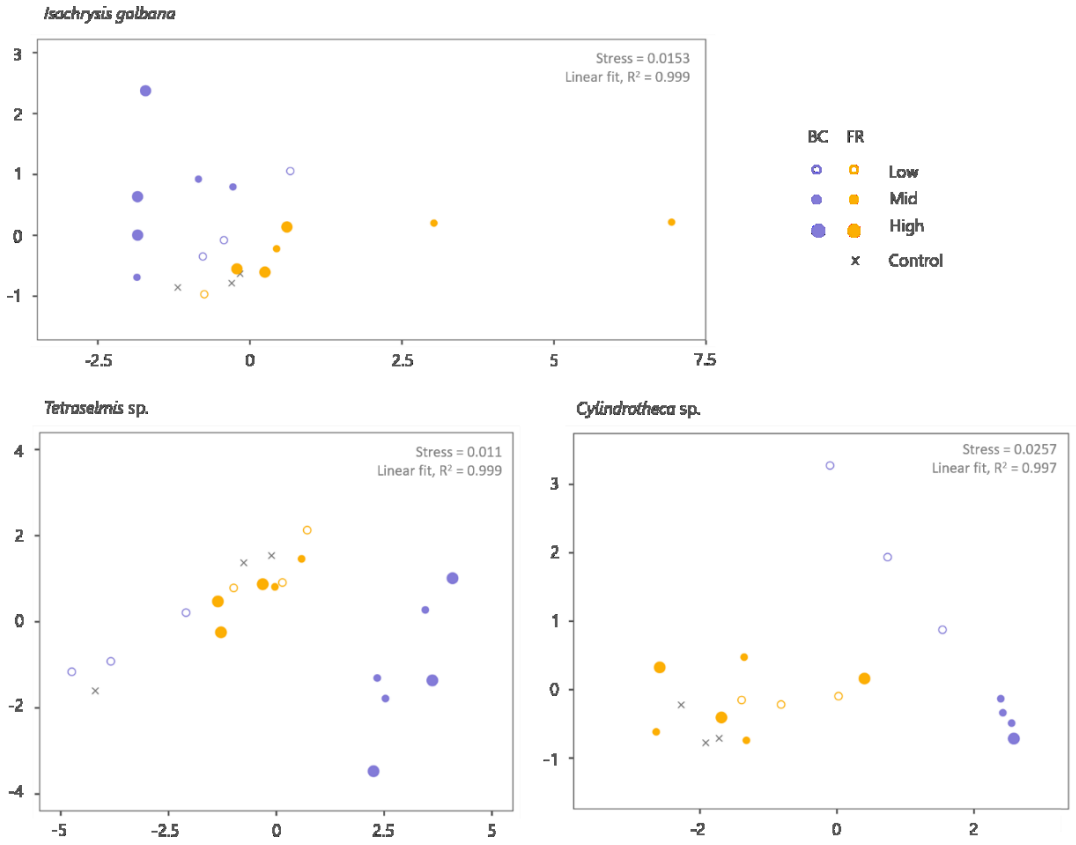


Figure 5. nMDS of pigment composition and content for each algal species based on Euclidean distances. Colour indicates coating (BC in violet and FR in orange; controls are represented with a grey x), and symbol and size represent lixiviate concentration (hollow dots indicate low concentration, small filled dots mid concentration and big filled dots high concentration).

3.5 Biomarkers of oxidative stress: enzymatic activities and lipid peroxidation (LPO)

Measurements of enzymatic activities and LPO levels are presented as SM. There results were still used as inputs to understand biological responses at different levels using an integrative index (see Integrated Biological Response (IBR) index results). Broadly, species responded differently to lixiviate exposure, with no unified response among them. The microalgae *I. galbana* and *Cylindrotheca* sp. experienced a proportional reduction in protein content with increasing concentrations of BC coating lixiviate, an effect that was not observed in the case of *Tetraselmis* sp. Catalase activity was enhanced with respect to the controls under all exposure treatments in *I. galbana* and *Cylindrotheca* sp., while kept as control levels in *Tetraselmis* sp. As regards GST activity, *Cylindrotheca* sp. did not show a clear response, while *I. galbana* experienced a reduction in its activity under FR coating treatments and no differences with respect to the control under BC coating lixiviate exposures. *Tetraselmis* sp. however tended to have lower GST values under any of the treatments compared to the control. Finally, LPO levels were generally higher under BC

exposure in all the three species and more variable, as well as comparable to controls, under FR coating treatments.

3.6 *Cellular metal concentration*

Results from cellular metal sorption do not show significant differences across treatments, with the exception of the microalgae *Cylindrothenca* sp., for which paint and concentration are significant factors (p-values = 0.0001 and 0.0045, respectively). The nMDS plots (Figure 6) show the cellular composition of 12 selected metals for each algae and treatment (Table S5 and S6 from 3. Supportive materials from the Annex).

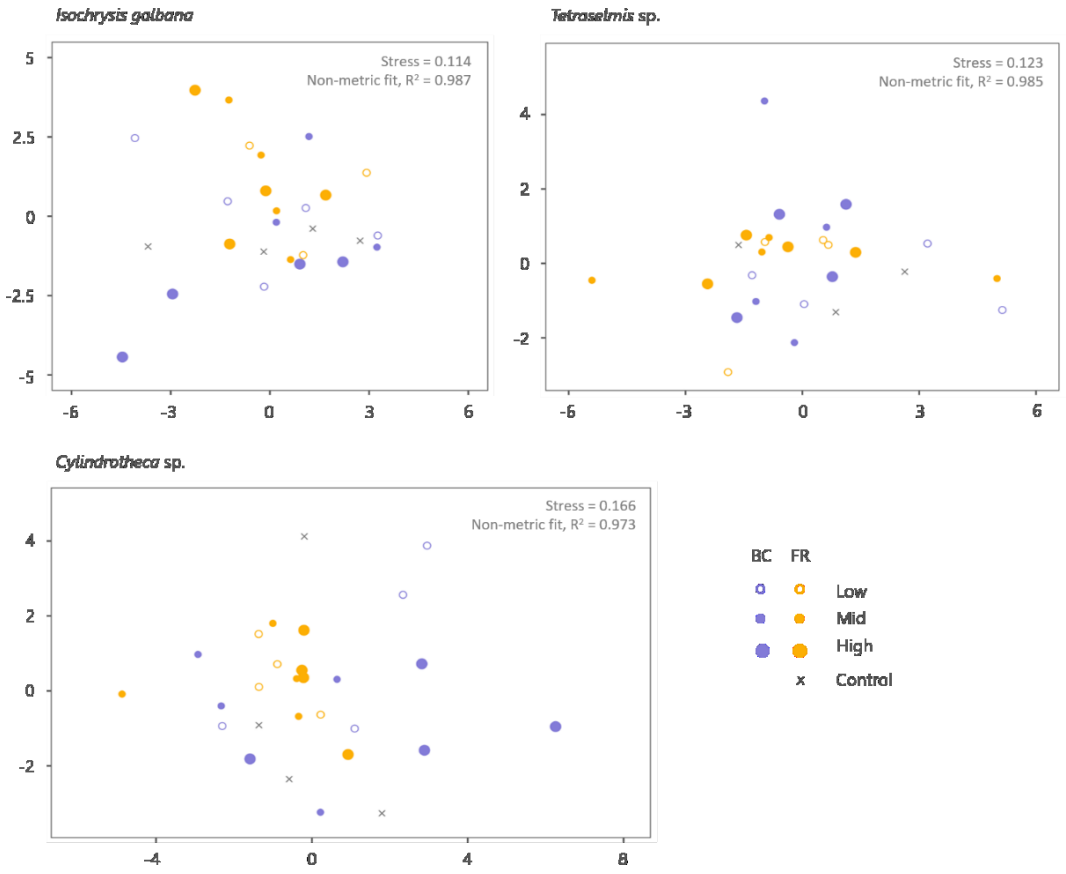


Figure 6. nMDS of cellular metal sorption for each algal species based on Euclidean distances. Colour indicates coating type (BC in violet and FR in orange; controls are represented with a grey x), and symbol and size represent lixiviate concentration (hollow dots indicate low concentration, small filled dots mid concentration and big filled dots high concentration).

Cylindrotheca sp. in particular shows a clear cluster of FR treatments, indicating high similarities of the samples under that treatment, regardless concentration. In the case of *I. galbana*, there is an observable tendency towards two separated clusters based on coating (in fact, p-value =

0.0568 for coating factor). In the contrary, *Tetraselmis* sp. does not show significant responses nor tendencies, indicating no differences in metal accumulation across treatments.

Only when certain metals, such as copper, are individually analyzed can accumulation be actually observed. In fact, Cu is accumulated in all three species under BC coating treatments (Figure 7). *Cylindrotheca* sp. had the most marked response of Cu accumulation, while *I. galbana* accumulated only under high BC lixiviate concentrations. The accumulation in *Tetraselmis* sp. showed to be more variable, although the tendency is also well evidenced.

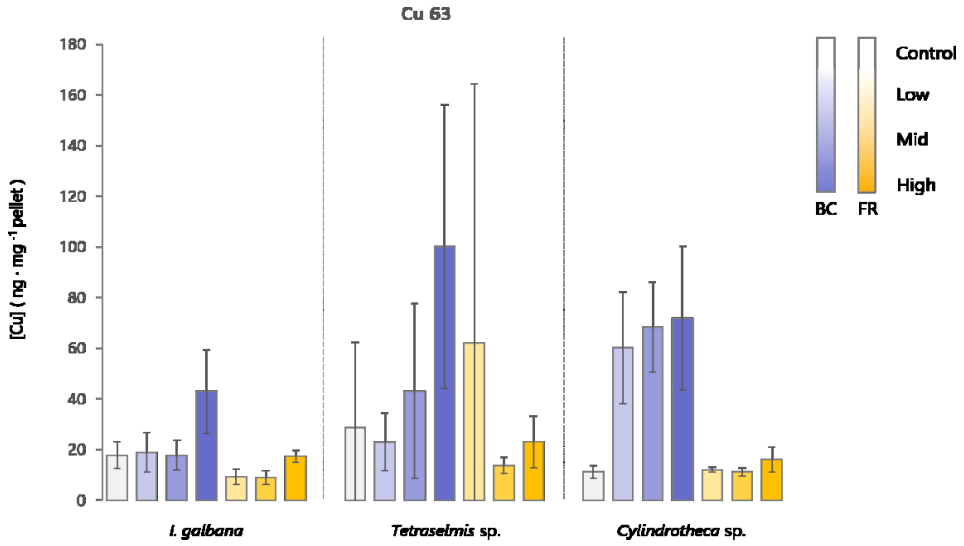


Figure 7. Cellular copper concentrations in the three microalgae species under different coating lixiviate treatments. Colour indicates coating (violet scale for BC and orange scale for FR; controls in white background), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration).

3.7 Integrated Biological Response (IBR) index

Star plots (Figures 8, 9 and 10) graphically illustrate multivariate response data of the selected biomarkers (growth rate; photosynthetic efficiency, here as FLUO; protein content; catalase activity and LPO level) highlighting areas proportionally to the stress level, and evidencing the main variables influencing on it. All three microalgal species showed stress levels when exposed to coatings at any concentration. In particular, there is an evident progressive increase in the stress levels with increasing BC concentrations for all three cases, with a variable effect on the responses. Under FR exposure, the observed stress is mostly linked to subcellular responses, such as LPO, catalase activity or protein content, while major physiological endpoints, such as growth and photosynthetic activity, are not affected. IBR t-scores indicated significant differences from the

mean value for the treatment with the highest BC coating lixiviate concentration in all three species, as well as the treatment with mid concentration of BC for *Cylindrotheca* sp. (Figures 8, 9 and 10).

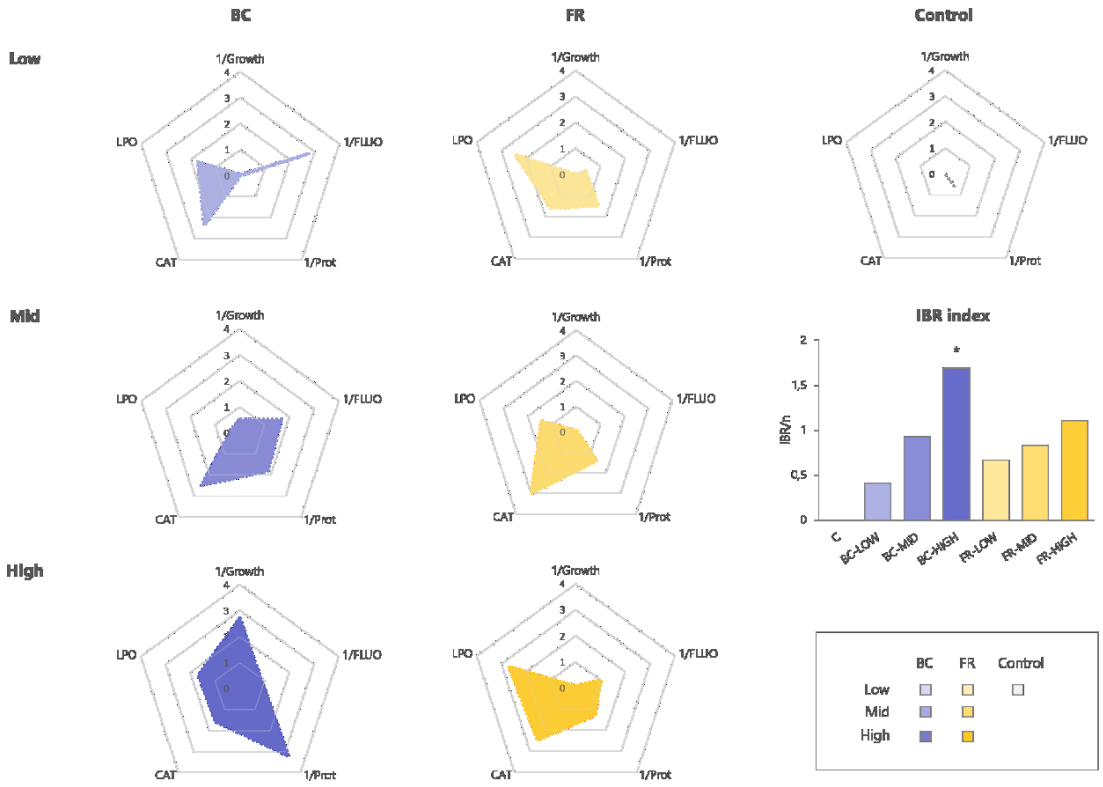


Figure 8. Star-plots based on mean values of 5 biomarkers (growth; fluorescence yield, 'FLUO'; protein content, 'Prot'; catalase activity, CAT; and lipid peroxidation, LPO) for each experimental group for *Isochrysis galbana*. The highlighted areas of the star-plots were used to calculate the IBR index (bar plot) and significant differences from the overall mean are calculated with the t-score and marked with an asterisk. Colour indicates coating (violet scale for BC and orange scale for FR; controls are represented in grey), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration).

4. Discussion

This study assessed the toxicological effects of two commercially available antifouling coatings on different microalgae species, including planktonic non-target species and a benthic target species. The aim was to understand how different species belonging to distinct phyla responded to the exposure to antifouling coating lixiviates, studying it from a biological multi-level approach.

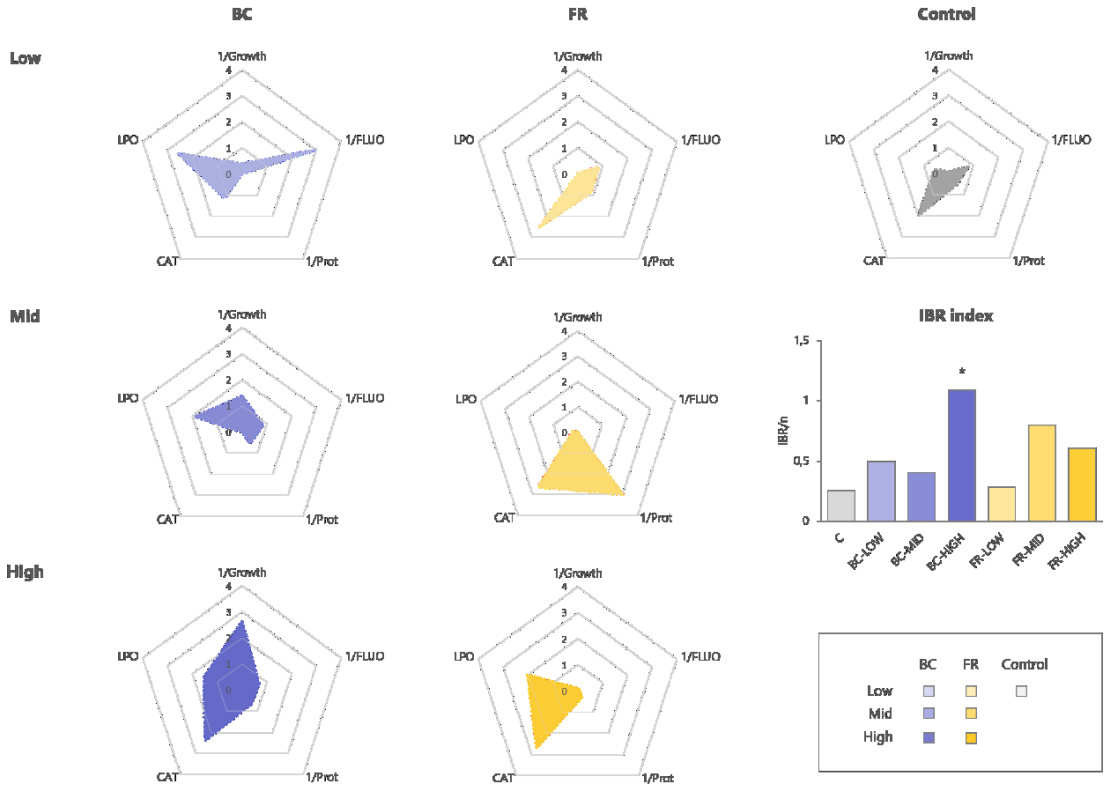


Figure 9. Star-plots based on mean values of 5 biomarkers (growth; fluorescence yield, 'FLUO'; protein content, 'Prot'; catalase activity, CAT; and lipid peroxidation, LPO) for each experimental group for *Tetraselmis sp*. The highlighted areas of the star-plots were used to calculate the IBR index (bar plot) and significant differences from the overall mean are calculated with the t-score and marked with an asterisk. Colour indicates coating (violet scale for BC and orange scale for FR; controls are represented in grey), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration).

The selected BC coating has dicopper oxide, in combination with zinc oxide as the main active compounds, while the alternative non-toxic FR coating contains zinc chromate as a component of the inner paint coating, isolated by an external silicone coat. Excessive concentrations of metals can alter the activity of biologically important molecules and interfere with key physiological processes (Balzano *et al.*, 2020). Besides, they can substitute essential metals ions in certain biomolecules and trigger over-production of reactive oxygen species (ROS) (Balzano *et al.*, 2020; Cavelletti *et al.*, 2022). Therefore, exposure to metal contaminants can lead to a wide range of biological responses.

The current work shows that the species responded differently when exposed to the selected antifouling treatments. *Tetraselmis* sp. arose as the most tolerant species, while *Cylindrotheca* sp. was the most sensitive one. In the studied species, BC coating had a marked effect, although the

magnitude of the measured responses was species-specific. It is noteworthy to mention that the alternative FR coating did have slight effects on the studied algae, mostly limited to subcellular responses.

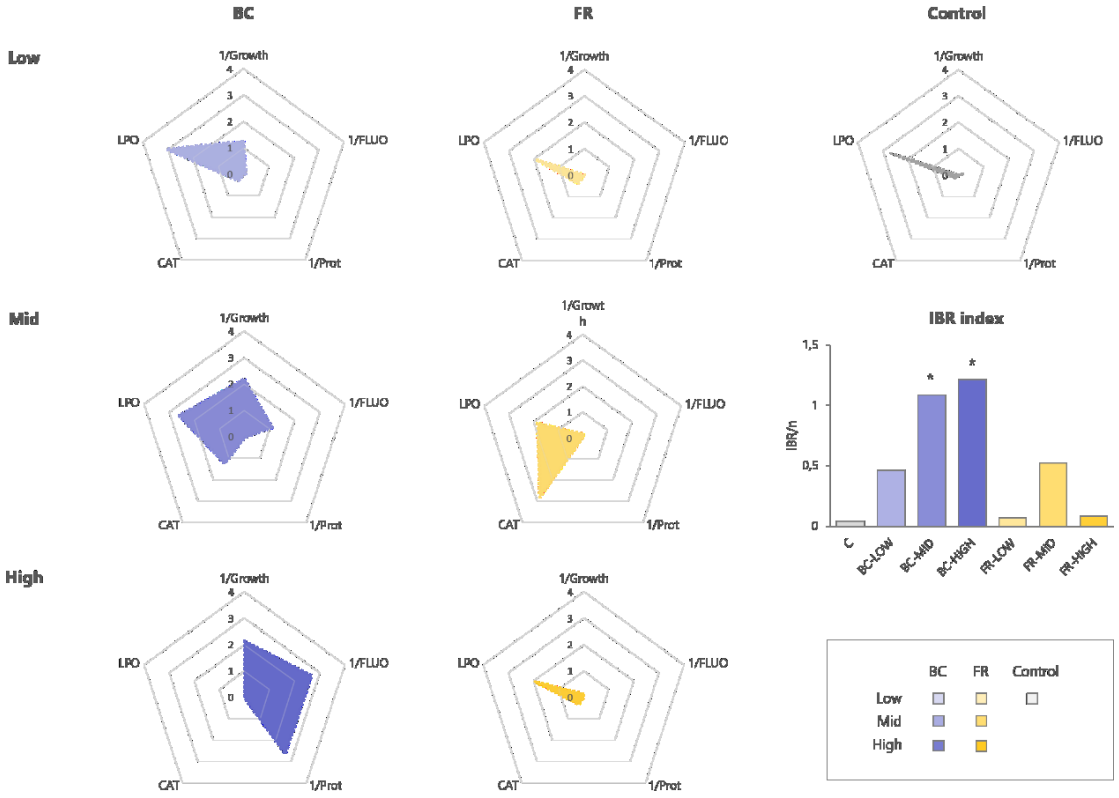


Figure 10. Star-plots based on mean values of 5 biomarkers (growth; fluorescence yield, 'FLUO'; protein content, 'Prot'; catalase activity, CAT; and lipid peroxidation, LPO) for each experimental group for *Cylindrotheca* sp. The highlighted areas of the star-plots were used to calculate the IBR index (bar plot) and significant differences from the overall mean are calculated with the t-score and marked with an asterisk. Colour indicates coating (violet scale for BC and orange scale for FR; controls are represented in grey), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration).

4.1 *Effects on culture growth*

One of the most evident responses of the current study is the effect of the exposure to coating lixiviates on culture growth. Microalgae exposed to BC coating lixiviates experienced a growth inhibition. However, this effect occurred at different BC lixiviate concentrations, *Tetraselmis* sp. being the most tolerant species, followed by *I. galbana* and, finally, *Cylindrotheca* sp., which was especially sensitive. The growth of the latter one, particularly, was much slower when compared

to the other two species, even for the controls. Although this could relate to its biological characteristics (i.e. frustule formation, available surface and density-dependent growth; Stevenson *et al*., 1991), the experimental design and methodological approaches could have contributed to underestimate to some extent the overall growth in all the experimental replicates of this species, not accounting for all the adhered cells. Still, the differences across treatments for this species are remarkable. As regards the alternative non-toxic coating FR, the opposed response was observed, proving to allow growth of target and non-target species even at exposures of 75% of lixiviate, the highest tested concentration in the current study, in most cases with similar rates to control ones.

Growth inhibition by antifouling biocides has been reported on different microalgal species for diverse active compounds (Devilla, 2005; Lim *et al*., 2006; Gatidou & Thomaidis, 2007; Levy *et al*., 2008; Dupraz *et al*., 2018), with records of different sensitivities across species of chlorophytes, haptophytes and heterokontophytes (Dupraz *et al*., 2018). In fact, authors such as Dupraz *et al*. (2018), reported higher tolerances of *Tetraselmis suecica* to organometals (ZnPT and CuPT) and metals such as copper, when compared to the haptophyte *Tisochrysis lutea* and the diatom *Skeletonema marinoi*. Levy *et al*. (2008) also reported higher sensitivity to copper of the diatom *Phaeodactylum tricornutum* in comparison to *Tetraselmis* sp. All these outcomes go in line with the results of the current study. Copper is the main active compound of the tested BC coating and has proven to be toxic for selected microalgae species, although the observed effects are species-specific, hampering growth at relatively low lixiviate concentrations.

Growth inhibition assays can be considered toxicity screening assays and, as an endpoint, reflect major physiological impairment and remarkable toxicological effects. In presence of elevated heavy metal concentrations, and particularly copper, various effects, other than an overall reduction in growth rates, have been described in microalgae, such as diminished photosynthetic efficiency and respiration, changes in cell and organelles size and morphology, to mention some (Cavalletti *et al*, 2022; Xiao *et al*., 2023). In areas of high boat concentrations, where biocides and heavy metals tend to accumulate, these major physiological impairments could lead to the domination of tolerant species and elimination of sensitive ones, shifting the composition of local microalgal communities (Lim *et al*., 2006; Devilla *et al*., 2005), which has been identified as a response at taxocenosis level in benthic diatoms from metal-polluted environments (Martínez *et al*., 2021).

4.2 Effects on photosynthetic efficiency

Photosynthetic efficiency is another major measurable physiological endpoint responsive to contaminant exposure, including metals, in plants and algae (Clijsters & Van Assche, 1985; Souri *et al.*, 2019; Cavalletti *et al.*, 2022; Xiao *et al.*, 2023). The pulse-amplitude modulation (PAM) fluorometric method has been successfully adapted from plants to measure the effects of copper exposure on microalgae and has been proposed as a sensitive approach in aquatic toxicology (Juneau *et al.*, 2002; López-Rodas *et al.*, 2010, Pérez *et al.*, 2010).

Heavy metals can interfere with the photosynthetic apparatus (Souri *et al.*, 2019, Cavaletti *et al.*, 2022). The role of copper in photosynthesis is well known, as it is a constituent of the plastocyanin protein, the electron donor acting as a transfer agent between cytochrome f and photosystem I (PSI). However, Cu at excessive or at deficient concentrations can be detrimental for the photosynthetic apparatus (Barón *et al.*, 1995; Souri *et al.*, 2019). In fact, the results of our study evidence alterations in photosynthetic efficiency when exposed to metal biocides from BC coating lixiviates, while FR exposed treatments maintained baseline photosynthetic levels. Particularly, *Cylindrotheca* sp. shows clear response to BC coating lixiviate, having a reduced photosynthetic efficiency with increasing BC lixiviate concentrations, backing up the outcomes from growth inhibition tests as the most sensitive species. The opposed response was observed for *Tetraselmis* sp., whose photosynthetic performance was not altered by the exposure to BC coating lixiviates and maintained similar levels as the controls and FR exposed samples, confirming its higher tolerance. The response of *I. galbana* was less clear, with an overall reduction in photosynthetic efficiency when exposed to BC coating lixiviates, although with higher performance at higher concentrations. Even if for this microalga species the growth at mid and high BC concentrations was compromised, the photosynthetic efficiency does not follow the same pattern, a response that can also be observed for *Tetraselmis* sp. To better illustrate this outcome, Figure 11 was pieced together for discussion purposes, combining in a descriptive way growth and photosynthetic efficiency responses. Prior studies have demonstrated growth inhibition together with maintained photosynthetic activity as a species-specific response (Stauber & Florence, 1987), and Purbonegoro *et al.* (2018) reported an observable increase in cell size due to impaired cell division and accumulation of photosynthetic products.

4.3 Effects on total pigment content

Chlorophylls and carotenoids are the main pigment classes involved in the capturing of light and photosynthesis. Alterations in pigment composition can negatively impact the photosynthetic function and lead to major physiological consequences. Heavy metals, specifically, can interfere

in the pigment biosynthesis or, in the case of divalent cations (Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} or Pb^{2+}), substitute the Mg^{2+} in the porphyrin ring of the chlorophyll structure (Souri *et al.*, 2019; Cavalletti *et al.*, 2022; Xiao *et al.*, 2023). In this work, an overall change in the pigment composition was observed under BC coating lixiviate exposure for all three microalgae species, while FR values remain similar to those from the control. Although, overall, most carotenoid pigments decreased with exposure to BC lixiviates, a tendency to increased chlorophyll-a content was observed under this treatment, particularly evident in the case of *I. galbana*. This increase in chlorophyll-a content backs up the above-discussed results on photosynthetic efficiency, suggesting that *I. galbana* does not divide when certain concentration of contaminants are present, but can cope with those concentrations ensuring photosynthetic activity (Figure 11). Similarly, an increasing tendency of chlorophyll content can be observed for *Tetraselmis* sp., which showed constant photosynthetic efficiency levels across treatments. Remarkably, an unknown chlorophyll-like pigment appears in *Tetraselmis* sp, solely under mid and high BC concentrations.

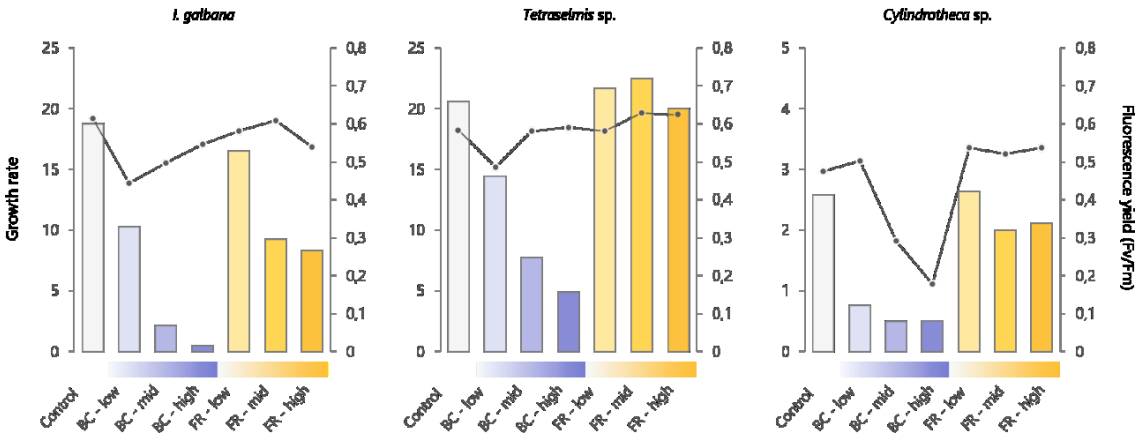


Figure 11. Descriptive graph combining responses of growth rate (bar plot, left y-axis) and photosynthetic efficiency (lines, right y-axis) for the three microalgae species under exposure to different antifouling lixiviates treatments. Colour indicates coating (violet scale for BC and orange scale for FR; controls in white background), and colour intensity indicates lixiviate concentration (from light to bright intensity according to increasing lixiviate concentration).

This response is of particular interest since, to our knowledge, is the first time that has been recorded. The role of chlorophylls in metal exposure as a protective pigment has not been much explored, being most of the studies focused on carotenes as antioxidant defenses (Cirulis *et al.*, 2013; Danouche *et al.*, 2022). These observed responses in *I. galbana* and *Tetraselmis* sp. contrast with most literature studies, which consistently reported a decrease in chlorophyll content under heavy metal exposure accompanied with an increase in carotenoid values, as a protective mechanism against oxidative stress (Cirulis *et al.*, 2013). A drastic decrease in chlorophyll-a content

was only observed for *Cylindrotheca* sp. under mid and high BC concentrations, which adds upon theoberved growth response under those treatments and relates to the low biomass of the cultures.

4.4 Biomarkers of oxidative stress

Enzymes are part of the defense strategies against stressful conditions, which include neutralization and detoxification of harmful substances, such as contaminants and byproducts of the metabolism. Electron transport activities in chloroplasts and mitochondria have the inevitable consequence of ROS formation (Gao *et al.*, 2017; Khorobrykh *et al.*, 2020). In particular, photosynthetic electron transfer chain (PETC) is considered the main source of ROS in chloroplasts (Khorobrykh *et al.*, 2020). To cope with ROS formation and keep them at bay, all aerobic organisms have ROS-scavenging mechanisms. These same mechanisms are triggered under stress conditions, to deal with additional ROS formation. For example, certain metals, like Cu, under exceeding concentrations can catalyze the generation of toxic ROS (Cavalletti *et al.*, 2022). Changes in enzyme activity above baseline levels are considered a biomarker of stress in cells (Valavanidis *et al.*, 2006). Additionally, production of lipid oxidation by-products is another biomarker of oxidative stress, responsive to the increase of ROS level above the threshold value in relation to the exposure to heavy metals (Danouche *et al.*, 2022).

Observed responses to different lixiviates were fragmented and species-specific, suggesting different mechanisms to handle oxidative stress, observations that had also been reported in prior studies, although with more marked responses (Lozano *et al.*, 2014; Gao *et al.*, 2017). In particular, Lozano *et al.* (2014) reported increased CAT activity under Cu exposure for the diatom *P. tricornutum* and the cryptophyceae *Rhodomonas salina*, but not for the diatom *Cylindrotheca closterium*. Besides, Cavelletti *et al.* (2022) performed a profound literature review focusing on the effects of Cu toxicity, biological responses and potential bioremediation application of microalgae. The results of our study do not show so such clear responses and are mostly observable trends. The tendency of *I. galbana* to higher LPO levels and a trend of increased CAT activity under exposure to any of the coatings indicates certain levels of oxidative stress. Enzymatic defenses of CAT and GST, however, were not triggered in *Tetraselmis* sp., although LPO levels increased under BC coating lixiviate exposure, indicating oxidative stress evidenced by TBARs markers. Due to the limited range of selected biomarkers of oxidative stress, other enzymatic and non-enzymatic responses may have gone unexplored for the selected species. In fact, other authors suggested a combination of biochemical and enzymatic pathways triggered to cope with heavy metal exposure in the freshwater microalgae *Scenedesmus obliquus*, including levels of antioxidant

compounds such as proline, ascorbate or g α,α-diphenyl-β-picrylhydrazyl (DPPH); enzymatic activity of catalase, ascorbate peroxidase or superoxide dismutase, as well as lipid peroxidation levels (Danouche *et al*., 2020). Considering the overall tolerance and physiological performance of *Tetraselmis* sp., the molecular mechanisms allowing it may have been left aside the scope of the present work. A broader battery of molecular biomarkers should be considered in order to elucidate the mechanisms involved in ROS-scavenging pathways in the studied species, counting on designs such as the proposed by Danouche *et al*. (2020).

Moreover, the responses of *Cylindrotheca* sp. and high variability within treatments suggest unsuitable methodological approach for this species. Total organic biomass in diatoms may be blurred by the weight of the external inorganic frustule and the actual initial organic pellet weight could be too small for proper testing. Even under balanced growth, diatom cultures do not always show a direct biomass correlation with growth and, indeed, under prolonged asexual reproduction growth, the average size decreases, although for short-term bioassays researchers tend to disregard it (Wood *et al*., 2005). However, our experience shows important limitations related to the inorganic component of the final pellet and the growth traits of the selected diatom, *Cylindrotheca* sp.

4.5 Cellular metal concentration

Microalgae have consistently been proposed for heavy metal phycoremediation purposes, due to their intrinsic characteristics (Cavalletti *et al*., 2022; Priya *et al*., 2022). They pose key features that make them suitable for this purpose, including inexpensive growth requirements, high surface-volume ratio and, therefore, uptake capacity, high sorption capacity even if unalive, and sequestration, compartmentation and tolerance capabilities (Kumar *et al*., 2015). Adsorption of metals to the cell walls is related to the electrostatic interactions with functional macromolecular groups present on the cell surface, while cellular uptake depends on active transportation across the cell membrane in the case of hydrophilic molecules, like heavy metals (Kumar *et al*., 2015), although sorption, sequestration and tolerance capacities vary across species. Intrinsic biological characteristics of certain species can contribute to these differences and make some species more suitable for the purpose of bioremediation and metal uptake, although authors have proposed different mechanisms (Levy *et al*., 2007; Monteiro *et al*., 2012).

The current study reveals subtle differences when exposed to the two coatings considering multiple metal concentrations. Yet, individual analysis of the main metal of interest, i.e. copper, shows differing cellular sorption according to treatment and microalgal species. Differences across treatments are mostly evidenced in the diatom *Cylindrotheca* sp., while *Tetraselmis* sp. shows

greater values, but higher variability. The study lead by Levy *et al.* (2007) pointed out differences among species, *Tetraselmis* sp. being the one adsorbing relatively more copper per cell than any other alga. Quigg *et al.* (2006) also observed variability among species, but the diatom *Thalassiosira weissflogii* accumulated the most copper per cell, accompanied by high accumulation in *Tetraselmis* sp. too. Altogether, these results contribute to the hypothesis by Levy *et al.* (2007) that suggest that cell wall type does not determine the sorption of metals, nor the species sensitivity to metal exposure. However, due to methodological differences with these studies, more accurate comparisons are difficult.

4.6 Integrated Biological Response (IBR) index

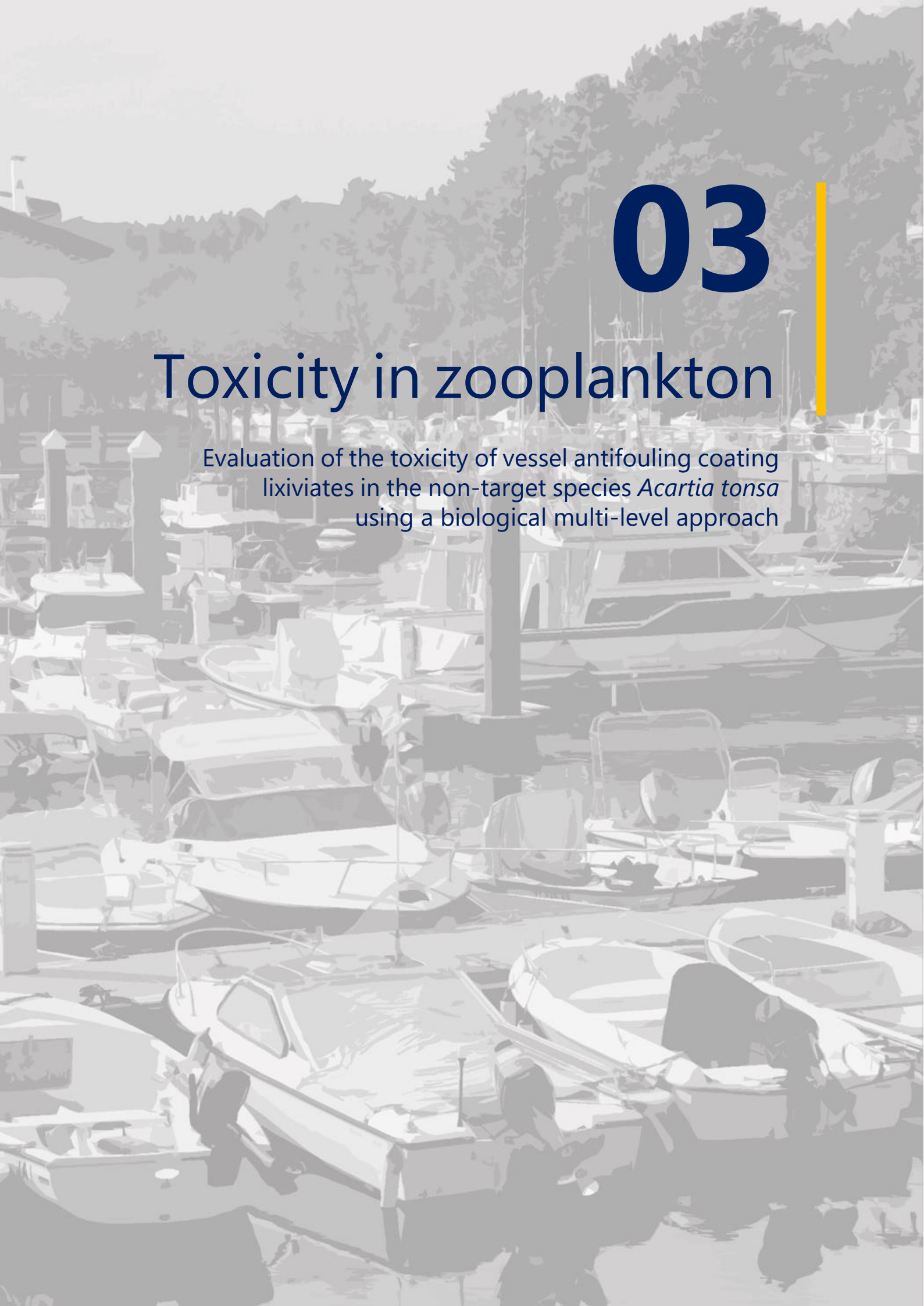
Overall, our results suggest that, at low BC concentrations, subcellular responses are mainly triggered, while physiological effects are observable only when the concentration gets too high to cope with by means of molecular mechanisms. Interestingly, FR coating does not unchain responses measurable at physiological level, and its effects remain restricted to subcellular responses. Even if this is generally met for the studied species, there are evident differences in sensitivity across them. Interspecies sensitivity to metals is yet not fully understood and many are the mechanisms proposed by different authors, with some pointing out to biological traits as the main drivers of tolerance (surface to volume relation, Quiggs *et al.*, 2006; cell wall and its composition, Xiao *et al.*, 2023), while others suggest that uptake rates, internal binding mechanisms and/or detoxification processes draw the differences across species (Levy *et al.*, 2007; Levy *et al.*, 2008; Xiao *et al.*, 2023).

Although mechanisms underlying species-specific responses, in particular sensitivity and tolerance, are yet to be figured out, their implications can have important environmental consequences. Sensitivity and tolerance of non-target species to antifouling compounds can lead to changes in community assemblages (Morín *et al.*, 2012; Martínez *et al.*, 2021), while resistance to these compounds has been proposed as relevant factor to be considered when studying vectors of species introductions, such as biofouling of vessel hulls (Costas *et al.*, 2013, Culver *et al.*, 2021; Santos-Simón *et al.*, 2024). Microalgae tolerance to antifouling compounds has been observed in prior studies (López-Rodas *et al.*, 2010) and attributed to a spontaneous rare mutation in the case of the TBT antifouling compound, raising concerns on adaptation to resistant strains potentially linked to the introduction of microalgal species, including toxic ones (Costas *et al.*, 2013). These outcomes have direct impact on local biodiversity and pose biosecurity risks, highlighting the importance of comprehensive approaches to further understand complex

responses at multiple levels of biological organization that need to be integrated in biofouling management plans.

5. Conclusions

The multi-taxa and biological multi-level approach applied in this study provided an integrative overview of the biological responses occurring in microalgae exposed to antifouling coatings and have the potential to be a valuable tool in aquatic toxicology. Our study shows that antifouling coating lixiviates directly affect both target and non-target microalgae species, although the magnitude of the responses varies according to the species. Observable physiological impairments were reported under traditional biocide-based coating lixiviates, while alternative foul-release coatings triggered responses restricted to subcellular levels. Effects on non-target species pushes into the limelight the usage of toxic antifouling compounds, while differences in sensitivities raise concerns on shifts in community assemblages in areas of higher boat densities where certain contaminants tend to accumulate.



03

Toxicity in zooplankton

Evaluation of the toxicity of vessel antifouling coating lixiviates in the non-target species *Acartia tonsa* using a biological multi-level approach

Abstract

Concerns on potential environmental effects of antifouling coatings have pushed into the limelight the use of biocides, putting down to scrutiny their indirect effects on non-target species and the overall impact on environmental health, backed-up by prior laboratory evidence. In the current work a non-target zooplankton model species, the copepod *Acartia tonsa*, was exposed to lixiviates from two available antifouling coatings, a traditional biocide-based (BC) and an alternative non-toxic foul-release (FR). The effects of exposure were assessed from a biological multilevel approach. Lethal toxicity assays were performed with the water lixiviates of two paints and their individual active components to elucidate toxicity mechanisms and to establish sub-lethal concentrations for subsequent experiments on fecundity and molecular responses. While FR lixiviate did not cause mortality, BC lixiviate induced it even at relatively low lixiviate concentrations (12.9 % of coating lixiviate after 24 h). Lethality assays with the active components revealed unnecessary load of zinc to paint formulations, as well as toxicity of other compounds. Fecundity assays with BC lixiviates showed no effect on the total number of reproductive females; however, the total amount of eggs and the eggs per reproductive female were negatively impacted. At molecular level, 24 h exposure to BC lixiviates tended to induce an upregulation on antioxidant response related genes, while FR lixiviate caused the opposed response. Effects on the chitin pathway and nervous system functioning were also observed. Overall, the biological multilevel approach applied in the current study came out as a valuable tool, revealing a comprehensive frame of responses to antifouling coating lixiviate exposure in copepods. The findings suggest that *A. tonsa* is responsive to AF exposure inducing critical endpoints at low BC concentrations of the tested range, leading to observable sub-lethal effects on reproduction and molecular levels. Further work is required to understand the implications on the studied pathways, particularly in the chitin pathway and nervous system functioning, with additional routed related with fecundity.

Keywords | antifouling coatings · copepod · bioassay · target gene expression · LC50 · fecundity

Resumen

La creciente preocupación sobre los posibles efectos ambientales de los revestimientos antiincrustantes han puesto en el punto de mira el uso de compuestos biocidas, sometiendo a escrutinio sus efectos indirectos sobre especies no diana y el impacto general sobre la salud ambiental, respaldado por evidencias previas obtenidas en estudios de laboratorio. En este trabajo, una especie modelo de zooplancton, el copépodo *Acartia tonsa*, fue expuesta a lixiviados de dos revestimientos antiincrustantes disponibles, uno tradicional basado en biocidas (BC) y uno alternativo de liberación de bioincrustaciones (FR). Los efectos de la exposición se evaluaron desde un enfoque biológico multinivel. Se realizaron ensayos de toxicidad letal para cada una de las pinturas, así como de sus componentes activos individuales con el objetivo dilucidar los mecanismos de toxicidad y establecer las concentraciones sub-letales a utilizar en experimentos posteriores sobre fecundidad y respuestas moleculares. Mientras que el lixiviado FR no causó mortalidad, el lixiviado BC la indujo a concentraciones relativamente bajas (12.9 % de lixiviado tras 24 h de exposición). Los ensayos de letalidad con los componentes activos revelaron una carga innecesaria de zinc en las formulaciones de pintura, así como la toxicidad de otros compuestos. Los ensayos de fecundidad con lixiviados BC no mostraron ningún efecto sobre el número total de hembras reproductivas; sin embargo, la cantidad de huevos puestos por dichas hembras se vio afectada negativamente. A nivel molecular, la exposición durante 24 horas a lixiviados BC causó la regulación positiva de los genes relacionados con la respuesta antioxidante, mientras que el lixiviado FR provocó la respuesta opuesta. También se observaron efectos en la vía de la quitina y el funcionamiento del sistema nervioso. En general, los análisis de enfoque biológico multinivel aplicadas en este estudio resultaron ser una herramienta valiosa que reveló un marco integral de respuestas a la exposición a lixiviados de revestimientos antiincrustantes en copépodos. Los hallazgos sugieren que *A. tonsa* responde a la exposición a AF, llegando a inducir respuestas críticas a bajas concentraciones del lixiviado BC dentro del rango probado, conllevando también a efectos sub-letales observables en la reproducción y las respuestas moleculares. Se requiere más investigación para comprender las implicaciones en las vías estudiadas, particularmente en la vía de la quitina y el funcionamiento del sistema nervioso, con rutas adicionales relacionadas con la fecundidad.

Palabras clave | patente antiincrustante · copépodo · bioensayo · expresión de genes diana · LC50 · fecundidad

Riassunto

Le crescenti preoccupazioni sui possibili effetti ambientali dei rivestimenti antivegetativi hanno messo al centro dell'attenzione l'uso di composti biocidi, portando sotto esame i loro effetti indiretti su specie non bersaglio e l'impatto complessivo sulla salute ambientale, supportato da precedenti prove ottenute in laboratorio. Nel presente lavoro, una specie modello di zooplancton, il copepode *Acartia tonsa*, è stata esposta ai percolati di due rivestimenti antivegetativi comerciali, un rivestimento tradizionale a base di biocidi (BC) e un rivestimento alternativo non tossico (FR). Gli effetti dell'esposizione sono stati valutati mediante un approccio multilivello, selezionando diversi parametri biologici di risposta. In particolare, sono stati effettuati test di tossicità letale per ciascuna vernice, nonché per i singoli componenti attivi, per chiarire i meccanismi di tossicità e stabilire le concentrazioni subletali da utilizzare in successivi esperimenti sulla fecondità e sulle risposte molecolari. Mentre il percolato FR non ha causato mortalità, il percolato BC ha indotto mortalità a concentrazioni ache relativamente basse (a 12.9 % di percolato dopo 24 h). I test di letalità con i componenti attivi hanno rivelato un carico eccessivo di zinco nelle formulazioni delle vernici, nonché la tossicità di altri composti. I test di fecondità con i percolati di BC non hanno mostrato alcun effetto sul numero totale di femmine riproduttrici; tuttavia, il numero di uova deposte da questi femine riprodottive è stato influenzato negativamente. A livello molecolare, l'esposizione per 24 ore al percolato BC tendeva a indurre una sovraregolazione dei geni relazionati alla risposta antiossidante, mentre il percolato FR ha suscitato la risposta opposta. Sono stati osservati anche effetti sulla via della chitina e sulla funzione del sistema nervoso. Nel complesso, i test con approccio multilivello applicati in questo studio si sono rivelati uno strumento utile, rivelando un quadro completo di risposte all'esposizione dei percolati di rivestimenti antivegetativi. I risultati suggeriscono che il copepode *A. tonsa* reagisce all'esposizione di AF, inducendo risposte critiche a basse concentrazioni di BC dell'intervallo testato, portando a effetti subletali osservabili a livello riproduttivo e molecolare. Sono necessarie ulteriori ricerche per comprendere le implicazioni sui percorsi molecolari studiati, in particolare sulla via della chitina e sulla funzione del sistema nervoso, con ulteriori indagini legati alla fertilità.

Parole chiave | vernice antivegetativa · copepodo · bioassay · espressione di geni bersaglio · LC50 · fecundità

Laburpena

Anti-inkrustazio gainestaldurak ingurumenean izan ditzaketen eraginek kezka gero eta handiagoak sustatzen dituzte, konposatu bioziden erabilera jopuntuan jarriz. Hauek xede ez diren espezieetan eta ingurumen-osasunean dituzten zeharkako efektuak direla eta, laborategian lortutako ebidentziekin lotuta, azterketa zehatzagoa behar dute. Lan honek, zooplankton-espezie bat, *Acartia tonsa* kopepodoa, eredu biologiko giza hartuta, komertzialki eskuragarri dauden gainestalduren lixibatuen toxikotasuna aztertzen du, bata biozidetan (BC) oinarritutako ohiko gainestaldura eta bestea ordezko gainestaldura ez toxiko (FR) bat. Esposizioaren ondorioak maila anitzeko ikuspegi batetik ebaluatu dira, interesekoak diren erantzun biologiko desberdinak hautatuz. Hilkortasun proba toxikologikoak egin ziren pintura bakoitzeko, baita haien osagai aktiboekin ere, alde batetik, toxikotasun-mekanismoak argitzeko eta, bestetik, ondorengo esperimentuetan erabiliko ziren kontzentrazio sub-hilgarriak ezartzeko, ugalkortasunaren eta erantzun molekularren inguruko esperimentuetan erabiliko zirenak zehazki. FR lixibatuak ez zuen hilkortasunik eragin, BC lixibatuak, ordea, hilkortasuna eragin zuen kontzentrazio nahiko baxuetan (lixibatuaren kontzentrazioa 12.9 % izanda 24 h ostean). Osagai aktiboekin egindako hilkortasun frogek pintura-formulazioetan alferrikako zink karga zegoela agerian utzi zuten, baita formulazioetako beste konposatuen toxikotasuna ere. Ugalkortasun-azterketek erakutsi zuten BC lixibatuak ez zuela eraginik eme ugalkor kopuruan; hala ere, hauek errundako arrautza-kopuru totalean eragin negatiboa zuten. Maila molekularrean, 24 orduko esposizioak BC lixibatupean erantzun antioxidatzaileari lotutako geneen gorako erregulazioa eragin zuen eta FR lixibatuak berriz, kontrako erantzuna sortu zuen. Gainera, kitinaren metabolismo-bidean eta nerbio sistemaren funtzioan aldaketak ere ikusi ziren. Orokorrean, tresna erabilgarritzat har daiteke lan honetan jarraitutako maila anitzeko esperimentazioa, erantzunen esparru zabala estaltzen baitute. Aurkikuntzek iradokitzen dute *A. tonsak* AF esposizioari ihardesten duela, frogatutako BC kontzentrazio tarte baxuetan erantzun kritikoak eraginez, ugalketa- eta molekula-mailako efektu sub-hilgarriak eraginduz. Lana honek ikerketa gehiago behar dela nabarmentzen du aztertutako bideetan, bereziki kitinaren bideetan eta nerbio-sistemaren funtzioan, ikerketa osagarriekin batera ugalkortasunaren inguruan.

Gako-hitzak | anti-inkrustazio gainestaldura · kopepodo · bioentsegua · itu-geneen adierazpena · ugalkortasuna · LC50

1. Introduction

The application of antifouling measures is essential to control the unwanted settlement and growth of organisms on artificial hard substrates and to ensure correct functioning of systems susceptible of being colonized (Lewis, 1998; Dürr & Thomason, 2009), as described previously in this thesis (see General Introduction). However, environmental concerns related to the use of traditional AF coatings based on biocides (BC coatings) (Yebra *et al.*, 2004; Dafforn *et al.*, 2011; Amara *et al.*, 2018; de Campos *et al.*, 2021) and uncertainties on their actual performance (Culver *et al.*, 2021) have push their use into the limelight, being some commonly used biocides currently under scrutiny. The necessity of biofouling prevention measures ensuring environmental safety coatings have given raise to the development of alternative non-toxic substitutes. Non-stick or foul-release coatings (FR) focus on altering the properties of the surface of interest (Magin *et al.*, 2010; Tian *et al.*, 2021) and are mostly based on silicone elastomers, fluoropolymers or a combination of both (Wezenbeek *et al*, 2018). Although there are other alternatives available (Wezenbeek *et al*, 2018; Tian *et al.*, 2021) and explored (Kyei *et al.*, 2020), FR coatings are becoming a common substitute and a valuable alternative to BC coatings (Lagerström *et al.*, 2022).

The previous chapter (see II. Toxicity in microalgae) evidenced the effects of the selected AF coatings in microalgae at different biological levels of organization, including physiological endpoints (culture growth rates and photosynthetic efficiency) and sub-cellular responses (pigment content, activity of antioxidant enzymes, lipid peroxidation levels and cell metal concentration). These results contribute to existing data on the effects to non-target organisms (Katranitsas, 2003; Karlsson *et al.*, 2010; Ytreberg *et al.*, 2010; Oliveira *et al.*, 2017; Amara *et al.*, 2018) and tolerance of certain species to metal exposure (Floer *et al.*, 2004; Piola & Johnston, 2006), particularly copper.

The results of the prior chapter, altogether with the existing uncertainties and environmental concerns associated to the use of BC, lead us to soundly hypothesize that the widely used BC coatings indirectly impact non-target marine zooplanktonic species and that alternative non-toxic coatings, aimed to dodge these effects, are presumably a safer environmental choice in biofouling prevention. Therefore, the main goal of this chapter was to assess the effects of selected AF coatings available for leisure boats, belonging to different typologies (BC and FR coatings), on the copepod *Acartia tonsa* Dana, 1849, a widely distributed planktonic species.

The order Calanoida is a successful taxa of copepods, with both marine and freshwater species. *Acartia tonsa* is a species of the family Acartiidae belonging to this order. It is considered a model species in ecotoxicology (Khattat & Farley, 1976; Lopes *et al.*, 2021; Rotolo *et al.*, 2021), being it a

very well-studied organism with multiple advantages that make it a suitable organism for laboratory bioassays. Besides its wide distribution and ecological relevance, its intrinsic biological characteristics, which facilitate the experimental manipulation, make of *A. tonsa* a good choice as test organism. For the purpose of this research, it was selected as a non-target model zooplankton species.

Biological responses to metal pollution has been well described at different levels in copepods, from acute and chronic toxicity assays of survival and development (Charry *et al*., 2019; Biandolino *et al*., 2018) to reproductive endpoints (Biandolino *et al*., 2018; Hussain *et al*., 2020) and molecular mechanisms of toxicity coping (Lauritano *et al*., 2012; Kadiene *et al*., 2020). Furthermore, other key physiological processes, such as moulting and nervous system functioning, have been studied using molecular approaches (Lee *et al*., 2015; Kadiene *et al*., 2020).

In this study, bioassays were carried out under controlled lab conditions, focusing on survival, reproductive and molecular endpoints, constituting a biological multilevel integrative approach in AF testing. Mortality was used for toxicity screening and for the establishment of narrower sub-lethal exposure ranges for the study of the effects on fecundity and molecular responses. The analysed molecular responses relate to 1) toxicity coping mechanisms including general stress responses using a selected heat-shock protein (*hsp90*), oxyradical metabolism and detoxification pathways (glutathione-S-transferase, *gst; ferritin, fer;* and superoxide dismutase, *sod-II*) and 2) key physiological processes like moulting and nerve system functioning through selected genes involved in those pathways (chitin-deacetylase, *cda*; and acetylcholine receptor, *ach-r*).

2. Materials and methods

2.1 Copepod culture

Copepod egg cysts of *A. tonsa* were purchased (Algova ©) and kept at 4 °C until the culture preparation. Following the protocol provided by the manufacturer, hatching was induced at 23 °C ± 1°C after transferring 5 mL of the mixed solution of copepod cysts into one litre of filtered seawater (0.22 µm mesh size). The egg culture was kept at a constant temperature, with a light cycle of 16:8, salinity of 33 practical salinity unit (PSU) and vigorous aeration for a minimum of 48 hours. After confirming successful hatching, the content of the beaker was transferred into a larger container of 5 L, at a temperature of 20 °C ± 1 °C and gentle aeration, while the rest of the parameters were maintained. At this stage, copepods were fed daily with a mixture of 1:1 of *Isochrysis galbana* Parke, 1949 and *Tetraselmis chui* Butcher, 1959. Tank maintenance was done once a week.

2.2 Antifouling coatings

Two commercially available antifouling coatings were selected from the market, corresponding to different categories: a biocidal coating (BC) and a foul-release coating (FR). The specifications of each coating can be found in the chapter I. Chemical characterization. Briefly, BC coating contained between 25-50 % in weight of dicopper oxide (Cu_2O) and less than 10 % in weight of zinc oxide (ZnO) as active compounds. Among the other components, the coating contained colophony, naphtha, xylene and ethylbenzene (see Table 1 of the chapter I. Chemical characterization). The FR coating was composed of two layers of coatings: 1) an external layer of silane composites (between 2-8% in weight of methyl tris(methylethylketoximine)silane and 0.1-2% in weight of 3-Aminopropyltrimethoxysilane), and 2) an inner isolated paint coating that contains zinc chromate, xylene, talc, propan-2-ol; 2-methylpropan-1-ol and phosphoric acid (see Table 2 of the chapter I. Chemical characterization).

2.3 Preparation of the lixiviate and contaminant solutions

Lixiviates were prepared as in chapters I. Chemical characterization and II. Toxicity in microalgae, using sandblasted and coated 14x14 cm PVC plates, and incubated in a glass container with 1L of filtered sea water (0.22 µm mesh size), with the plate fixed and the coated side facing downwards and completely submerged. Subsequently, the container was placed on a shaker at 150 rpm for 24 h. Lastly, water samples were taken from the final lixiviate preparation for chemical analysis prior to the start of the lixiviate exposure experiments.

In order to test the individual components and potential synergies, the coating active compounds were used as single contaminants. However, due to the methodological limitations in the solubilisation of Cu_2O and ZnO, CuCl (Copper (II) chloride dehydrate, > 99 %; Sigma Aldrich) and $ZnCl_2$ (Zinc chloride, ≥ 98 %; Sigma Aldrich) were used instead as the source for Cu and Zn. Finally, methyltris(methylethylketoximine)silane (Ref. 10-S12075; Fluorochem) was used as the main component of the external FR coating. In all cases, a stock solution was prepared and thereafter diluted to the final experimental concentrations (Table 1).

2.4 Chemical analysis of the lixiviates

Metal concentrations on lixiviates were quantified using inductive coupled plasma mass spectrometry (ICP-MS) as established in the chapter I. Chemical characterization.

2.5 Toxicity screening through mortality assays

The assessment of lethality was carried out according to the international standard of water quality for the determination of acute lethal toxicity to marine copepods (ISO 14669 from ISO, 1999), slightly adapted for manipulation purposes. Five different concentrations and a control were set (see Table 1), with 3-4 replicates each and exposure was done under static conditions. The experiment was performed using 12 well plates, with a volume of 4 mL and 5 individuals per well, regardless their sex, , since previous studies did not detected sex-related mortality differences in *A. tonsa* exposed to environmental contaminants (Hafez *et al.*, 2021). Twenty-four hours prior to the beginning of the experiments, the culture was washed and fed, while during the course of the experiment, individuals were kept unfed ensuring waterborne exposure (Hafez *et al.*, 2021). Mortality was checked after every 24 hours during the exposure experiments, considering an individual dead when there was no movement nor response to gentle stimulation using a pipette. The test was considered valid when the mortality recorded for controls was less than 10 %.

Table 1. Summary of the design of the exposure experiments for different endpoints using *A. tonsa* adults.

Bioassay type	Test compound	Concentrations	Duration	Exposure
Toxicity screening (lethal toxicity assays)				
Lixiviate exposure	BC lixiviate	0, 10, 30, 50, 80, 100 %	96 h	Static
	FR lixiviate	0, 10, 30, 50, 80, 100 %	96 h	Static
Compound testing	$ZnCl_2$	0, 20, 40, 60, 80, 100, 200 µg/L	72 h	Static
	CuCl	0, 50, 100, 200, 400 µg/L	72 h	Static
	$ZnCl_2$ + CuCl	0, 25, 50, 100, 200, 500 µg/L $ZnCl_2$	72 h	Static
		0, 50, 100, 200, 400, 1000 µg/L CuCl	72 h	Static
	Silane	0,50, 150, 200, 400, 600, 700 mg/L	72 h	Static
Fecundity				
	BC lixiviate	0, 3, 8 % of BC lixiviate	7 days	Semi-static
Differential gene expression				
	Control	50 % bare PVC	24 & 48 h	Static
	BC lixiviate	3 and 6 %		
	FR lixiviate	50 %		

2.6 Fecundity assay

The experiment was divided into two main phases, exposure and individual female testing. First, gravid females were selected from the culture using a stereoscope (Nikon SMZ745T) with a magnification of x10. Additionally, 1 adult male per every 5 females was selected, with the aim of ensuring female fertility along the experiment (Holste & Peck, 2006; Hafez *et al.*, 2021). In the exposure stage, the experiment was performed using 30 mL containers, each containing five females and one male, in a semi-static exposure condition for a period of 7 days. Copepods were exposed to two sub-lethal lixiviate concentrations (3 % and 8 %) plus a control, with 3 replicates

each. During this phase, copepods were fed daily with *T. chuii* at 3000 cells/mL to maintain the females in optimal gravid stage and containers were cleaned gently by removing faecal pellets, dead copepods and algae. After every 48 h, the content of the containers was renewed.

Once the exposure stage was over, females were transferred to an incubation chamber for the individual testing phase. The chamber consisted on a modified falcon tube, which had its conical end removed and substituted with a 200 µm mesh-size net, semi-submerged in a 200 mL container with clean filtered sea water. This design allowed having two separated compartments: the spawning chamber that hosted an individual female within the modified falcon tube and the brooding chamber, where the eggs are released and collected afterwards (Hafez *et al*. 2021). Data on reproductive females and egg production rate was recorded, in particular, the total number of reproductive females (presented as reproductive female ratio), the total number of eggs relativized to the total number of reproductive females (presented as eggs per reproductive female), and the total number of eggs per test group.

2.7 Differential transcription of target genes

2.7.1 Exposure, sample collection and RNA extraction

In order to analyse differential expression of target genes in *A. tonsa*, copepods were exposed to selected sub-lethal concentrations of coating lixiviates for 24 and 48 hours, determined previously through lethal toxicity bioassays (see section 2.5). An experiment consisting of three treatments plus a control per exposure time was set up, with five replicates per treatment. Each replicate contained a total volume of 200 mL and between 35 to 40 copepods. Once the exposure time was over, copepods were collected using a 200 µm mesh-size filter and preserved in RNA*later* (Invitrogen, Thermo Fisher Scientific), immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. RNA extraction was carried out using RNeasy Minikit (Qiagen) and following the protocol by the manufacturer. Before starting, RNA later from samples was removed aid by a syringe, leaving the minimum volume possible. Subsequently, buffer lysis was added and samples were homogenized (Precellys 24, Bertin Technologies). Homogenates were then centrifuged at maximum speed (8000 g) and the supernatant collected for the extraction procedure. RNA extraction was carried out using RNeasy spin columns using all sample volume to maximize the RNA yield.

2.7.2 RNA quality and cDNA synthesis

RNA integrity and concentration were measured using the RNA 6000 Nano Kit for Bioanalyzer 2100 (Agilent Technologies, USA). Although most samples did not meet the RNA Integrity Number

(RIN) score, RNA integrity was confirmed through the presence of the 18S and the 28S RNA peaks. RNA purity was additionally supported with measurements of sample absorbance ratios of 260/280 nm using a UV-spectrophotometer (Epoch, Biotek, USA). All samples showed acceptable 260/280 ratios, (between 1.8 and 2.1) indicating RNA purity. Finally, cDNA synthesis was carried out using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, USA) and following the manufacturer's first-strand cDNA synthesis protocol with random primers on SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific).

2.7.3 Selection of genes of interest (GOI), primer design and PCR amplification

A battery of genes of interest (GOI) were selected (Table 2) based on previous studies, including relevant target biological pathways such as control of development, general stress responses, oxyradical metabolism, detoxification pathways and nerve system functioning. Chitin deacetylase was the only transcript designed *de novo* using Cluscal Omega from alignments of *A. tonsa* genome sequence (accession number LS044924.1) and the corresponding annotated gene of *Acartia pacifica* (accession number KT754625.1), searching for conserved regions, which were then used for specific primer design. Successful amplification was confirmed by PCR (Thermo Cycler, BioRad) in a 25 µL reaction volume (details in the section 2.3 Protocols from the Annex). For negative controls 2.5 µL of ultra-pure RNA free water were added instead. PCR products were run on agarose gel electrophoresis and visualized using a gel imaging system (GBox, Syngene). The obtained transcript was sequenced using Sanger Sequencing (General Genomics Service Sequencing and Genotyping Unit; SGIker, UPV/EHU) and published in GenBank (reference PP595814.1).

Table 2. Battery of selected genes of interest (GOI) with their corresponding function, corresponding forward (Fw) and reverse (Rv) primer sequence, melting temperature (Tm), primer concentration, amplification efficiency (E %) and their respective reference.

GOI name	Function		Primer sequence (5' to 3')	Tm (°C)	[primer] (pmol · µL^{-1})	E %	Reference
β-actin	House-keeping	Fw	TTGGGTATGGAGTCCTGTGG	59.4	0.8	91	Zhou *et al.*, 2020
		Rv	CCTGGATACATAGTGGTGCC	59.4			
Elongation factor	House-keeping	Fw	AGGTTAAGTCCGTGGAGATG	57.3	0.8	93	Soloperto *et al.*, 2022
		Rv	ACTGGCTTGTTCTTGGAGTC	57			
Chitin deacetylase (*cda*)	Moulting	Fw	TTCTTCAATCACTGCCCCC	62.1	0.57	85	Current study
		Rv	TCCTCCCTTCTGTCCAACTC	62.1			
Glutathione S-transferase (*gst*)	Detox (phase II)	Fw	TGCTTGATTCACTTCTACAAGAGA	57.6	0.8	101	Hafez *et al.*, 2021
		Rv	GTCACCATCAACAACAGTTGGA	58.4			

Heat-shock protein 90 (*hsp90*)	Stress	Fw	GTCACATCCCAGTATGGTTGG	59.8	0.8	95	Nilsson *et al.*, 2014
		Rv	CCATGGTGGAGGTGTCACGG	63.5			
Ferritin (*fer*)	Storage & oxidative stress	Fw	ACGCTTGCACTGATAATCCA	55.3	0.8	87	Nilsson *et al.*, 2014
		Rv	AGTTCTACCGTGACGCATCC	59.4			
Superoxide dismutase [Cu–Zn] (*sod-II*)	Oxidative stress	Fw	CTGGTCTCGATGATGGCCTC	61.4	0.8	91	Rotolo *et al.*, 2024
		Rv	ATGACGTTCACCCTTGGCAT	57.3			
Acetylcholine receptor (*ach-r*)	Nervous system	Fw	GCTGACGAAGAGTTTGCCAA	57.3	0.8	89	Rotolo *et al.*, 2024
		Rv	ATGTAGAAGTTGCCCTCGCC	59.4			

2.7.4 RT-qPCR: standard curves and GOI expression

Real-Time quantitative PCRs (RT-qPCR) were conducted in MicroAmp Optical 384-well reaction plate in ViiA7 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, USA), using the specific primers at the specified conditions and concentration shown in Table 2. FastStart Universal SYBR Green Master (ROX) mix was used in a final reaction volume of 10 µL. Reaction volume contained 5 µL of the mix, 0.1 µL of each specific primer (forward and reverse), 2.8 µL of ultra-pure water and 2 µL of cDNA. The amplification consisted of the following steps and conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles composed of 15 s at 95 °C and 1 min of annealing at the corresponding melting temperature (Tm) of each primer (Table 2). Product specificity and absence of primer dimers was checked with a dissociation curve, after the PCR cycles. All qPCR assays were conducted in triplicates, and non-template controls (NTC) were included for each gene and plate.

A six 1:2 serial dilution standard curve in triplicates was used to calculate the amplification efficiency of each primer pair, using a pool of cDNA of random experimental samples. All primer pairs had an amplification of at least 85 % efficiency (Table 2). The stability of two candidate reference genes (β-actin and Elongation factor) was assessed in treatment and control samples through RT-qPCR and calculated with the NormFinder (Andersen *et al.*, 2004). β-actin was selected as the best reference gene for this study and it was used to normalize the relative expression ratio of each GOI and calculating the gene expression level of treatment conditions with respect to that from control conditions, using the Pfaffl equation (Plaffl *et al.*, 2004).

2.8 Statistical analysis

The software RStudio (`R version 4.1.2`; R Core Team, 2022) was used to perform the statistical analysis. Mortality at different exposure times was assessed using PROBIT analysis ('`ecotox`' package; Hlina *et al.*, 2021), calculating LC50s for the lixiviates and test compounds. Significance was defined based on the overlapping of the 95% confidence interval limits.

Data from the fecundity test was first checked for normality and homogeneity of variance using residuals' distribution visualization, Levene's (Levene, 1960) and Shapiro Test (Shapiro & Wilk, 1965) from the '`car`' (Fox & Weisberg, 2019) and '`stats`' (R Core Team, 2022) packages, respectively. Fecundity data failed to meet these assumptions and thus, permutational ANOVA ('`lmPerm`' package; Wheeler & Torchiano, 2016) for two factors was used instead, which additionally overcomes issues related to small sample size.

Lastly, relative gene expression levels were determined using Pfaffl method (Pfaffl *et al.*, 2004) and transcript levels were log2 transformed for visualization and data normalization. Statistical differences across treatments, exposure times and the interaction of the two factors were checked using two-way ANOVA when the data of the response variables met the assumptions of normality and homogeneity of variances as described above and permutational ANOVA ('`lmPerm`' package; Wheeler & Torchiano, 2016) was used otherwise. Pairwise tests were carried out for significant factors, using Tukey contrasts ('`broom`' package; Robinson *et al.*, 2023) for parametric data and Wilcox Test ('`stats`') for non-parametric data.

3. Results

3.1 Chemical analyses

The concentration of selected metals from coatings lixiviates is summarized in the Table 3. Differences between coatings in concentrations of Cu and Zn are evident. . Levels of both biocidal metals were much higher in the BC coating paint than in the FR coating paint. However, other metals with known toxicity such as Cr and As were also present in the lixiviates, as seen in previous chapters.

Table 3. Summary of selected metal concentrations in coating lixiviates.

Bioassay type	[Cu] (µg · L $^{-1}$)	[Zn] (µg · L $^{-1}$)	[Cr] (µg · L $^{-1}$)	[As] (µg · L $^{-1}$)
BC coating	1467.28	188.654	missing	33.71
FR coating	23.58	31.64	missing	37.71

Due to issues with the water samples for chemical analyses, only those regarding toxicity screening are shown (the remaining are yet to be done). Prior chemical data from other sections of this thesis serve as reference and provide sufficient data to understand the chemical aspects intrinsic to the assays of this chapter.

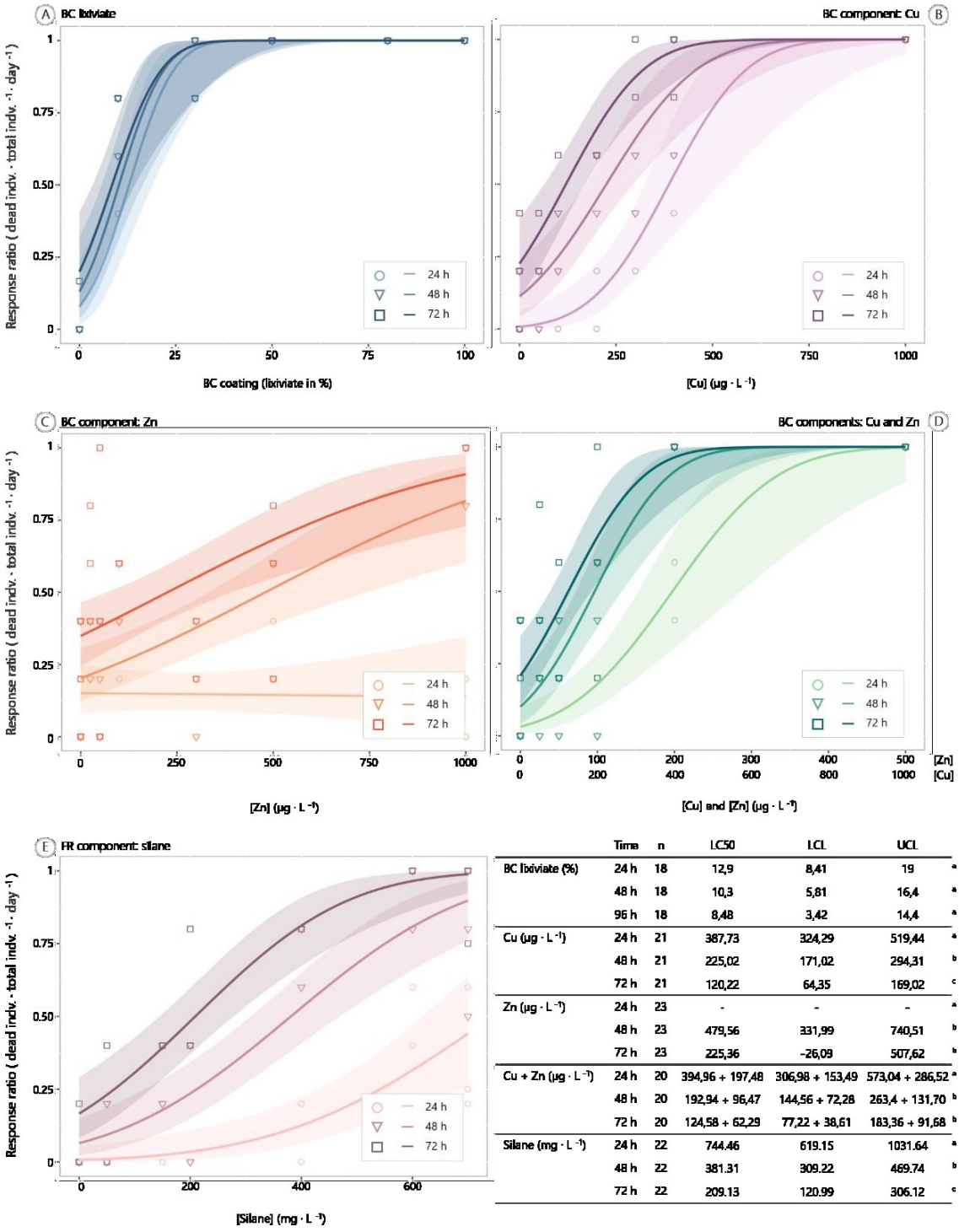
3.2 L Toxicity screening through mortality assays

Lethal toxicity assays on *A. tonsa* varied according to the test contaminant. Figure 1 includes determined LC50s for each test condition and the corresponding confidence intervals per

exposure time. FR lixiviate did not cause mortality and, thus, LC50 could not be determined. In contrast, BC lixiviate showed severe toxicity and LC50 was reached at a lixiviate concentration of 12.8 % during the first 24 hours and decreases during the exposure with time (Figure 1A). Considering the chemical characterization of the metal content of the BC lixiviate (Table 3), a concentration of 12.9 % would contain 189.28 μg · L^{-1} of Cu and 24.34 μg · L^{-1} of Zn.

To understand the toxicity of active compounds of each AF coating, they were individually tested, and, in the case of Cu and Zn also in combination, through mortality assays (Figure 1: B, C, D and E). The calculated LC50 for Cu was 387.73 μg · L^{-1} after 24 hours and this concentration decreased with time, with values of 225.02 and 120.22 μg · L^{-1} after 48 and 72 hours, respectively. As regards zinc, 24 h exposure did not cause enough mortality to calculate the LC50, and only longer exposures induced it, showing a LC50 of 479.56 μg · L^{-1} after 48 h and of 225.36 μg · L^{-1} after 72 hours. Interestingly, the LC50 values for Cu and Zn individually were significantly higher than their estimated respective concentrations in the LC50 of BC lixiviates, suggesting additive toxicity of other paint components.

Furthermore, the mixture scenario with the combination of Cu and Zn did not exacerbate significantly lethality. In fact, LC50 values for Cu in the mixture do not significantly differ from those of Cu alone, being these 396.96; 192.94 and 124.58 μg · L^{-1} after 24, 48 and 72 hours, respectively. Different concentrations of Zn did not significantly increase the toxicity of the mixture. Regarding silane, the main component of the external silicone layer from FR coatings, the lethal effects occurred at relatively high concentrations (Figure 1E), with concentrations of 744.46; 321.31 and 209.13 mg · L^{-1} after 24, 48 and 72 hours of exposure, respectively.



	Time	n	LC50	LCL	UCL	
BC lixiviate (%)	24 h	18	12,9	8,41	19	a
	48 h	18	10,3	5,81	16,4	a
	96 h	18	8,48	3,42	14,4	a
Cu (µg · L⁻¹)	24 h	21	387,73	324,29	519,44	a
	48 h	21	225,02	171,02	294,31	b
	72 h	21	120,22	64,35	169,02	c
Zn (µg · L⁻¹)	24 h	23	-	-	-	a
	48 h	23	479,56	331,99	740,51	b
	72 h	23	225,36	-26,09	507,62	b
Cu + Zn (µg · L⁻¹)	24 h	20	394,96 + 197,48	306,98 + 153,49	573,04 + 286,52	a
	48 h	20	192,94 + 96,47	144,56 + 72,28	263,4 + 131,70	b
	72 h	20	124,58 + 62,29	77,22 + 38,61	183,36 + 91,68	b
Silane (mg · L⁻¹)	24 h	22	744.46	619.15	1031.64	a
	48 h	22	381.31	309.22	469.74	b
	72 h	22	209.13	120.99	306.12	c

Figure 1. PROBIT curves of lethal toxicity for the tested lixiviates and active compounds at different exposure times. A: BC lixiviate; B: copper (main active compound of BC coating); C: zinc (booster co-biocide in BC coating); D: mixture of Cu and Zn; and E: silane (main component of FR external coat). Lethal toxicity curve for FR lixiviate is not represented due to lack of mortality even at 100% lixiviate concentration. The statistical outputs are included, specifying the LC50 values and their confident intervals (level 95%; lower confidence level, LCL; upper confidence level, UCL); equal letters group non-significant differences across exposure times for each of the tested compounds independently.

3.3 Fecundity bioassays

Exposure to the BC lixiviate did not significantly affect the ratio of reproductive females (pr meaning probability) (pr = 0.4137 for factor treatment) (Table 4, Figure 2A), but a reduction in this ratio was observed in all experimental groups with time (pr = 8.5e-05), pointing to lower presence of active reproductive females by the end of the 72 h, as they spawn. The number of eggs laid per active reproductive females (Figure 2B) was lower in BC exposed groups than in controls, an effect that irregularly varied with time. However, issues with an experimental replicate and an imbalanced design (unequal number of reproductive females per replicate) blurred statistical significance (pr = 0.1895 for the factor treatment) (Table 4). The exposure condition caused a dramatic reduction in the number of eggs laid in BC exposed groups compared to the control (Figure 2C). This response was both treatment and time dependent (pr = 0.0026 for factor treatment and pr = 0.0124 for factor time) (Table 4). If the egg production is analysed daily (Figure 2D), a difference in the total cumulative number of eggs was indeed observed, being the greatest differences after 72 h.

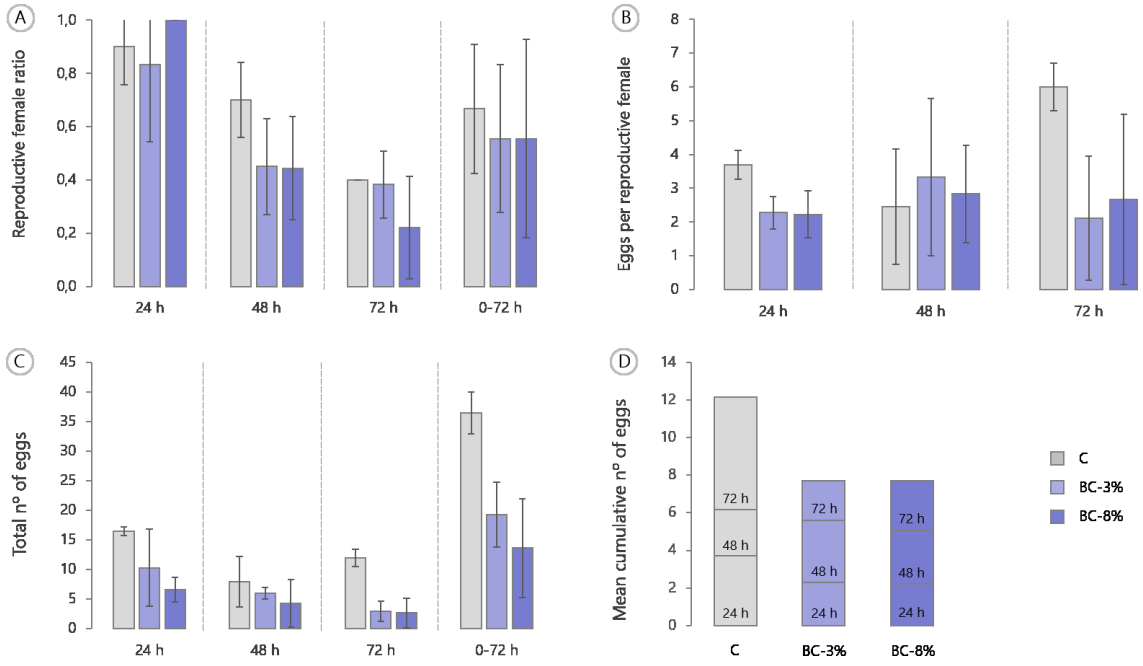


Figure 2. Summary of fecundity parameters per treatment and time. A: Reproductive female ratio; B: eggs per reproductive female; C: total number of eggs; and D: cumulative number of eggs. Colour indicates treatment (grey: control; light blue: low BC lixiviate concentration; and dark blue: high BC lixiviate concentration). Error bars indicate standard deviation.

Table 4. Statistical outputs of the permutational ANOVA test for fecundity endpoints. Significant factors for each variable are marked with an asterisk (*). *DF*: degrees of freedom; *R SS*: RStudio sum of squares; *R mean sq*: RStudio mean sum of squares; *Iter*: iteractions; *Pr(Prob)*: p-value estimate.

	Ind. variable	DF	R SS	R mean sq	Iter	Pr(Prob)	
Reproductive female ratio	Treatment	2	0.05556	0.02778	1e+06	0.4137	
	Time	2	1.32259	0.6613	1e+06	8.5e-05	*
	Treatm : Time	4	0.13553	0.03388	1e+06	0.3805	
	Residuals	15	0.45148	0.0301			
Eggs per reproductive female	Treatment	2	9.840	4.9198	1e+06	0.1895	
	Time	2	3.274	1.6371	1e+06	0.5476	
	Treatm : Time	4	14.303	3.5757	1e+06	0.2932	
	Residuals	15	39.489	2.6326			
Total number of eggs	Treatment	2	216.056	108.028	1e+06	0.0026	*
	Time	2	137.476	68.738	1e+06	0.0124	*
	Treatm : Time	4	42.528	10.632	1e+06	0.4576	
	Residuals	15	167.167	11.144			

3.4 Differential gene expression

Differential expression levels for the analysed target genes were compared among treatments within each exposure time and between exposure times for the same treatment (Figure 3). Overall, selected genes responded differently to the treatments, being this factor significant for all genes except for *hsp90* (Table 5). In fact, *hsp90* did not show significant expression changes among treatments nor exposure times, except a slight trend to downregulated transcription levels in FR exposed group at 24 and 48 h and in BC high dose exposed group at 48 h. Genes related to oxidative stress, i.e. *gst*, *fer* and *sod-II*, showed a common response pattern (Figure 3; Table 5). These three genes had an initial upregulation after exposure for 24 h to BC lixiviates, with a dose dependent trend. After 48 h, the response is attenuated and expression levels were similar to control and FR treatment groups, even with a tendency to downregulation for the highest concentration of BC coating in the cases of *fer* and *gst*. Interestingly, FR treatment caused a downregulation in all these three genes and levels were kept constant through time, showing a significant opposed response to that from BC coatings after 24 hours of exposure. As regards *cda* and *ach-r* transcription levels were downregulated after 24 h in BC exposed groups regardless concentration, but did not vary in FR exposed groups when compared to the control. As observed with the oxidative stress related genes, a compensatory response was observed after 48 h in *cda* and *ach-r*, being transcription levels similar to control and FR group levels, but showing a trend to upregulation in low BC concentration group (Figure 3; Table 5).

Table 5. Statistical outputs of differential gene expression analysis. Significant factors for each gene are marked with an asterisk (*). Analyses are divided in blocks according to the data distribution of each gene and the corresponding test performed: ANOVA [1] and Permutational ANOVA[2]. *DF*: degrees of freedom; *R SS*: RStudio sum of squares; *R mean sq*: RStudio mean sum of squares; *Iter*: iteractions; *Pr(Prob)*: p-value estimate.

	Ind. variable	**DF**	**R SS**	**R mean sq**	**F value**	**p-value**	
cda [1]	Treatment	3	18.52	6.17	5.058	0.00612	*
	Time	1	57.39	57.39	47.019	1.56e-07	*
	Treatm : Time	3	28.58	9.53	7.806	0.00057	*
	Residuals	29	35.40	1.22			
gst [1]	Treatment	3	10,461	3.487	5.426	0.00493	*
	Time	1	10.630	10.630	16.542	0.00039	*
	Treatm : Time	3	6.458	2.153	3.350	0.03427	*
	Residuals	26	16.708	0.643			
sod-II [1]	Treatment	3	5.830	1.9433	4.613	0.00931	*
	Time	1	1.766	1.7657	4.191	0.04980	*
	Treatm : Time	3	2.997	0.9989	2.371	0.09096	
	Residuals	29	12.218	0.4213			
	Ind. variable	**DF**	**R SS**	**R mean sq**	**Iter**	**Prob**	
ach-r [2]	Treatment	3	7.4112	2.4704	1e+06	0.02414	*
	Time	1	10.958	10.958	1e+06	0.00047	*
	Treatm : Time	3	8.2167	2.7389	1e+06	0.01552	*
	Residuals	24	15.708	0.6545			
hsp90 [2]	Treatment	3	2.8552	0.95175	1e+06	0.1113	
	Time	1	0.3215	0.32153	1e+06	0.3974	
	Treatm : Time	3	1.6225	0.54084	1e+06	0.3118	
	Residuals	29	12.626	0.43539			
fer [2]	Treatment	3	5.0535	1.68448	1e+06	0.01748	*
	Time	1	1.1375	1.13748	1e+06	0.11558	
	Treatm : Time	3	3.9320	1.32067	1e+06	0.04203	*
	Residuals	29	12.344	0.42565			

4. Discussion

The current study assessed, from an integrative biological multilevel approach, the responses of a model non-target species, the copepod *Acartia tonsa*, exposed to two types of antifouling (AF) coatings: biocide based (BC) and foul-release (FR). This work investigates the effects of coating lixiviates obtained from coated PVC plates at different biological levels: 1) toxicity screening and determination of lethal toxicity effects of the lixiviates and their main active compounds; 2) sub-lethal effects of the toxic coating on fecundity; and 3) molecular responses at transcriptional level of oxidative stress related genes (*gst*, *fer* and *sod-II*) and general stress (*hsp90*), as well as chitin metabolism (*cda*) and nervous system functioning (*ach-r*) related genes.

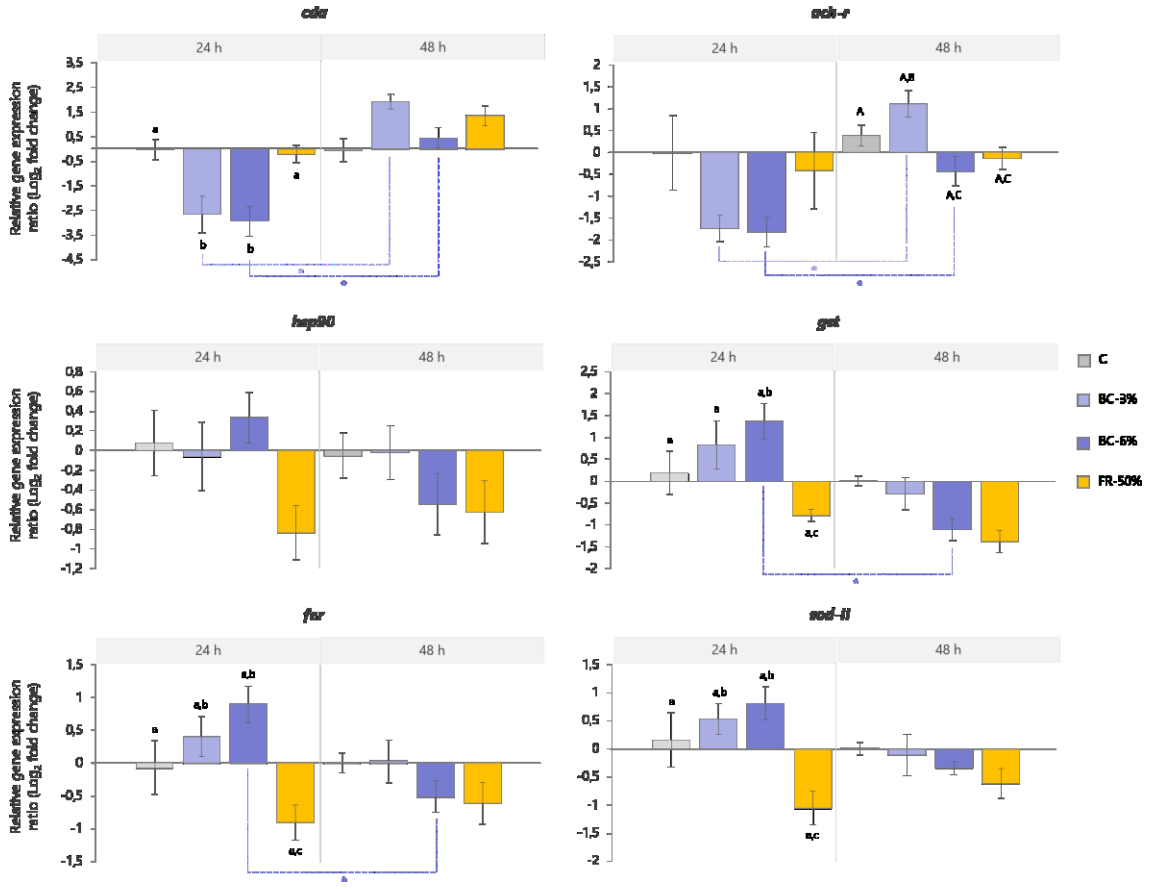


Figure 3. Relative gene transcription levels for each of the selected genes of interest (GOI) per treatment and exposure time, expressed as the mean Log2 fold change from reference genes. Colours indicate treatment (grey: control; powder blue: low BC lixiviate concentration; violet: high BC lixiviate concentration; and amber yellow: FR lixiviate). Error bars indicate standard error. Post-hoc tests were done independently for every gene and relevant comparisons were selected and illustrated as follows: equal letters group treatments with non-significant differences (lower case letters visualize differences across treatments after 24 hours of exposure, while caps are used to indicate differences after 48 hours of exposure). Asterisks (accompanied with dashed line grouping) indicate significant differences in transcription levels for specific treatments across exposure times.

The chemical composition of AF coating lixiviates differed greatly in terms of metal concentrations, particularly of Cu, Zn and Cr, as observed in chemical data from previous chapters and according to their formulation and action mechanism. While FR coating lixiviate did not cause mortality, BC coating lixiviate showed high mortality at relatively low lixiviate concentrations, being 12.9 % of lixiviate the established LC50 after 24 h. Remarkably, the LC50 of the BC lixiviate for *A. tonsa* was found to be much lower than those calculated through additional mortality assays for other target fouling organisms like the amphipod *Monocorophium insidiosum* (Crawford, 1937)

and the pediveliger larvae of the mussel *Mytilus galloprovincialis* Lamarck 1819 (LC50 after 24 h exposure of 77.9 % and 67.9 %, respectively, see section 1. Additional results of the Annex), suggesting certain degree of tolerance of some fouling species and adding to existing data (Piola & Johnston, 2006; Culver *et al.*, 2021), while raising concerns on the sensitivity of non-target species.

Active compounds of the coatings were tested individually in *A. tonsa* to elucidate the underlying toxicity of the coatings. Copper and Zn are the main active compounds of BC coatings and, as expected, Cu was the most toxic (Chapman & Riddle, 2005; Holan *et al.*, 2016). Mixture effects with both Cu and Zn were tested and the addition of Zn did not increase the toxicity of Cu alone in *A. tonsa*, suggesting that Zn did not booster the overall toxic effects. Zinc is commonly added as a binder and pigment (Yebra *et al.*, 2004), but also to broaden the toxicity spectrum and overcome the tolerance of copper seen in certain species (Paz-Villarraga *et al.*, 2022), a fact that was not observed for *A. tonsa* under the current experimental conditions. Interestingly, the LC50 calculated for the BC coating lixiviate contains an estimated concentration less than half of the determined LC50 for the Cu and Zn mixture or their individual test condition alone, suggesting that other paint components (mainly low molecular weight aromatic compounds, such as naphtha, ethylbenzene and xylene) contribute to the toxicity of the BC lixiviate (Paixão *et al.*, 2007; Nascimento *et al.*, 2020), other than just the active compounds (Karlsson *et al.*, 2010). However, little is known on the synergistic or additive effects that those chemicals included in coating formulations with non-biocidal purposes could have. Most studies focused on the toxicity assessment of the active compounds (mainly Cu), including co-biocides and their mixtures (Bellas, 2008; Wendt *et al.*, 2016; Jung *et al.*, 2017; Elia *et al.*, 2022), but leaving aside the potential effects of other components in the formulation. Our results add upon the importance of testing whole products instead of isolated active compounds (Karlsson *et al.*, 2010), and emphasises the need of investigating the effects of other compounds in the paint formulations.

AF coating lixiviates were used in subsequent exposure experiments with *A. tonsa* to assess sub-lethal effects on fecundity and gene transcription levels. Reproduction is a valuable endpoint in ecotoxicology due to its relevance at population level (Biandolino *et al.*, 2018). Prior studies have reported important reproductive effects of metals in copepods (Soroldoni *et al.*, 2017; Biandolino *et al.*, 2018; Hussain *et al.*, 2020). The current study shows that, although the total number of reproductive females did not change under BC lixiviate exposure in comparison to control, a decrease in the total number of eggs laid per reproductive female and whole treatment group was detected under BC lixiviate exposure. Despite the statistical limitations of the fecundity experiment, results show clear diminishing tendencies in the fecundity of exposed *A. tonsa*

females. These findings are consistent with previous results that reported reduced naupliar production per female of the copepod *Tigropus fulvus* exposed to copper (Biandolino *et al.*, 2018) and other reproductive endpoints, including egg production rate and hatching success (Hussain *et al.*, 2020). Our results raise concerns of potential impacts of BC coatings in non-target planktonic organisms such as copepods at population levels, particularly in areas of high boat density, were AF leaches could locally increase concentration of biocides such as Cu and Zn (Brooks & Waldock, 2009; Ytreberg *et al.*, 2010).

Molecular responses were also investigated with the aim of understanding how target pathways were affected by the exposure to BC and FR lixiviates. Exposure to external stressors, such as transition metals, may produce an imbalance in the levels of reactive oxygen species (ROS) and induce oxidative stress (Valavanidis *et al.*, 2006). Antioxidant defences of organisms prevent cellular damage caused by excessive concentration of ROS by regulating the synthesis and activity of certain antioxidant enzymes. Genes like *gst*, *fer* and *sod-II* are involved in detoxification processes, playing a key role in the antioxidant metabolism of animals, and have been proposed as target biomarkers of oxidative stress in *Acartia* (Hafez *et al.*, 2021; Lautitano *et al.*, 2021; Rotolo *et al.*, 2023). The results of the current study showed that *gst*, *fer* and *sod-II* had a unified response to BC lixiviate exposure, showing similar patterns at the transcription levels through time. An exposure of 24 h to BC lixiviate provoked a marked tendency of upregulation in their expression, followed by a compensation response after 48 h, with which transcription levels returned to control values. This quick upregulation response of *gst*, *fer* and *sod-II* is in agreement with similar effects in in antioxidant and oxyradical metabolism reported in copepods exposed to metals (Lee *et al.*, 2008a; Park *et al.*, 2019; Kadiene *et al.*, 2020). Contrarily, FR exposure caused a downregulation of the three oxidative stress genes studied, regardless exposure time, although, in this case too, remained as a marked tendency with no significant differences in oxyradical metabolism gene transcription levels if compared to controls. Yet, the opposed responses of BC and FR treatments lead to significant differences between them. Downregulation of oxidative stress related genes has been reported previously, suggesting that it could be associated to particularly stressful conditions or to compensate up-regulation of other genes or pathways (Rotolo *et al.*, 2024). However, in our study, there was no significant upregulation in any of the analysed target genes under FR exposure; although other pathways outside the scope of this work could be affected. Therefore, further investigation is required to elucidate the implications of FR exposure in this regard.

Additionally, effects of lixiviate exposure on other key pathways, such as chitin metabolism and nervous system functioning, were investigated in exposed copepods. Chitin deacetylases (CDAs,

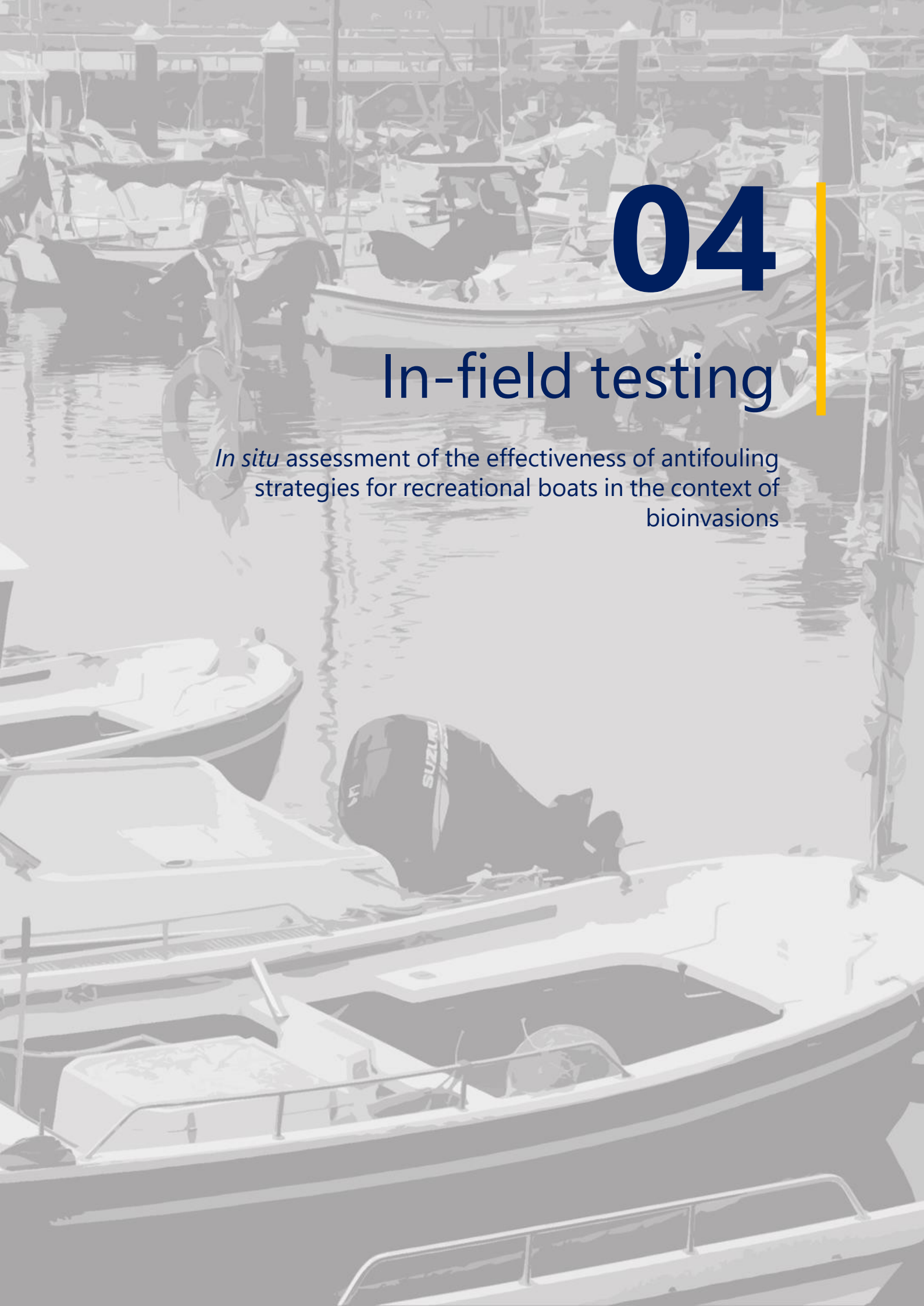
EC 3.5.1.41) are classified in the carbohydrate esterase family 4 (CE4) in the CAZY database (Carbohydrate Active Enzymes; Lombard *et al.*, 2014; Grifoll-Romero *et al.*, 2018). They are chitin-modifying metalloproteins widely distributed in microorganisms, fungi, plants and other chitin-producing organisms such as arthropods, in which CDAs play a fundamental role in many processes, including moulting, structuration and permeability of the cuticle (Li *et al.*, 2021). The results of our study evidence a strong downregulation of *cda* in *A. tonsa* after 24 h exposure to BC lixiviate. Downregulation of chitin deacetylases were also observed in the copepod *Tigriopus japonicus* after Cu exposure (Ki *et al.*, 2009), as well as in several development-related genes in the same species after exposure to manganese, including ecdysone receptors and various cuticle proteins (Kim *et al.*, 2013), while other authors have reported opposed responses (i.e. upregulation) for *cda* expression for *A. tonsa* after Ni exposure (Rotolo *et al.*, 2024). Yet, data from experiments on whole life cycle exposure to common contaminants, including copper, showed developmental effects, e.g. extension of naupliar phase in *T. japonicus* (Lee *et al.*, 2008b), providing additional evidences using a different approach from gene expression. These phenotypical developmental alterations could be further explained considering that metal ions are known to act as activators or inhibitors of enzyme activity and, in the case of CDAs, certain metals such as Zn^{2+} can act as catalyst at given concentrations or inhibitors if the threshold is trespassed. Other metals like Cu^{2+} or Ni^{2} can substantially inhibit the activity of these enzymes, although most of these specificities have been studied for fungi (Ghormade *et al.*, 2010; Grifoll-Romero *et al.*, 2018). Our results, together with those from the literature, highlight the necessity to better understand the role and functioning of CDAs in the moulting pathway of arthropods, particularly marine crustaceans, alongside the underlying regulation mechanism of the respective *cda*-s genes under metal pollution scenarios. This could eventually help to comprehend the impacts of BC coatings in the development of key crustacean species, such as planktonic copepods.

Acetylcholine receptors (AChR) are integral membrane proteins responding to acetylcholine neurotransmitter, mediating chemical neurotransmission at neuromuscular junctions and in the nervous system (Sarkar *et al.*, 2006). Their number and activity in post-synaptic cells determines, to some extent, the chemoreceptive mechanism and signal transmission and, therefore, these proteins are target of biocidal products (Ihara *et al.*, 2017), together with others from the pathway (e.g. acetylcholinesterase, AChE) (Lee *et al.*, 2015). The current study showed an initial marked downregulation of *ach-r* transcription levels after 24 h exposure to BC lixiviates, followed by a compensatory response after 48 h. Exposure to biocides or metallic compounds were reported to exert downregulation of AChR and AChE in copepods, suggesting potential effects in the nervous system of these crustaceans (Lee *et al.*, 2015; Rotolo *et al.*, 2024). Altogether, these results

contribute to the existing evidence of indirect effects of antifouling coatings in non-target species, like the planktonic copepod *A. tonsa*, unchaining sub-lethal effects at molecular level and potentially causing concerning effects.

5. Conclusion

The selected biological multilevel approach provided a wired overview of the range of effects caused by exposure to antifouling coatings in a non-target planktonic model species. The combination of toxicity screening with AF derived lixiviates and the individual active components of each coating evidenced excessive load of zinc and toxicity of other compounds in BC paint formulations, which shall be identified with further research and accounted in AF assessments. The results on fecundity highlight the need of deeper understanding of the effect of BC exposure on the reproduction capacity of these organisms with additional experimentation, including molecular endpoints. Differential gene transcription levels, indeed, unveiled effects of BC lixiviates on important physiological pathways, including the chitin pathways and nervous system functioning. These results deserve further attention and shall be complemented with other laboratory assays at different levels.



04

In-field testing

In situ assessment of the effectiveness of antifouling strategies for recreational boats in the context of bioinvasions

Journal publication:

Santos-Simón, M., Ferrario, J., Benaduce-Ortiz, B., Ortiz-Zarragoitia, M., & Marchini, A. (2024). Assessment of the effectiveness of antifouling solutions for recreational boats in the context of marine bioinvasions. *Marine Pollution Bulletin*, 200, 116108.

Additional publication, which includes data from this project:

Guerra-García, J. M., Revanales, T., Saenz-Arias, P., Navarro-Barranco, C., Ruiz-Velasco, S., Pastor-Montero, M., Sempere-Valverde, J., Chebaane, S., Vélez-Ruiz, A., Martínez-Laiz, G., Santos-Simón, M., ... & Ros, M. (2023). Quick spreading of the exotic amphipod Laticorophium baconi (Shoemaker, 1934): another small stowaway overlooked? *Mediterranean Marine Science*, 24, 644-665.

Congress contributions:

Santos-Simón, M., Ferrario, J., Benaduce-Ortiz, B., Ortiz-Zarragoitia, M., & Marchini, A. (2022). "Testing effectiveness of different antifouling and boat maintenance practices in the context of bioinvasions". *Marine Symposia on Marine Vegetation, Coralligenous, Dark Habitats and Non-Indigenous Species*. SPA/RAC, Genova, Italy.

Santos-Simón, M., Ferrario, J., Benaduce-Ortiz, B., Ortiz-Zarragoitia, M., & Marchini, A. (2022). "Assessment of the effectiveness of antifouling solutions for recreational boats in the context of marine bioinvasions". *2nd IMO-GloFouling R&D Forum on Biofouling Management and Prevention*. International Maritime Organization Headquarters, London, UK.

Santos-Simón, M., Ferrario, J., Benaduce-Ortiz, B., Ortiz-Zarragoitia, M., & Marchini, A. (2023). "Antifouling solutions for recreational boats: effectiveness and implications for fouling management" | "Recent detection of an old introduction: the case of *Laticorophium baconi* in the Ligurian Sea". *XI. International Conference on Marine Bioinvasions*. Baltimore, USA.

Abstract

The recreational boating sector is a major vector for the introduction of non-indigenous species (NIS) via biofouling. Despite applying control measures to prevent the growth of fouling communities, most vessels are NIS carriers. This study assessed the effectiveness of different antifouling strategies in a manipulative experiment by testing two common coating typologies (biocide-based and foul-release coatings), accompanied with simulated maintenance practices. The experiment was carried out in the Gulf of La Spezia (Italy) using recruitment plates, which were photographed monthly and samples collected at two different periods. Results showed significant differences among antifouling treatments regarding community structure, diversity, coverage and biovolume of the sessile component, alongside a significant decrease in the performance of biocide-based coating with time. Interestingly, peracarid NIS/native species ratio was higher for biocide-based treatments, suggesting potential biocide resistance. This study highlights the urgent need to develop common and feasible biofouling management plans and provides insights towards identification of best practices for recreational vessels.

Keywords | antifouling · biofouling · non-indigenous species · manipulative experiment · image analysis · bioinvasion management · recreational vessel.

Resumen

La náutica de recreo es uno de los principales vectores de introducción y dispersión de especies exóticas marinas a través del biofouling. A pesar de aplicar medidas para la prevención y el control del crecimiento de las comunidades de fouling, muchas de las embarcaciones son portadoras de especies exóticas. En este estudio se evalúa la efectividad de diferentes estrategias antifouling mediante un experimento manipulativo de campo, para lo que se seleccionaron dos pinturas antiincrustates (una a base de biocidas y otra alternativa), acompañadas de prácticas de mantenimiento simuladas. El experimento se condujo en el Golfo de La Spezia (Italia) empleando paneles para el crecimiento de bioincrustaciones. Dichos paneles se fotografiaron mensualmente y las muestras se recogieron en dos periodos diferentes. Los resultados indican diferencias significativas entre los distintos tratamientos antifouling en lo que respecta a la composición de la comunidad, la diversidad, la cobertura y volumen del componente sésil, junto con una disminución relevante de la funcionalidad en el tiempo del antiincrustante a base de biocidas. Destaca la notoria presencia de especies exóticas de crustáceos peracáridos en los tratamientos con patentes a base de biocidas, lo que sugiere una potencial resistencia a sus compuestos activos. Este estudio resalta la urgencia de desarrollar planes comunes de gestión del fouling y proporciona nuevas bases para la identificación de buenas prácticas aplicables a las embarcaciones de recreo.

Palabras clave | antiincrustante · bioincrustaciones · especies exóticas · experimento manipulativo · análisis de imagen · gestión de invasiones biológicas · embarcaciones de recreo.

Riassunto

Il settore della nautica da diporto è uno dei principali vettori per l'introduzione di specie non indigene via il biofouling. Nonostante l'applicazione di misure di controllo per prevenire la crescita di comunità incrostanti, la maggior parte delle navi sono portatori di specie alloctone. Questo studio ha valutato l'efficacia di diverse strategie antivegetative in un esperimento manipolativo testando due tipologie di rivestimento comuni (rivestimenti a base di biocidi e a rilascio di fouling), accompagnati da pratiche di mantenimento simulate. L'esperimento è stato effettuato nel Golfo di La Spezia (Italia) utilizzando pannelli come sustrato per la crescita del biofouling. I pannelli sono stati fotografati mensilmente e i campioni sono stati raccolti in due periodi diversi. I risultati mostrano differenze significative tra i trattamenti antivegetativi per quanto riguarda la struttura della comunità, la diversità, la copertura e il biovolume della componente sessile, oltre a una significativa diminuzione nel tempo delle funzionalità del rivestimento a base di biocidi. Inoltre, è notoria la elevata presenza relativa di peracaridi alloctoni nei trattamenti a base di biocidi, suggerendo una potenziale resistenza biocida. Questo studio evidenzia l'urgente necessità di sviluppare piani comuni e fattibili di gestione delle biofouling e fornisce informazioni sull'individuazione delle migliori pratiche per le navi da diporto.

Parole chiave | antivegetativo · biofouling · especie non-indigene · esperimento manipolativo · analisi di immagine · gestione delle bioinvasioni · imbarcazioni a scopo ricreativo.

Laburpena

Aisialdirako nautika inkrustazio biologikoen bidez itsas espezie exotikoak sartzeko eta barreiatzeko bektore nagusietako bat da. Inkrustazio biologikoen aurkako neurriak aplikatu arren, ontzi askok espezie exotikoak daramatzate. Ikerketa honetan, anti-inkrustazio estrategia ezberdinen eraginkortasuna ebaluatzen da egoera errealak antzeratuz manipulazio-esperimentu baten bitartez. Horretarako, bi anti-inkrustazio gainestaldura (AF) aukeratu ziren (bat biozidetan oinarritutakoa, BC; bestea alternatiboa, FR), mantentze-praktika simulatuekin batera. Esperimentua La Speziako Golkoan (Italia) egin zen, panelak erabiliz bioinkrustazioak hazteko substratu gisa. Hilero, panelen argazkiak atera ziren eta, azkenik, laginak bi aldi ezberdinetan jaso ziren. Emaitzek AF tratamendu desberdinen arteko alde nabarmenak adierazten dituzte komunitatearen osaerari, aniztasunari, estaldurari eta osagai sesilaren bolumenari dagokionez. Hala, BC gainestalduraren funtzionalitatea murrizketa garrantzitsua jasaten du denboran zehar. Interesgarria da BC gainestaldurekin tratatutako unitate esperimentalek erakusten duten perakaridoen espezie exotikoen presentzia altua, haien konposatu aktiboekiko erresistentzia potentziala iradokitzen duena. Azterketa honek inkrustazio biologikoak kudeatzeko plan komunak garatzearen premia nabarmentzen du eta aisialdiko ontziei aplika daitezkeen praktika onak identifikatzeko oinarri berriak ematen ditu.

Gako-hitzak | anti-inkrustazio · inkrustazio biologikoa · espezie exotikoa · manipulazio-esperimentua · irudi analisia · inbasio biologikoen kudeaketa · aisialdirako itsasontziak.

1. Introduction

The unwanted settlement and growth of organisms on artificial hard substrates partially or totally exposed to aquatic environments, namely biofouling, is a major issue for vessel navigation performance and artificial marine infrastructures, as well as for global biosecurity (Yebra *et al.*, 2004; Dafforn *et al.*, 2011; Schultz *et al.*, 2011; Davidson *et al.*, 2016). In fact, because of the increased drag in fouled surfaces, higher fuel consumption, hydrodynamic resistance and higher risk of structural damage are expected, leading to reduced operational efficiency and significantly increased costs (Schultz *et al.*, 2011). Besides, ships' fouling, together with ballast water, is considered a major human-mediated vector for marine bioinvasions at global level (Hewitt *et al.*, 2009; Georgiades *et al.*, 2020; Ros *et al.*, 2023).

In particular, recreational boats have been identified as one of the largest unregulated vectors of introduction and spread of non-indigenous species (NIS) (Clarke-Murray *et al.*, 2011; Ferrario *et al.*, 2017; Ashton *et al.*, 2022) and, thus, a target for global biosecurity (IMO, 2022). Recreational boating is, indeed, mainly responsible for secondary spread of NIS and offers frequent opportunities for transfers and high connectivity between locations, including areas of conservation and special interest (Ulman *et al.*, 2019; Ashton *et al.*, 2022). Despite the role of boats in the invasion dynamics, biosecurity measures are mostly focused on commercial vessels and ballast waters (Bailey, 2015; Drake *et al.*, 2021), while biofouling of recreational boats is mostly based on recommendations and good practices that rely on self-management (IMO, 2012; GEF-UNDP-IMO GloFouling Partnerships, 2022).

Overall, effective antifouling measures (AFM) to control fouling development usually require combining chemical, physical and even cultural approaches to maximize their performance (Wezenbeek *et al.*, 2018; Xie *et al.*, 2019; Culver *et al.*, 2021).

Chemical approaches consist in the application of an antifouling coating (AF), whose action mechanism vary according to the coating category (Dafforn *et al.*, 2011; Wezenbeek *et al.*, 2018). They contain active compounds, such as enzymes or biocides, which hamper recruitment and/or affect early survival of foulers. On the other hand, physical approaches include not only a wide variety of cleaning devices and maintenance practices, but also coatings that alter surface properties and reduce the attachment strength, for instance, foul-release (FR) coatings (Xie *et al.*, 2019). Yet, biocide-based (BC) is the most widespread coating typology and, currently, copper represents the most common active compound in combination with booster co-biocides (Jones & Bolam, 2007; Ytreberg *et al.*, 2010). However, there is still uncertainty regarding their actual performance (Culver *et al.*, 2021) and potential indirect effects on the environment (Amara *et al.*,

2018; de Campos *et al.*, 2021). Moreover, some target species have shown resistance to the biocides (Floer *et al.*, 2004; Piola & Johnston, 2006), potentially favouring their spread. Hence, alternatives that dodge the limitations of BC coatings are being explored, like the above-mentioned FR coatings, which have reached the market and are becoming a valuable alternative to the traditional BC coatings. Finally, cultural tactics integrate aspects of boat maintenance, such as planning and timing, adapting the frequencies of different maintenance activities (Culver *et al.*, 2021), presence of supporting infrastructure for correct waste management, regulations and awareness, among others.

Different combinations of those approaches, however, can negatively alter the performance of selected AFM and increase environmental risks. For example, in-water cleaning of hulls can release propagules and viable fragments of biofouling taxa (Hopkins & Forrest, 2008; Kim *et al.*, 2023), as well as favour the leaching of coating material (Schiff *et al.*, 2004; Turner, 2010; Ralston & Swain, 2023) or antifouling paint particles (APPs), that keep acting as a source of biocides (Almeida *et al.*, 2023). Thus, investigating the performance of different strategies is essential for a good biofouling management plan (Davidson *et al.*, 2016).

The Mediterranean Sea is the second most visited destination worldwide for nautical and recreational tourism (Cappato, 2011; Ramieri *et al.*, 2022). Previous studies in this region have shown that most boaters regularly apply antifouling measures with, at least, a yearly coating and cleaning frequency, out of which 50% did so through professional services (Ferrario *et al.*, 2016; Martínez-Laiz et al., 2019; Ulman *et al.*, 2019). In addition to this, several Mediterranean boaters reported to carry out periodical manual cleaning during the boating season; these are usually carried out in-water with soft sponges, while the boat is anchored offshore. However, 71% of the nearly 600 boat hulls surveyed by Ulman et al. (2019) across the Mediterranean hosted at least one NIS.

Altogether, we can reasonably hypothesize that the commonly recommended antifouling strategies, even if regularly followed, may be insufficient in the Mediterranean context. This study addresses this point by experimentally assessing the effectiveness of different antifouling strategies, based on commonly adopted boaters' practices. In particular, we apply and evaluate the performance of two selected antifouling coating typologies complemented with manual cleaning. The research aims to better understand the dynamics of fouling communities and their response to antifouling treatments, with a particular limelight on NIS recruitment, that serve as a baseline for future management plans applicable to the recreational boating sector.

2. Materials and methods

2.1 Study site

The experiment was conducted in the Gulf of La Spezia, located in the eastern side of the Ligurian coast, North-West Mediterranean Sea (Italy) (Figure 1). It has a semi-enclosed configuration given by the presence of a dam that protects the inner Gulf area (Gasparini *et al.*, 2009). It hosts different urban settlements and many anthropogenic activities, including the presence of different industrial facilities, such as a big commercial and touristic harbour, a military base, several marinas, an electric power-plant and aquaculture installations for both finfish and shellfish (Gasparini *et al.*, 2009). In particular, Fezzano and Le Grazie, located in the western part of the Gulf (Figure 1), were selected for the study, based on the results of a previous NIS monitoring in the area (Tamburini *et al.*, 2021).

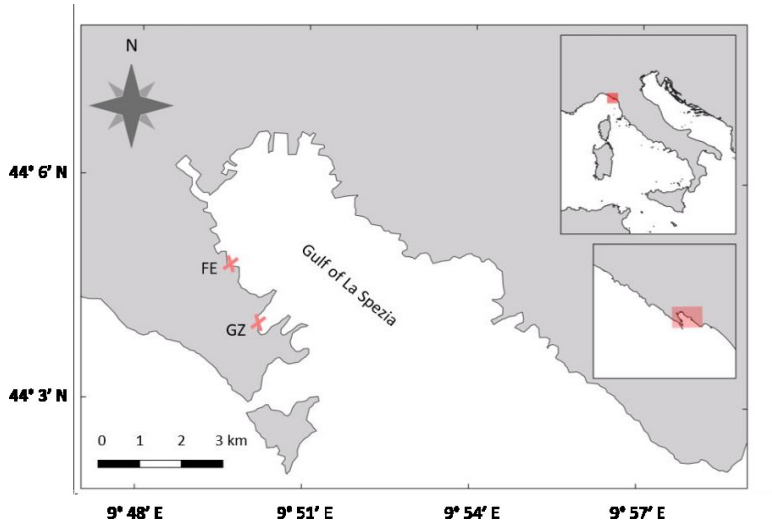


Figure 1. Overview of the study area and location of sampling sites, the marinas of Fezzano (FE) and Le Grazie (GZ).

2.2 Experimental design and sampling method

The effects of coating typology, maintenance practices and seasonality were examined, adapting the protocol developed by the Smithsonian Environmental Research Center for the purpose of the research (Marraffini *et al.*, 2017; Tamburini *et al.*, 2021). In total, 48 PVC plates with a dimension of 14 x 14 cm were sanded, coated if required, and attached to a brick, ensuring downward orientation of the coated surface (section 3. Supportive material of the Annex), and deployed randomly at 1 m depth in floating pontoons or concrete docks in the selected marinas in May 2021. The effect of coating typology (factor "coating": three levels, including uncoated controls, referred to as C) was tested on two commercially available paints: a) a traditional biocide-based coating (BC) and b) a foul-release coating (FR) (specifications in Table 1 and 2, I. Chemical

characterization). Two sampling periods (factor "season": two levels) were established, a first one immediately after the high boating season (T1: August 2021) and a second one at the end of the low season (T2: February 2022), before the preparations for the upcoming high season. Throughout the duration of the experiment, half of the plates were cleaned periodically (factor "maintenance": two levels) by means of a soft sponge, simulating the common behaviour of Mediterranean boaters (monthly during T1; once every 2 months during T2). The remaining half was left untouched (no maintenance, NM; maintenance, M). The combination of factors (coating and maintenance) resulted in six antifouling strategies (C+NM, C+M, BC+NM, BC+M, FR+NM, FR+M) that were tested for two different submersion periods (T1, T2), by deploying 4 plates per treatment and time (Figure 2). Plates were photographed monthly before any treatment-related manipulation.

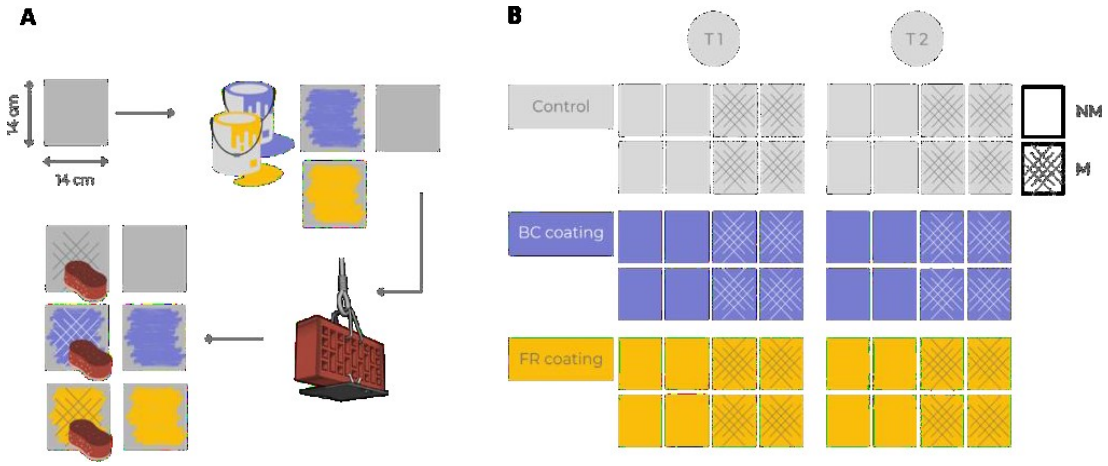


Figure 2. A) Experimental procedure showing the coating, deployment and maintenance steps. B) Overview of the experimental design showing the combination of factors that result in different antifouling strategies. Colours indicate coating typology: control, biocide-based coating (BC) and foul-release coating (FR). Maintenance practices are indicated by the pattern (plain/patterned): uncleaned (NM) and cleaned (M) plates. Plates were sampled at two different periods (T1 and T2) and, in total, there were six AF strategies, with 4 plates per period each.

2.3 Biological sample analysis

Plates were retrieved and stored in zip-bags containing 70% ethanol and preserved in the lab until their analysis. By means of a stereoscope (WILD Heerbrugg), fouling communities were morphologically analysed. For sessile assemblages, percentage cover of each the sessile species was assessed with a manual point count method, using a 7 x 7 point grid plus an extra random point (Chang *et al.*, 2018). Although the adopted protocol is specifically designed for sessile fouling species, in this study we decided to also identify the associated mobile organisms. These

were retrieved for specimen identification and direct counting of the individuals with a particular focus on peracarid crustaceans, due to the high occurrence of NIS within this taxon (Martínez-Laiz *et al.*, 2019). Finally, the total biovolume of the sessile assemblages alone (excluding the mobile component) was determined by measuring the volume difference after adding the sample to an initial known volume (results included in the section 1. Additional results of the Annex).

Both sessile and mobile components were identified to lowest taxonomic level possible, their relative abundances noted and classified according to their potential origin (native, non-indigenous or cryptogenic; Chapman & Carlton, 1991), supported by available literature.

2.4 Image analysis

Monthly photographs were analysed in terms of coverage with the software CPCe (Coral Point Count with Excel extensions) (Kohler & Gill, 2006), using a uniform gridded layout design of 49 points equally distributed in the plate are of the image (see Figure S16 of the section 3. Supportive material of the Annex). This design, for resolution purposes, resembled the point-count method described in the previous section. All images were digitally analysed by assigning a value of `cover`, `bare` or `slime` to each point and data was then used to calculate the percentage coverage of each plate per month, allowing to visualize its evolution through time. Only the values designed as `cover` were used for the calculation.

2.5 Statistical analyses

Statistical analyses were conducted using RStudio (`version 4.2.2`; R Core Team, 2022) and its following packages: `vegan` (Oksanen *et al.*, 2022), `car` (Fox & Weisberg, 2019), `rcompanion` (Mangiafico, 2023), `indicspecies` (Cáceres & Legendre, 2009), lmPerm (Wheeler & Torchiano, 2016) and `ggplot2` (Wickham, 2016).

Differences in community composition based on the treatment type were tested using a multivariate approach with Permutational Analysis of Variance (PERMANOVA) (Anderson, 2001) and supported with the Analysis of Similarities (ANOSIM) (Clarke, 1993), both based on a dissimilarity matrix (Bray & Curtis, 1957). Furthermore, dispersion within treatment groups was checked with PERMDISP (Anderson, 2006). For the PERMANOVA, the function `adonis2` of the `vegan` package was used, applying the permutation test under the reduced model following a sequential addition of terms and 9999 free permutations. Non-metric Multidimensional Scaling (nMDS) was used as a representation of the pairwise distances among treatments and the species contributions and their association with specific treatments were determined by the Indicator Species Analysis, using the function `multipatt` of the package `indicspecies`.

Differences among treatments in univariate response variables such as coverage, diversity and NIS/native abundance ratio were tested with univariate analysis. Prior to the test, normality and homogeneity of variances were checked with Levene's (Levene, 1960) and Shapiro tests (Shapiro & Wilk, 1965), respectively. Whenever the assumptions were met, ANOVA (Chambers & Hastie, 1992) was performed, otherwise, permutational ANOVA (Wheeler & Torchiano, 2016) was applied. Both multivariate and univariate analyses were done with untransformed data.

3. Results

We retrieved 44 out of the 48 deployed PVC plates all four lost plates corresponding to the T2 sampling period. Biofouling organisms were classified into 129 taxa, out of which 76 were identified to species level, considering the sessile and mobile components of the community. Full species lists can be found in Table S2 and Table S3 in the first section (1. Additional results) of the Annex for sessile and mobile components, respectively.

3.1 Sessile community

Throughout the whole experiment, 59 sessile taxa had colonised the plates, out of which 47 were already present in uncoated plates during the first 3 months of submersion. In total, nine NIS species were identified (*Paraleucilla magna* Klautau, Monteiro & Borojevic, 2004; *Branchiomma luctuosum* (Grube, 1870); *Hydroides dirampha* Mörch, 1863; *Hydroides elegans* Haswell (1883); *Amathia verticillata* (delle Chiaje, 1822); *Celleporaria brunnea* (Hincks, 1884); *Tricellaria inopinata* d'Hondt & Occhipinti Ambrogi, 1985; *Watersipora arcuata* Banta, 1969; *Styela plicata* (Lesueur, 1823)) and 37 native. Bryozoans and annelids represented the most abundant macrogroups of the sessile assemblages for the entire duration of the experiment (average abundance of bryozoans and annelids in control plates: 36.88 ± 4.19 % and 34.38 ± 3.68 %, respectively during T1; 21.53 ± 1.38 % and 33.68 ± 4.94 %, respectively during T2), while tunicates gained presence with time. However, the specific composition of those assemblages changed significantly with time, coating type and their interaction (p-values < 0.05; Table 1; Figure 3).

Location (Fezzano vs Le Grazie) did not result a significant factor affecting the sessile community composition (p-value > 0.05), thus, it was excluded in the subsequent tests, as the communities of the two marinas can be considered highly similar (see also Tamburini *et al.*, 2021). In fact, coating emerges as one of the main factors shaping the community composition, clearly clustering together samples of BC coated plates apart from any other coating treatment during T1, regardless maintenance (Figure 3). However, the dissimilarity pattern becomes blurrier with time and BC's communities become more similar to untreated and FR-treated plates (Figure 3).

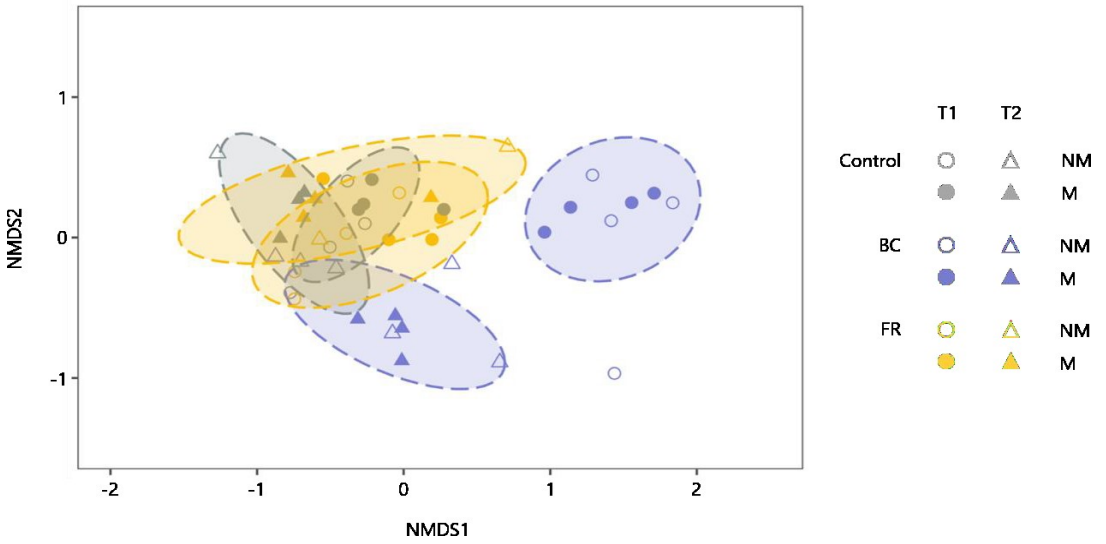


Figure 3. nMDS of the sessile communities for T1 (dots) and T2 (triangles). Colours represent the coating factor: grey = control (C), violet = biocide-based coating (BC) and amber yellow = foul-release coating (FR). Shape filling corresponds to maintenance practices: empty = unmaintained (NM); filled = maintained

Table 1. Statistical summary of PERMANOVA test on community composition of both the sessile and mobile components. Significant factors (p-value < 0.05) are marked in bold and an asterisk.

	Ind. variable	DF	R SS	R2	F value	p-value (>F)	
Sessile component	Coating	2	2.3366	0.20839	7.0453	**0.0001**	*
	Maintenance	1	0.3098	0.2763	1.8680	0.6606	
	Time	2	1.0838	0.09666	6.5359	**0.0001**	*
	Coating : Time	1	1.2734	0.11357	3.8395	**0.0001**	*
	Coating : Maint	2	0.2981	0.02658	0.8987	0.5596	
	Maint : Time	1	0.2727	0.02432	1.6446	0.0977	
	Coating : Maint :Time	2	0.3316	0.02957	0.9998	0.4312	
	Residuals	32	5.3065	0.47327			
Mobile component	Coating	2	0.8748	0.05754	1.5988	**0.0438**	*
	Maintenance	1	0.4408	0.02900	1.6114	0.0871	
	Time	1	2.0488	0.13477	7.4891	**0.0001**	*
	Location	1	0.6524	0.04291	2.3847	**0.0109**	*
	Coating : Maint	2	0.5091	0.03349	0.9305	0.5439	
	Coating : Time	2	0.6053	0.03982	1.1064	0.3205	
	Coating : Loc	2	0.3633	0.02390	0.6640	0.8930	
	Maint : Time	1	0.6380	0.04197	2.3321	**0.0110**	*
	Maint : Loc	1	0.6293	0.04140	2.3005	**0.0123**	*
	Time : Loc	1	0.7349	0.04834	2.6862	**0.0058**	*
	Coating : Maint : Time	2	0.5594	0.03680	1.0224	0.4251	
	Coating : Maint : Loc	2	0.4838	0.03183	0.8843	0.6155	
	Coating : Time : Loc	2	0.4767	0.03136	0.8712	0.6359	
	Maint : Time : Loc	1	0.2883	0.01897	1.0538	0.3817	
	Coating: Maint : Time : Loc	2	0.1517	0.00998	0.5547	0.9052	
	Residuals	21	5.7450	0.37792			

The Indicator Species Analysis (Table 2) identifies during T1 the bryozoan *Amathia gracilis* (Leidy, 1855) as the species most strongly related with BC coating, while the bryozoan *Schizoporella errata* (Waters, 1878) is the most related to FR coated plates. However, for T2, the most related species to BC coating changed (Table 2), being of particular interest the encrusting bryozoan *Watersipora subtorquata* (d'Orbigny, 1852).

Table 2. Statistical summary of the Indicator Species Analysis of the sessile communities with coating as grouping factor for both T1 and T2 with a significance level of 0.05.

Remarkable species per coating treatment	**T1**		**T2**	
	Statistic	**p-value**	**Statistic**	**p-value**
Group C (n° spp = 8)				
Anomia ephippium Linnaeus, 1758	0.785	0.0002	0.519	0.0339
Branchiomma luctuosum	-	-	0.394	0.0008
Bugula neritina (Linnaeus, 1758)	-	-	0.671	0.0031
Ciona sp.	-	-	0.578	0.051
Cradoscrupocellaria bertholletii (Audouin, 1826)	0.502	0.0457	-	-
Crisia denticulata (Lamarck, 1816)	0.472	0.0064	-	-
Savignyella lafontii (Audouin, 1826)	0.451	0.0141	0.599	0.0075
Simplaria sp.	-	-	0.679	0.0055
Group BC (n° spp = 2)				
Amathia gracilis (Leidy, 1855)	0.411	0.0078	-	-
Watersipora subtorquata (d'Orbigny, 1852)	-	-	0.75	0.0009
Group FR (n taxa = 2)				
Actiniidae	0.784	0.0007	-	-
Schizoporella errata (Waters, 1878)	0.540	0.023	-	-

Results on plate surface coverage (Figure 4 and 5) and recruited biomass' volume (see section 1. Additional results of the Annex) support previous differences among treatments, BC coated plates being, irrespective of maintenance, significantly less fouled (lower percentage cover and biomass values) during T1 than the other two coating treatments (p-values < 0.05; Table 3). However, biofouling coverage in BC treatments considerably increases with time, while under FR treatments of unmaintained plates a decrease there is an observable decrease (Figure 4 and 5), although the loss of some plates increase variability and blur this effect. Diversity seems unaffected by both coating and time, maintenance being the only significant factor (p-value = 0.0383; Table 3; Figure 4), which tends to increase the diversity in cleaned plates. The serpulid *H. elegans* and the bryozoans *C. brunnea* and *T. inopinata* were the most frequent NIS found in the samples and *W. subtorquata* the most common cryptogenic species present. While differences in the overall sessile composition of communities were shaped by the coating typology, permutational ANOVA on NIS/native ratios as response variable determined maintenance as the factor driving differences

among treatments (p-value = 0.03351; Table 3), being NIS presence higher in uncleaned plates, despite coating factor (Figure 4). However, this effect dissipates with time.

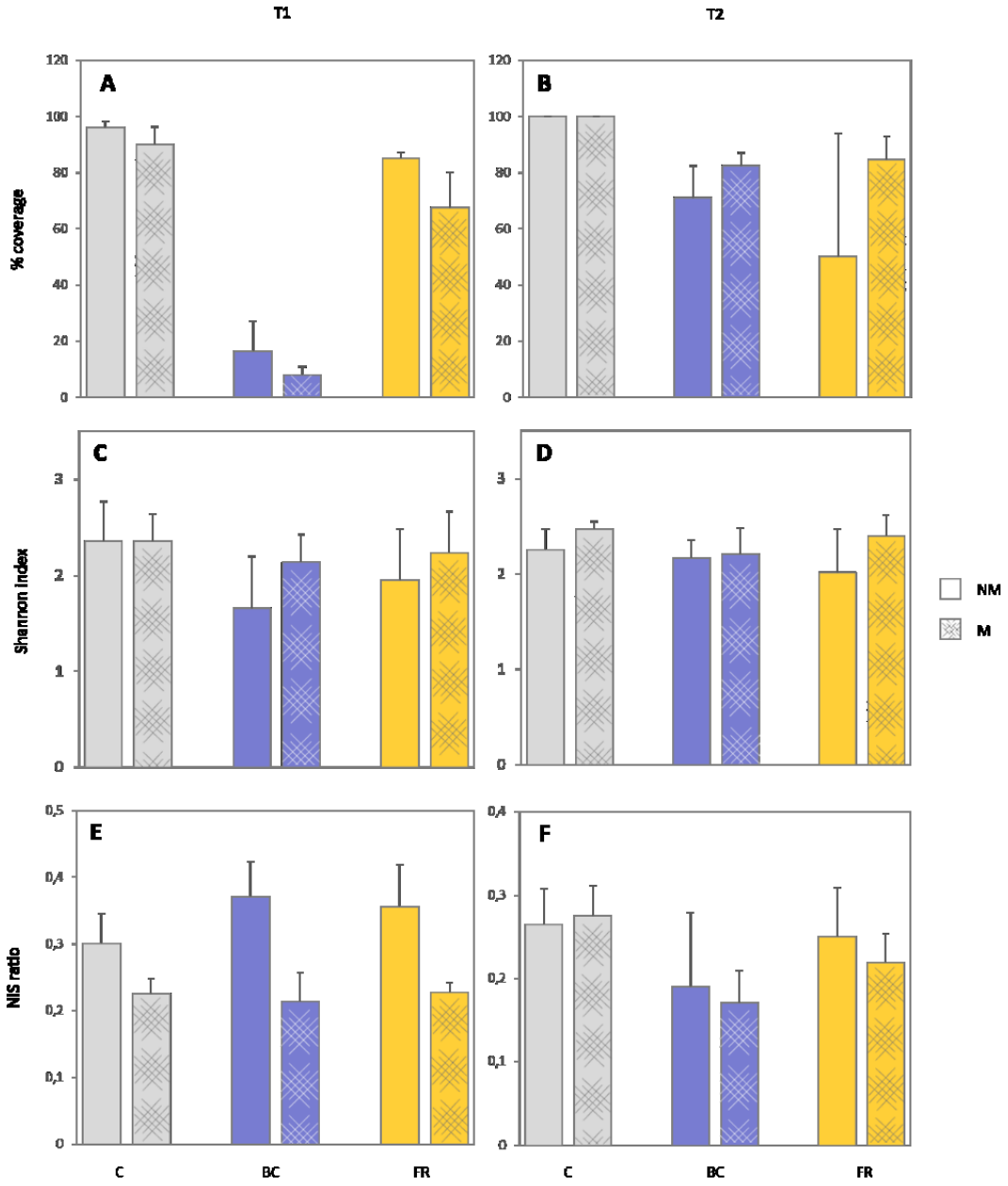


Figure 4. Coverage (A, B), Shannon diversity index (C, D) and NIS/native ratio (E, F) of sessile communities per treatment and time (left = T1; right = T2). Colours represent the coating factor: grey = control (C), violet = biocide-based coating (BC) and amber yellow = foul-release coating (FR). Bar pattern corresponds to maintenance practices: plain = unmaintained (NM); patterned = maintained (M). Variability is represented by the standard error. Summary of the statistical tests available in Table 3.

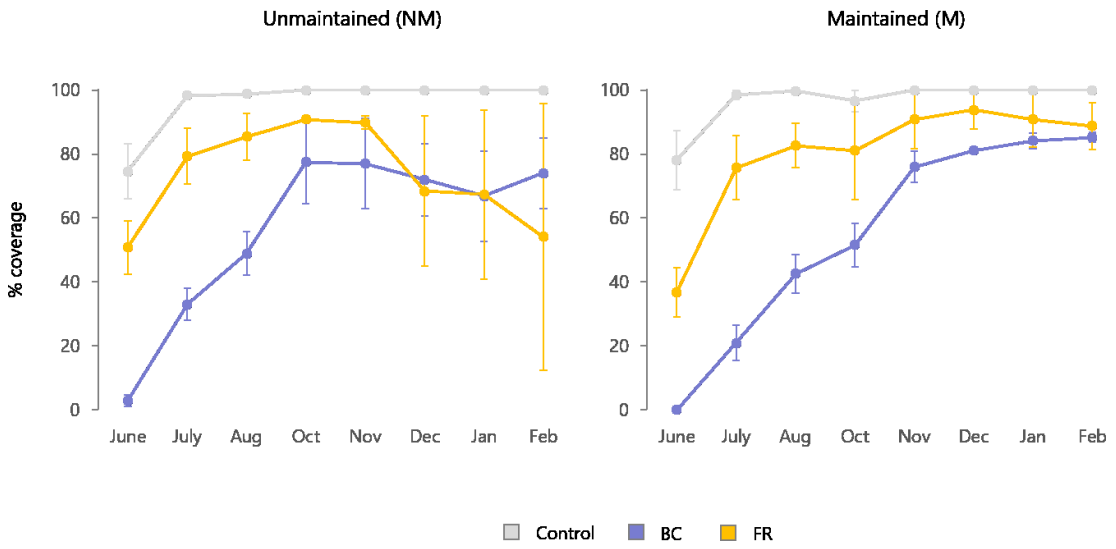


Figure 5. Monthly evolution of plate coverage per treatment. The graph on the left represents unmaintained (NM) plates and the one on the right maintained (M) plates. Colours indicate the coating factor: grey = control (C), violet = biocide-based coating (BC) and amber yellow = foul-release coating (FR). Note that data for September is missing.

3.2 Mobile community

Regarding the mobile component, Figure 6 describes qualitatively the contribution of the major macrogroups, peracarids being the most abundant taxon across samples, accounting for at least the 85 % of the individuals during T1, although their relative abundances decreased with time. In fact, there are clear differences among mobile assemblages for T1 and T2 (p-value = 0.0001, Table 1), with a lower dominance of peracarids and clear shift towards more heterogeneous communities with time (Figure 6). In mobile assemblages, coating is a significant factor (p-value = 0.0439; Table 1), however, its effect is less evident than in sessile assemblages, and maintenance does not seem to have an impact. For longer submersion periods, there are no evident effects of any of the treatments (Figure 7). However, location seems to be an important factor for the mobile communities (p-value = 0.0109; Table 1).

Analysing in deeper the mobile component, 33 species were identified in the peracarid community, out of which 8 were categorized as NIS, including *Jassa slatteryi* Conlan, 1990; *Caprella scaura* Templeton & Mills, 1836; *Stenothoe georgiana* Bynum & Fox, 1977; *Grandidierella japonica* Stephensen, 1938; *Paranthura japonica* Richardson, 1909; *Paracerceis sculpta* (Holmes, 1904); *Mesanthura romulea* Poore & Lew Ton, 1986 and *Laticorophium baconi* (Shoemaker, 1934). Besides, four species were categorized as cryptogenic: *Ericthonius brasiliensis* (Dana, 1853); *Ericthonius puntatus* (Bate, 1857); *Elasmopus rapax* Costa, 1853; *Caprella equilibra* Say, 1818 (see

Marchini & Cardeccia, 2017 for their classification as cryptogenic). The relative abundances of these species categories are descriptively illustrated in Figure 8 according to treatments and time, being particularly remarkable the high proportion of NIS species in BC coated plates during T1.

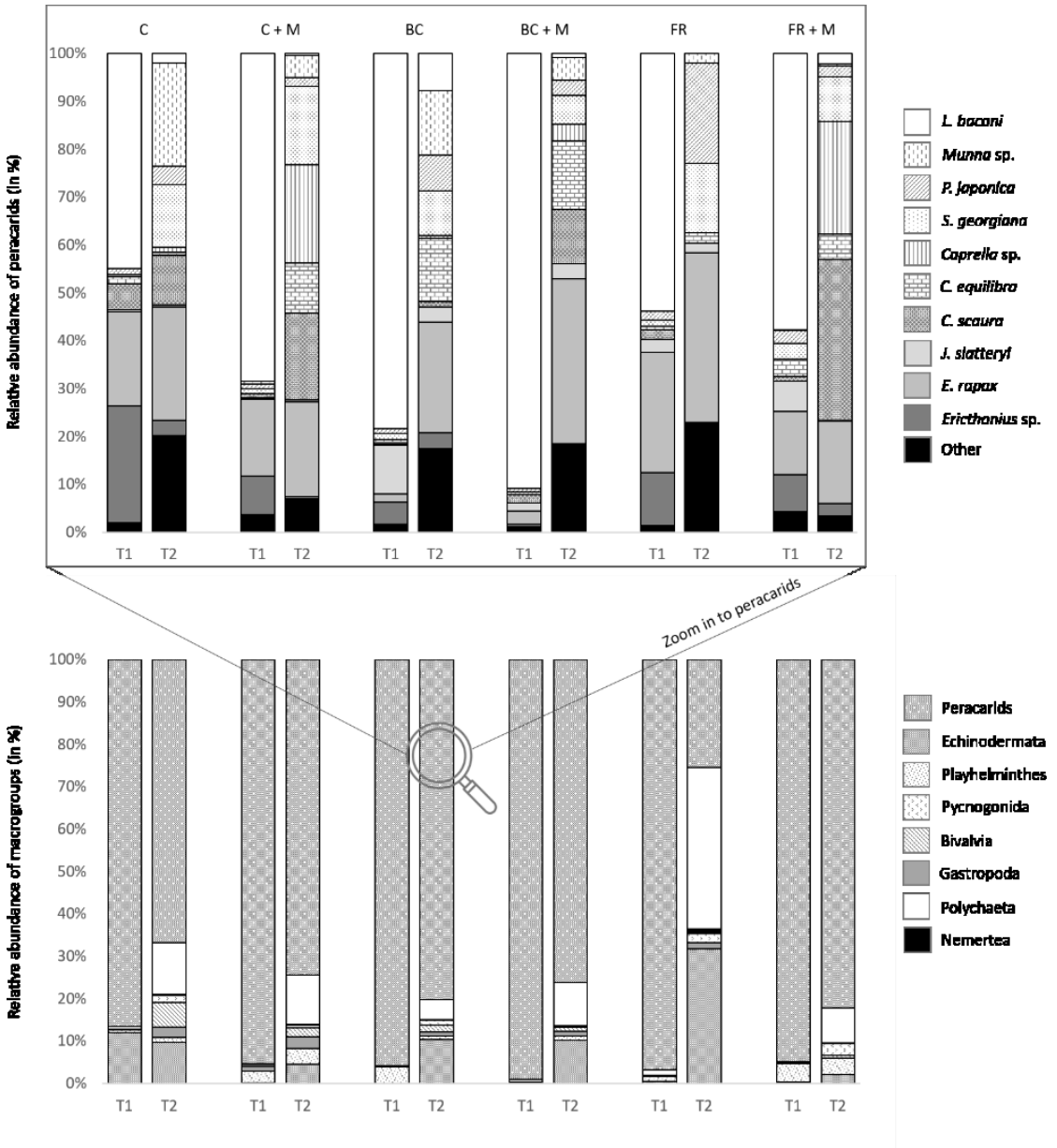


Figure 6. Descriptive stacked bar chart of relative abundances of the mobile taxa categorized in macrogroups (left) and of peracarid species of the fouling community per treatment type and time. Peracarid species with relative abundances inferior to 10 % are considered as a unique group, namely, 'Other'.

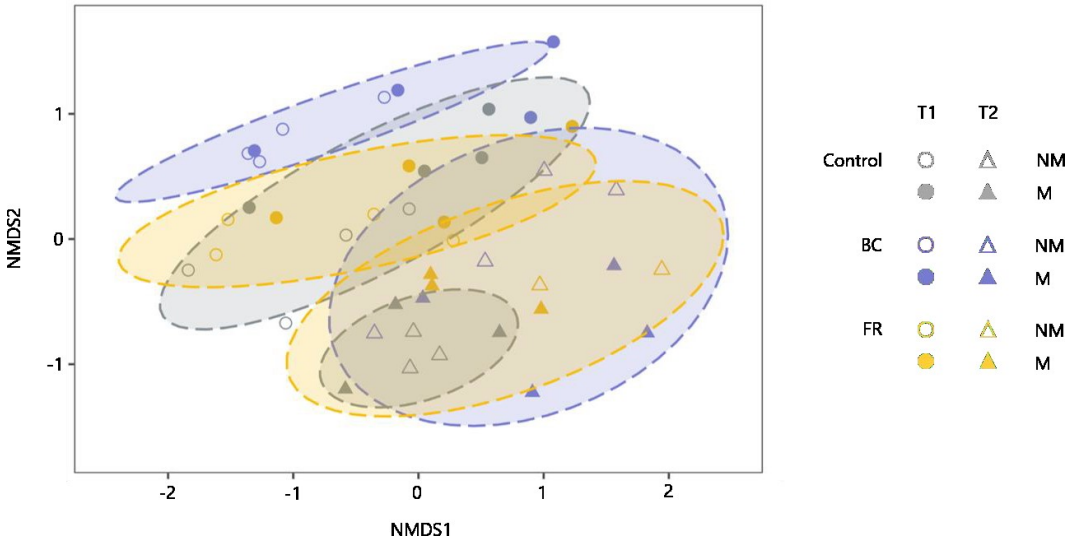


Figure 7. nMDS of the mobile communities for T1 (dots) and T2 (triangles). Colours represent the coating factor: grey = control (C), violet = biocide-based coating (BC) and amber yellow = foul-release coating (FR). Shape filling corresponds to maintenance practices: empty = unmaintained. (NM); filled = maintained (M).

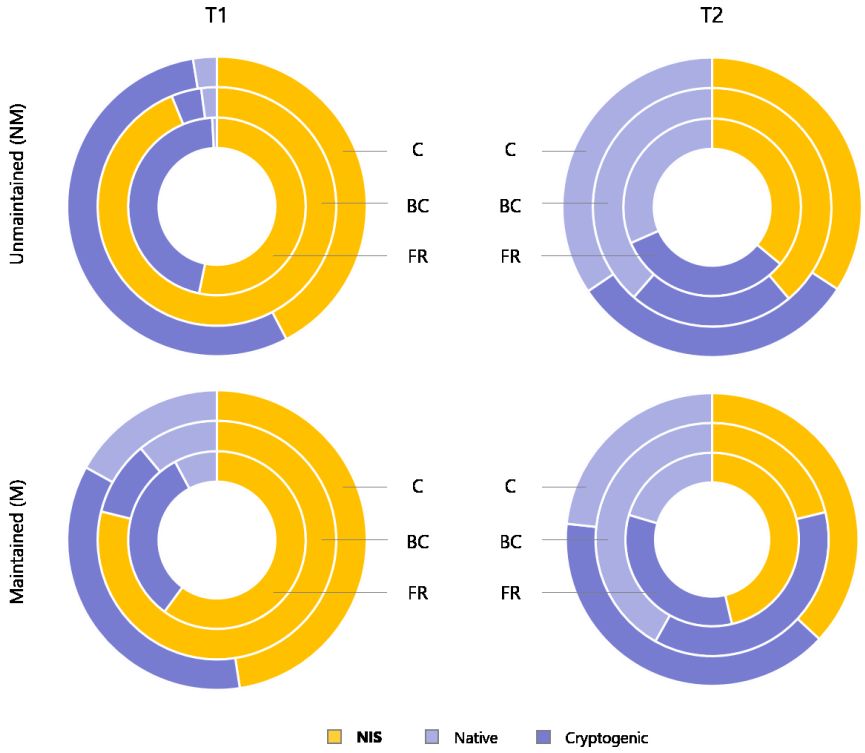


Figure 8. Relative abundances of peracarid NIS (yellow), native (powder blue) and cryptogenic (darker blue) species per treatment and time (left = T1; right = T2).

In fact, coating, time and their interaction have significant effects on NIS/native peracarid ratio (p-values < 0.05; Table 3), intending 'native' from a conservative approach that includes cryptogenic species within the category. During T1 this is significantly higher for BC coated samples, if compared to any other coating treatment, regardless maintenance (Figure 9). *Laticorophium baconi* represents most of the NIS countings across all treatments, but densities remain particularly high under BC treated plates (Figure 6). This effect disappears with time, with no significant differences for T2 among treatments. The dominance and remarkable abundances of *L. baconi* in BC samples is supported also by the Shannon Index values of the peracarid communities (Figure 9), significantly lower than the rest of the treatments during T1 (Table 3), indicating lower richness and a high relative abundance of that species.

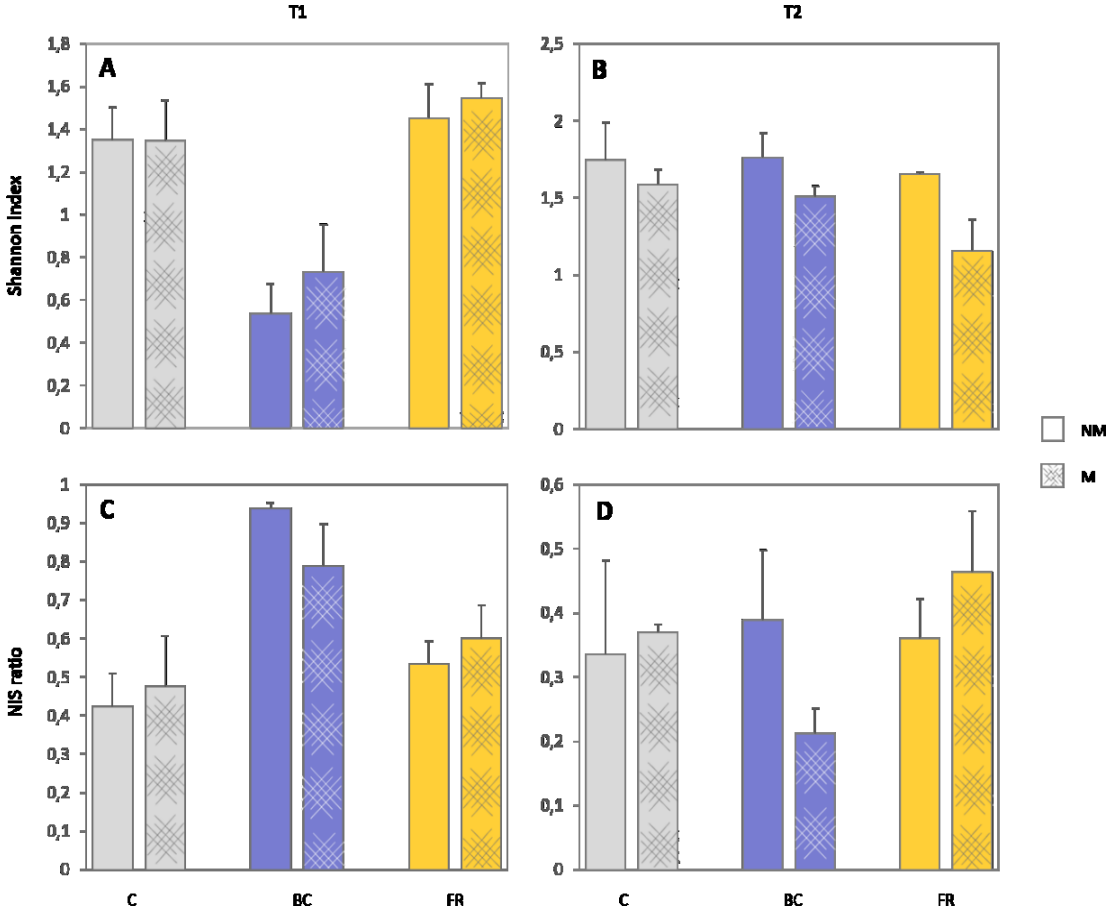


Figure 9. Shannon diversity index (A, B) and NIS/native ratio (C, D) of peracarid communities per treatment and time (left = T1; right = T2). Colours represent the coating factor: grey = control (C), violet = biocide-based coating (BC) and amber yellow = foul-release coating (FR). Bar pattern corresponds to maintenance practices: plain = unmaintained (NM); patterned = maintained (M). Variability is represented by the standard error. Summary of the statistical tests available in Table 3.

Table 3. Statistical outputs of the analysis of univariate responses, for both sessile and crustacean communities. Significant tests ($p < 0.05$) are marked in bold. The test is indicated for each

		Sessile community					Peracarid community			
	Ind. variable	**DF**	**R SS**	**R mean sq**	**F value**	**p-value**	**R SS**	**R mean sq**	**Iter**	**p-value**
Diversity (Shannon Index	Coating	2	0.743	0.3717	2.912	0.0689	1.2175	0.60874	1e+06	**0.00895**
	Maintenance	1	0.596	0.5958	4.667	**0.0383**	0.1147	0.11472	1e+06	0.31469
	Coating : Maint	2	0.251	0.2505	1.962	0.1709	0.0548	0.02739	1e+06	0.78432
	Time	1	0.101	0.0506	0.396	0.6761	1.7590	1.75904	1e+06	**0.00033**
	Coating : Time	2	0.147	0.0734	0.575	0.5683	2.2054	1.10269	1e+06	**0.00043**
	Maint : Time	1	0.012	0.0118	0.092	0.7634	0.4228	0.42277	1e+06	0.06152
	Coating : Maint : Time	2	0.228	0.1140	0.893	0.4193	0.0829	0.04145	1e+06	0.69152
	Residuals	32	4.085	0.1277			3.5439	0.11075		
	Statistical test					ANOVA				Permutational ANOVA
	Ind. variable	**DF**	**R SS**	**R mean sq**	**Iter**	**p-value**	**R SS**	**R mean sq**	**F value**	**p-value**
NIS ratio	Coating	2	0.0082	0.00410	1e+06	0.6512	0.2271	0.1135	3.804	**0.0330**
	Maintenance	1	0.0467	0.04668	1e+06	**0.0335**	0.0085	0.0085	0.284	0.5975
	Coating : Maint	2	0.0063	0.00317	1e+06	0.7155	0.8551	08551	28.649	**7.1e-06**
	Time	1	0.0316	0.03125	1e+06	0.0780	0.1479	0.0740	2.478	0.0999
	Coating : Time	2	0.0261	0.01307	1e+06	0.2659	0.4886	0.2443	8.185	**0.0014**
	Maint : Time	1	0.0299	0.02994	1e+06	0.0852	0.0001	0.0001	0.002	0.9609
	Coating : Maint : Time	2	0.0014	0.00070	1e+06	0.9293	0.0020	0.0010	0.033	0.9676
	Residuals	32	0.3034	0.0095			09551	0.0298		
	Statistical test					Permutational ANOVA				ANOVA
	Ind. variable	**DF**	**R SS**	**R mean sq**	**Iter**	**p-value**				
Coverage	Coating	2	20062.5	10031.2	1e+06	**2.2e-16**				
	Maintenance	1	57.4	57.4	1e+06	0.6786				
	Coating : Maint	2	221.7	110.8	1e+06	0.7191				
	Time	1	4573.2	4573.2	1e+06	**0.0008**				
	Coating : Time	2	11036.3	5518.2	1e+06	**2.9e-05**				
	Maint : Time	1	1780.7	1780.7	1e+06	**0.02767**				
	Coating : Maint : Time	2	917.6	458.8	1e+06	0.26487				
	Residuals	32	10580	330.6						
	Statistical test					Permutational ANOVA				

4. Discussion

The present study assessed the performance of different AF strategies that combined chemical and physical factors differently, aiming to elucidate their individual contribution to fouling management. Overall, BC coating performance decreased in a relatively short period, with an efficiency time lesser than half of what stated by the manufacturer. FR coating showed low efficiency under stationary conditions, performing similarly to uncoated plates. These findings confirm what previously reported in other studies (Davidson *et al.*, 2020; Culver *et al.*, 2021; Lagerström *et al.*, 2022). Regarding maintenance, it was only responsible of controlling particular components of the fouling, with little overall control of the total fouling biomass, as discussed more in detail below.

4.1 Sessile community

The high similarity among communities observed in the two investigated sites is easily explainable by the geographical proximity of the two marinas, promoting high connectivity of free-swimming larval stages of fouling taxa.

Results pointed out coating type as the main responsible of differences in sessile community composition and coverage among the different AF strategies. Both image and laboratory analyses allowed to understand the effects of coating in biofouling recruitment, providing complementary information, although with slight differences, mostly for T1 samples, due to resolution and point positioning. Biocide-based coating exhibits the greatest efficiency in the short-term; however, its performance considerably decreases with time, as clearly evidenced by the results on coverage and community composition (see also Culver *et al.*, 2021). Regarding FR coatings, they perform similarly to control plates, without significantly reducing total recruitment in terms of coverage and biovolume. Samples treated with FR exhibited a notable variability that can be explained by the spontaneous detachment of biofouling due to its weight (Figure S14 and Figure S15, section 3. Supportive material of the Annex), which should be more evident under dynamic conditions (Davidson *et al.*, 2020). Actually, untouched FR plates ended up containing lower biovolume values than those periodically cleaned (Figure S4, section 1. Additional results of the Annex), since the later ones did not accumulate enough biomass for self-release to happen. Yet, the strong variability observed among FR samples could also be responsible of blurring the effects of the coating typology, especially with time.

Dissimilarities in the assemblages of BC plates support the stated differences among treatments and suggest coating-specific composition of fouling communities. In fact, the Indicator Species Analysis greatly relates the presence of *W. subtorquata* to BC coated plates, implying some degree of resistance that favours colonization (Piola & Johnson, 2006) and, what is more, a facilitator effect as it acts as a foundation for other species to settle (Floerl *et al.*, 2004). Supportive material of this phenomenon (Figure S14. E) can be found in the third section (3. Supportive material) of the Annex.

As regards sessile NIS, coating fails to control their presence, being NIS/native ratios similar across coating treatments. In fact, NIS like *H. elegans* and *T. inopinata,* as well as the cryptogenic *W. subtorquata,* have shown tolerance to copper (Piola & Johnston, 2006; Piola & Johnston, 2008; Dafforn *et al.*, 2011). Cleaning with a soft sponge, instead, does reduce the NIS presence during the first 3 months, regardless of coating, but does not reduce NIS in the long term. Abrasive methods could be more effective against cementing and encrusting organisms, like the main

sessile NIS found in our plates (*H. elegans*, *T. inopinata* and *C. brunnea*) or the crytogenic *W. subtorquata*, but are less feasible to be conducted on a frequent basis. However, other studies have reported that cleared surfaces attract more organisms (Ralston & Swain, 2023) and, particularly, NIS, showing a positive correlation between disturbance and susceptibility to invasions (Altman & Whitlatch, 2007; McQuaid & Arenas, 2009). According to the intermediate disturbance hypothesis (Connell, 1978), more diverse fouling communities could also be expected on cleaned plates. Interestingly, our results show, indeed, a tendency to more diverse sessile communities in maintained plates (tendency to higher Shannon Indexes), however, in this case, the NIS/native species ratios were lower, contrasting with the previously mentioned susceptibility to invasions on disturbed surfaces.

Although maintained BC plates did not show greater decrease in their performance, as expected due to potential coating damage and faster loss of active compounds, interactions between maintenance and coating performance still need to be clarified.

4.2 Mobile community

Contrarily to what observed for the sessile component, the associated mobile communities significantly differed between locations. Prior studies (Martínez-Laiz *et al.*, 2019; Saenz-Arias *et al.*, 2022) have repeatedly shown high beta-diversity in peracarid assemblages, often linked to local-scale variability in environmental characteristics (Kenworthy *et al.*, 2018). Besides, peracarids, indeed, lack pelagic larvae and instead present extended parental care (Thiel, 2003), resulting in low dispersion capacity and gregarious life-style. Some of them, such as corophiids and ischyrocerids, even lead a semi-sessile life (Moore & Eastman, 2015), contributing to prodigious population densities locally (Thiel, 2003; Moore & Eastman, 2015).

Statistical results highlight that coating type significantly affects mobile community composition; and together with time, it is the main factor driving differences among communities. In fact, mobile species in fouling communities strongly depend on the presence of three-dimensional complex structures that host them (Martínez-Laiz *et al.*, 2022; Tempesti *et al.*, 2022), which increases with time and explains the difference in the composition of the communities (Vicente *et al.*, 2021). Although manual removal of the coverage could affect recruitment of vagile organisms and lead to the differences across treatments, maintenance does not seem to have an impact on the community composition of these assemblages.

Among the mobile organisms, peracarids were the most abundant group and *L. baconi* a remarkable species, due to the considerable densities and the significance of its finding. *Laticorophium baconi* was first described in the coast of California (Shoemaker, 1934), in the

northeast Pacific Ocean, its likely native range, and considered exotic in the coast of East Asia, Australia and Atlantic Ocean (Hirayama, 1986; Valerio-Berardo & De Souza, 2009; Ahyon & Wilkens, 2011). *Laticorophium baconi* has recently been reported in European Mediterranean waters (Gouillieux & Sauriau, 2019), although its occurrence could date back earlier, unreported due to misidentification with *Apocorophium acutum* (Chevreux, 1908). Its presence has also been documented in other European coasts, Western Australia and New Caledonia (Guerra-Garcia *et al*., 2023).

This study shows the impressive colonization capacity of *L. baconi* on bare surfaces: it resulted the most abundant species in T1 plates, with notable abundances in BC coated plates, which are of particular concern, as they correspond to the high season of boating and thus, the period of greatest mobility and connectivity between locations (Ulman *et al*., 2019). These great abundances are reflected in the NIS/native ratio and Shannon Index values of the different treatments, which reflect a low diversity and a NIS/native ratio near one, implying the dominance of *L. baconi* in BC freshly coated plates. In all treatments, the presence of *L. baconi* decreases with time, with the development of the fouling communities and arrivals of other species, coinciding with low season and winter period. These results, together with those from *W. subtorquata*, add to the pre-existing reports on NIS tolerance to biocides, particularly to copper (Floerl *et al*., 2004; Piola *et al*., 2006; Culvier *et al*., 2021), which could not be effective enough in limiting their dispersion.

4.3 Challenges and future perspectives

Many factors contribute to fouling dynamics and, when it comes to its prevention, additional factors, such as the strategies followed, need to be included in the equation. Establishing clear links among abiotic factors in a certain context, boat type, including travel habits, and antifouling efficiency, is key for effective biofouling management (Acosta *et al*., 2010; Parretti *et al*., 2020). Furthermore, certain environmental parameters, such as salinity or temperature (Singh & Turner, 2009; Lagerström *et al*., 2020) are known to affect biocide-leaching rate and, thus, determine coating performance and suitability of paint selection. Additionally, cleaning practices and tools can also play a role in determining coating functionality (Oliveira & Granhag, 2020). Although in-water cleaning can be effective in reducing the overall biofouling coverage, unintended side effects that pose biosecurity risks and environmental hazards might happen: a) some species may persist (Davidson *et al*., 2008); b) viable propagules or complete individuals may be released; c) spawning could be triggered; and d) increased biocide discharge and diminished coating performance could be expected (Morrisey *et al*., 2013; Kim *et al*., 2023).

The interaction among all these factors contributes to uncertainty on the efficiency of different biofouling management approaches and their combination. In warm seas, like the Mediterranean, where temperatures are favourable in extended periods for biofouling growth, and salinity levels are relatively higher, antifouling performance could be compromised (Kiil *et al.*, 2001; Dobretsov, 2009). Indeed, our study suggest that the widely used BC coatings have lower performance than expected under the current experimental conditions. Coatings that rely on surface alterations rather than chemical leaching could be a potential alternative.

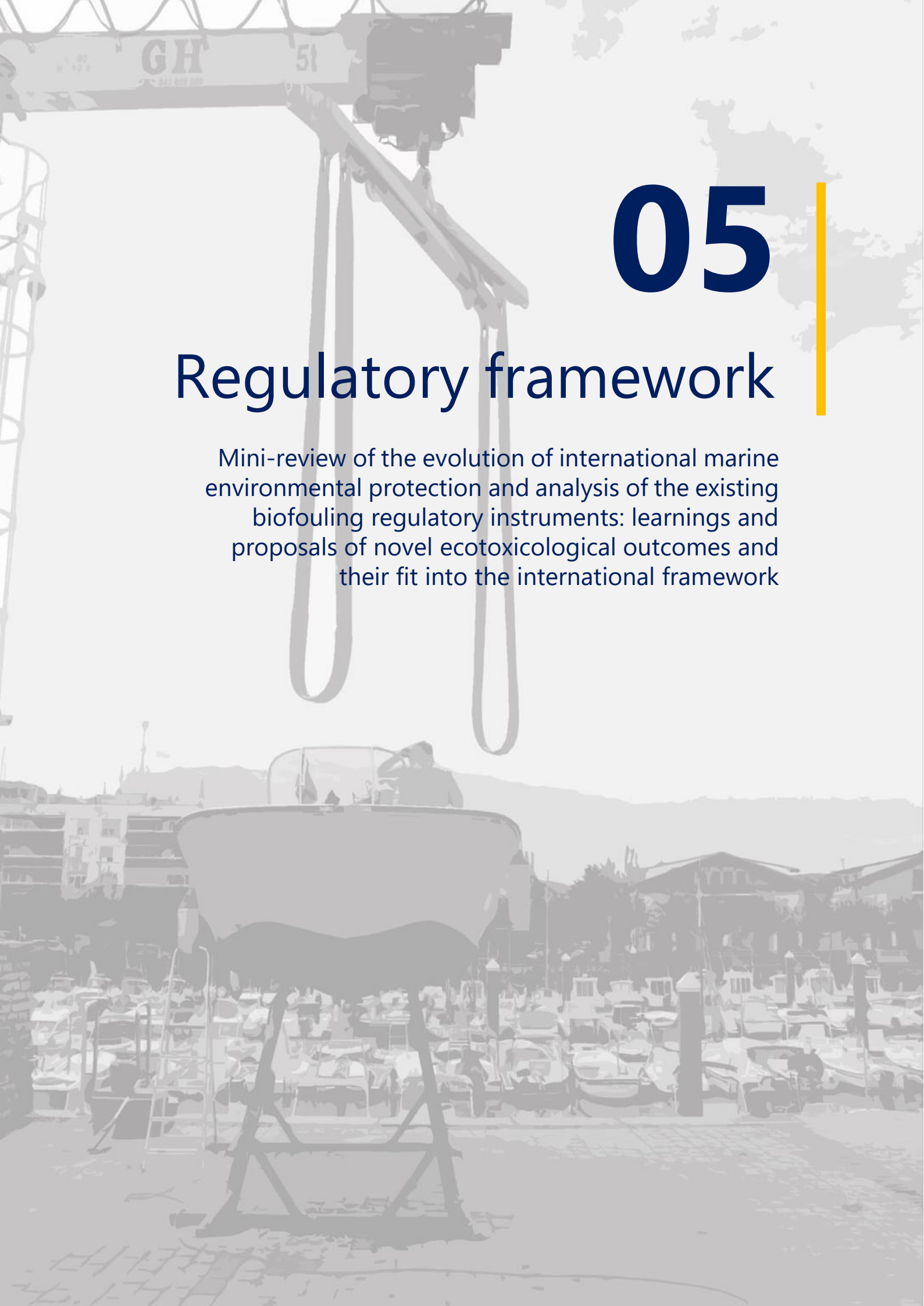
Likewise, the duration of stationary lay-up periods are a determining factor for the development of fouling communities and, thus, a limitation for the optimal performance of AF coatings. While coatings are designed to perform best under movement, lay-ups are common and unavoidable in the sector of recreational boating; hence, understanding their implications for biofouling management is essential to develop workable post-lay-up approaches (Davidson *et al.*, 2020). The results of this study reflect fouling dynamics under different treatments in motionless conditions, which is an important factor to consider that limits the outcome to idle period simulation. Overall, understanding the complex interactions involved in biofouling management is crucial to develop effective control plans, biosecurity standards, risk assessment procedures and realistic regulations, in accordance to the regional context (Sylvester *et al.*, 2011).

Despite the evidences of fouling from recreational vessels as an important vector for the spread of NIS (Ferrario *et al.*, 2017; Ashton *et al.*, 2022), there is a lack of a regulatory framework specifically addressing the issue and establishing clear fouling management protocols. Boating, yachting and other forms of maritime tourism constitute an important sector (Cappato *et al.*, 2011), particularly in the Mediterranean, and many efforts of sustainable development focus on it (Ramieri *et al.*, 2022). However, their role in the spread of NIS has been overlooked to the extent that biofouling management within this sector relies on guidelines and good practices (MEPC, 2011; MEPC, 2023). Additionally, the wide commercial offer of solutions with different approaches, the lack of standards and knowledge on the factors influencing fouling dynamics, remarkably contribute to the haziness of the problem and leave the responsibility to boat owners.

5. Conclusions

The tested antifouling strategies are insufficient to control biofouling development under the current experimental conditions. Coatings performed as expected in the short term, during summer season. However, after longer idle-periods, the performance of BC coatings dropped significantly, without reaching half of its service life. It is of particular concern the potential tolerance of certain species, which puts at risks biosecurity strategies. Thus, there is an urgent

need for efficient antifouling technologies and development of management policies that tackle the issue of biofouling from an integrative approach (economic, ecological, and cultural perspectives), considering the factors influencing the effectiveness of AF strategies, with a special focus on recreational boats, implementation feasibility and assessment standards.



05

Regulatory framework

Mini-review of the evolution of international marine environmental protection and analysis of the existing biofouling regulatory instruments: learnings and proposals of novel ecotoxicological outcomes and their fit into the international framework

Abstract

Marine biofouling is undeniably a relevant issue for most sectors within the blue economy. Its development is associated with remarkable impacts for the involved industries and is linked with major environmental concerns. Although national biofouling regulations have been issued by few countries in the recent past, these remain isolated initiatives. At a global scale, there is currently no international legally binding framework on biofouling, despite it undeniably represents a bottomless pit of costs and risks. This chapter focuses on the evolution of international marine environmental protection legislation and analyses existing regulatory instruments linked to the matter, with particular emphasis on the few regional in-force regulations on biofouling, accompanied by examples of their enforcement. Lastly, the main gaps and challenges for the development of a regulatory framework on biofouling are identified and listed, accompanied with the major learnings and proposals derived from the experimental outcomes of the previous chapters of this work.

Keywords | biofouling · antifouling · legislation · implementation · guidelines · IMO

Resumen

El desarrollo de incrustaciones biológicas (bioincrustaciones) marinas es sin duda un problema relevante para la mayoría de los sectores de la economía azul. Su desarrollo está asociado a impactos cuanto menos significativos para las industrias involucradas y está vinculado a importantes riesgos ambientales. Recientemente algunos países han promulgado normativas nacionales sobre bioincrustaciones, sin embargo, éstas siguen siendo iniciativas aisladas. En una escala global, actualmente no existe un marco legal internacional vinculante sobre las bioincrustaciones con normativas y medidas específicas, a pesar de ser un indudable pozo sin fondo de costes y riesgos. Este capítulo se centra en la evolución de la legislación internacional de protección del medio marino y analiza los instrumentos regulatorios existentes vinculados a la materia, con especial énfasis en los excepcionales casos regionales con regulaciones vigentes sobre dichas bioincrustaciones, acompañados de ejemplos de su implementación. Por último, se identifican y enumeran las principales brechas y desafíos para el desarrollo de un marco regulatorio sobre bioincrustaciones, respaldados por los principales aprendizajes y propuestas derivadas de los resultados experimentales de los capítulos anteriores de este trabajo.

Palabras clave | bioincrustaciones · antiincrustante · legislación · implementación · directrices · OMI

Riassunto

Lo sviluppo del biofouling marino è, senza dubbio, un problema rilevante per la maggior parte dei settori dell'economia blu. Il suo sviluppo è associato ad impatti quantomeno significativi per le industrie coinvolte ed è legato a importanti rischi ambientali. Nel recente passato in alcuni Paesi è stato approvato un regolamento nazionale sul biofouling, ma rimangono pochi casi isolati. A scala globale, attualmente non esiste un quadro giuridico internazionale vincolante sul biofouling, con normative e misure al riguardo, nonostante questo problema abbia dimostrato di avere innegabili costi e rischi. Questo capitolo si concentra sull'evoluzione della legislazione internazionale per la protezione dell'ambiente marino e analizza gli strumenti normativi esistenti legati alla materia, con particolare attenzione ai casi regionali eccezionali con normative attuali sul biofouling, accompagnati da esempi della loro implementazione. Infine, vengono identificate ed elencate le principali lacune e sfide per lo sviluppo di un quadro normativo sul biofouling, supportate dai principali insegnamenti e proposte derivati dai risultati sperimentali dei capitoli precedenti di questo lavoro.

Parole chiave | biofouling · antivegetativo · legislazione · implementazione · linee guida · OMI

Laburpena

Zalantzarik gabe, inkrustazio biologikoen (biofouling-aren) garapena arazo garrantzitsua da ekonomia urdineko sektore gehienentzat. Inplikatuta dauden industrietan, haren garapena inpaktu esanguratsuekin lotuta dago, baita ingurumen-arrisku garrantzitsuekin ere. Duela gutxi zenbait herrialdek inkrustazio biologikoen inguruko araudi nazionalak ezarri dituzte; hala ere, horrelako ekimenek isolatuak izaten jarraitzen dute. Mundu mailan, gaur egun ez dago inkrustazio biologikoei buruzko nazioarteko lege-esparru loteslerik, araudi eta neurri zehatzekin, kostu eta arrisku-jario galanta bada ere. Kapitulu honek itsas ingurunea babesteko nazioarteko legediaren bilakaeran zentratzen da eta gai horri lotuta dauden arau-tresnak aztertzen ditu, indarrean dauden inkrustazio biologikoei buruzko eskualde-araudien salbuespenetan arreta jarriz, horien ezarpenaren adibideekin batera. Azkenik, inkrustazio biologikoen inguruko arau-esparru bat garatzeko hutsune eta erronka nagusiak identifikatu eta zerrendatzen dira, lan honen aurreko kapituluetako emaitza esperimentaletatik eratorritako ikaskuntza eta proposamen nagusiek lagunduta.

Gako-hitzak | inkrustazio biologikoa · anti-inkrustazio · legedia · inplementazioa · gidalerroak · NIE

1. Introduction

Marine biofouling is undeniably a relevant issue for most sectors within the blue economy. Its development is associated with remarkable impacts for the involved industries and is linked with major environmental concerns.

1.1 Marine biofouling in numbers: economic costs

Biofouling is a global and cross-sectorial issue that accounts for millions of euros annually. If we focus on maintenance derived expenses, at a time, it was estimated that US Navy costs tied to biofouling for hull cleaning, paint removal and repainting, toxic water and grit disposal, OSHA health requirements, labour associated with corrective measures and other maintenance measures were approximately 100-200 million US$ a year (Alberte *et al*., 1992). For the Cantabrian fishing fleet, the economic impact estimation of biofouling represents approximately the 3 % of the intermediate consumption of the ship with respect to its fish production, and almost 40 % of the costs for spare parts, repair and maintenance of the ship (Trueba-Castañeda *et al*., 2021). This authors calculated the total average annual costs of maintenance of the underwater hull to be 9 220 € per ship and 1 244 700 € for the total fishing fleet of Cantabria. In oil and gas industry, the cost to manually clean these platforms of accumulated organisms is approximately 30 000 – 100 000 US$ per cleaning cycle (Page *et al*., 2009). The amount increases remarkably when the platforms reach their final stage and need to be decommissioned, with cost estimates for cleaning them ranging from 50 000 US$ for the smallest platforms (Category I, one well, shallow water) to over US$100 million (Category V deep water multi-well) (National Research Council, 1985; Page *et al*., 2009). Finally, although it is difficult to determine the exact cost associated to biofouling in the aquaculture sector, estimates indicate that between 5-10 % in industry value is spent in dealing with fouling related issues every year. This typically accounts for 20–30 % of total operating costs and can be translated in 260 million € annually only for Europe (Lane & Willemsen, 2004; Dürr & Watson, 2009). In particular, for the sector of marine salmon aquaculture, the cost per farm site and production cycle ranged between 420 000 – 493 600 US$ (Bloecher & Floer, 2020).

Additional costs on fuel consumption, which are directly related with fouling development and surface roughness, can be included in the equation in cases of mobile elements such as vessels. According to the study by Schultz *et al*. (2011), heavy slime, considered a level typical of the Arleigh Burke-class destroyers (DDG-51, representative vessel of the US Navy), can increase fuel consumption by 10.3 % and fuel costs by approximately 1.15M US$ per ship per year. Similarly, the IMO calculated an increase in 25 % of fuel consumption and GHGs emissions in a bulk carrier

with 0.5 mm thick biofilm covering 50 % of the submerged surface (GEF-UNDP-IMO GloFouling Partnerships Project & GIA for Marine Biosafety, 2022).

The introduction of non-indigenous species is another major economic concern, as it can account for remarkable costs derived from direct and indirect impacts of the introduced species. Efforts are now being done to integrate direct measurable costs and quantify ecological losses. Globally, cumulated costs associated to aquatic invasive alien species (IAS) accounted for 345 billion US$ based on 5682 records from the expanded InvaCost database, with an observable increase in various orders of magnitude over the last years, peaking to at least 23 billion US$ only in 2020 (Cuthbert *et al.*, 2021).

1.2 Marine biofouling in numbers: ecological costs

Biofouling poses important environmental and biosecurity risks related to the introduction and spread of NIS. The translocation of organisms outside their natural range is considered one of the main threats for global biodiversity and can have important local economic costs associated to it, as seen above. In particular, the introduction of NIS poses a risk to the intrinsic value of biodiversity itself, with further effects on ecosystem services (Çinar *et al.*, 2014) and associated biosecurity risks whose impacts can go as far as pathogen translocation, and public and domestic or farmed animal health concerns (Georgiades *et al.*, 2021).

A recent in-depth report by Scianni *et al.* (2017) gathered and updated global marine NIS introductions, hereby summarized for the purpose of illustrating in numbers the impacts of biofouling as a NIS vector. According to this report, tidal waters of North America host 450 marine and estuarine NIS that considered to be established, of which 44 to 78 % are attributable to shipping, either by ballast water or by biofouling. Other studies focused on regional estimates, also mentioned in the report, indicate that biofouling is responsible of up to 58 % of the established coastal and estuarine NIS invertebrates and algae in Puget Sound in Washington State (Davidson *et al.*, 2014a), 60 % in California (Ruiz *et al.* 2011) and 78 % in Hawaii (Davidson *et al.*, 2014b). As for the Mediterranean Sea, aside of embracing the largest number of species for its size on the planet (Coll *et al.*, 2010), it also hosts the highest know number of NIS in the world, with estimates pointing out to nearly 1000 species, most of which arrived through the Suez Canal (Zenetos *et al.*, 2022). In this particular context, biofouling of recreational boats has been repeatedly suggested as major vector for the secondary spread of NIS, offering frequent opportunities for transfers and high connectivity between locations (Ulman *et al.*, 2019; Ahston *et al.*, 2022).

Biofouling clearly poses important losses, both at economic and ecological levels; however, the regulatory framework directly addressing this issue is scarce, if not almost inexistent. Current legislation unequally addresses different sectors, leaving some of these completely neglected. It is of particular concern the case of recreational boating, lacking of regulatory and enforcement tools that allow a solid legislative and implementation framework. Still, European waters host over 6 million recreational boats and approximately 10 000 marinas are home to over 1 million boats, providing berths both inland and in coastal areas.

Despite biofouling of recreational boats being the main focus of this thesis, at the light of today's global legislative scenario, which lacks a specific regulatory instrument on the matter, this chapter: 1) reviews the evolution of international environmental protection, 2) analyses the current regulations related to biofouling and its control and 3) provides evidences and learnings, intended as proposals addressing identified gaps, aiming for their integration in existing biofouling management plans, which could ultimately lead to a comprehensive biofouling policy framework.

2. Addressing marine environmental threats derived from shipping

The global nature of shipping industry and the motile essence of boats of all classes implies that vessel derived marine pollution needfully requires to be dealt at international level. Threats and risks associated to shipping are rather broad; however we will focus on the usage of antifouling systems and the environmental risks of biofouling itself. Still, a summary of the international regulatory instruments is herewith considered fundamental, mostly sourced from Harrison (2017) and Tanaka (2023). A supportive figure (Figure 1) was additionally created with the purpose of accompanying the text explanations on the chronological development of the discussed regulatory instruments.

2.1 International Regulatory Framework

At a global level, the most important legal instrument in the modern law of the sea relies in the United Nations Convention on the Law of the Sea, adopted in 1982 (UNCLOS, 1982), viewed as a legal framework within which to carry all the activities in the oceans and seas. Aside of establishing a legal worldwide order for the oceans and seas, it is also comprehensive legal framework for the protection of the marine environment.

It took over a decade to negotiate the convention and equally long to its entry into force (EIF). In fact, efforts to develop a regulatory instrument on the law of the seas started years before, in 1958, with the first United Nations Conference on the Law of the Sea leading to important international treaties. However, they soon caused disagreements and dissatisfaction in the

international community, due to imbalanced rights and obligations, as well as deficient marine environmental protection measures. In the light of catastrophic events, such as the *Torrey Canyon* incident in 1967, it was more than evident that there was an urgent need to address environmental emergencies and promote preventive and protective measures on its regard (Figure 1). At that moment, the International Maritime Organization (IMO), officially established and operational in 1958, worked only as a forum for cooperation on shipping regulation, with no specific competences on environmental matters themselves. It all fostered a gathering in Stockholm in 1972 to discuss deficiencies in the existing UNCLOS I (1958), ask for stronger protection of the world's marine environment and demand a regulatory body with competences in all the related affairs. Finally, the question of marine pollution was raised at the *United Nations Conference on the Human Environment* (United Nations, 1972), which concluded with the *Stockholm Declaration and Action Plan for the Human Environment* (Figure 1).

This UN Conference sets the starting line in marine environmental protection, by recognizing the human fundamental right and solemn responsibility to protect and improve the environment for present and future generations (United Nations, 1972). It propelled a series of changes that "Having considered the need for a common outlook and for common principles to inspire and guide the peoples of the world in the preservation and enhancement of the human environment, (…)" (United Nations, 1972) culminated with:

1. The recognition of deficient environmental protection by the delegates present at the UN Conference.
2. The creation of the United Nations Environment Programme (UNEP, 1972)
3. The convening of the third and last United Nations Conference on the Law of the Seas in 1973 and the negotiations for provisions on the protection of the marine environment, integrated in the final UNCLOS text as Part XII of the Convention, adopted in 1982 (United Nations, 1982).
4. The broadening of the IMO competences in 1975, through the modification of its constituent instrument, to include shipping related environmental regulations. Eventually, a permanent Marine Environmental Protection Committee (MEPC) was then established, born as a dedicated forum to consider the development of environmental legal regimes (IMO Convention, after the amendments of 1975).

According to the Part XII UNCLOS (1982) States have the obligation to protect and preserve the marine environment. In this regard, "States shall take, individually or jointly as appropriate, all measures consistent with this Convention that are necessary to prevent, reduce and control

pollution of the marine environment from any source, using for this purpose the best practicable means at their disposal and in accordance with their capabilities, and they shall endeavour to harmonize their policies in this connection". Not only shall the states take measures, but shall also "cooperate on a global basis and, as appropriate, on a regional basis, (…) for the protection and preservation of the marine environment", which is considered a fundamental principle under this convention (Tanaka, 2023) (Figure 1).

First steps specifically regarding and internationally addressing vessel-sourced pollution of the marine environment were initially focused on oil pollution, with the *1954 International Convention on Pollution of Sea by Oil*, namely 1954 OILPOL Convention (United Nations, 1954; Harrison, 2017). Nonetheless, similar to the UN Conference of 1958, this treaty was soon considered deficient in many aspects, falling to cover other types of pollution from the same source. Besides, it was considered to be troublesome to amend, and therefore tricky to keep it updated.

The 1954 OILPOL Convention cleared the way to the *International Convention on the Prevention of Pollution from Ships* (MARPOL Convention, 1973), as amended by the adoption of a Protocol in 1978, and still today, is the major treaty to regulate marine pollution sourced from ships, currently with 161 parties to it, making it 99.89 % of the gross tonnage of the world's merchant fleet (IMO, 2024) (Figure 1). This convention addresses the prevention of "pollution of the marine environment by the discharge of harmful substances or effluents containing such substances". It defines harmful substances as "any substance, which, if introduced into the sea, is liable to created hazards to humans, to harm living resources and marine life, to damage amenities or interfere with other legitimate uses of the sea". Vessel-source pollution can be either operational or accidental and, to date, the convention contains six Annexes covering particular categories of vessel-sourced pollution and providing detailed technical standards, both operational and accidental (Tanaka, 2023):

- Annex I: Regulations for the prevention of pollution by oil;
- Annex II: Regulations for the control of pollution by noxious liquid substances in bulk;
- Annex III: Regulations for the prevention of pollution by harmful substances carried by sea in packaged form;
- Annex IV: Regulations for the prevention of pollution by sewage from ships;
- Annex V: Regulations for the prevention of pollution by garbage from ships;
- Annex VI: regulations for the prevention of air pollution from ships.

Today, UNCLOS and MARPOL make up the two major global international treaties addressing the protection of the marine environment and the prevention of its pollution. At smaller scale, specific

directives on environmental protection aim to set quality standards and mechanisms to achieve those. In particular, the Water Framework Directive (WFD, Directive 2000/60/EC) "set out rules to halt deterioration in the status of EU water bodies and achieve good status for Europe's waterbodies, including coasts, rivers, lakes and groundwater". Daughter directives saw light from this initial one, including the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC).

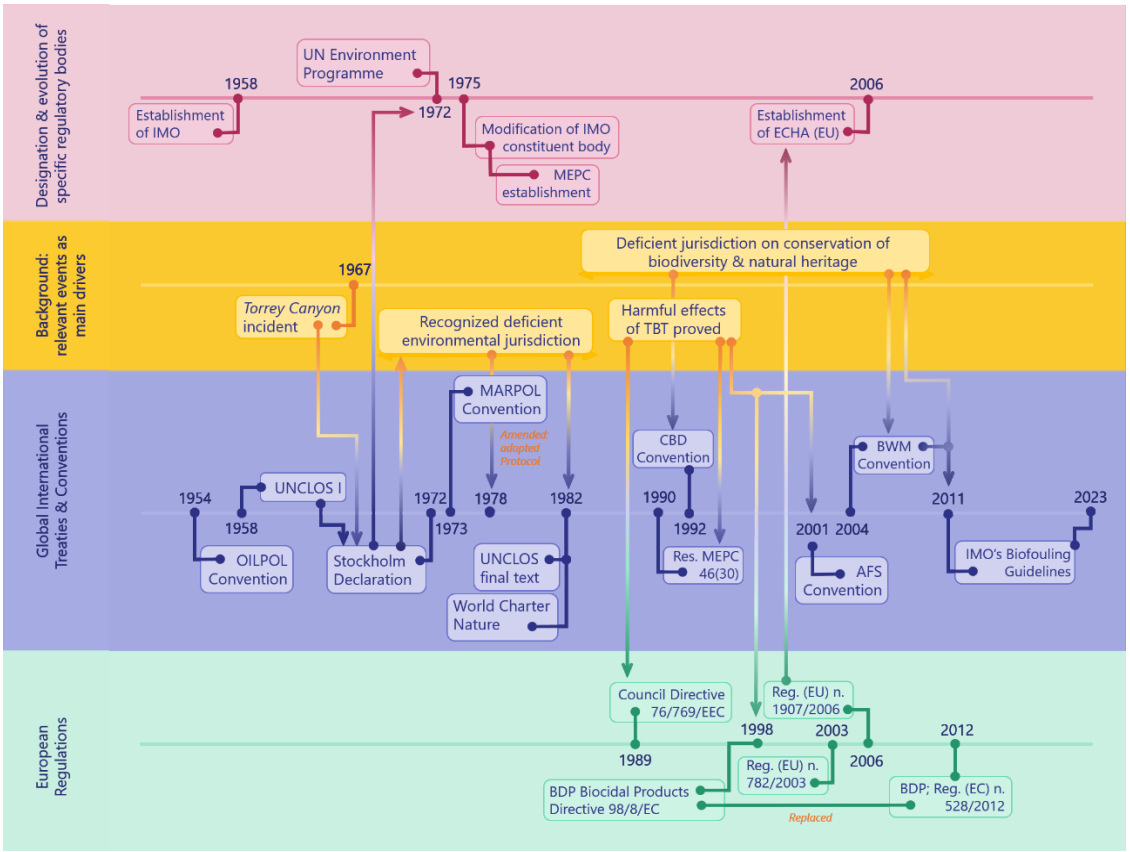


Figure 1. Descriptive graph illustrating the chronological development of the regulatory instruments discussed in the chapter. The graph is composed of four colour coded sections refering to: 1) the designation and evolution of specific regulatory bodies (pink, upper section); 2) background indicating relevant events and main drivers (yellow, second upper section), 3) global international treaties and conventions (blue, third upper section); and 4) European regulations (green, bottom section). The historical background is provided to contextualize the convening of international meetings, the creation of competent bodies and the development of regulatory instruments and their amendments or substitutions. The interactions are indicated with an arrow (effect arrow), pointing the direction of the effect and colour coded indicating de transition between sections.

Other regional efforts have also been taken, including the Regional Convention for the Conservation of the Red Sea and Gulf of Aden Environment (1982); the Convention for the Protection of the Marine Environment of the Wider Caribbean Region (Cartagena Convention, 1983); the Convention for the Protection of the Marine Environment of the North-East Atlantic

(OSPAR Convention, 1992); the Convention for the Protection of the Marine Environment of the Baltic Sea (Helsinki Convention, 1992); the Convention for the Protection of the Marine Environment and the Coastal Region of the Mediterranean (Barcelona Convention, 1995), among others. The definitions and provisions of the mentioned treaties and conventions are of broad scope, thought in such a way to be applicable to all sources of pollution, making them flexible enough to face new environmental threats as they emerge. They provide global, broad and flexible frameworks to back-up specific regulatory instruments thereafter developed.

3. Current policies and legislation on antifouling systems

Regulations on antifouling systems saw light after undeniable evidences of serious environmental impacts derived from the use of tributyltin-based AF paints. In the 1960s, the usage of TBT containing paints was very much spread due to their high efficiency related to its toxicity. Yet, the effects were observable beyond target species, with impacts in the surrounding environments and the species they host. These effects included imposex in gastropods and consequent reduction in reproductive capability, deformation and abnormalities in oysters, as well as bioaccumulation potential and persistence in sediments (Bryan & Gibbs, 1991; Stroben *et al*., 1995; Santillo *et al*., 2002; Price & Readman, 2013). Gradually, supported by the scientific evidence, restrictions on its use arrived (Council Directive 76/769/EEC, 1989; MPEC Resolution 46(30), 1990), leading ultimately to its global ban in 2001 with the adoption of the *International Convention on the Control of Harmful Anti-fouling Systems on Ships* (AFS Convention, IMO, 2001) and the European Union (EU) Regulation (EC) No 782/2003, aiming for its effective elimination from ships by 2008 (Figure 1).

The AFS Convention emerges from the decision to develop a self-standing treaty, rather than a new Annex to the MARPOL Convention, based on practicalities (Harrison, 2017). The AFS Convention also introduces an important obligation to the Parties, that of "taking appropriate measures to promote and facilitate scientific and technical research on the effects of antifouling systems as well as monitoring such effects", permitting the proposal of new antifouling systems to the Annex 1 of the Convention, which lists prohibited AF systems. To this end, the Convention sets a group of technical experts to review proposals and to report decisions to the Committee, which ultimately dictates the final resolution. Amendments to the Annex are adopted using a tacit approach, which ensures quickness throughout the process, and from the precautionary approach providing that "where the Committee is of the view that there is a threat of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason to prevent a decision to proceed with the evaluation of the proposal" (Article 6(3)).

At European level, the Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) establishes a general framework for the legislation of substances "ensur[ing] a high level of protection of human health and the environment as well as the free movement of substances, on their own, in preparations and in articles, while enhancing competitiveness and innovation". In this aim, it establishes a European Chemicals Agency (ECHA), created as an independent central entity for the effective management of the technical, scientific and administrative aspects of this Regulation at Community level.

Specific regulations concerning biocides in particular were first gathered under the Biocidal Products Directive (BDP; Directive 98/8/EC) and later on replaced by the Regulation (EC) No 528/2012. These provisions regulate the usage of biocidal products and the authorisation for placing them on the market, including biocides as antifouling agents (product type 21). Biocidal product is there defined as "any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action"; always ensuring that "they are sufficiently effective and have no unacceptable effect on the target organisms such as resistance, or, in the case of vertebrates, unnecessary suffering and pain. Furthermore, they may not have, in the light of current scientific and technical knowledge, any unacceptable effect on human health, animal health or on the environment. Where appropriate, maximum residue limits for food and feed should be established with respect to active substances contained in a biocidal product to protect human and animal health" (Regulation (EU) No 528/2012).

Currently, unauthorised biocidal antifouling products banned from use include TBT (IMO, 2001; Regulation (EC) 782/2003; Regulation (EC) 536/2008) and Irgarol (cybutryne) (MEPC 76/3/7 (2021); Commission Implementing Decision (EU) 2016/107, 2016), both listed under the Annex I of the AFS Convention. However, at national level, domestic regulations can extend the limit of usage to additional substances and, therefore, the list of unauthorised substances can differ across countries (Thomas & Brooks, 2010; Price & Readman, 2013). Copper is still widely used, cuprous oxide being the main active agent used in the market (Jones & Bolam, 2007; Ytreberg *et al*., 2010; Amara *et al* 2018). Nonetheless, increasing concerns regarding its environmental impacts have made copper go under scrutiny (Ytreberg *et al*., 2010; Dafforn et a., 2011; Oliveira, 2017; Amara *et al*., 2018). Consequently, some countries are starting to limit its use and/or concentration as biocide in AF coatings, including the Danish Environmental Protection Agency (DEPA, 2011; DEPA, 2024) in the Baltic region; as well as certain US states such as California (California Regional Water

Quality Control Board for San Diego Region, 2005; and Los Angeles Region, 2015) and Washington (Washington State Legislature, 2011). Additionally, the United States Environmental Protection Agency (US EPA) is also promoting the shift towards safer alternatives that exclude the use of copper by financing projects such as the one in San Diego bay ('Copper Reduction Program', Port of San Diego, 2024) and is currently working on a 'Clean Boating Act' (US EPA, 2024).

Surprisingly, in Europe, despite having specific directives that address the issue of marine environmental status, with tailored assessments designed for its monitoring, the reporting of certain substances is regarded as voluntary. As an example, HELCOM considers reporting copper concentrations as voluntary, limiting mandatory metal indicators to just three, these being mercury, cadmium and lead (HELCOM, 2018). Proposals to have copper included as a core indicator in HELCOMs third holistic assessment (HOLAS III) have been raised (Lagerström *et al*., 2021), together with the load compilations from shipping and leisure boats (Ytreberg *et al*., 2022), after it being identified as a remarkable source of pollution linked with AF usage (Ytreberg *et al*., 2022; Directorate-General for Environment, 2023).

4. Current policies and legislation on biofouling

Thus far, it has been addressed the legislation on the usage of certain chemical substances, including also the case of biocides in antifouling paint formulations. These regulations focused on the chemical aspect of biofouling control, aiming for environmental protection and pollution prevention. Therefore, their focal point is the assessment of chemical risk and limitation, when applicable, of certain substances to safeguard the overall marine environmental health. Even if they relate to the issue of biofouling, they do not state the need of controlling biofouling nor provide measures to do so. In fact, currently there is no international legally binding framework on biofouling, which, despite being a bottomless pit of costs and risks, to date, at a global scale, remains largely unregulated, although a few virtuous exceptions exist, as later discussed below.

Current voluntary management of biolfouling is a common practice, yet it focuses on drag reduction and fuel saving, but without specifically targeting the biodiversity conservation goals. As a result, it only partially addresses the NIS introduction problem, and some commonly employed antifouling practices, e.g. in-water cleaning, may be ineffective for certain taxa (Floerl *et al*., 2005) or may even result in dissemination of biofouling propagules and promotion of new NIS introductions (Tamburri *et al*., 2021). The need for an international regulatory framework on biofouling is strictly linked to the conservation of biodiversity and the concept of biosecurity. Although the view that "[hu]man has a special responsibility to safeguard and wisely manage the heritage of wildlife and its habitats (…)" (United Nations, 1972 though the Stockholm Declaration),

the concept of biodiversity in international rules emerged a decade later with the World Charter for Nature (WCN, 1982), a non-binding instrument adopted by the UN General Assembly, as a set of principles of conservation. The first global binding treaty on biodiversity arrived in 1992 with the Convention on Biological Diversity (CBD; UNEP, 1992) (Figure 1). The CBD Convention established a general framework for the conservation of biological diversity and the sustainable use of its components, demanding the integration of biodiversity conservation into (cross)sectorial plans, programmes and policies (Art. 6 and 10), including the obligation to introduce environmental impact assessment (EIA) plans for individual projects. Particular measures to ensure in-situ conservation are stated in the Article 8, in which, among others, "each Contracting Party shall, as far as possible and as appropriate (…) prevent the introduction of, control or eradicate those alien species which threaten ecosystems, habitats or species". However, the CBD itself does not perform a regulatory role and the Parties, ultimately, are to take further action to regulate activities, to determine the measures to fulfil its obligation and to ensure the implementation. In this case to the CBD establishes a broad scope and acts as an umbrella treaty that backs-up further and specific legislation. The Conference of the Parties (COP) was established as the main body in charge of ensuring its implementation, with the power to adopt further legally binding measures and provision to the Convention. Maintaining an oversight function, the COP leaves regulation to third actors, while ensuring the adoption of measures with the scope of the Convention. An example of this would be the *International Convention for the Control and Management of Ships' Ballast Water and Sediments* (Ballast Water Convention or BWM; IMO 2004), led by the IMO (Figure 1).

Under the umbrella of the CBD, more regulations are still needed, as the international legal framework on biological diversity and its conservation is of a broad scope. Its application to the marine environment is particularly challenging because of the complex jurisdictional regime that governs it, as well as the intrinsic difficulties to study it and the greater scientific uncertainty surrounding certain marine ecosystems (Harrison *et al.*, 2017).

4.1 IMO's guidelines

As stated above, "each Contracting Party shall, as far as possible and as appropriate (…) prevent the introduction of, control or eradicate those alien species which threaten ecosystems, habitats or species" (Art. 8 of the CBD; UNEP, 1992). Therefore, vectors for the introduction of marine non-indigenous species (NIS) shall be targeted by specific regulations and, because of the nature of the marine environment, only international level regulations can effectively address the issue.

The IMO has, during the last two decades, addressed the issue of NIS introduction related to shipping, first with the BWM Convention in 2004 and after with guidelines focused on biofouling as the vector of introductions. The case of BWM Convention is herewith considered of particular interest, as may serve as its development is paving the way to a potential future international regulation of biofouling. Therefore, it is worth stopping to understand the regulatory process behind this Convention. During the 1980s, after the initial steps on the protection of the marine environment (focused on pollution), increasing concerns on biological diversity were emerging, including those related to marine alien species. As mentioned above, a series of events (WCN; CBD Convention) succeeded, and the IMO too took steps forward to address the issue. In 1993, the IMO finally adopted the *Guidelines for Preventing the Introduction of Unwanted Aquatic Organisms and Pathogens from Ships' Ballast Water and Sediment Discharges*. It was a non-binding instrument aimed at giving guidance to States on measures that could be taken on the matter. Further efforts first leaned into the creation of a new Annex to MARPOL Convention, but eventually, in 1997, the IMO decided to work towards a new self-standing treaty, which saw light in 2004, and entered in force only in 2017, but with a time frame of seven years for parties to implement it within National Regulations (deadline in 2024). The BWM Convention includes measures to be taken by both coastal and flag States and sets regulations for ballast water management, according to vessel dimension and construction date. It also states the requirement of a record book containing all the ballast water operations, including reasons of discharge. Ships built after certain date are required to have approved ballast water management systems that ensure the treatment of these waters on board (BWM Convention, Regulation D-3; IMO, 2004), and to date, the approved systems include more than a hundred (IMO, 2023). While it established regulations and minimum standards for ballast water management, the Convention also recognized the right of the coastal States to take 'more stringent measures' on the matter (BWM Convention; IMO, 2004 Art. 2(3)) and encourages them to implement continuous monitoring (Art. 6). Lastly, the Convention established the obligation of providing technical assistance for developing countries to support them with all the duties. In particular, the IMO, together with other partners, does so under the umbrella of a specific programme (GEF-UNDP-IMO GloBallast Partnership Project).

Regarding biofouling management, coordinated international efforts to address it started in 2006, after formally raising the matter at the IMO (Scianni *et al.*, 2021), which was placed on the agenda of the IMO Marine Environment Protection Committee in 2007, leading to the establishment of an IMO correspondence group on biofouling the year after (Georgiades *et al.*, 2020). The first voluntary *Guidelines for the Control and Management of Ships' Biofouling to Minimize the Transfer*

of Invasive Aquatic Species arrived in 2011 (MEPC, 2011) and were extended to leisure boats with the *Guidance for Minimizing the Transfer of Invasive Aquatic Species as Biofouling (Hull Fouling) for Recreational Craft* (MEPC, 2012). These guidelines have been updated (MEPC, 2023b), after the launch of a global project on the topic (GEF-UNDP-IMO GloFouling Partnerships Project). These guidelines propose the development and application of two vessel-specific tools: the Biofouling Management Plan (BFMP) and the Biofouling Record Book (BFRB), and overall, these steps mirror those taken for the BWM Convention.

To date, these are the only international documents that set a baseline for biofouling control, although the provisions of these guidelines remain voluntary. The complexity of the issue of biofouling, characterized by clear challenges (see below), lessens the pace of the regulatory process. Still, exemplary cases with enforced legislation on the matter exist and are the proof that biofouling regulation, albeit tricky, is an achievable goal and a necessary process.

4.2 State of California, USA

Based on scientific evidence and existing data on vessel maintenance, operational practices and biofouling surveys, California's Legislature amended the Marine Invasive Species Act of the state in 2007, which addressed ballast water but not biofouling itself; placing a mandate on the California State Lands Commission (CSLC) with the aim of developing and adopting regulations on biofouling management (California Legislature 2007) (Scianni *et al*., 2021). The CSLC started working on specific regulations in 2010, supported by an advisory technical group, which finally arrived in 2017 as California Biofouling Regulations (California State Lands Commission, 2017). The process was accompanied of stakeholder engagement, outreach campaigns, industry meetings, etc. before the entry into force in October of the same year. However, this provisions apply only to larger vessels, specifically to "vessels 300 Gross Registered Tons or above that carry, or are capable of carrying, ballast water that arrive at a California port". Similar to what proposed by the IMO guidelines, these vessels require having a BFMP and BFRB as well as submitting an 'Annual Vessel Reporting Form'. If vessels are found to violate those requirements during an inspection, 60-day grace period is given to correct the deficiencies that, once over, if the deficiencies prevail, the vessel will receive a Notice of Violation.

4.3 New Zealand

By the time that the CBD entered into force in 1993, New Zealand (NZ) was launching a legislative tool to protect their local biodiversity, addressing the issue of *pests*. The Biosecurity Act of 1993 is an "act to restate and reform the law relating to the exclusion, eradication, and effective management of pests and unwanted organisms" which are defined as "any organism that a chief

technical officer believes is capable or potentially capable of causing unwanted harm to any natural and physical resources or human health" (Biosecurity Act; New Zealand Legislation, 1993). It provided the background for the development of further regulations addressing specific vectors. In particular, a targeted research and risk analysis period between 2004 and 2007 led NZ to identify biofouling-related NIS introduction as a key priority for the country's biosecurity (Scianni *et al*., 2021). A consultation was launched on biosecurity management (Georgiades *et al*., 2020) and translated into a consultation paper (MPI, 2010), followed by further research and cost-benefit analysis (Branson, 2012), and ultimately culminating with the development of *Craft Risk Management Standard for Biofouling on Vessels Arriving to New Zealand* (CRMS-BIOFOUL) by the Ministry for Primary Industries (MPI) in 2014 and adopted that same year. The entry in force considered a four-year adaptation period in which compliance was voluntary. In 2018, it finally became of mandatory implementation (MPI, 2018), in this case, for all vessel types, including recreational boats. Recently, the CRMS-BIOFOUL has been updated into the *Craft Risk Management Standard* (CRMS) *for Vessels* (MPI, 2023).

The MPI regards that "marine pests and diseases introduced to NZ on vessel hulls (biofouling) are a threat to our marine environment and resources. All vessels arriving in NZ must provide evidence of biofouling management prior to arrival". Therefore, vessel operators are required to take preventive biofouling measures prior to the arrival to NZ territory (excluding innocent or transit passage as defined in UNLCOS) and sets a minimum outcome to be met. In particular, vessel operators or any other person in charge shall provide, at least 48 hours prior to the vessel's entry into NZ territory, the MPI with 1) vessel details, 2) voyage details and 3) biofouling information, as stated in the section 1.4 of the CRMS for vessels (MPI, 2023). The CRMS relies on IMO's guidelines as a model of good practices and, currently, the MPI is working on a document with the "Approved Biosecurity Treatments" (MPI-STD-ABTRT). In case of non-compliance, the MPI may a) require a hull inspection upon arrival to NZ territory; b) restrict the itinerary in NZ; c) restrict the entry of the vessel into NZ territory; or d) ask for vessel cleaning within 24 hours by an approved provider in NZ, all at the expense of the vessel owner or operator.

To ensure compliance with the CRMS for vessels, the MPI has a fully dedicated site (MPI, 2024a) with all the required information, complemented with additional resources and tools that facilitate boat owners (MPI, 2024b) and operators to prepare their entrance in NZ territory.

4.4 Australia

The Biosecurity Act of 2015 is an "Act relating to diseases and pests that may cause harm to human, animal or plant health or the environment, and for related purposes" (Office of

Parliamentary Counsel, 2015). In 2021, a Regulation Impact Assessment (RIS) was carried out, aiming to provide policy options to improve the regulation of biosecurity risk associated with biofouling on vessels arriving into Australian territory. The decision was to rely on proactive biofouling management practices. The Biosecurity Act 2015 was amended in 2021 (*Biosecurity Amendment (Biofouling Management) Regulations 2021*) and in force since 2022. The Department of Agriculture, Fisheries and Forestry (DAFF), in charge of its administration, implemented an 18-month education phase after extensive consultation with stakeholders.

The *Australian Biofouling Management Requirements* (ABFMR) (DAFF, 2023), in particular, "set[s] out vessel operator obligations for the management of biofouling when operating vessels under biosecurity control within Australian territorial seas. These requirements apply to all operators of vessels subject to biosecurity control and provide guidance for vessel operators on best practice biofouling management". It established the requirement of submitting a vessel pre-arrival report (PAR) for commercial vessels through the department's Maritime and Aircraft Reporting System (MARS) at least 12 hours prior to its entrance in Australian territory and not before 96 hours. Vessel operators shall report if they can demonstrate compliance with one of the established options of biofouling management: a) implementation of an effective BFMP and BFRB (as in IMO's guidelines); b) cleaning of all biofouling within 30 days prior to the arrival; or c) implementation of an alternative biofouling management method pre-approved by the DAFF. Vessel operators that cannot demonstrate compliance with one of the three proactive biofouling management options will be subject to further pre-arrival reporting questions through MARS (DAFF, 2023). MARS is therefore used by the DAFF as a tool to target vessel interventions and assess biosecurity risks associated with vessel biofouling. Finally, verification upon arrival is carried out to certify compliance with the stated requirements. Reporting for non-commercial vessels is regarded as optional, through a non-commercial vessel pre-arrival report (NCV PAR).

4.5 Norway

The case of Norway can be considered the most recent step forward by a State to regulate biofouling (DTR 2024/9003/NO), led by the Norwegian Maritime Authority (NMA) (NMA, 2024a), following IMO's guidelines for the control and management of ships' biofouling to minimize the transfer of invasive aquatic species. The aim of the regulation "to prevent the introduction of hazardous invasive species to Norway through hull biofouling resulting from international shipping, and to prevent the further spread of hazardous non-indigenous species in Norwegian waters". Similarly to California State, this provisions apply only to certain vessels types and '*mobile offshore units*'. Specifically, it applies to "Norwegian passenger ships, cargo ships and barges

certified for foreign voyages, as well as for mobile offshore units and for fishing vessels with trade area Bank Fishing I or greater trade area when they are in Norwegian territorial waters, (…), in Norwegian [EEZ] and on Norwegian Continental Shelf". However, they share with other regulations the requirement of a BFMP and a BFRB, as well as the obligation of implementing a biofouling control and management system. Additionally, they provide an alternative option to the BFMP and to the control system and state the conduction of inspections as independent assessments and entry requirements.

A first regulatory document was drafted and notified to the EU Commission in March 2024, which was in standstill until June, when no conflict with the community regulations was found (TRIS-EC, 2024). Although its entry into force was initially planned for 2024, the consultation period between those months of March and June provided feedback and information that resulted in the decision of reassessing initial regulation draft and incorporate changes (NMA, 2024b). Currently, its entry into force is expected by July 2025 (NMA, 2024a).

5. Implementation and enforcement: from adoption to action

Generally, the implementation and enforcement of international shipping standards relies on three main jurisdictional mechanisms: 1) the flag State jurisdiction, 2) the coastal State jurisdiction and 3) the port State enforcement jurisdiction.

Flag States have primary responsibility to implement international standards and national regulations, it being a basic principle of the law of the sea that flag States have jurisdiction over their ships for all matters, regardless where they are in the world (UNCLOS, Art. 94; United Nations 1982). Since the practice may be challenging, flag States bear due diligence obligation to take all appropriate measures to prevent violations of regulations. Instruments to ensure it are inspecting and emitting certificates, as well as investigations of suspected violations. However, due to certain practices, such as flagging out (i.e. registration under the flag of another State without nationality link, usually to open registries of States with lower standards), the IMO had to implement mechanisms to deal with it. First, by the establishment of a Flag State Implementation Sub-Committee and finally, with the Member State Audit Scheme (MSAS) approved in 2005, by which all Member States shall be audited on the implementation of the standards.

Coastal States have an important role in prescribing and enforcing international standards in the areas in close proximity to their coast, yet, these may depend on the location of the vessel (territorial waters or exclusive economic zone, EEZ) and the type of standard. In the territorial sea, the coastal State is given the power to enforce its own laws and regulations (UNCLOS, Art. 220; United Nations, 1982). Still, their power is mostly limited to discharge and operational standards,

as well as protection of particular ecological features, and always ensuring the right of innocent passage. The coastal State may do so by taking special navigational measures, for example prescribing sea-lanes or traffic separation schemes, among other regulations. In the EEZ, the coastal State their power is additionally limited and dependant on the degree of harm or threat to the marine environment (UNCLOS, Art. 220; United Nations, 1982) and adoption of navigational measures in this area need the approval of a competent international organization (UNCLOS, Art. 211(6); United Nations, 1982). Further interventions, including inspections and judicial proceedings need to be backed-up by evidence of violation.

Port State enforcement jurisdiction refers to the situation in which action is taken against a vessel for a violation of international or national standards, including those that have taken place at sea before the ships has entered the port. UNCLOS describes three main scenarios in which States may exercise this type of enforcement in relation to pollution. The first scenario regards a vessel that is voluntarily in port and suspected of violation of standards. The port State in this case is enforcing a form of quasi-territorial jurisdiction (Molenaar, 2015; Harrison, 2017). The port State may also enforce 'effects jurisdiction' when a violation (e.g. discharge) has been committed beyond its waters, but eventually affected its own (UNLCOS, Art. 218, United Nations, 1982). The second scenario regards the port State bringing proceedings against a vessel or gather evidence of a incompliance on behalf of another State, either an affected coastal State or a flag State (see MARPOL Convention, Art. 6(5)). The third and last scenario regards violations of applicable international rules and standards in high seas (UNCLOS, Art. 218; United Nations, 1982) on behalf of the international community as a whole, seen as an innovative provision establishing a 'universal jurisdiction' (Birnie *et al.*, 2009, Harrison, 2017). Although not explicitly mentioned in the Conventions, a port State shall also set and enforce standards or conditions that must be met by a vessel to enter the port, considered a matter of general international law (Harrison, 2017). A port State can do so by denying access to its ports, regarded mostly as an exercise of sovereignty, or by sanctioning any violation (lack of information regarding the voyage, penalize false information, etc.).

5.1 Examples of enforcement of national biosecurity regulations

After the entry in force of New Zealand's and Australia's respective legislations on biofouling management, a few cruise vessels have been denied entry permission in ports of both countries, due to unmet biofouling standards, which were considered to be higher than allowed levels. New Zealand, for example, routinely inspects vessels biofouling levels, even for domestic ones (Georgiades & Kluza, 2020). The affected cruise vessels included the Viking Orion, the Coral

Princess, the Seven Seas Explorer and the Queen Elisabeth (The Maritime Executive, 2023). The Viking Orion in particular, was denied three scheduled stops in NZ during its itinerary in late December 2022, after which it headed to Tasmania (Australia), where officials again denied its entry. It then headed to Adelaide (Australia) where, according to Australian Fisheries Management Authority (AFMA), the vessel required hull cleaning to remove existing biofouling and prevent the introduction of potential pests, to its own expenses (Maishman & Murphy, 2023; Safety4Sea, 2023). Additionally, due to the caused disruptions in the scheduled itinerary, an "adjusted offer of compensation" to the passengers was claimed (Maishman & Murphy, 2023) in all cases.

The case of the Viking Orion and the other three cruises are examples of port State enforcement jurisdiction by Australia and New Zealand falling within the first described scenario and, in this particular case, stating unmet national standards on biosecurity. After denial of entry, finally, the vessel operator or authority accepted to undergo with cleaning procedures to meet those standards at their own expenses. Mechanisms or reporting (pre-arrival documentation) and inspection were applied to dictate the conclusion that vessels did not comply with the national biosecurity standards. Other than the expenses of cleaning, operators had to deal with claims by passengers that saw their voyage itinerary altered.

6. Gaps and challenges

6.1 Regulations on biofouling and antifouling systems

Having reviewed the main regulatory instruments, it is evident that the main gap regarding biofouling and its management is the lack of a global international binding framework that ensures the application of measures and approved antifouling treatments in order to control or, at least, minimize the introduction of non-indigenous species.

BWM Convention is, somehow, the sibling convention of what some day will be the Biofouling Convention. As already described before, the origins of BWM Convention resemble the current steps done for biofouling. In both cases, first regulatory attempts started with non-binding instruments that provided guidance to both flag and coastal States. Today's guidelines for biofouling consider a management plan and a record book as the main tools, which are key instruments of the BWM Convention. They also consider vessel design as a key factor to manage fouling, which can be considered analogous to that from vessels built up containing ballast water treatment systems in the BWM Convention. Additionally, biofouling guidelines describe different AF measures, while referring to various considerations on the selection, installation or application, and their maintenance. It refers to the AFS Convention as supportive, despite it only lists two banned substances; conversely, a list of approved technologies similar to that from BWM

Convention and NZ's CRMS for vessels (MPI, 2023) is missing. Furthermore, guidelines describe factors to be taken into account for the selection of AF coatings, but do not provide a roadmap to the final choice, as done for example by the National Institute for Public Health and the Environment of the Ministry of Health, Welfare and Sport of the Dutch Government in the RIVM Report for recreational boats (Wezenbeek *et al.*, 2018). In fact, the current plethora of available market products and solutions only contributes to blurry the choices of boat owners. Recently, under the scope of the GEF-UNDP-IMO GloFouling Partnerships Project, the lack of clarity on available solutions has being recognized as a major gap to be addressed and in that same document, clear efforts have been done to tackle it (GEF-UNDP-IMO GloFouling Partnerships Project, 2022).

6.2 Cultural measures for effective regulation enforcement

Ensuring commitment from the involved stakeholders implies adopting further measures other than just enforcing. Cultural tactics generally have been understood as practices that prevent or delay pest outbreaks, including site selection, scheduling and planning management tactics and increasing efficacy by removing sources of the pest (Culver *et al.*, 2012; Culver *et al.*, 2021); yet, they can include other measures (see Introduction) such as access to information, outreach programmes, improvement of services and maintenance facilities, training courses and technical assistance, etc. Some of these measures have accompanied the implementation of international measures and examples of it are the BWM Convention, which explicitly established the obligation for technical assistance (Art. 13(1); IMO 2004), leading to programmes as the one launched on the topic (GEF-UNDP-IMO GloBallast Partnership Project) or the implementation the CRMS by the MPI, with targeted outreach campaigns (Scianni *et al.*, 2021). It requires specific resources to be allocated for the purpose assigned to a) infrastructure improvement, b) outreach programmes to enhance engagement and c) technical assistance and information points.

6.3 Aligning drivers of interest

Motivations behind the need of biofouling control and its regulation vary according to the interested party and it can be challenging to set common minimum standards satisfying their demands. The mini-review by Davidson *et al.* (2016) concluded that, despite unified wills to regulate and manage biofouling exist, the resolution of the gathered information and the areas of utmost concern compose the main discrepancies across parties, i.e. involved industries, port authorities, biosecurity managers and the academic community. Cost saving, safety at sea, biosecurity, biodiversity and conservation are not always shared priorities and fall within interests of particular sectors only. While industry seeks optimization of operational performance and

associated cost savings, authorities and environmental managers biosecurity risk reduction is the main driver. However, overcoming the discrepancy among those drivers of interest appears reasonable and feasible. Greater uncertainties surround the sector of recreational boating, as there has been less emphasis on understanding them, as well as less awareness on the matter. As other stakeholders incorporate to the equation, different drivers may emerge.

6.4 Finding the balance

Balancing risks has never been an easy task and establishing acceptable environmental risks is no exception. Defining a middle ground in biofouling management implies counterpoising biosecurity risks to those from antifouling systems' implementation, both under the umbrella of environmental protection (GEF-UNDP-IMO GloFouling Partnerships Project, 2022). Additionally, measures need to be feasible and practical, while meeting the established minimum environmental standards. Due to recent concerns on the effects of certain biocides, antifouling technologies are shifting toward alternative non-toxic solutions and, as seen above, certain regional governments are promoting projects in doing so ('Copper Reduction Program', Port of San Diego, 2024). However, currently existing biofouling management strategies are not protective of biosecurity goals (Davidson *et al*., 2016) and, although some exceptions exist (DAFF, 2023; MPI, 2023), the scale is unbalanced.

6.5 Geopolitics

Effective environmental protection and regulation requires targets to be reconciled with social, economic, cultural, and political needs (Katsanevakis *et al*., 2015). In practice, where regions have inherent geopolitical complexity and a wide range of priorities, like the case of the Mediterranean Sea, challenges arise, hampering the development of a common shared legal framework. The Mediterranean is an interesting case study from the jurisdictional perspective as, once declared, the EEZ of the over 20 countries leave no space for High Seas. Coastal States have "sovereign rights for the purpose of exploring and exploiting, conserving and managing the natural resources, whether living or non-living, of the waters superadjacent to the seabed and of the seabed and its subsoil, and with regard to other activities for the economic exploration and exploitation of the zone" (UNCLOS, Art. 56; United Nations, 1982). Furthermore, coastal States have legislative and enforcement jurisdiction with regard to the protection and preservation of the marine environment, including matters defined by international law (UNCLOS, Art. 56(1)b; United Nations, 1982). This is a double-edged sword: even if it offers an unprecedented opportunity for environmental jurisdictional matters (Katsanevakis *et al*., 2015), disputes over EEZ's

boundaries entail legal uncertainty over the complex jurisdictional scenario of the region and the applicable rules and standards aiming for effective environmental protection (Andreone, 2022).

7. Learnings and proposals

7.1 Lessons learned

The experimental outcomes of the current thesis demonstrated that antifouling paints, in particular the one containing biocides (BC), were toxic for non-target organisms, causing major physiological impairments even at the lowest tested concentrations. The alternative non-toxic coating, FR, showed very low effects in the tested organisms and limited to subcellular changes, mostly just as observed tendencies. Furthermore, some of the tested target species demonstrated higher tolerance to BC coatings, a fact that was backed-up by the field experiment. This tolerance phenomenon, however, has major biosecurity implications, since some of the observed species are considered non-indigenous species (NIS). Besides, the in-field performance testing experiment suggested that, under the followed experimental design, the BC coating did only reduce the coverage in the short term, but hosted higher NIS ratios during the high boating season; and failed to meet performance goals in the long term.

Additionally, chemical experiments showed that the studied environmental factors affected the release of metals from the coatings to the water, potentially affecting the toxicity, durability and performance of the tested coatings, which are of particular importance for suitable AF selection and risk mitigation under a climate-changing scenario.

7.2 Synthesizing

The main identified gaps can be summarized into the following points:

- Uncertainty of the performance of some AF products and their environmental effects.
    - Uncertainty on the required biocidal load, generally too high.
    - Uncertainty on their effects in non-target species.
    - Uncertainty on the influence of location and associated environmental factors and the operational profile of the boat.
- Lack of approved AF technologies, similar to that for ballast water treatments, rather than just substances listed in Annex 1 of AFS Convention.
- Lack of roadmap to suitable AF strategy selection, particularly for recreational boats.
- Lack of services and facilities for enhanced management of biofouling.
- Feasibility of some products and services for the sector of recreational boats.

- Lack of engagement due to low perceived risk and scarce knowledge on the topic in certain sectors or stakeholders.

The main identified challenges can be summarized into the following points:

- Applicability of certification and inspection regimes for recreational boats.
- Alignment between industries, authorities, scientific community and final users
- Regulation and enforcement in regions, such as the Mediterranean, with multiple nations and overcoming geopolitical issues that may arise.
    - For example, 23 (some may argue 22) coastal States of Europe, Africa and Asia surround the Mediterranean.
- Implementation and enforcement in certain sectors, e.g. recreational boating.

7.3 Proposals to some of the existing gaps

Creating a list of approved antifouling products and technologies could positively contribute to a shift in the market and greater regulation enforcement regarding the products, taking as a reference the procedures of ballast water treatments. Environmental safety certifications and periodical revisions on the available solutions, particularly those containing biocides, could contribute to ease the choice of more environmentally friendly alternatives while redirecting the global antifouling market towards developing sustainable alternatives and fuel associated technological innovation. Additionally, requirements to include suitability of use of a product, i.e. information on environmental conditions and geographical location, boat area, etc., could help to enhance the performance of the product, contribute to environmental protection and facilitate the choice of appropriate solutions. These measures could contribute to reducing the total biocidal load of some products or adequate it to certain environments. Furthermore, certified sellers, appliers and managers could improve product selection and application, as well as waste reduction and management, extending durability of the selected systems and minimizing environmental risks.

An additional supportive measure for suitable AF selection would be the development of a roadmap to guide users in the decision-making process. Currently, the information available tends to summarize the types of AF systems (Cao *et al.*, 2011; Tian *et al.*, 2021; GEF-UNDP-IMO GloFouling Partnerships Project, 2022) and the most recent contribution to a guided decision could be that from Wezenbeek *et al* (2018), whose decision-tree has been updated here in Figure 2, to cover the latest findings, including those from the present work. Figure 2 has been conceived as a proposal to assist in the decision of coating selection, considering multiple factors, such as the operational profile, maintenance, boat area and environmental conditions.

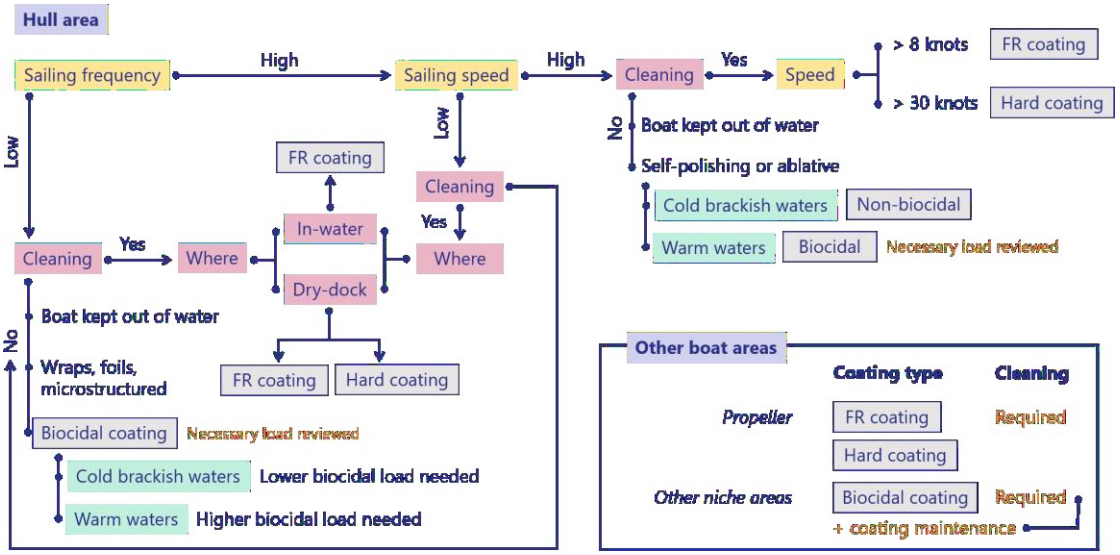
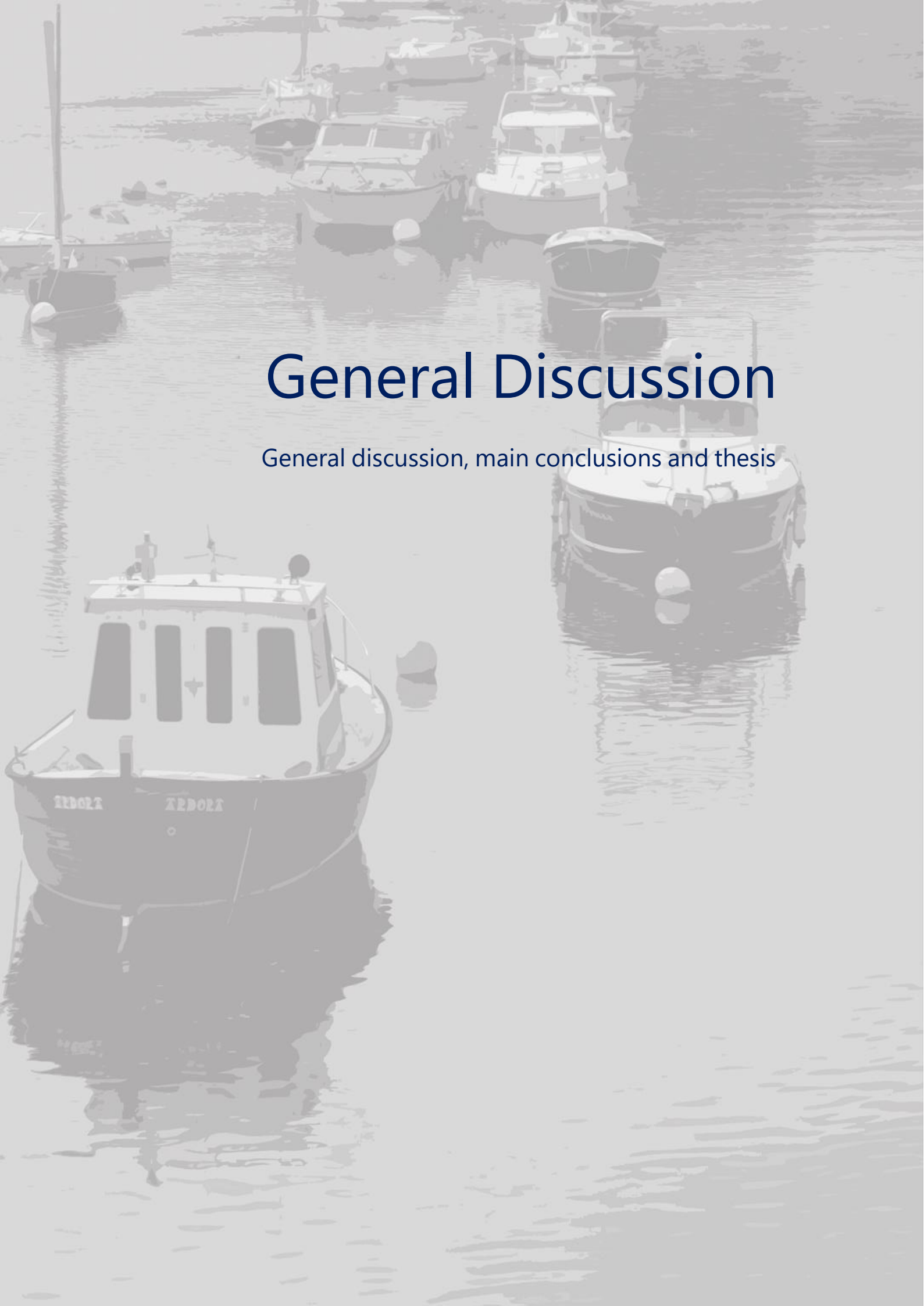


Figure 2. Decision-tree for suitable antifouling coating selection for recreational boat owners. The tree includes updates to the proposal by Wezenbeek *et al.* (2018). Colour indicates the class of factor on the decision process: yellow for operational profile; pink for maintenance (cleaning); green for environmental factors and powder blue for boat area. The final coating choice is indicated in grey background.

Ensuring access to this type of information by final users is key to optimize AF measure selection. Creating a network of information and promoting dissemination activities would, ultimately, help to increase awareness and promote commitment among boat owners. In fact, tools such as the guidelines by the IMO (GEF-UNDP-IMO GloFouling Partnerships Project, 2022) with clear language and illustrative information, are essential to translate policy and scientific outcomes to final users. Still, enhanced engagement from the stakeholders is necessary, which could be achieved by allocating funds to infrastructure improvement and to the design of outreach campaigns with different goals. Accessible cleaning and waste management facilities in ports and marinas area must to reduce pollution and biosecurity risks, backed-up by management and risk reduction plans. Besides, having technical staff in charge of applying those plans and of assisting final users in their maintenance activities, accompanied by training courses could contribute further to reach this goal. Finally, clear procedures for vessel inspections and surveys are required where regulations are in force, similar to those proposed by Georgiades & Kluza (2020), although further steps could be done by marina and port managers as implied responsible authorities. This later step also requires investing in a network of certified personnel and important efforts need to be done regarding engagement and outreach, as mentioned above. In summary, efficient biofouling management would be nourished by different type of actions, as listed above, which requires an important reshaping of it was conceived until now, and includes 1) clear and accessible information, 2) engagement activities, 3) infrastructure improvement, 4) solid network of certified personnel and product sellers and 5) implementation of management plans.



General Discussion

General discussion, main conclusions and thesis

General Discussion

Biofouling management is a challenging (historical) matter and, to date, there is no panacea for its control (Yebra *et al.*, 2004). In the plethora of available antifouling (AF) measures, traditional biocide-based (BC) coatings dominate the market and are the most widely used option, being copper (Cu), specifically as cuprous oxide, the main active compound (Finnie & Williams, 2009; Howell & Behrends, 2009; Dafforn *et al.*, 2011). Because of the release of active compounds to the environment, AF coatings have been pointed out as important sources of metal pollution, and even as the largest individual anthropogenic sources of Cu to the Baltic Sea (Ytreberg *et al.*, 2022). Concerns on the impacts of biocide loads into the marine environment (Ytreberg *et al.*, 2010; Amara *et al.*, 2018; de Campos *et al.*, 2021) have propelled limitations on Cu usage regionally (California Regional Water Quality Control Board, 2005; DEPA, 2011; Washington State Legislature, 2011; California Regional Water Quality Control Board, 2015; DEPA, 2024) and the search for more sustainable antifouling alternatives. Silicone-based foul-release (FR) coatings have reached the market and are the main substitutes to traditional BC ones (Candries & Anderson, 2003; Lagerström *et al.*, 2022). The ream of AF choices is broad (Yebra *et al.*, 2004; Cao *et al.*, 2011) and so is the diversity of fouling organisms that have been identified, accounting for over 4 000 fouling species (Lewis, 1998; Finnie & Williams, 2009), representing a wide range of sensitivities to AF substances. However, although antifouling testing is a common procedure, there is no unified standardized approach for all the AF categories, nor an integrative approach combining different testing methods. Methodological procedures in laboratory conditions can be very diverse (Briand *et al.*, 2009), but recent efforts to standardize these procedures for certain AF typologies and organisms have been developed (ISO, 2020), including specific projects on technology demonstration (IMO-NORAD, 2024). Yet, most of the studies focus on a single approach, such as in lab or in field; or have limited scope. The application of integrative approaches is essential to comprehensively assess the toxicity and performance of AF coatings.

This doctorate project integrated chemical, toxicological and ecological approaches for AF testing, with a suite of laboratory and field experiments. The main outcomes were, eventually, discussed in the light of the current legislative framework, highlighting challenges and limitations and stating the main learnings and proposals to address some of the existing gaps.

The chemical characterization of the selected AF coatings (BC, FR) is covered in the first chapter (I. Chemical characterization), backed-up with a chemical behaviour assessment under different temperature and salinity scenarios. The chapter describes a standard procedure on lixiviate preparation and characterization, which was thereafter applied for laboratory experimentation.

Lixiviates from the selected AF coatings were clearly distinct in composition according to their respective formulations. It was observed that incubation parameters during lixiviate preparation affected their final composition. Copper release resulted mostly influenced by salinity (Howell & Behrends, 2009), while zinc and chromium release mainly by temperature. Remarkably, chromium concentrations were significantly higher in lixiviates of the double-layer FR coating. Results evidenced that environmental factors could alter the efficiency, durability and toxicity profiles of AF coatings, which is of particular importance for suitable AF selection and risk mitigation under a climate-changing scenario (Cabral *et al.*, 2019). In fact, the effects of environmental factors in coating performance could explain, to some extent, the reduced efficiency of the BC coating in-field, as discussed below.

The toxicity profiles of the selected coatings were studied in the second and third chapters (II. Toxicity in microalgae; III. Toxicity in zooplankton), using target and non-target species. Additional toxicity screening assays on other two key target species were done and presented in the annexes (Box 1). Both for microalgae and zooplankton, an initial toxicity screening assay was conducted, allowing to determine critical lixiviate concentrations and to narrow down the concentration range for further testing at sub-effect (sub-lethal and sub-inhibition) concentrations. Toxicity to microalgae was assessed from a multi-taxa and multilevel approach, using pelagic (*Isochrysis galbana* and *Tetraselmis* sp.; non-target) and benthic (*Cylindrotheca* sp.; target) species and a battery of endpoints at different biological levels, including physiological and subcellular responses. Growth and photosynthetic efficiency were the chosen physiological endpoints, and pigment content, enzymatic activities (CAT, GST), lipid peroxidation level (LPO) and cellular metal sorption were the selected subcellular biomarkers. Results evidenced clear differences in the toxicity profiles of the two tested antifouling coatings and the integration of the responses provided a defined overview of the effects of lixiviate exposure. Particularly, FR did not unchain responses measurable at physiological level and its effects remain restricted to subcellular responses. Oppositely, exposure to BC lixiviates induced growth inhibition in all the three microalgae species and affected photosynthetic efficiency in *I. galbana* and *Cylindrotheca* sp. There were marked differences in species sensitivities, but, generally, at low BC concentrations, subcellular responses are mainly triggered, while physiological impairments were observable only when the concentration gets too high to cope with by means of molecular mechanisms. These measurable effects under exposure to any of the treatments deserves particular attention, especially in the case of emerging alternatives. Silicone based eco-friendly alternatives have been suggested as valuable substitutes and their indirect effects to other marine organisms remains considerably below to those from BC based ones, although some effects have been reported

(Lagerström *et al.*, 2022). As regards species sensitivities, *Cylindrotheca* sp. was especially responsive to BC exposure, while *Tetraselmis* sp. dealt better, tolerating higher BC concentrations. The phenomena of sensitivity and tolerance can have important environmental implications, mainly in areas of high boat densities, as the can lead to changes in community assemblages (Morín *et al.*, 2012; Martínez *et al.*, 2021). Additionally, tolerance to antifouling coatings is an important factor to consider in biosecurity plans, as it can contribute to the introduction on non-indigenous species (NIS) with direct impacts on local biodiversity and overall health of the environment, including cases of toxic microalgae and/or with the potential to cause harmful algal blooms (Costas *et al.*, 2023) and pathogens (Georgiades *et al.*, 2021).

Toxicity in zooplankton was assessed using a planktonic model species, *Acartia tonsa*, following a biological multilevel approach in a non-target species. As before, initial toxicity screening assays were performed with lixiviates as test contaminants and, in this case, chosing mortality as the critical endpoint. Results were used to narrow down concentrations for the study of sub-lethal effects, including fecundity endpoints and molecular responses based on differential transcription of target genes. Additional toxicity screening assays were carried out using individual active components of the coatings to elucidate the underlying toxicity mechanisms. While FR coating lixiviate did not cause mortality, lethal toxicity assay with BC showed high mortality at relatively low lixiviate concentrations. Individual component testing showed that copper was more toxic than zinc and that the mixture Cu + Zn did not increase the mortality, being equal to that from copper alone and indicating an unnecessary load of zinc. Zinc is commonly added as a binder and pigment (Yebra *et al.*, 2004), but also to broaden the toxicity spectrum and overcome the tolerance of copper seen in certain species (Paz-Villarraga *et al.*, 2022). However, parallel toxicity screening assays on target species (pediveliger larvae of the mussel *Mytilus galloprovincialis* and the fouling amphipod *Monochoropium insidiosum*; Annex, Box 1) revealed higher tolerance thresholds, confirming that some species are more resistant to copper and zinc fails to increase the toxicity spectrum. Lixiviate concentrations responsible of the mortality of the 50 % of the individuals (LC50) contained less than half of the needed copper concentration to reach the LC50 given by copper alone or the Cu + Zn mixture, suggesting toxicity of other components of the paint formulation. Sub-lethal responses to lixiviate exposure were further investigated at reproduction and molecular levels. Reproduction is a valuable endpoint in ecotoxicology due to its relevance at population level (Biandolino *et al.*, 2018) and exposure to BC lixiviates showed that, although the number of reproductive females is not affected, the mean number of eggs laid by these reproductive females decreases, as well as the total number of eggs of the whole female pool population. These experimental outcomes raise concerns of potential impacts at population levels.

Lastly, molecular responses were studied, in which genes like *gst*, *fer* and *sod-II,* involved in detoxification processes and proposed as target biomarkers of oxidative stress (Hafez *et al.*, 2021; Rotolo *et al.*, 2023), were chosen together with the chitin-pathway gene *cda* and *ach-r* of the nervous system. Oxidative stress genes were responsive to lixiviate exposure, triggering unified responses of upregulation for BC and downregulation for FR after 24 h exposure. Interestingly, the chitin pathway was significantly affected by changes in *cda* transcription levels, inducing strong downregulation under 24 h BC exposure and similar tendency for *ach-r*, responses that could have important physiological implications. Overall, these outcomes confirm *A. tonsa* as a suitable planktonic non-target model in biological multi-level testing of antifouling toxicity and prove appropriate the selected battery of responses. Furthermore, results evidence the toxicity of BC coatings greater for the selected non-target species than for certain target ones.

In the fourth chapter, (IV. In-situ performance) the realistic effectiveness of the selected antifouling coatings in terms of fouling colonization and, specifically, protection from NIS was tested in two marinas located in the Gulf of La Spezia (Liguria, Italy) by means of experimental units with a hard PVC standard surface. The application of antifouling coatings was accompanied with other antifouling maintenance practices, i.e. manual cleaning, in a manipulative experiment with two sampling periods (end of high boating season after 3 months of submersion, end of the low boating season after 9 months of submersion). In the short term, the test BC coating proved to be effective in controlling the development of fouling communities, showing significantly lower coverage of the test units compared to control plates, while fouling level of the FR coating was comparable to control plates. However, after longer submersion periods, BC coating had a remarkable decrease in their performance (contrasting indications of product label), an effect that was not observed in FR treated plates, which constantly ensured easy detachment of biomass, even with evidences of self-detachment. Interestingly, although BC coating performed well in the short time with little coverage, the few organisms detected were mainly NIS, in particular, the amphipod *Laticorophium baconi* and the cryptogenic bryozoan *Watersipora subtorquata*. This outcome suggested copper tolerance and, in the case of the amphipod, is backed-up by the laboratory experiments on *M. insidiosum* (Box 2), a similar species belonging to the same family and present in BC experimental units of the Bay of Biscay. Additionally, the high correlation of the presence of the encrusting cryptogenic bryozoan *W. subtorquata* and BC treatments also suggest tolerance to copper and, what is more, a facilitator effect, as it grows forming a mat that allows settlement of other organisms. Copper tolerance in fouling species has already been described (Floerl *et al.*, 2004; Piola & Johnston, 2006; Culver *et al.*, 2021), but it happening shortly after the application of antifouling coatings and during the high boating season, which is the period of

greatest mobility and connectivity between locations, is of great concern and raises biosecurity alarms. Overall, antifouling coating type was the main factor shaping the fouling communities in terms of structure and composition, primarily in the short term. Long idle periods can lead to decreased coating performance; however, they are common and unavoidable for recreational boats, thus, effective fouling management should consider them (Davidson *et al.*, 2020). Besides, the environmental conditions can determine performance and durability of the coatings, as seen above. In the Mediterranean, high temperatures and high biofouling pressure in summer, as well as high salinity, can contribute to minor performance and unbalanced biocide release.

The outcomes of this work shed light into effective biofouling management, providing proposals to be considered and highlighting issues of special concern and priority areas to be covered with further research. Still, there are some uncertainties on the environmental effects of antifouling measures, particularly those relaying in chemical mechanisms, stressing the need of more efforts on the matter.

Despite the relevance of biofouling in economic and environmental terms, the lack of an international binding regulation on the matter entails uncertainty over effective environmental protection. The last chapter (V. Regulatory framework) discusses current legislation gaps and challenges, while taking into account the main learnings from the experimental outcomes of this project and results in the design of updated decision trees, developed to assist final users in the selection of suitable antifouling strategies according to key factors of boats' operational profile and surrounding environmental factors. In summary, the followed holistic approach in AF testing proved a valuable tool to identify priority areas and risks that can contribute to the development of appropriate legislation and to build realistic management tools, such as decision trees.

Conclusions

1. The developed procedure for AF coating lixiviates and their chemical characterization proved suitable and served as a standard procedure to carry out further experimental testing on toxicological profiles of AF lixiviates.

2. Both AF coatings leached metals, even the selected alternative FR coating. Environmental factors, such as temperature and salinity, affect the release of metals from the coatings, although differently according to the chemical formulation of the AF coating. Therefore, environmental conditions have the potential to alter AF efficiency, durability and toxicity profiles, which are of particular importance for suitable AF selection and risk mitigation under a climate-changing scenario.

3. AF coating lixiviates showed different toxicological profiles. While the tested BC coating proved toxic at very low concentrations inducing critical endpoints both in microalgae and zooplankton, the FR coating did not cause major physiological impairments. Generally, narrower sub-effect concentrations derived from toxicity screening assays did trigger subcellular responses under exposure to any of the coating types, a fact that deserves special attention. The selected battery of biological endpoints revealed useful and provided an integrative picture of the overall effects of AF exposure across multiple taxa.

4. The type of response and magnitude was species-specific, unveiling, in the studied taxa, phenomena of sensitivity and tolerance, which are of utmost importance due to the potential environmental effects, stressing the need of further investigating sensitivity-related changes in community assemblages and biosecurity risks of tolerance-related introduction of species.

5. In-field performance testing evidenced the action mechanisms of the selected coatings, which hosted remarkably different biofouling communities. BC coating resulted effective in the short term, but without reaching half of its service life with optimal performance. The FR coating behaved alike controls and had a constant performance profile, ensuring easy detachment and even self-detachment. Maintenance practices only help reducing sessile NIS species in the short term, without otherwise affecting the recruitment of other foulers.

6. The field experiment unveiled a remarkable presence of non-indigenous species during the high boating season under BC treatment, indicating copper tolerance in *Laticorophium baconi* and *Watersipora subtorquata*, and even facilitation effect by the later one. The tolerance of the amphipod was further backed-up by the outcomes of the additional toxicity screening assays with its close relative *Monocorophium insidiosum*. Both tolerance and facilitation phenomena have major implications for biosecurity standards and reveal the limitations of BC coatings.

7. The application of integrative approaches is essential to comprehensively assess the toxicity and performance of AF coatings. Combining chemical characterization, toxicity testing and in-field performance testing from a multi-taxa and biological multi-level

approach provided a broad understanding of the toxicity profiles and performance of the selected coatings and helped to identify associated environmental risks.

8. The current uncertainties and challenges in biofouling management leave a gap that entails legal uncertainty over effective environmental protection, therefore highlighting the urgent need of regulatory instruments on the matter. The development of holistic investigations on the topic confirmed to be valuable approaches to address these existing uncertainties, resulting in the identification of priority areas and risks, and in the design of useful management tools for final users, such as integrative decision trees on suitable selection of AF strategies.

Thesis

Multi-taxa biological multi-level approach proved a valuable tool, demonstrating distinct toxicological profiles of the tested antifouling coatings and unveiling sensitivity and tolerance responses to classic chemical measures of biofouling control (i.e. biocide-based coatings), which, additionally failed to meet *in situ* performance goals. The alternative product for physical control of biofouling (i.e. foul-release coating), although not completely innocuous, showed very low toxicity and optimal *in situ* performance, even in the long term, with evidences of self-detachment. The current tools of biofouling regulation are insufficient, but holistic studies can help to pave the way to efficient management and provide valuable insights that can be translated into tangible instruments for final users.

Annex

Additional results, protocols & supportive materials

Original picture: Aitor Puente

1. Additional results

1.1 Integrative outputs from toxicity assays in microalgae

Principal Component Analysis (PCA) was used to describe the experimental data according to all the biological responses (variables) using the package '`FactoMineR`' (Lê et al., 2008) with scaled data (`scale.unit = TRUE`). To handle missing data due to mismatch in replicate size (Figure 1), the package '`missMDA`' (Josse & Husson, 2016) was used to impute the missing values of the data frame. The PCA plot included ellipses with 0.85 confidence level.

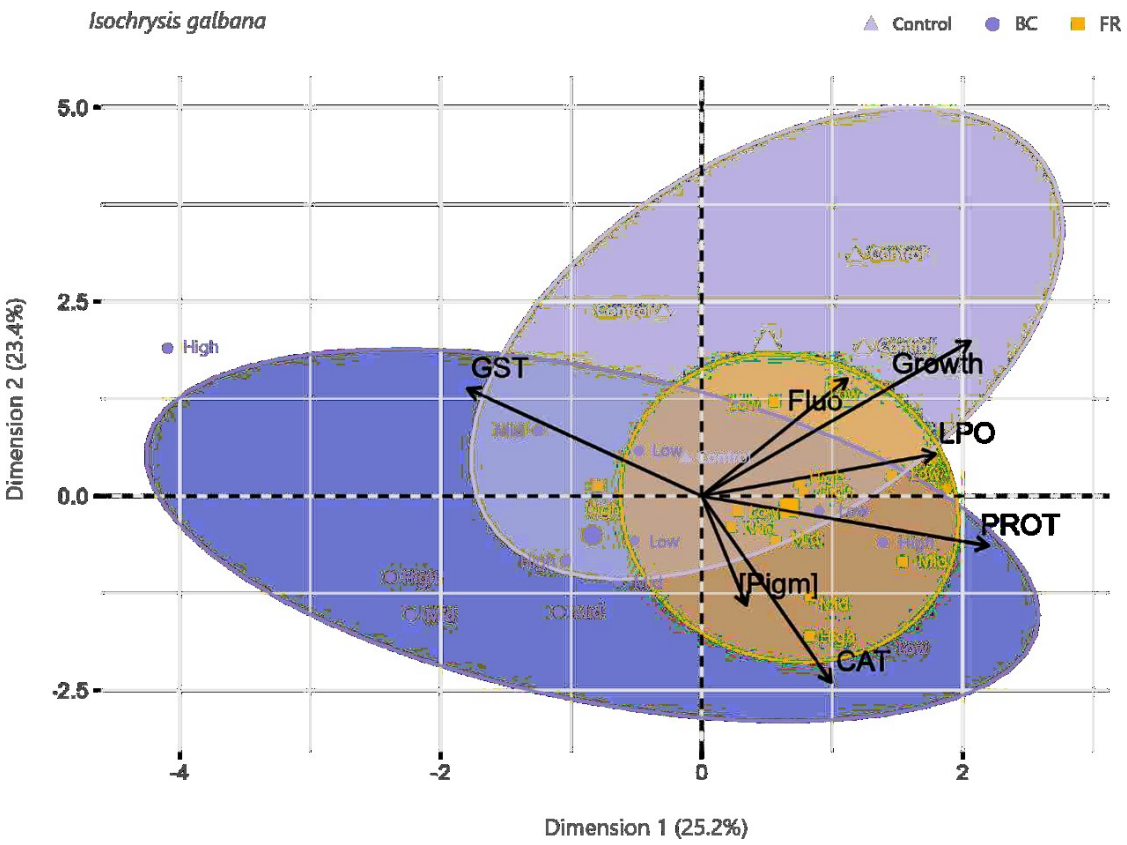


Figure S1. Principal Component Analysis (PCA) plots of toxicity assays with AF lixiviates in *I. galbana*. The response variables are indicated with a black arrow and the coating factor is represented with colours: grey = control (C), violet = biocide-based coating (BC) and orange = foul release coating (FR). Concentration is indicated as point labels (scores ID), corresponding to low, mid and high concentrations. The percentage of contribution of each component is also noted.

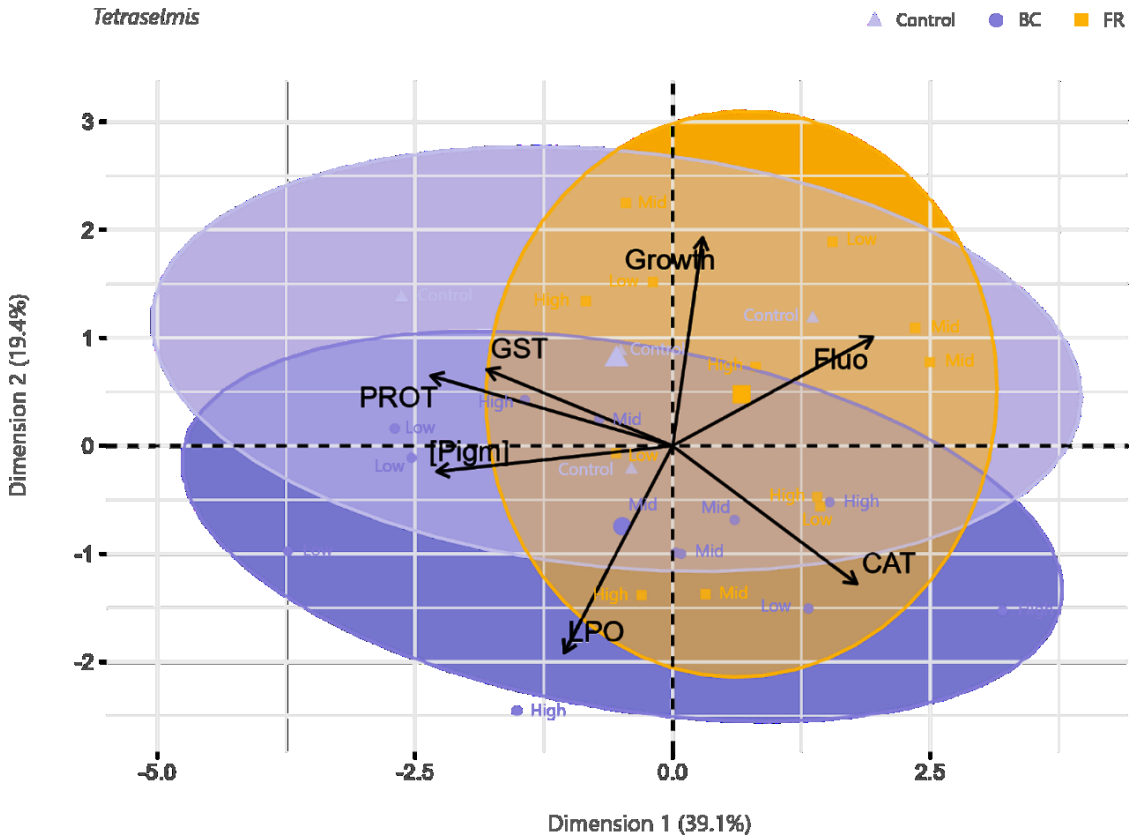


Figure S2. Principal Component Analysis (PCA) plots of toxicity assays with AF lixiviates in *Tetraselmis* sp. The response variables are indicated with a black arrow and the coating factor is represented with colours: grey = control (C), violet = biocide-based coating (BC) and orange = foul release coating (FR). Concentration is indicated as point labels (scores ID), corresponding to low, mid and high concentrations. The percentage of contribution of each component is also noted.

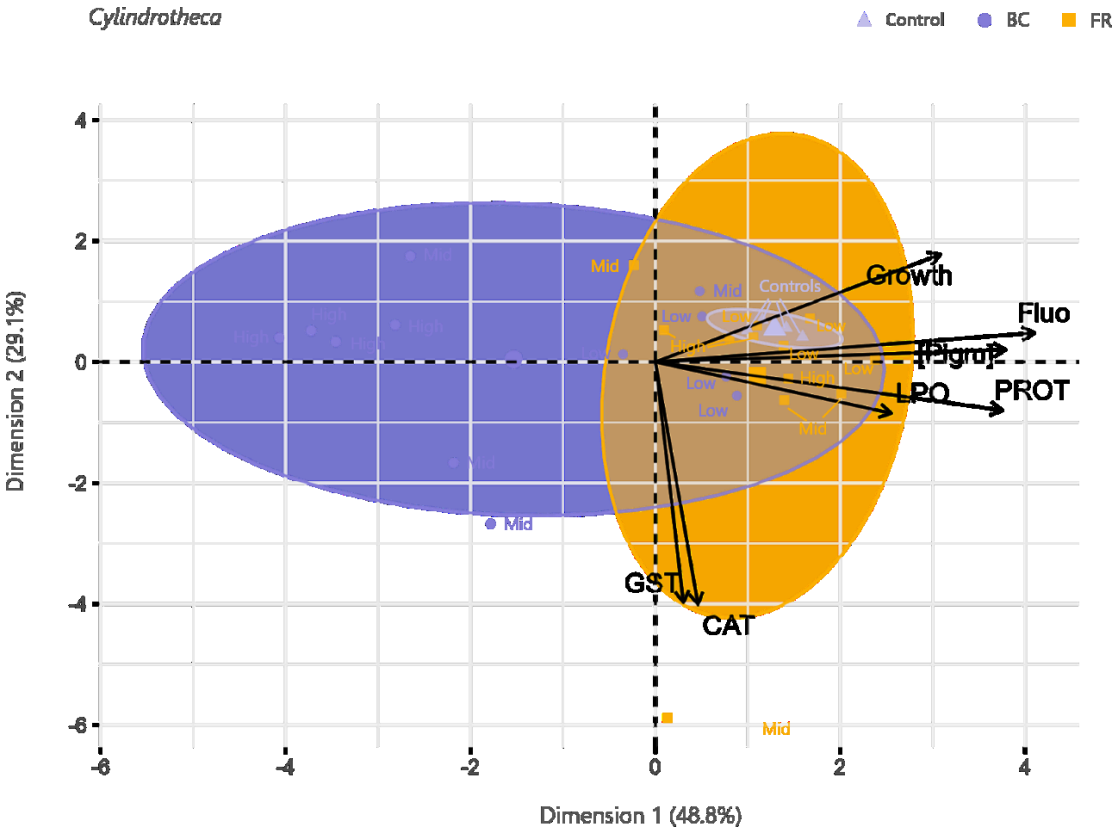


Figure S3. Principal Component Analysis (PCA) plots of toxicity assays with AF lixiviates in *Cylindrotheca* sp. The response variables are indicated with a black arrow and the coating factor is represented with colours: grey = control (C), violet = biocide-based coating (BC) and orange = foul release coating (FR). Concentration is indicated as point labels (scores ID), corresponding to low, mid and high concentrations. The percentage of contribution of each component is also noted.

1.2 Additional toxicity screening assays on target species

Additional toxicity assays were performed in *Monocorophium insidiosum* (Crawford, 1937) and *Mytilus galloprovincialis* Lamarck, 1819 from lab-cultured specimens. Methodological information on culturing and lethal toxicity assay can be found in section '*2. Protocols*' of this annex.

Box S1. Lethal toxicity assays with additional test organisms, both target species: *Monocorophium insidiosum* (Crawford, 1937) and *Mytilus galloprovincialis* Lamarck, 1819.

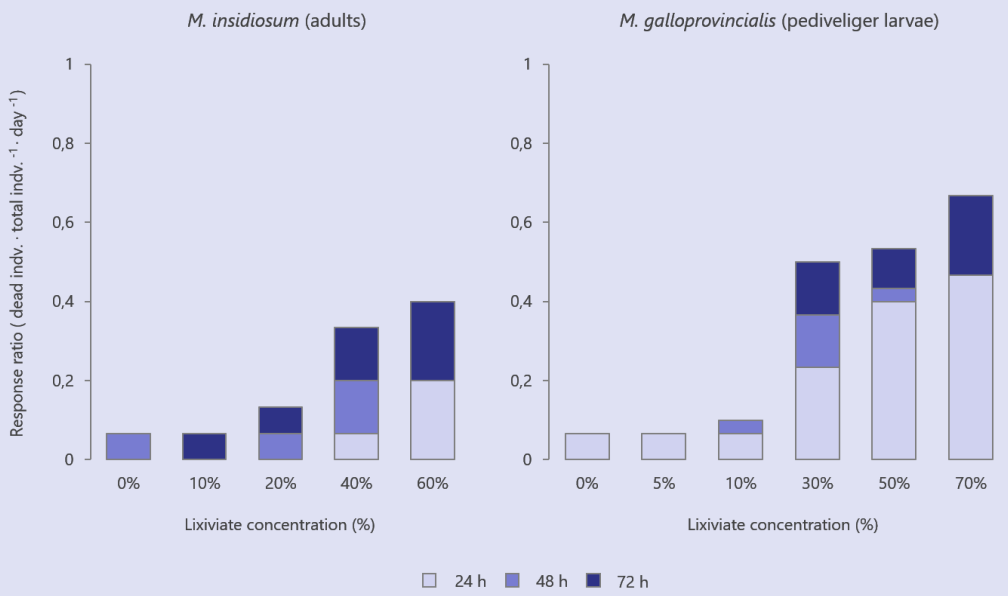


Table S1. Summary of the outcomes of PROBIT analysis. LC50s are highlighted in bold. The asterisk indicates a rare decrease in toxicity due to earlier mortality recorded in controls.

Test species	Time	Prob (P)	n	Dose (%)	LCL	UCL
M. insidiosum	24 hours	50	15	**77,9**	61,6	701
		90	15	107	78	1397
	48 hours	50	15	**102 ***	63,8	-
		90	15	175	101	-
	72 hours	50	15	**66,6**	48,4	145
		90	15	122	83,8	316
M. galloprovincialis	24 hours	50	18	**67,9**	55,5	91,2
(pediveliger larvae)		90	18	125	98,7	181
	48 hours	50	18	**63,7**	51,6	86,1
		90	18	124	97,2	182
	72 hours	50	18	**47,5**	39,8	58
		90	18	92,2	77,1	119

1.3 In-field experiment

Complete species list

Data archived in Mendeley data repository (DOI: 10.17632/w82cx6g48w.1). This section includes a species list of sessile (Table S2) and mobile (Table S3) components collected from PVC plates in the experimental site of La Spezia Gulf (Ligurian Sea) in August 2021 (T1) and February 2022 (T2). These data generate the graphs of community patterns shown in Figure 3 and Figure 6 of the chapter IV. In-field testing.

Table S2. Species list of the sessile components

ID	Original description	Phylum	Code
Un-ID sea anemone		Cnidaria	Actiniidae
Amathia gracilis	(Leidy, 1855)	Bryozoa	Ama_gra
Amathia sp.		Bryozoa	Ama_sp
Amathia verticillata	(delle Chiaje, 1822)	Bryozoa	Ama_ver
Amphibalanus amphtrite amphitrite	(Darwin, 1854)	Arthropoda	Amphi_amphi
Anemonia sulcata	(Pennant, 1777)	Cnidaria	Ane_sul
Anomia ephippium	Linnaeus, 1758	Mollusca	Ano_ephi
Ascidia sp.		Chordata	Ascidia_sp
Ascidiella sp.		Chordata	Ascidiella_sp
Botryllus schlosseri	(Pallas, 1766)	Chordata	Botry_sch
Branchiomma luctuosum	(Grube, 1870)	Annelida	Branchio_luc
Branchiomma sp		Annelida	Branchio_sp
Bugula fulva	(Ryland,1960)	Bryozoa	Bugu_ful
Bugula neritina	(Linnaeus, 1758)	Bryozoa	Bugu_neri
Celleporaria brunnea	(Hincks, 1884)	Bryozoa	Cel_bru
Ciona sp.		Chordata	Ciona_sp
Clathrina clathrus	(Schmidt, 1864)	Porifera	Cla_cla
Clavelina sp.		Porifera	Cla_sp
Clytia sp.		Cnidaria	Cly_sp
Conopeum seurati	(Canu, 1928)	Bryozoa	Cono_seu
Cradoscrupocellaria bertholletii	(Audouin, 1826)	Bryozoa	Crado_bert
Crisia denticulata	(Lamarck, 1758)	Bryozoa	Cri_den
Crisia eburnea	(Linnaeus, 1758)	Bryozoa	Cri_ebu
Crisia sp.		Bryozoa	Cri_sp
Un-ID crinoid		Echinodermata	Crinoidea
Cryptosulla palasiana	(Moll, 1803)	Bryozoa	Crypt_palla
Diplosoma sp.		Chordata	Diplo_sp
Eudendrium recemosum	(Cavolini, 1785)	Cnidaria	Eude_race
Eudendrium sp.		Cnidaria	Eude_sp
Filicrisia sp.		Bryozoa	Fili_sp
Hydroides dianthus	(Verril, 1873)	Annelida	Hydro_dn
Hydroides dirampha	Mörch, 2863	Annelida	Hydro_dr
Hydroides elegans	(Haswell, 1883)	Annelida	Hydro_el
Hydroides sp.		Annelida	Hydro_sp
Janua sp.		Annelida	Janua_sp

Kirchenpaueria halecioides	(Alder, 1859)	Cnidaria	Kirchen_hal
Limaria hians	(Gmelin, 1791)	Mollusca	Lima_lima
Mytilus galloprovincialis	Lamarck, 1758	Mollusca	Myt_gal
Obelia sp.		Cnidaria	Obe_sp
Paraleucilla magna	Klautau, Monteiro & Borojevic, 2004	Porifera	Para_magna
Pennaria disticha	Goldfuss, 1820	Cnidaria	Pen_dis
Perforatus perforatus	(Bruguière, 1789)	Arthropoda	Per_per
Phallusia mammillata	(Cuvier, 1815)	Porifera	Pha_mam
Un-ID porifera		Porifera	Porifera
Protula sp.		Annelida	Protula_sp
Sabella spallanzanii	(Gmelin, 1791)	Annelida	Sab_spa
Sabella sp.		Annelida	Sabella_sp
Salmacina sp.		Annelida	Salma_sp
Savignyella lafontii	(Audouin, 1826)	Bryozoa	Savi_lafo
Schizoporella errata	(Waters, 1878)	Bryozoa	Schizo_err
Simplaria sp.		Annelida	Simplaria_sp
Spirobranchus sp.		Annelida	Spiro_sp
Spirobranchus triqueter	(Linnaeus, 1758)	Annelida	Spiro_trique
Un-ID stolidobranchia		Chordata	Stolidobranchia
Styela plicata	(Lesueur, 1823)	Chordata	Styela_pli
Sycon sp.		Porifera	Sycon_sp
Tricellaria inopinata	d'Hondt & Occhipinti, 1985	Bryozoa	Trice_ino
Watersipora arcuata	Banta, 1969	Bryozoa	Wat_arc
Watersipora subtorquata	(d'Orbigny, 1852)	Bryozoa	Wat_sub

Table S3. Species list of the mobile components

ID	Original description	Phylum	Code
Acanthocardia sp.		Mollusca	Acanthocardia_sp
Achelia echinata	Hodge, 1864	Arthropoda	Ach_echinata
Achelia hispida	Hodge, 1864	Arthropoda	Ach_hispida
Alvania sp.		Mollusca	Alvania_sp
Amphilochus picadurus	J.L. Barnard, 1962	Arthropoda	Amph_picad
Amphipholis squamata	(Delle Chiaje, 1828)	Arthropoda	Amph_squamata
Anoplodactylus sp.		Arthropoda	Anoplodactylus_sp
Apocorophium acutum	(Chevreux, 1908)	Arthropoda	Apo_acutum
Arca noae	Linnaeus, 1758	Mollusca	Arca_noae
Asterina gibbosa	(Pennant, 1777)	Echinodermata	Ast_gibbosa
Calliostoma sp.		Mollusca	Calliostoma_sp
Caprella equilibra	Say, 1818	Arthropoda	Cap_equi
Caprella scaura	Templeton, 1836	Arthropoda	Cap_scau
Caprella sp.		Arthropoda	Caprella_sp
Ceratonereis sp.		Annelida	Ceratonereis_sp
Chondrochelia savignyi	(Kroyer, 1842)	Arthropoda	Chon_savig
Neoischyrocerus inexpectatus	(Ruffo, 1959)	Arthropoda	Cox_inexp
Cymodoce truncata	Leach, 1814	Arthropoda	Cym_trunc
Dynamene edwardsi	(Lucas, 1849)	Arthropoda	Dyn_edw
Elasmopus rapax	A. Costa, 1853	Arthropoda	Elas_rapax
Ericthonius sp.		Arthropoda	Ericthonius_sp
Ericthonius brasiliensis male	(Dana, 1853)	Arthropoda	Eri_brasi (M)
Ericthonius punctatus male	(Spence Bate, 1857)	Arthropoda	Eri_punct (M)
Exogone sp.		Annelida	Exogone_sp
Gibbula sp		Mollusca	Gibbula_sp
Gnathia sp. juvenile		Arthropoda	Gnathia_juv
Grandidierella japonica	Stephensen, 1938	Arthropoda	Gra_japo
Harmothoe sp.		Annelida	Harmothoe_sp
Hexapleomera bultidactyla	Esquete & Fernandez-Gonzalez, 2016	Arthropoda	Hex_bultid
Hexaplex trunculus	(Linnaeus, 1758)	Mollusca	Hex_trunc
Hiatella arctica	(Linnaeus, 1767)	Mollusca	Hiat_arctica
Iphimedia vicina	Ruffo y Schiecke,1979	Arthropoda	Iph_vicina
Jassa slatteryi	Conlan, 1990	Arthropoda	Jas_slatt
Laticorophium baconi	(Shoemaker, 1934)	Arthropoda	Lati_baco
Lembos websteri	Spence Bate, 1857	Arthropoda	Lem_webst
Leptochelidae		Arthropoda	Leptochelidae
Limaria tuberculata	(Olivi, 1792)	Mollusca	Lim_tuberculata
Leucothoe denticulata	A. Costa in Hope, 1851	Arthropoda	Leu_dent
Lysianassa pilicornis	Heller, 1866	Arthropoda	Lysi_pili
Mesanthura romulea	Poore & Lew Ton, 1986	Arthropoda	Mesa_rom
Microdeutopus stationis	Della Valle, 1893	Arthropoda	Microd_stati
Mimachlamys varia	(Linnaeus, 1758)	Mollusca	Mimac_varia
Monocorophium acherusicum	(A. Costa, 1853)	Arthropoda	Mon_acheru
Munna sp.		Arthropoda	Munna_sp
Musculus costulatus	(Risso, 1826)	Mollusca	Musc_costulatus

Nemertea		Nemertea	Nemertea
Nereididae		Annelida	Nereididae
Nudibranch		Mollusca	Nudibranch
Ocinebrina sp.		Mollusca	Ocinebrina_sp
Ophiactis virens	(M. Sars, 1859)	Echinodermata	Oph_ virens
Ophiactis balli	(W. Thompson, 1840)	Echinodermata	Oph_balli
Paracerceis sculpta	(Holmes, 1904)	Arthropoda	Par_sculp
Paranthura japonica	Richardson, 1909	Arthropoda	Par_japo
Perinereis sp.		Annelida	Perinereis_sp
Petricola lithophaga	(Retzius, 1788)	Mollusca	Petr_lithophaga
Phtisica marina	Slabber, 1769	Arthropoda	Phti_mari
Phyllodocidae		Annelida	Phyllodocidae
Platyhelminthes		Platyhelminthes	Platyhelminthes
Polyophthalmus pictus	(Dujardin, 1839)	Annelida	Polyoph_pictus
Pusillina sp.		Mollusca	Pusillina_sp
Stenothoe elachista	Krapp-Schickel, 1975	Arthropoda	Ste_elach
Stenothoe georgiana	Bynum & Fox, 1977	Arthropoda	Ste_georg
Stenothoe valida	Dana, 1852	Arthropoda	Ste_valida
Salvatoria sp.		Annelida	Salvatoria_sp
Sphaerosyllis sp.		Annelida	Sphaerosyllis_sp
Spionidae		Annelida	Spionidae
Syllidae		Annelida	Syllidae
Tellinidae		Mollusca	Tellinidae
Trophonopsis sp.		Mollusca	Trophonopsis_sp
Veneridae		Mollusca	Veneridae

Additional results

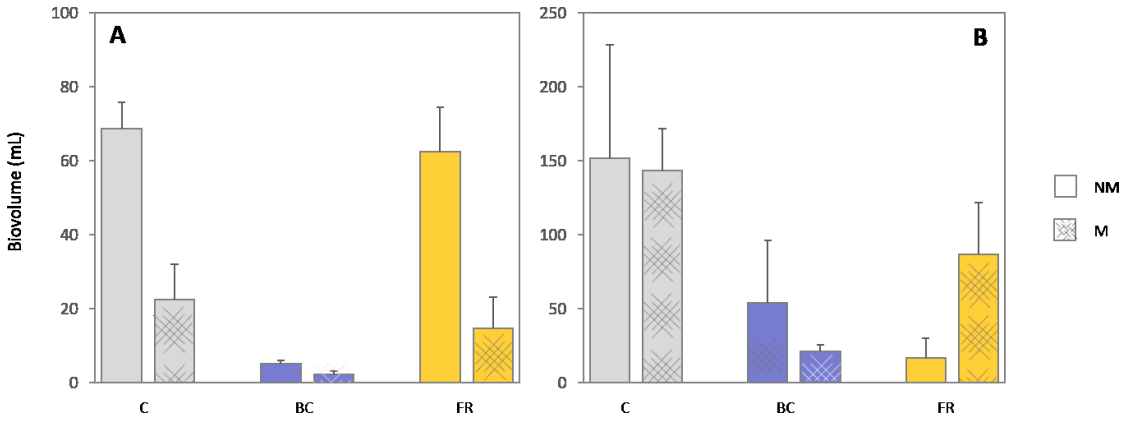


Figure S4. Biovolumes of sessile communities per treatment and time (A = T1; B = T2). Colours represent the coating factor: grey = control (C), violet = biocide-based coating (BC) and amber yellow = foul release coating (FR). Bar pattern corresponds to maintenance practices: plain = unmaintained (NM); patterned = maintained (M). Variability is represented by the standard error.

2. Protocols

2.1. Lab culturing

2.1.1 Microalgae cultures

Axenic stock cultures of *Tetraselmis* sp. and *I. galbana* can be purchased (Marine Science Station of Toralla (ECIMAT)) or, obtained from the Basque Microalgae Culture Collection (BMCC), as *Cylindrotheca* sp. (strain BMCC 385). Stock manipulation is done meticulously in a clean environment (Telstar Mini-V/PCR laminar flux cabinet) to avoid cross contamination. Additionally, material is periodically cleaned with H_2O_2 and glass flasks are rinsed with HCl (37 %) to ensure proper removal of organic rests from prior cultures.

Stocks are initiated in small Erlenmeyer flasks and transferred to larger volumes as they grow, until enough algal amount is reached to start culturing in big reactors (Figure S5). Filtered (0.2 µm) seawater is used at a given volume based on the container capacity and enriched with commercial F/2 media (Fritz Pro Aquatics F/2 algae food), in the ratio specified by the manufacturer (30 mL of each product part per 227 L of culture). Algae cultures are grown in an acclimatized room and kept at 21 ± 2 °C, with an artificial photoperiod of 16:8 under white LED illumination (100 µmol · s^{-1} · m^{-2}) and filtered aeration. Cultures are diluted frequently upon necessity, once they get too concentrated and factors start being limiting. Large cultures are used for feeding purposes and separated stocks are kept as back-up.

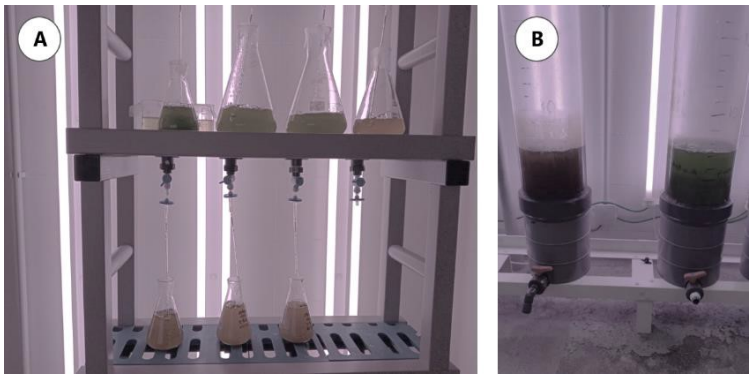


Figure S5. A) Microalgae stock cultures, B) microalgae production in reactors to upscale production for feeding purposes.

Toxicity assays on microalgae

Exposure media was prepared accordingly for each treatment and concentration in a 250 ml Erlenmeyer glass flask. Seawater was filtered by using 0.22 µm pore size nitrocellulose filters (FisherScientific) and sterilized afterwards in a bath at 80°C for an hour. Media was directly added to the experimental flasks, at 1 ml per 1L ratio, using f/2-silica enriched medium ('easy algae f/2

modified + sicilca' product from Fitoplancton marino). The inoculation densities varied for each species and were calculated according to their biovolume (Sun & Liu, 2003). Flasks were kept in an isolated incubator at a constant temperature of 21 ± 1°C, with a photoperiod of 16:8 under a white LED (100 µmol · s-1 · m-2) light and were manually shook three times per day. The duration of the exposure was of 72 h, throughout which daily values of culture growth were measured manually by means of a Neubauer cell-counting chamber.

2.1.2 Copepod cultures

Acartia tonsa culture is initiated from cysts, directly purchased to the supplier Algova ©. Dormant cysts are induced hatching as indicated by the manufacturer. In short, after thoroughly shaking the product, 5 ml were dosed to 1 L of filtered (0.2 µm) seawater with vigorous aeration and a temperature of 23 ± 2 °C. The culture was indirectly illuminated with an artificial light cycle of 16:8. Hatching was checked after 48 h and, if necessary, left another 24 h under vigorous aeration conditions. Once hatching has been confirmed, aeration can be reduced, nauplii transferred to a larger container (4 – 5 L) and fed with small-sized microalgae like *Isochrysis galbana*. After the first week, larger microalgae, such as *Tetraselmis* sp. can be included, in a ratio 1:1, enough to just slightly colour the culture water. Feeding is done once every two days and the evolution of the culture is checked. Cleaning is done once a week using a small syphon to remove ¾ of the culture water, attaching to the end a 45 µm mesh-size to avoid suction of nauplii and larger sized copepod(ite)s (Figure S6). The water was then replaced by clean filtered seawater and, if required the tank was changed for a clean one.

Figure S6. Simplified syphon illustration for cleaning and nauplii collection. Graph created in BioRender

When adult state is reached and start reproducing, nauplii stages are separated from the main culture to a *hatchery*, by collecting them using the syphon (Figure S6), in this case with a 150 µm mesh-size that allowed nauplii to be collected but avoided the uptake of organisms in later developmental stages.

2.1.3 Mussel larvae cultures

Culturing of *Mytilus galloprovincialis* larvae starts by inducing spawning in adult individuals with a heat shock, by changing temperature from 14 to 26 °C. During the temperature change, mussels are individualized in glass containers in a water bath with the desired temperature (26 °C). Spawning individuals are transferred to the initial temperature for about 30 min before starting the fertilization step (Figure S7).

- Female gametes are filtered using a filter with a mesh size of 150 µm and male gametes using a 30 µm mesh size.
- Egg concentration is estimated for each of the females using a Neubauer and sperm activity is checked under the microscope, together with egg quality.
- Optimal egg concentration is 800 eggs/mL, probably requiring dilution of the initial spawned stocks.
- Pools of 3 males are done to fertilize females in a ratio equal to 1 mL of sperm solution per 10 mL of egg solution.
- Fertilization is allowed in glass containers with large surface and a total volume of 150 mL. Finally, fertilization is checked after 30 min and concentration of fertilized eggs estimated using the Neubauer (Figure S7 E).

Larvae are kept at a concentration of 2 larvae/mL in tanks of 25L. The tanks have several windows ranging between 3x8 and 5x10 cm covered with a 30 µm mesh-size nylon filter, which allows water exchange, and are kept under flow through conditions in bigger containers with continuous water replacement (Figure S7 C and D). Larvae are fed every day with microalgae at the specified concentrations by Helm *et al.*, (2004):

- Initial stages (Day 1 to day 5): *Isochrysis galbana* at a concentration of 10000 cell/mL
- Later stages (from day 6 onwards): Combination of *I. galbana* and *Tetraselmis* sp.
- The equivalency between algal cells of different species based on their volume and density (see Helm *et al.*, 2004) is used in the proportion:

  1 cell *I. galbana* = 0.1 cells of *Tetraselmis* sp.

  50000 cell/mL *I. galbana* and 500 cell/mL of *Tetraselmis* sp.
- While feeding (1 hour/day) the tanks water renewal is closed.

The larvae concentration needs to be checked periodically using a glass-counting chamber of 1 mL (Sedgewick Rafter Counting Cell) and a minimum of 4 replicates. For toxicity assays on antifouling larvae at pediveliger are used.

Toxicity assays in pediveliger lavae

When larvae reached the pediveliger stage, individuals were collected for toxicity assays with BC coating lixiviates. The experiment was carried out in 24-well plates, with 5 treatments plus a control and three replicates per treatment. Each well contained 10 individuals. The experiment lasted 72 h and mortality was checked every 24 h by means of an inverted microscope and considering dead irresponsive individuals to gentle pipetting or with no gill aeration. PROBIT analysis was applied to determine the lethal concentration 50 (LC50).

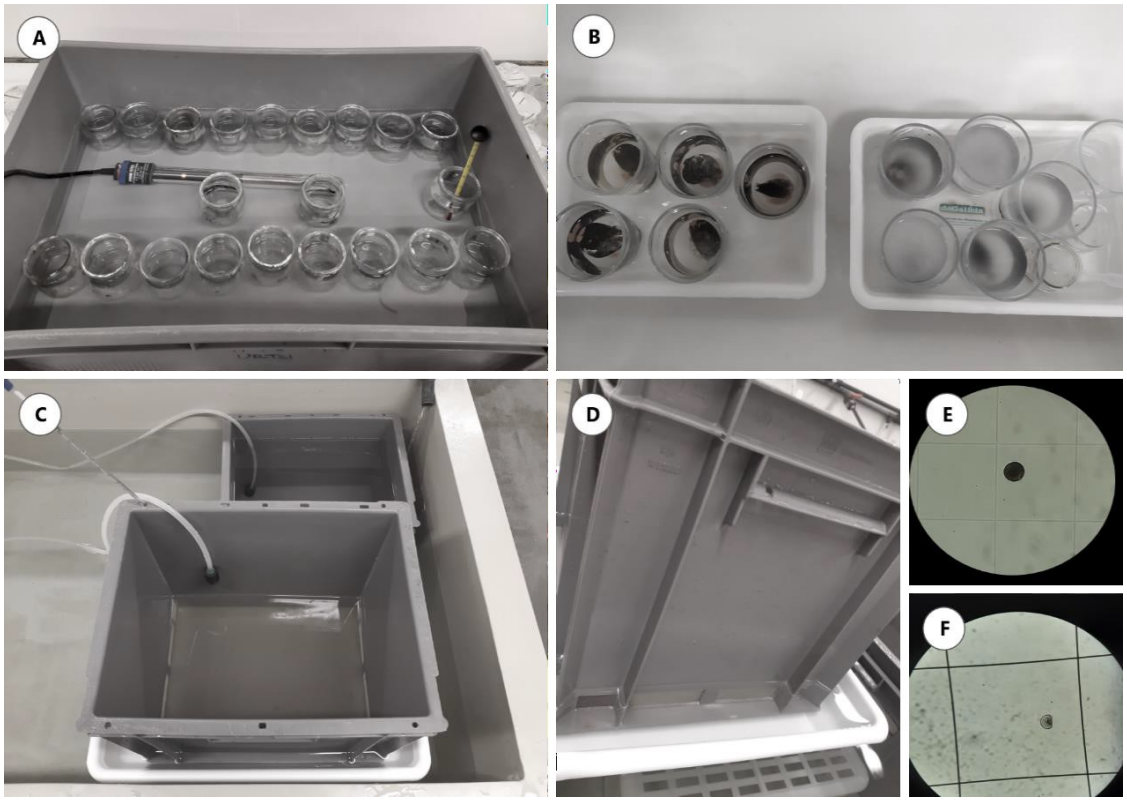


Figure S7. A) Spawning induction in adult mussels, B) spawned females (left tray) and males (right tray), C) larvae rearing tanks in a flow through system, with blocked flow for feeding in the tank lower in the picture, D) larvae rearing tank with the overview of the window (with the nylon filter) allowing water exchange, E) fertilized egg and F) mussel veliger larvae.

2.1.4 Amphipod cultures

Individuals from *Monocorophium insidiosum* are collected from fouling samples of recreational marinas, in particular from deployed experimental units in the recreational marina in Santurtzi, and transferred to the lab. The culture can be started in 2 L plastic containers with large available surface and filtered (0.2 µm seawater). Ten individuals (males and females) were enough to start the culture. Organic detritus from lab aquaria (microalgae agglomerates from lab cultures, mainly) is provided as food source and organic material for tube construction. Additionally, live

microalgae can also be given, as will afterwards be part of the deposited organic material. Pieces of field-collected macroalgae (*Ulva* sp.) can serve as protection and surface for tube construction. The culture is gently aerated and kept at a constant temperature of 21 ± 2 °C, with an artificial light cycle of 16:8. Water is renewed once a week, but the tank is not changed. Feaces and other depositions can be removed gently with a pipette. Once the culture has grown enough (Figure S8 A and C), adult individuals can be used for toxicity assays.

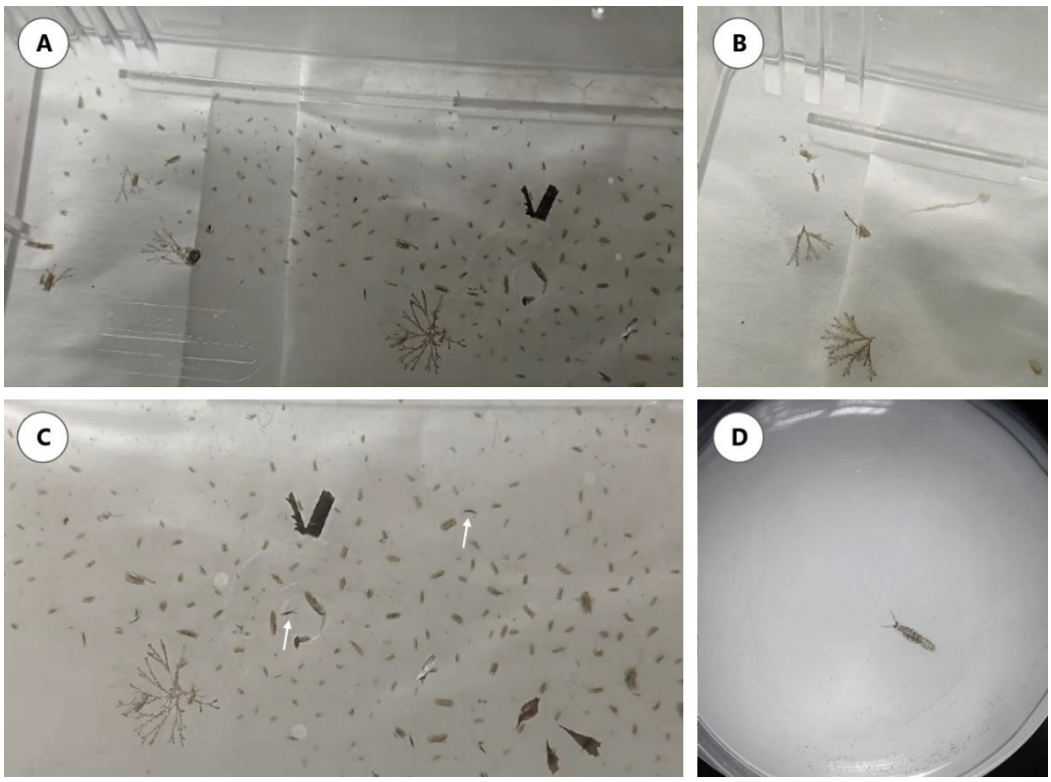


Figure S8. A) and C) grown laboratory culture of *M. insidiosum* showing multiple tube constructions and individuals (white arrows), B) detail of an individual during early stages of the culture, D) isolated individual in an experimental flask.

Toxicity assays in amphipods

Lethal toxicity assays were done to test the toxicity of BC lixiviates in a corophiid species, *M. insidiosum*, whose presence was observed in BC coated experimental units deployed in Spain and which is most available close relative to the resistant corophiid *L. baconi* found in the field experiment performed in Italy. The exposure was done in small flasks (wider than taller) containing a total volume of 30 ml. For the assay, 5 concentrations with 3 replicates each were set. Each replicate contained 5 adult amphipods. The experiment lasted 72 h and mortality was checked every 24 h, considering dead irresponsive individuals to gentle pipetting or, in case of doubt, checked under the stereoscope for gill aeration. PROBIT analysis was applied to determine the lethal concentration 50 (LC50).

2.2. Biochemistry assays in microalgae

2.2.1 Sample collection

Once the exposure period of 72 h was over, the content of the experimental flasks was centrifuged for at least 10 minutes to collect algal pellets that were then weighted (and weights noted), frozen in liquid nitrogen and stored at -80 °C. Centrifugation speeds varied across algal species, being 2700 rpm for *I. galbana* and *Cylindrotheca* sp., and 3500 rpm for *Tetraselmis* sp.

- Weight the empty labelled Eppendorfs / cryotubes. It is convenient to have separated pellets for each procedure, i.e. enzymatic activities and lipid peroxidation.
- Centrifuge large volumes in (labelled) falcon tubes and remove supernatant, refill with the corresponding samples and repeat until the experimental flasks are emptied.
    - In the last cycle, remove supernatant leaving approximately 1 ml and resuspend. Transfer the volume to the corresponding labelled Eppendorf / cryotube and centrifuge again.
    - Remove all the supernatant leaving just the pellet and weight it. Freeze it in liquid nitrogen immediately after and store all samples at -80 °C until analysis.
- IMPORTANT: for enzymatic activities a minimum pellet of 0.025 g is needed and for LPO 0.02 g at least are recommended.

2.2.2 Sample preparation

Start preparing the required reagents:

- K-phosphate 0.05 M (pH = 7.0): add 1.70 g of KH_2PO_4 and 2.175 g of K_2HPO_4 to 500 ml of miliQ water.
    - Tip: since adjusting the pH (using HCl or NaOH) will require adding extra volume is better to mix the components to 450 ml and afterwards add the remaining volume of water.
- BHT = (2,6-Di-tert-butyl-4-methylphenol) 4% in methanol (3,167 g of BHT in 96.01 ml of methanol)

Unfreeze the samples to be processed and add K-phosphate buffer 0.5 M (pH = 7.0) in a ratio 10 ml per pellet gram. Keep the samples in ice and sonicate them in 3 cycles of 10 sec each at a power of 50 W. Avoid sample heating.

After homogenizing the samples, centrifuge them:

- For LPO:
    - Centrifuge homogenates at 10 000 xg for 10 min at 4 °C

    - Collect the supernatant and transfer it to a 2 ml tube. Add BHT (4 % in methanol) in a ratio 4 µl per 200 µl of sample.
- For enzymatic activities:
    - Centrifuge homogenates at 10 000 xg for 20 min at 4 °C.
    - Collect the supernatant and transfer it to a new tube.

2.2.3 Protein quantification

Total protein quantification is necessary to relativize the measurements of enzymatic activities. Quantification of protein content in samples will be done using the Bradford method and based on a standard curve using albumin bovine serum (ABS).

Prepare the reagents and standard curve (which can be done during the 20 min centrifugation of samples for enzymatic activities):

- Start by accommodating Bradford dye reagent (fridge) to room temperature.
- Dissolve 0.1 g of ABS in 1 ml of K-phosphate buffer 0.05 M. Leave it dissolve by itself at room temperature.
- Prepare the dilutions for the standard curve. They will serve as standard samples
- Map the 94-well plate

Prepare the plate:

- Dilute the samples if needed (important to note down in case of doing so).
- Pipette 5 µl of sample (standard samples and test samples) according to the mapping and in triplicates.
- Add 250 µl of Bradford dye reagent in sample containing wells. Remove bubbles.
- Read the plate at 595 nm (the programme includes 5 min plate incubation before the reading)
- Use the equation of the standard curve to calculate protein content in test samples.

2.2.3 Catalase

Reagents need to be prepared fresh the same day of the measurements:

- K-phosphate 0.05 M (pH = 7.0): add 1.13 g of KH_2PO_4 and 5.97 g of $Na_2HPO_4 \cdot 12H_2O$ to 500 ml of miliQ water. Adjust the pH to 7.0 (see above).
- 0.03 M H_2O_2 in K-phosphate 0.05 M (pH = 7.0): 20 ml of the recently prepared buffer + 49.6 µl of H_2O_2 (30 %) for one whole plate. This reagent is photosensitive, cover it with aluminium foil.

Procedure:

- The microplates for this measurement are specific for UV light readings. Map the plate
- Pipette 15 µl of test samples in triplicates, use the initial buffer (of sample prep) for blanks
- Add 135 µl of K-phosphate 0.05 M (pH = 7.0)
- Add the reagent: pipette 150 µl of H_2O_2 0.03M
- Read the absorbance at 240 nm using the programme Gen5 and selecting (or creating) a protocol with the specifications.

Note: the programme and protocol used did not count for readings through time; therefore, the units are not respective of time and act as single snapshots of the activity.

2.2.4 Glutathione-S-transferase

Reagents need to be prepared fresh the same day of the measurements:

- K-phosphate 0.2 M (pH = 7.9): add 8.71 g of K_2HPO_4 and 6.8 g of KH_2PO_4 to 500 ml of miliQ water. Adjust the pH to 7.9 (see above).
- K-phosphate 0.1 M (pH = 6.5): add 4.355 g of K_2HPO_4 and 3.4 g of KH_2PO_4 to 500 ml of miliQ water. Adjust the pH to 6.5.
- 10 mM GSH in K-phosphate 0.1 M (pH = 6.5): dissolve 0.0251 g of GSH (L-glutathione) in 10 ml of miliQ water. Keep it on ice.
- 60 mM CDNB in ethanol –Dissolve 0.0827 g of CDNB (1-chloro-2,4-dinitrobenzene) in 20 ml of 99% ethanol. Add 30 ml of ultrapure water. Photosensitive reagent, cover it with aluminium foil, keep on ice and always make fresh. Careful when managing the CDNB.

Procedure:

- Map the plate and prepare the reaction solution:
    - 25 ml of K-phosphate 0.2 M (pH = 7.9) + 750 µl CDNB 60 mM + 750 µl of GSH
- Pipette 50 µl of test samples in triplicates, use the initial buffer (of sample prep) for blanks
- Add 250 µl of the reaction solution
- Read the absorbance at 340 nm using the programme Gen5 and selecting (or creating) a protocol with the specifications.

Note: the programme and protocol used did not count for readings through time; therefore, the units are not respective of time and act as single snapshots of the activity.

2.2.5 Lipid peroxidation

Reagents need to be prepared fresh the same day of the measurements:

- TCA 12 %: dissolve 1.2 g of TCA in 10 ml H_2O (enough for 20 samples)
- 60 mM Tris-HCL with 0.1 mM DTPA: dissolve 1.89 g of Tris-HCl and 0.0007 g of DTPA (diethylenetriaminepentaacetic acid) in 200 ml miliQ water.
- TBA 0.73 %: dissolve 0.365 g in 50 ml miliQ water (enough for 100 samples). Place it on a heated shaker to dissolve it better.

Prepare the samples by adding to 2 ml tubes:

- 150 µl of the homogenate reserved for LPO (already mixed with BHT)
    - Use the initial buffer (sample prep) as blank
- 500 µl of cold TCA 12 %
- 400 µl of Tris-HCl 60 mM
- 500 µl of TBA 0.73 %

Procedure:

- Place the tubes (open, with loose caps during boiling) at 100 °C for 60 min.
- Cool the samples in ice
- Centrifuge the tubes at 11500 rpm for 5 min at 25 °C
- Take all the supernatant without removing the pellet and transfer it to new Eppedorfs, mix them gently by pipetting
- Transfer 300 µl of supernatant in triplicates to a microplate.
- Read the absorbance at 535 nm

2.3 Differential expression of target genes in copepods

2.3.1 Exposure and sample collection

Once the exposure time is over, copepods are collected using a 200 µm mesh-size filter and preserved in RNAlater (Invitrogen, Thermo Fisher Scientific), immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Samples contain at least 35-40 copepods.

2.3.2 RNA extraction

RNA extraction is carried out using RNeasy Minikit (Qiagen) and following the protocol by the manufacturer. All the RNA extraction steps are done at room temperature.

Samples are left unfreeze in ice. Using syringe with a small needle, carefully remove the RNA later from the cryovials without removing copepods in the process. To do so, place the needle at side of the wall of the cryovial and then start extracting the RNA later carefully. If a copepod is taken in the process by the syringe, push it back in the cryotube and try extracting the RNA later again.

After removing the maximum amount of RNA later, add 600 µl of RLT lysis buffer and beads for tissue homogenization:

- Homogenize the samples using a Precelis tissue disruptor (Precellys 24, Bertin Technologies) at 6500 rpm during 45 seconds twice
- Place the samples in ice for 4 – 5 minutes to let them cool
- Perform another two cycles of 45 seconds each at 6500 rpm and place them in ice

Centrifuge the samples for 3 minutes at maximum speed, collect the supernatant into new RNA-free tubes, add 1 volume of ethanol 70 % to each sample and mix it thoroughly by pipetting.

Mount the RNeasy spin columns in 2 mL collection tubes and label them accordingly. Add 700 µL from each sample and centrifuge at 8000 G for 15 seconds.

- Empty the collection tubes
- Repeat this process to get the maxim yield with the remaining supernatant from the previous step using the same spin column.

Start the washing steps of the procedure:

- Add 700 µL of RW1 buffer to the spin-column (pink) and centrifuge at 8000 G for 15 sec. Discard the flow through from the collection tube.
- Add 500 µL of RPE buffer to the spin-column and centrifuge at 8000 G for 15 sec. Discard the flow through from the collection tube.

- Add 500 µL of RPE buffer to the spin-column and centrifuge at 8000 G for 2 min. Discard the flow through from the collection tube.
- Optional step: place the spin-column into a new tube and centrifuge for 1 min at 8000 g in order to dry the membrane.

Collect the RNA:

- Place the spin-column into a 1.5 mL collection tube.
- Add 30 µL of RNAse free water and centrifuged at 8000G for 1 min to elute the RNA collected.

2.3.3 RNA quality check and quantification

It is carried out by using Aligent RNA 6000 Nano Kit. The full manufacturer's protocol can be found here: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90034_RNA6000Nano_KG.pdf

Before starting we need to be sure that the gel and the ladder are prepared. The ladder is prepared upon the arrival of the kit, by pipetting the ladder in RNase- free vial and heat denaturing it for 2 min at 70 °C, and immediately cooling it down in ice. Prepare aliquots and store them at -70 °C.

The gel preparation is done based on usage and once prepared lasts for a month. Allow all reagents to equilibrate to room temperature for 30 minutes before use.

- Place 550 µl of Agilent RNA 6000 Nano gel matrix (red cap) into the top receptacle of a spin-filter (in the kit, the Eppendorf with a membrane).
- Place the spin-filter in a microcentrifuge and spin for 10 minutes at 1500 g ± 20 % (= 4000 rpm for Eppendorf microcentrifuge).
- Aliquot 65 µl filtered gel into 0.5 ml RNase- free microfuge tubes that are included in the kit. Store the aliquots at 4 °C. Once prepared the gel lasts for a month in good conditions.

In order to use it, we need to mix it with a dye (photosensitive, to be used in that same day).

- Allow all reagents to equilibrate to room temperature for 30 minutes before use. Protect the dye concentrate from light while bringing it to room temperature. Once tempered, vortex the dye concentrate (blue cap) for 10 sec.
- Take one of the filtered gel aliquots of 65 µl and add 1 µl of dye. Store back in the dark the gel concentrate.
- Vortex thoroughly and visually inspect proper mixing of gel and dye. Spin tube for 10 minutes at room temperature at 13000 g (= 14000 rpm for Eppendorf microcentrifuge).

Once ready, the chip can be loaded and can host up to 12 samples each.

- Place the chip on the chip priming and pipette 9 µl of the gel- dye mix at the bottom of the third well (Figure S9, marked with a circled G) and dispense the gel- dye mix.
- Make sure that that the plunger is positioned at 1 mL and then close the chip priming station. Set the timer to 30 seconds and press air into the well until the metallic holder keeps it in place. Start the timer for 30 sec. Release the mechanism once the time is over and wait for 5 sec. The chip is now primed.

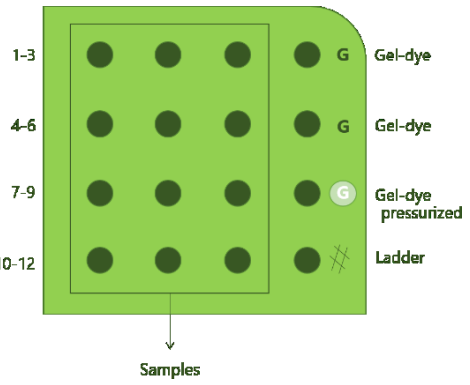


Figure S9. Schematic representation of the chips of the Aligent RNA 6000 Nano Kit, with well names and sample location.

- Add 9 µl of the gel- dye mix to the upper two "G" wells (Figure S9).
- Add 5 µl of the marker (green cap) into each of the 12 sample wells and the ladder well. Do not leave empty wells. Unused wells need to be loaded with 5 µl of marker plus 1 µl of buffer that substitutes the sample.
- Before using, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process). To minimize secondary structure, heat denature (70 °C, 2 minutes) before loading on the chip and afterwards, pipette 1 µl of the RNA ladder into the well marked with the ladder symbol
- Pipette 1 µl of each sample into a well. Map the sample distribution.
- Set the timer to 60 seconds. Place the chip horizontally in the adapter of the IKA vortex mixer and run it for 60 sec at 2400 rpm. Start the analysis in the Bioanalyzer within the next 5 min.

Running the Bioanalyzer:

- Clean the electrodes with the cleaning chips, first filled with RNA zap (500 µl) and then with RNA free water (500 µl).
- Place the chip carefully into the receptacle and close the lid. The chip fits only one way.
- Select the assay method in the 2100 Expert programme (Eukaryotic total RNA nano series) and start reading.
- Do not forget to clean it after use with the cleaning chips as above.

2.3.4 First strand cDNA synthesis

This procedure for synthesis of cDNA considers the concentration of RNA of each sample (quantified by the previous section). Total RNA volume plus RNA free water volume make 12.7 µl

and their relative volumes were calculated to reach a final concentration of 50 ng / µl. Additionally 3 µl of random primers are added, making a final volume of 15.7 µl. The final samples are incubated at 65 °C for 5 min and cooled down at room temperature for 10 min (cDNA RT-I; last step of the programme is 10 min at 25 °C).

For the final reaction, the following components are added: 1 µl of RT buffer, 0.8 µl of dNTPs, 0.5 µl of RNAse block and 1 µl of RT enzyme (summing up 4.3 µl and making20 µl the final volume). This step can be simplified by preparing a master mix considering the volumes of the components and the total number of samples. Mix gently and incubate at 40-55 °C for 60 min (cDNA RT-II) and, finally, the reaction was terminated by incubating at 70 °C for 15 minutes and samples stored at -20 °C).

2.3.5 Real time q-PCR

Real-Time quantitative PCRs (RT-qPCR) is conducted in MicroAmp Optical 384-well reaction plate in ViiA7 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, USA), using FastStart Universal SYBR Green Master (ROX) mix in a total reaction volume of 10 µl.

Allow all samples and reagents (SYBR Master) to equilibrate to room temperature in ice for 30 minutes before use. The SYBR dye reagent is photosensitive and needs to be protected from any light source. The components for the reaction include: 5 µl of SYBR MM, 0.2 µl of target gene primer (0.1 µl forward and 0.1 µl reverse at a concentration of 80 pmol / µl), 2.8 µl of RNA free water and 2 µl of cDNA (sample).

- Calculate the corresponding volumes of SYBR mix, primers and water based on the total amount of samples. Consider the non-template controls (NTCs) and some extra samples to compensate for pipetting error.
- Mix the components maintaining the ratios into a unique master mix (MM) and keep it on ice protected from light.
- Map the plate, noting down the wells that will be used (samples in triplicates) and their distribution in the plate. Count for extra wells without sample as NTCs.
- Pipette 8 µl of the master mix to each of the wells to be used, including NTC wells. Make sure the MM does not stay in the walls of the well.
- Add 2 µl of cDNA from each sample in triplicates (using the stepper of the automatic pipette), following a mapped plan.
- Cover the plate with the Optical Adhesive Film, avoiding the formation of bubbles. Place the plate on a shaker for a minute.

- Place the plate in the ViiA7 Real-Time PCR System. Select the method SYBR, the reaction volume and melting temperature (Tm) of the primer. The programme allows to map the plate by specifying the gene(s), the sample wells and their labels, as well as the NTC wells.
- The amplification conditions are 50 ◦C for 2 min, 95 ◦C for 10 min, followed by 40 cycles of 95 ◦C for 15 s, and then 1 min of annealing at the corresponding Tm of each primer.

Prior to the quantification in the actual samples, a standard curve is prepared and used to calculate the amplification efficiency of the primers under the given amplification conditions. Therefore, per gene, one standard curve is required using a pool of cDNA from randomly selected samples. Usually, 6 curve points are enough and are done by serial dilutions of the pooled samples (1:2 to 1:32). The procedure goes as described above but instead of samples, the pooled sample and its dilutions are used, in triplicates.

2.3.6 Primer design, PCR and electrophoretic determination of amplification

Chitin deacetylase was the only gene designed *de novo* using Cluscal Omega from alignments of *A. tonsa* genome sequence (accession number LS044924.1) and the corresponding annotated gene of *Acartia pacifica* (accession number KT754625.1), searching for conserved regions, highlighted in the sequence of *A. tonsa* below (Figure S10). After blasting the sequence of A. pacifica against the whole genome of *A. tonsa*, a conserved region was identified and the primer was design based on the segment highlighted in blue, using Eurofins' Primer Design tool.

Successful primer design and amplification can be checked using cDNA samples of *A. tonsa* and standard PCR in a total reaction volume of 25 µl. The components for the reaction included: 2.5 µl of x10 buffer, 0.5 µl of dNTPs, 0.75 of Mg chloride, 0.5 µl of primers (i.e. 0.25 µl forward primer and 0.25 µl reverse primer at a concentration of 80 pmol / µl), 0.25 µl of Taq polymerase, 18 µl of water and 2.5 µl of cDNA.

- Prepare the reaction for each sample by mixing the components as stated above.
- Place them in the Thermal Cycler and select the programme. The amplification conditions are 95 °C for 2.5 min, followed by 40 cycles of 30 seconds at the corresponding Tm of each primer, and then 5.5 min at 72 °C.

Once the PCR is over, the samples are run in an electrophoresis gel to confirm successful amplification of the designed primers.

- Mix 1.5 g of agarose with 100 ml of diluted TAE(x1) buffer, remove it properly and microwave it for 1 minute. Afterwards, add 10 µl of dye (Safe-Green DNA stain).

- Mount the gel tray, including the comb(s) for the samples, and pour the gel into it. Let it cool down for at least 30 minutes
- Remove the structure and the combs and submerged the solid gel into the TAE buffer (x1) in electrophoresis device.
- Prepare the samples. Cut a piece of parafilm and pipette drops of 2 µl of loading dye (nº of samples plus a blank). Set an order (sample mapping) and add 5 µl of sample to each of the dye drops, mixing them properly by pipetting, leaving one blank.
- Start by loading the ladder into the gel by pipetting 5 µl into the first cell created by the comb. Continue loading the blank and samples drops into the gel in the following *cells*, according to the established order.
- Connect the cables and start running the gel. Run time can vary based on the size of the sequence, for big ones 20 mins can be enough to see clear bands corresponding to the amplified sequence.
- Visualize the results using the gel imager system (GBox, Syngene).

Once successful amplification is confirmed, the exact sequence of chitin deacetylase in *A. tonsa* (see below) was obtained using Sanger Sequencing (General Genomics Service Sequencing and Genotyping Unit; SGIker, UPV/EHU) (Figure S11) and published in GeneBank (accession number PP595814.1). Primers were redesigned using the exact sequence.

Figure S10. Result of sequence alignment (*A. pacifica* gene against from *A. tonsa* full genome). The sequence corresponds to *A. tonsa* with identical nucleotide matches highlighted. In yellow with lower density of identical matches and in blue with higher, therefore used for primer design.

TGTCCTTGTAAGCATAGGGGCCCATCAATCCCTTGGGGTCCTTCACCACGGTCCAACCT
CCCTGCTTGATATCAGCACAGATTTCGTAGTAGGCGAGGAAGCCGGCGGCACGGGTGAA
CTCACCGGCCTTGCCCTTCTGGCCGGCGGGCGCGTTGAGGTCGTGGTTGCTGGCGCTGT
CCAGGGTGAAGGCCTGGCCGTACAGTGGCATACCCATGACTAGCTTGTTGCGAGGAGCT
CCAGATTCGATCCAATAGTTGATGGTGTAATTCGTGTTGAAGTAGAAATATTCATCTTC
AGGGTGTTCATAGAAAGGAGCCACATGCCCTGTCTTCTTGTCCCAATGTCCATGATAGT
CGTAGGTCATCACAGCAATCCAGTCCAGGTCACGGGCGATGCTAGGAATATCATAGCCA
ACATCCATAATCTTCTTGCTGGGGGACACGGCTGCGGAGAGAAGGTATCCGCGGGGCGC
GAACGCCTCCTTCAACTCCCTGACCCAGGCGGCAAAGGCATCCTTGTCCTTGTACCTCT
CCTCCTTGCATTCAGTCTGCCAGCAGCTGGGATACTCCCAGTCAAGGTCCAGACCGTCA
AAGTTGTATTTGTGGATGAAGTCCATGACGTGTTTGATAAACTTGGCCCGGGCGGACGG
ATTGTTGACCAACCTACTGTACTTATCGCCCTGGCTGTCGTTCCAACCGCCAATAGCGA
CGGTAACCTTGATGCCGTACTTTTTAAATTCAGTGACCTTGCCGTAGAAGTCATTATCA
ATATCAGCCCATGAATCATGGGGCTTGAGCAGCAAGGTAGAGTAGTCAAGAACAGCAAA
TCCATAAACAATATGGGTACAAATGGTTGGGTCAATATCATCAGGTTTGTACTTTCCAA
TACCAGGCCTATACCAAGCCCAGTTGGTGAAGTAGCAGACAACCTTGTAGTCACCAGAC
AAGGGGCCCTGCAGGGGTGCAGCCTCGGTGGTTCCGGTAAATGGCTTCAATGGCTTTGG
TGTTGATGGCTTCCATGCTCCTTCATAATCTCCACTTTCCCATTGGCCGTTGTCGTAGT
CTCCACTCTCCCACTGGCCATTATCATAATCATTACCATTGTTGTTGTTGTCAGTGTTT
TCTCCACCACCCACTGATTCTACGCTTGTTGAGCTGTCACCATTGGAGGAATCGCCACC
GCCGTTGGAGGTACATCCCGCCAGTTCAGCATGGTTACATGACATGATTTTGGTGTCCC
AATGAGTACCTGCCTGACACTGGAACTTCTCAAACTTGCCGTGAACACAGAAAAGATAT
GATCCGCAATCATTAGGATTGGCCTGGAAGGCTCCTTCTTGGCAGGCGGCCTTGACAGT
CTTGTCATCCTGGTATACTTTACCTTCACAGCCAGCATTGATCGGCCAGTCACACAGGC
TCTTGGAGGCGCTGAAATGAAGTCCAGGGCTGCAGGATTGCTTCAGCTGGGCACCGTTC
ACGCACTGGTAGAATGAGGAACAGTCGCCAGGAACGTTGGAGTATTCGTTGCCGGAGCA
GGAGCGAGCTATCTCGCTGATGACAGCAATACCGACAGATTCAATTTCGTTATTCTCAA
CAATAATAGCTTCGTCCTCATGGTTAAAGATTGATCTTCTGTGACACTTGACATTATCC
TCCCAGTCACAGGTGAGTGCTAGTTGATCCCAGGCAAGGCCACCAGCACAAGAGTGAAG
CACCTTCACACCGTTCAAACATCTGTAATATTTCTGGCAGCTAGCAGGATGTCTGTAGT
ACTGTCCTTCCTGGCACTCTCCCTGTCCTCCTAAATTTTCTGCTGTAGTTTTGGTTGTT
GTTGTTGTAGTAGTAG

Primers
Fw = AGCATTGATCGGCCAGTCACAC
Rv = TCCGGCAACGAATACTCCAACG

Figure S11. Final exact sequence of chitin-deacetylase in *A. tonsa* and redesigned primers. GeneBank accession number PP595814.1

3. Supportive material

3.1 Community analysis

The effects of coating typology, maintenance practices and seasonality in biofouling dynamics were examined. To do so, fouling communities are obtained from passive collectors, namely PVC plates, using a brick as ballast (Figure S12), following the SERC protocol (Marraffini *et al.*, 2017; Tamburini *et al.*, 2021). Before submersion, PVC plates are treated with BC or FR paints, or not painted for control (Figure S13). Half of them were maintained, i.e. periodically cleaned with a sponge, resulting in the experimental design shown in Figure 2 of the chapter IV. In-field testing.

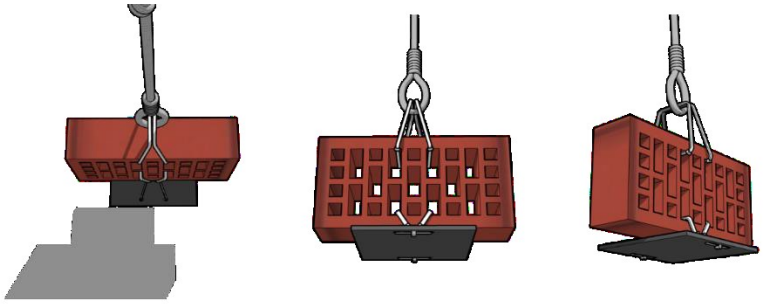


Figure S12. Three-dimensional schematic representation of the experimental units, following the original Smithsonian Environmental Research Center (SERC) protocol for the monitoring of fouling communities in ports. Images were created using the software SketchUp (credit to Fabian J. Koppes)

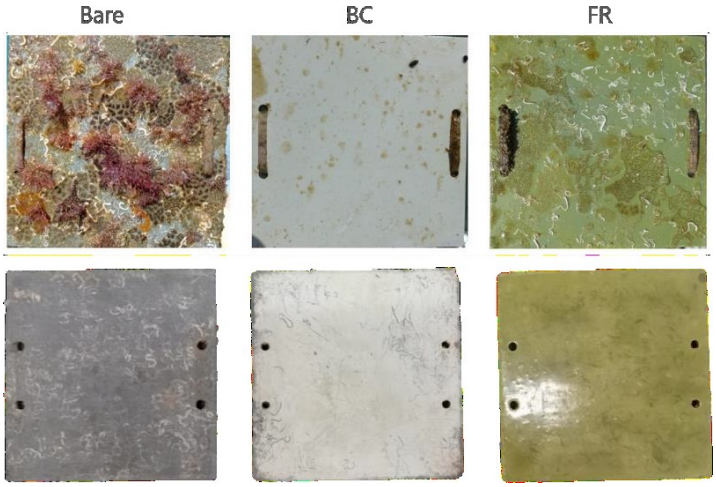


Figure S13. Pictures of plates after one month deployment (upper row) and after laboratory analyses and scrapping (lower row).

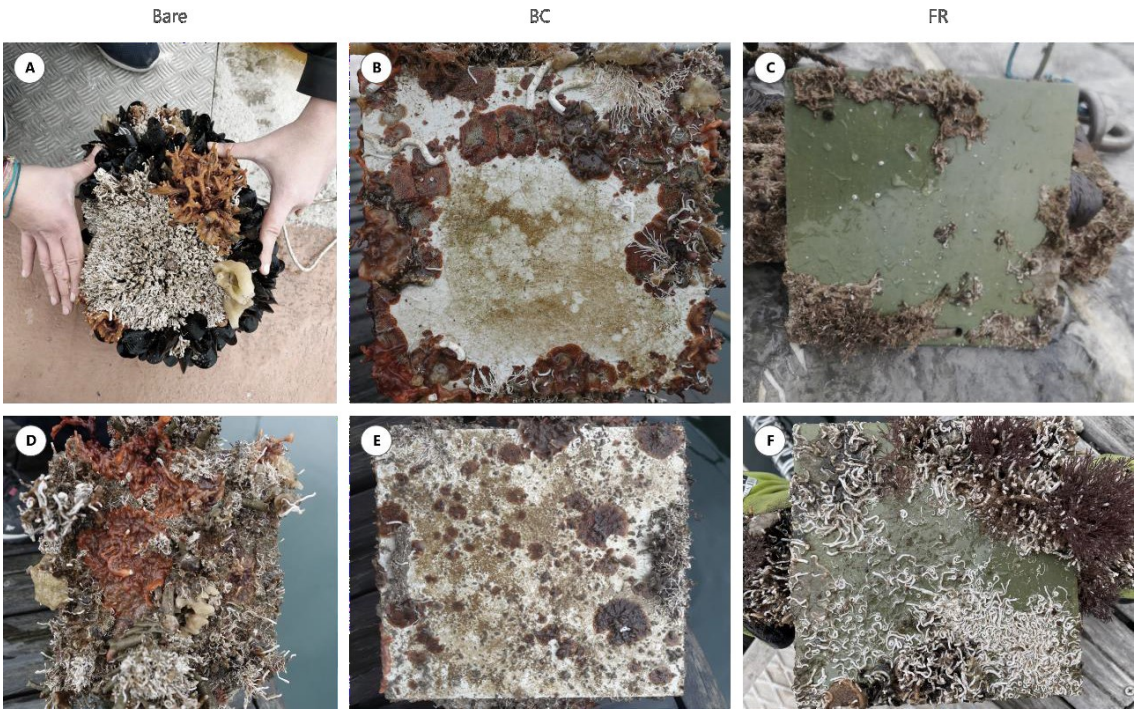


Figure S11. Selection of field pictures showing differences among treatments, including growth on BC coated plates and the facilitator effect (B and E) and self-cleaning effects in an FR coated plate (picture C).

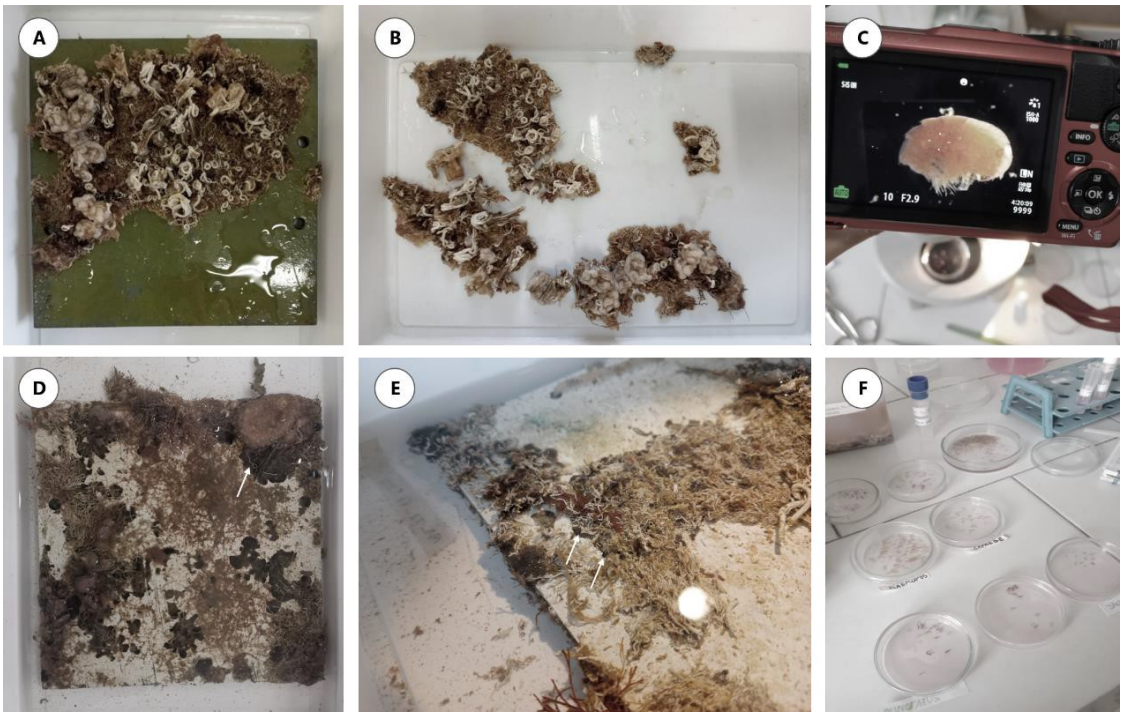


Figure S15. Selection of lab pictures. A) and B) correspond to FR treatments and show easy detachment in pieces of '*community sheets*'. D) and E) correspond to BC treatments, showing important coverage after 9 months and the facilitator effect by mats of incrusting bryozoans (white arrows). C) and F) are snapshots of mobile community identification process.

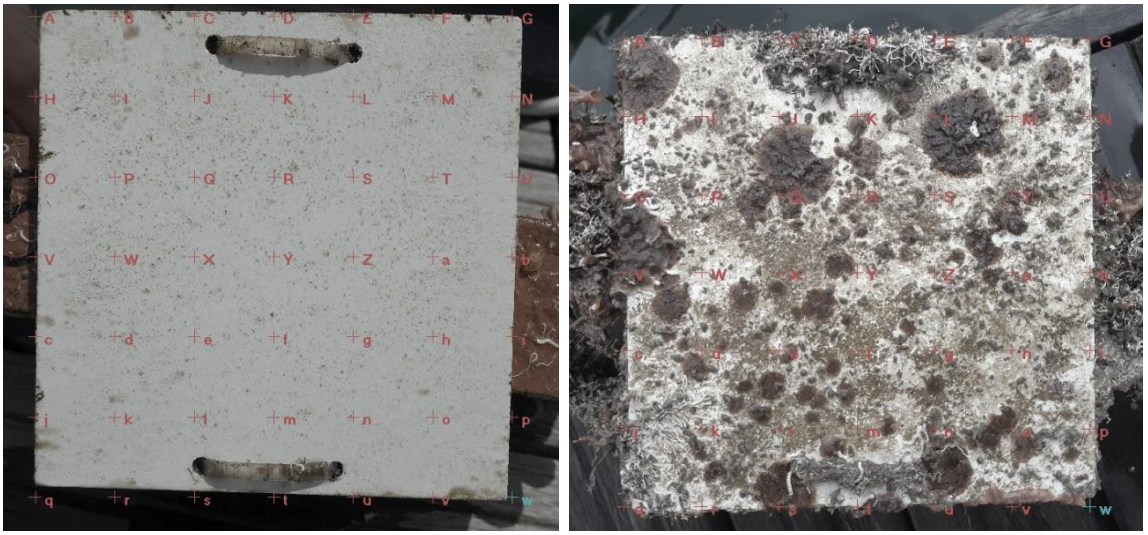


Figure S16. Uniform gridded (7 x 7) design showing the point display for image analysis in the software CPCe (Coral Point Count with Excel extensions) (Kohler & Gill, 2006). Based on a code created for the purpose, specific entries can be assigned to each point. For the analysis of coverage through time, the entries `cover`, `bare` or `slime` were employed and data was then used to calculate the percentage coverage of each plate per month. The picture on the left belongs to a BC treated plate after one month of submersion (month of June), mostly covered by a fine slime. The picture on the left belongs to the same plate after three months of submersion (month of August).

Table S4. Normalized metal concentrations (in µl · L^{-1}) in lixiviates prepared under different incubation scenarios (from the chapter I. Chemical characterization).

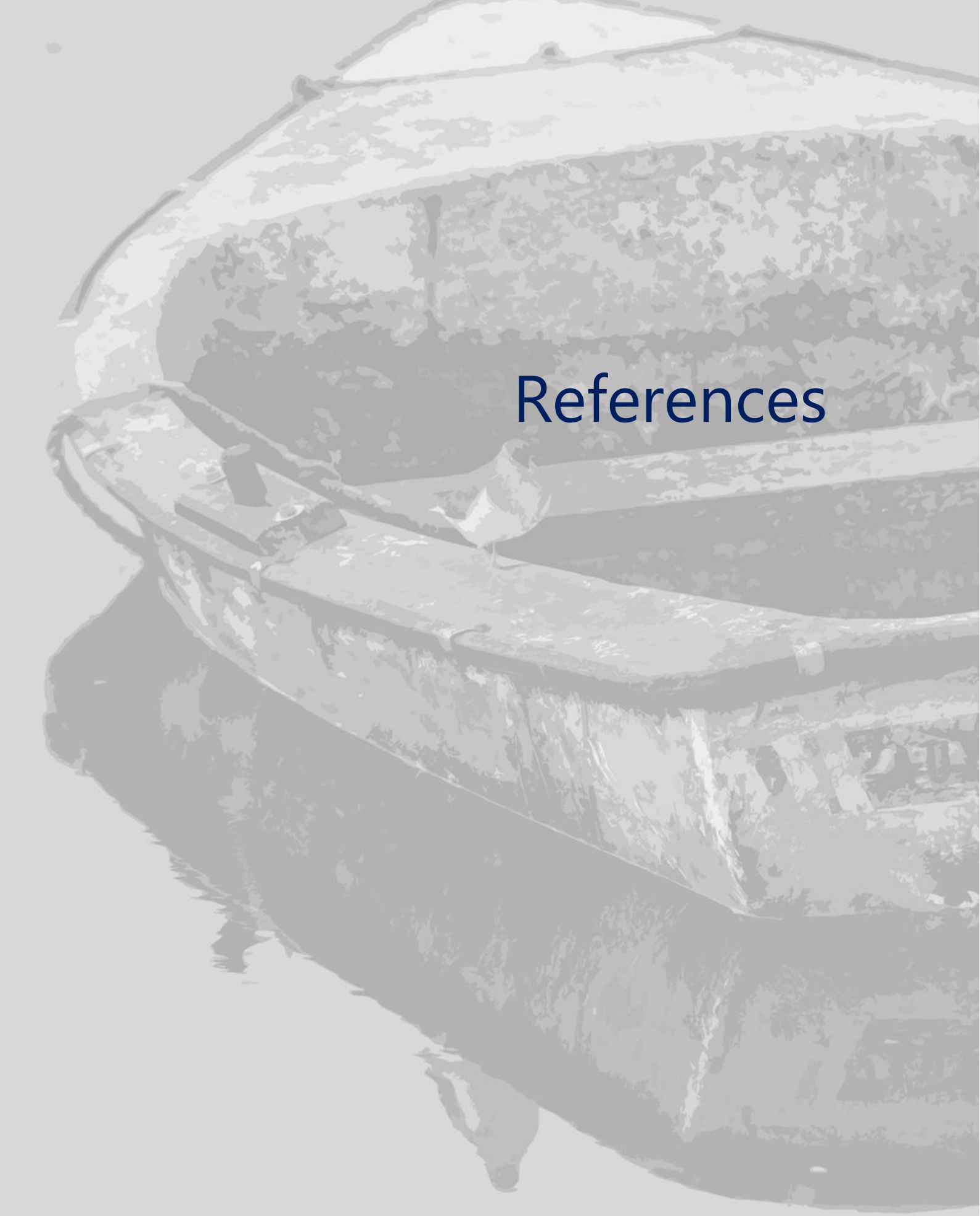
Coating	Temperature (° C)	Salinity (PSU)	[Cu]	[Zn]	[As]	[V]	[Cr]	[Mn]	[Fe]	[Ni]	[Li]	[Mo]	[Sn]	[Sb]	[Ba]	[Pb]
BC	30	33.5	1617.744	507.974	28.024	2.623	0.320	0.540	1.899	1.421	177.083	13.347	0.379	1.035	23.421	0.835
	30	33.5	1778.364	630.360	34.040	2.710	0.385	0.668	2.135	0.145	218.705	18.420	0.269	1.871	30.193	1.002
	30	33.5	1769.535	646.118	29.525	2.571	0.371	0.870	7.519	0.766	198.961	16.378	0.372	2.265	26.441	1.552
	14	33.5	2243.733	544.085	39.296	2.312	0.396	0.730	1.149	0.279	216.128	19.374	0.260	3.271	29.299	0.642
	14	33.5	2178.248	506.304	33.371	2.374	0.420	0.834	9.042	0.392	198.843	19.062	0.313	3.500	28.417	1.090
	14	33.5	1422.857	315.525	26.055	1.546	0.180	0.373	0.689	0.000	127.881	10.249	0.122	1.494	17.263	0.576
	20	33.5	1628.233	353.341	21.579	2.299	0.306	0.611	5.007	1.089	212.142	17.459	0.317	0.794	27.977	5.400
	20	33.5	2192.868	431.326	28.761	2.867	0.868	0.834	6.201	1.241	252.170	21.270	0.418	2.409	33.123	1.873
	20	33.5	2321.050	371.435	26.037	2.596	0.875	0.736	3.632	1.791	247.570	21.000	0.530	2.883	34.030	2.332
	20	37.5	2409.446	522.206	34.085	3.234	0.582	41.538	3.655	2.046	256.808	20.065	0.293	2.046	94.984	4.045
	20	37.5	2622.671	512.510	42.147	3.604	0.509	44.333	7.232	2.503	267.973	20.986	0.350	2.566	100.745	4.837
	20	37.5	1930.598	410.470	30.439	3.173	0.440	35.635	8.682	1.843	213.729	16.166	0.320	1.453	80.262	4.573
	20	20	1306.251	473.374	19.832	1.879	0.278	0.719	1.842	1.049	146.806	12.740	0.265	1.300	28.503	0.718
	20	20	1187.461	304.051	17.209	1.340	0.249	0.714	1.359	0.751	116.652	10.322	0.273	1.830	21.368	0.990
	20	20	1392.091	461.636	17.245	1.570	0.317	0.918	1.422	1.098	139.901	12.670	0.209	2.302	25.852	1.202
FR	30	33.5	186.434	196.701	85.983	6.508	132.281	1.277	5.670	0.289	227.862	18.205	0.846	0.856	36.474	3.338
	30	33.5	276.102	189.390	87.101	4.568	155.726	1.141	2.763	0.187	224.260	19.243	0.886	1.714	33.962	2.064
	30	33.5	292.334	282.815	78.205	4.423	244.304	1.226	3.203	0.598	242.933	21.234	0.811	1.801	37.390	2.135
	14	33.5	585.199	150.184	25.777	1.582	112.999	0.459	12.375	0.184	77.501	6.457	0.277	0.470	17.209	1.504
	14	33.5	432.623	203.461	17.638	1.835	108.741	0.287	2.744	0.300	95.204	6.981	0.340	0.202	19.070	1.731
	14	33.5	329.947	196.870	18.180	1.817	130.545	0.129	1.581	0.181	85.170	6.346	0.200	0.171	17.064	0.827
	20	33.5	274.506	189.753	66.241	3.910	116.711	0.871	1.948	0.315	189.668	17.358	0.489	1.733	28.255	16.244
	20	33.5	572.849	166.701	51.387	3.220	46.062	0.995	1.795	0.185	244.182	22.940	0.622	3.037	33.467	6.334
	20	33.5	613.071	208.015	43.786	2.946	107.339	1.050	3.104	0.272	223.936	21.829	0.554	3.626	32.184	3.282
	20	37.5	100.009	149.653	65.003	5.883	90.434	46.705	10.205	0.023	205.791	14.027	0.444	0.590	106.600	1.668
	20	37.5	83.956	285.839	65.320	6.118	206.870	44.200	12.719	0.129	195.583	13.493	0.382	0.598	102.401	1.690
	20	37.5	323.374	182.404	83.118	7.360	74.185	42.109	15.084	0.080	198.510	14.587	0.521	0.629	100.247	2.274
	20	20	100.030	145.419	27.121	2.695	91.355	0.423	0.000	0.000	228.381	18.530	0.537	2.668	28.628	5.378
	20	20	240.046	123.310	25.094	2.670	140.977	0.423	0.719	1.132	257.451	19.381	0.487	2.308	29.230	2.244
	20	20	369.978	224.676	27.163	2.838	188.310	0.439	0.766	0.000	271.949	21.455	0.586	2.432	32.327	3.100
Bare (sanded)	30	37.5	127.486	23.977	8.384	0.827	0.189	13.368	1.317	1.924	72.240	5.661	1.387	0.940	28.992	3.489
	30	37.5	91.520	23.591	7.281	0.886	0.144	13.547	1.201	1.897	74.298	5.722	1.355	0.734	28.381	3.504
	30	37.5	69.253	23.441	6.886	0.838	0.157	13.233	2.757	1.776	71.905	5.689	1.336	0.627	28.232	3.913
Bare	30	37.5	150.030	51.125	6.241	0.770	0.246	6.665	1.554	1.405	56.568	4.589	0.440	0.354	24.804	1.958
	30	37.5	184.348	66.831	7.299	1.040	0.094	8.925	0.526	1.965	73.263	5.790	0.529	0.434	31.734	2.531
	30	37.5	153.186	57.964	6.437	0.943	0.180	8.032	3.031	1.820	60.129	8.455	0.478	0.370	27.019	2.208

Table S7. Average cellular metal concentration values (in ng per g, relative to the weight of algal pellet) (from Chapter II. Toxicity in microalgae). In bold the selected metals for the multivariate analysis.

		Ti 47	**Cu 63**	Zn 66	As 75	**Cd 11**	V 51	**Cr 52**	**Mn 55**	**Fe 56**	Ni 60	Se 78	**Li 7**	**Al 27**	Mo 98	**Sn 120**	Sb 121	**Ba 137**	W 184	Tl 205	**Pb 208**
Isochrysis galbana	C	60.571	17.898	1852.811	1.702	0.644	0.640	6.132	22.126	3945.14	1.527	2.494	2.244	777.061	0.310	4.543	0.152	65.294	<LoD	<LoD	5.399
	BC - low	92.815	18.972	1277.979	1.569	1.895	0.404	5.474	19.571	3447.51	0.104	1.334	3.130	1145.30	0.401	2.021	0.032	81.329	<LoD	<LoD	17.665
	BC -mid	63.288	17.858	989.151	1.027	1.465	0.328	4.252	15.569	2871.59	1.359	1.264	2.492	821.694	0.284	0.563	0.110	62.550	<LoD	<LoD	10.434
	BC - high	57.130	42.971	<LoD	1.972	0.509	0.956	8.019	34.875	6570.17	<LoD	2.164	2.232	968.335	0.331	1.245	0.099	73.611	<LoD	<LoD	10.554
	FR - low	72.883	9.378	<LoD	1.379	1.702	0.617	5.426	19.888	3447.43	<LoD	<LoD	3.083	908.058	0.217	0.870	0.098	52.881	<LoD	<LoD	13.953
	FR - mid	147.060	9.065	1920.699	1.668	6.721	0.733	5.327	13.297	2337.52	0.741	<LoD	7.061	2010.46	0.924	1.724	0.276	76.613	<LoD	<LoD	23.616
	FR - high	101.085	17.421	1624.533	0.997	2.115	0.556	8.529	16.762	2714.53	0.226	<LoD	4.368	1484.92	0.776	3.182	0.250	71.324	<LoD	<LoD	5.052
Tretaselmis sp.	C	95.409	32.733	2602.437	3.148	4.614	0.603	3.915	30.890	2916.78	<LoD	2.394	3.243	1173.91	0.619	7.444	0.294	90.749	<LoD	<LoD	11.174
	BC - low	122.193	23.039	3422.697	3.526	9.737	0.677	1.859	31.889	2974.08	1.409	0.426	4.445	1476.10	0.344	2.490	0.346	89.377	<LoD	<LoD	26.312
	BC -mid	48.354	43.229	1698.041	2.323	2.087	0.848	4.589	18.366	3484.20	2.133	0.659	2.195	704.034	0.208	0.416	0.070	70.644	<LoD	<LoD	16.360
	BC - high	69.760	100.184	1253.931	2.882	4.056	0.784	2.539	17.874	3155.80	1.071	1.109	2.818	790.319	0.350	0.612	0.278	60.349	<LoD	<LoD	7.356
	FR - low	73.142	62.256	1722.789	3.318	2.780	0.769	4.120	30.786	3751.81	1.540	1.089	2.606	799.754	0.266	0.893	0.034	68.490	<LoD	<LoD	12.096
	FR - mid	84.751	13.849	3020.965	3.262	8.294	0.835	8.155	39.039	5929.79	2.819	2.687	3.240	884.905	0.292	1.998	0.109	82.725	<LoD	<LoD	10.563
	FR - high	69.984	23.144	3551.096	3.036	3.941	0.852	5.527	34.868	4413.31	<LoD	3.187	2.437	841.608	0.332	3.657	0.177	79.518	<LoD	<LoD	5.312
Cylindrotheca sp.	C	49.620	11.320	<LoD	0.945	1.114	0.483	5.327	46.001	2844.15	5.925	2.599	2.344	994.185	0.344	3.955	0.175	55.976	<LoD	<LoD	8.210
	BC - low	45.612	60.276	<LoD	1.015	1.439	0.612	4.834	44.180	3200.08	<LoD	2.193	2.461	846.382	0.240	0.424	0.114	69.684	<LoD	<LoD	33.176
	BC -mid	36.104	68.415	<LoD	0.887	0.715	0.586	4.090	14.803	2517.62	<LoD	1.469	2.848	583.289	0.361	1.004	0.051	76.782	<LoD	<LoD	2.242
	BC - high	58.605	71.901	<LoD	1.491	0.745	0.611	7.401	9.434	3024.08	<LoD	1.128	3.343	843.325	0.342	0.767	0.204	104.432	<LoD	<LoD	4.400
	FR - low	42.938	12.188	<LoD	1.001	0.483	0.504	4.794	57.130	3161.06	<LoD	1.292	2.248	566.859	0.230	0.572	0.044	54.701	<LoD	<LoD	9.256
	FR - mid	35.400	11.253	<LoD	0.808	0.576	0.377	4.698	42.132	3159.19	<LoD	1.061	2.110	502.486	0.258	0.879	0.126	52.638	<LoD	<LoD	5.437
	FR - high	50.293	16.122	<LoD	1.038	0.512	0.563	5.809	48.831	3555.37	<LoD	1.899	2.597	749.157	0.332	1.996	0.138	70.016	<LoD	<LoD	0.746

Table S6. Standard deviation values of cellular metal concentrations (in ng per g and relativized to algal pellet) (from Chapter II. Toxicity in microalgae). In bold the selected metals for the multivariate analysis.

		Ti 47	**Cu 63**	Zn 66	As 75	**Cd 11**	V 51	**Cr 52**	**Mn 55**	**Fe 56**	Ni 60	Se 78	**Li 7**	**Al 27**	Mo 98	**Sn 120**	Sb 121	**Ba 137**	W 184	Tl 205	**Pb 208**
Isochrysis galbana	C	23.460	5.218	1623.500	1.640	0.206	0.675	2.639	10.602	1922.13	<LoD	1.048	0.993	233.799	0.123	1.613	0.148	22.917	<LoD	<LoD	3.458
	BC - low	60.675	7.829	<LoD	1.134	1.403	0.289	3.446	9.142	1869.32	<LoD	<LoD	1.503	656.236	0.172	2.554	0.040	37.267	<LoD	<LoD	15.598
	BC -mid	44.450	5.834	<LoD	0.446	1.904	0.231	2.892	7.473	1806.20	<LoD	1.084	1.284	365.636	0.093	0.299	0.077	16.197	<LoD	<LoD	4.013
	BC - high	28.558	16.354	<LoD	1.108	0.755	0.461	4.941	16.870	3276.37	<LoD	2.498	0.524	540.770	0.121	0.327	0.038	25.535	<LoD	<LoD	14.584
	FR - low	34.184	2.905	<LoD	0.766	1.741	0.266	2.643	9.608	2051.77	<LoD	<LoD	1.028	364.660	0.135	0.410	0.080	12.065	<LoD	<LoD	16.572
	FR - mid	94.060	2.666	1483.172	0.572	8.638	0.214	2.279	6.957	1272.53	<LoD	<LoD	5.064	1057.44	0.960	0.085	0.149	14.769	<LoD	<LoD	22.070
	FR - high	54.437	2.249	1351.577	0.421	1.643	0.110	4.742	5.355	1139.56	<LoD	<LoD	1.487	585.765	0.860	1.229	0.089	9.202	<LoD	<LoD	1.013
Tretaselmis sp.	C	64.683	40.050	335.401	0.600	5.061	0.393	2.212	17.224	1504.09	<LoD	<LoD	2.173	749.249	0.637	0.830	0.120	15.089	<LoD	<LoD	3.849
	BC - low	102.227	11.301	2537.309	0.531	7.430	0.451	0.950	21.193	1779.77	<LoD	<LoD	3.162	899.802	0.250	3.797	0.004	8.489	<LoD	<LoD	20.279
	BC -mid	41.975	34.374	1345.646	1.453	1.703	0.690	2.038	13.534	3142.84	<LoD	0.080	1.478	514.458	0.150	0.287	0.057	50.343	<LoD	<LoD	22.787
	BC - high	51.338	56.128	1328.094	0.719	4.320	0.538	2.256	6.217	1994.45	<LoD	0.871	1.736	456.900	0.254	0.329	#¡DIV/0!	11.265	<LoD	<LoD	5.072
	FR - low	39.245	102.212	1157.665	0.782	2.146	0.609	2.409	8.711	2991.65	<LoD	1.387	1.342	431.542	0.081	0.085	0.024	1.806	<LoD	<LoD	7.438
	FR - mid	91.928	3.034	3036.263	1.105	11.370	0.504	7.854	25.134	4933.66	<LoD	0.164	3.025	1053.496	0.152	0.947	0.017	27.550	<LoD	<LoD	11.558
	FR - high	45.661	10.233	1178.593	0.574	4.011	0.340	2.313	27.576	2132.26	<LoD	0.963	1.037	517.922	0.132	0.997	0.120	17.547	<LoD	<LoD	5.550
Cylindrotheca sp.	C	20.793	2.492	<LoD	0.239	0.998	0.259	2.717	24.281	1006.81	<LoD	1.138	0.831	292.171	0.262	0.984	0.113	13.679	<LoD	<LoD	6.591
	BC - low	14.357	22.011	<LoD	0.177	0.935	0.250	1.136	31.952	1276.51	<LoD	3.280	0.359	371.221	0.058	0.112	0.091	26.330	<LoD	<LoD	28.489
	BC -mid	21.627	17.666	<LoD	0.221	0.809	0.300	1.291	3.980	827.74	<LoD	1.182	0.974	248.749	0.162	1.263	0.073	15.437	<LoD	<LoD	1.481
	BC - high	21.273	28.385	<LoD	0.334	0.392	0.196	4.027	4.172	1364.67	<LoD	0.445	0.827	259.561	0.215	0.334	0.271	34.048	<LoD	<LoD	4.268
	FR - low	15.217	0.951	<LoD	0.140	0.455	0.221	0.326	19.438	484.27	<LoD	1.220	0.491	152.934	0.054	0.114	0.033	6.705	<LoD	<LoD	8.785
	FR - mid	18.991	1.594	<LoD	0.350	0.220	0.157	1.892	27.518	1100.10	<LoD	0.346	0.746	277.877	0.091	0.219	0.077	20.179	<LoD	<LoD	5.896
	FR - high	16.844	4.912	<LoD	0.150	0.601	0.074	0.654	15.051	795.89	<LoD	0.512	0.603	233.105	0.153	0.209	0.078	17.930	<LoD	<LoD	0.280



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Original picture: Roberto Martínez

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