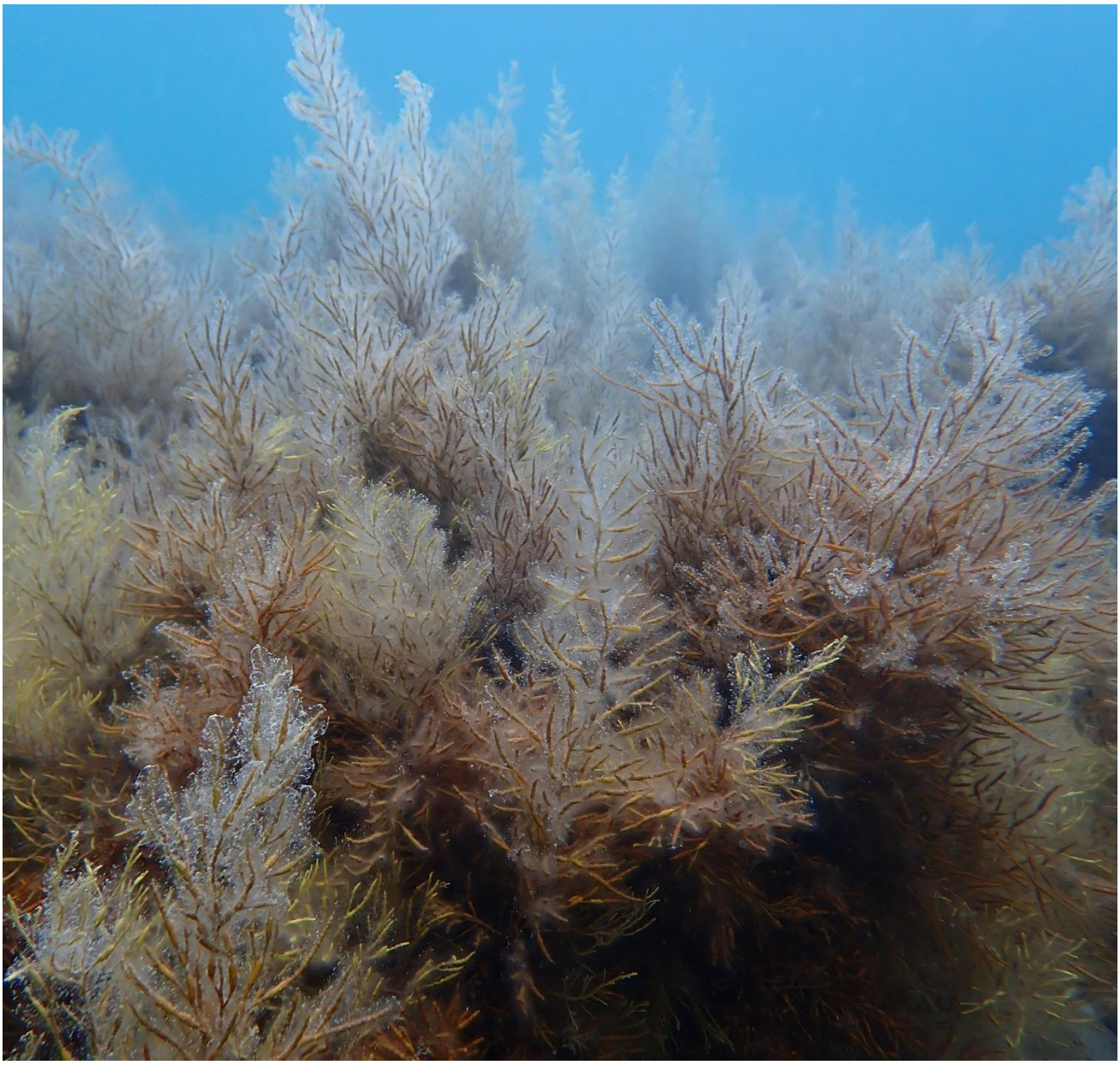
THREATS, CONSERVATION STRATEGIES AND RESTORATION EFFORTS FOR BROWN ALGAL FORESTS (*CYSTOSEIRA* S.L.) IN THE NORTHERN ADRIATIC SEA

Ana Lokovšek



Doctoral Dissertation
Jožef Stefan International Postgraduate School
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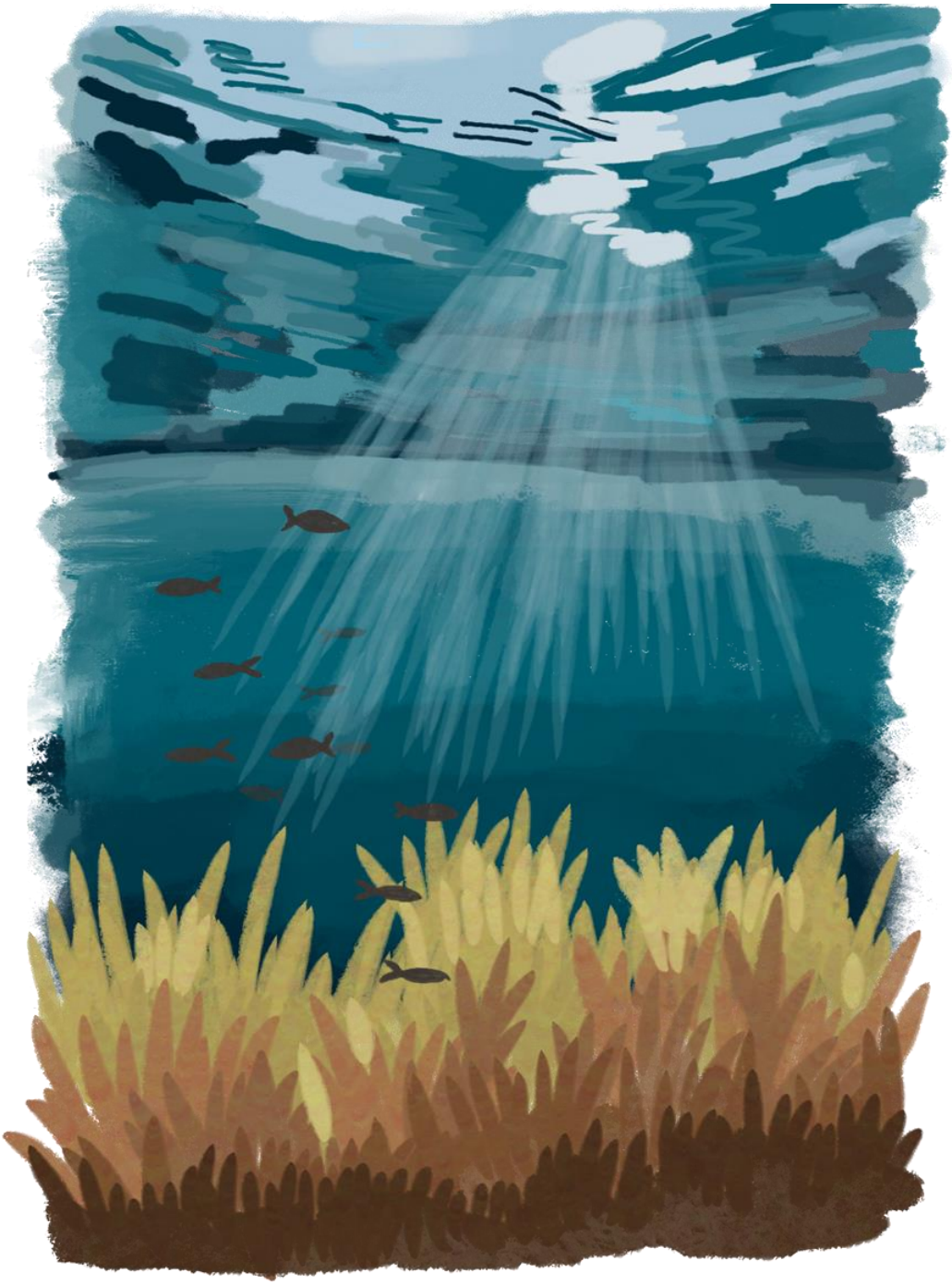
OGROŽENOST, STRATEGIJE OHRANJANJA IN PRIZADEVANJA ZA OBNOVO GOZDIČKOV RJAVIH ALG (*CYSTOSEIRA* S.L.) V SEVERNEM JADRANSKEM MORJU

Doktorska disertacija

Supervisor: Assist. Prof. Martina Orlando-Bonaca

Ljubljana, Slovenia, Februar 2025

"Only if we understand, can we care. Only if we care, we will help. Only if we help, we shall be saved."
(Jane Goodall)



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Abstract

In the Mediterranean Sea, one of the most important habitats, alongside dense seagrass meadows found on sedimentary bottoms, is formed by canopy-forming brown algae of the genus *Cystoseira sensu lato* on rocky shores. These long-living, slow-growing macroalgae possess a complex, tree-like morphology and form dense, structurally intricate habitats, so called marine forests. *Cystoseira* s.l. forests are known as biodiversity hotspots and areas supporting high primary productivity, enhancing ecosystem complexity, and providing essential habitats and food for numerous marine organisms. They also contribute significantly to nutrient cycling, shoreline protection, and carbon sequestration. However, due to a wide range of direct and indirect anthropogenic pressures, *Cystoseira* s.l. forests are in decline across the Mediterranean. This dissertation focuses on *Gongolaria barbata*, the most common *Cystoseira* s.l. species in the northern Adriatic Sea, where its forests have undergone severe degradation.

The overarching aim was to advance knowledge of the species, refine cultivation protocols, and explore restoration potential by identifying key ecological and methodological limitations and testing potential solutions. Five interconnected studies addressed key aspects of *G. barbata* ecology and restoration. The first study showed that ex situ cultivation can be streamlined through UVC sterilization and shortened laboratory phases, with early transfer to floating lantern net baskets, reducing costs while maintaining seedling survival. The second study demonstrated that seedlings are highly temperature-sensitive, with optimal growth at 15 °C and sharp declines above 20 °C, highlighting risks from warming seas and thermal anomalies. The third study investigated algal extracts, showing that certain formulations—such as those from the green microalga *Desmodesmus* sp.—can enhance seedling growth and resilience when applied at appropriate concentrations. The fourth study revealed that distinct *G. barbata* morphotypes represent phenotypic plasticity rather than genetic divergence. Finally, the fifth study characterized the *G. barbata* microbiome, uncovering body-part specificity and site-level variation, and suggesting that microbiome-aware practices can improve restoration outcomes.

Together, these findings provide a comprehensive understanding of *G. barbata* ecology and physiology, while offering practical guidance for restoration. They establish a scientific and methodological foundation for the recovery of *Cystoseira* s.l. forests and contribute to the development of an integrated, scalable, and climate-resilient framework for marine habitat restoration in the Mediterranean Sea.

Povzetek

Poleg morskih travnikov na sedimentnem dnu spadajo sestoji rjavih alg iz rodu *Cystoseira sensu lato* (v nadaljevanju cistozire) na kamnitih podlagah med najpomembnejše morske habitate v Sredozemskem morju Te počasi rastoče in dolgožive makroalge s kompleksno, drevesasto obliko steljke tvorijo goste, strukturno bogate habitate, tako imenovane podvodne gozdiče. Gozdiči cistozir veljajo za vroče točke biotske raznovrstnosti in območja visoke primarne produkcije ter povečujejo ekosistemsko kompleksnost in zagotavljajo ključen življenjski prostor ter vir hrane za številne morske organizme. Pomembno prispevajo h kroženju hranil, varovanju obale pred preperevanjem in spravilu ogljika. Zaradi različnih antropogenih pritiskov se gozdiči cistozir po celotnem Sredozemlju soočajo z izrazitim upadom.

Ta doktorska disertacija obravnava vrsto *Gongolaria barbata*, najpogostejšo predstavnico iz skupine *Cystoseira* s.l. v severnem Jadranu. Opazovanja v zadnjih desetletjih kažejo na izrazit upad in slabšanje stanja njenih populacij na tem območju. Glavni cilj tega dela je zato poglobiti poznavanje vrste, izpopolniti protokole njenega gojenja ter raziskati možnosti obnove habitata z identifikacijo glavnih omejitev in preizkušanjem možnih rešitev.

Doktorsko disertacijo sestavlja pet medsebojno povezanih študij, ki obravnavajo ključne vidike ekologije in obnove gozdičev *G. barbata*. V prvi študiji smo pokazali, da je mogoče *ex situ* gojenje poenostaviti z uporabo UVC-sterilizacije in skrajšanjem laboratorijske faze, kateri sledi zgodnji prenos v plavajoče mrežaste košare v morju. S tem zmanjšamo količino dela in stroške gojenja, hkrati pa izboljšamo preživetje mladih osebkov. V drugi študiji smo ugotovili, da so zgodnje razvojne faze *G. barbata* izrazito občutljive na temperaturo. Najboljšo rast in preživetje dosegajo pri 15 °C, medtem ko temperature nad 20 °C nanje delujejo negativno, kar kaže na tveganja, povezana s segrevanjem morja in pojavom temperaturnih anomalij. V tretji raziskavi podajamo spoznanja, da lahko nekateri algalni izvlečki, kot je izvleček zelene mikroalge *Desmodesmus sp.*, ob ustreznih odmerkih spodbudijo rast in preživetje mladih osebkov *G. barbata* ter povečajo njihovo odpornost na stres. V četrti študiji z genetsko analizo smo pokazali, da različni morfotipi vrste *G. barbata* ne pomenijo nujno genetskih razlik, temveč odražajo visoko fenotipsko plastičnost v različnih okoljskih razmerah. V peti študiji opredeljujemo mikrobiom vrste *G. barbata* in ugotavljamo, da velik del bakterijske združbe odraža bakterijsko združbo okolja, vendar se mikrobiom alge nekoliko razlikuje v različnih delih steljke in med lokacijami.

Ta spoznanja ponujajo celovito razumevanje ekologije in fiziologije vrste *Gongolaria barbata* ter hkrati nudijo praktične smernice za obnovo njenih gozdičkov. Utemeljujejo znanstvene in metodološke osnove za obnovo gozdičkov vrst *Cystoseira* s.l. ter prispevajo k razvoju integriranega in na podnebne spremembe odpornega okvira za obnovo morskih habitatov v Sredozemskem morju.

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Abbreviations

ANOVA	...	analysis of variance
ASV	...	amplicon sequence variant
bp	...	base pair
CC	...	climate change
CRM	...	certified reference material
DNA	...	deoxyribonucleic acid
EAC	...	East Adriatic current
DOC	...	dissolved organic carbon
EC	...	European Commission
EU	...	European Union
GF/F	...	glass fiber filter
MAP	...	Mediterranean Action Plan
MEA	...	Multilateral Environmental Agreement
MSFD	...	Marine Strategy Framework Directive
PCR	...	polymerase chain reaction
PERMANOVA	...	permutational analysis of variance
PSU	...	practical salinity unit
RNA	...	ribonucleic acid
SPAMI	...	Specially Protected Areas of Mediterranean Importance
SPA/BD	...	Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean
TDN	...	total dissolved nitrogen
UVC	...	ultraviolet C (shortwave UV radiation)
UV	...	ultraviolet
WAC	...	West Adriatic Current
WFD	...	Water Framework Directive

Symbols

%	...	Percent
°C	...	degree Celsius
Cm	...	Centimeter
G	...	Gram
H	...	Hour
L	...	Liter
M	...	Meter
mL	...	Milliliter
Mm	...	Millimeter
µL	...	Microliter
µm	...	Micrometer
N	...	sample size
P	...	probability value (statistical significance)
R^2	...	coefficient of determination
S	...	Second
SD	...	standard deviation
±	...	plus/minus (variation, error, tolerance)
×	...	multiplication, fold-chang

Glossary

Allelopathy – Biological interaction where one organism releases chemical compounds that influence the growth, survival, or reproduction of others.

Amplicon sequence variant (ASV) – High-resolution DNA sequence obtained from amplicon sequencing, representing a unique variant without clustering into OTUs.

Antheridium – Male reproductive structure in algae that produces and releases sperm cells (anterozoids).

Anterozoid – Motile, biflagellate male gamete in brown algae.

Base pair (bp) – Unit of measurement for DNA or RNA length, representing two complementary nucleotides bonded together.

Biofilm – Structured community of microorganisms attached to a surface and embedded in extracellular polymeric substances.

Biostimulant – Substance of biological origin that enhances growth, survival, or stress tolerance in plants or algae.

Canopy-forming algae – Large brown algae forming surface canopies that structure benthic habitats, similar to terrestrial forests.

Carbon sequestration – Long-term storage of carbon in oceans, soils, or vegetation, reducing atmospheric CO_2.

Conceptacle – Specialized reproductive cavity in brown algae containing gametangia and releasing gametes.

Copy number variation – Differences in the number of copies of a gene among individuals or populations.

Detritus – Dead organic matter derived from plants or animals, forming an important part of the marine food web.

Epibiont – Organism that lives attached to the surface of another living organism, often algae or seagrass leaves, or on abiotic substrata.

Eutrophication – Nutrient enrichment of water bodies, often leading to excessive algal growth and oxygen depletion.

Fertilization membrane – Sticky membrane surrounding the zygote in brown algae, facilitating adhesion to the substrate.

Fucales – Order of brown algae characterized by a unique reproductive system where the haploid phase is retained within superficial cavities on a diploid thallus, and its spores function as gametes. Members of this order typically possess robust structures including holdfasts, stipes, blades, and air bladders, and they play significant ecological roles in intertidal and shallow subtidal zones.

Gamete – Reproductive cell (egg cell or spermatia) involved in sexual reproduction.

Gamete release – Liberation of egg cells or spermatia (antherozoids) from algal conceptacles into the surrounding water for external fertilization.

Germling – Early developmental stage of a macroalga, arising from a zygote.

Grazing pressure – Intensity of herbivore consumption on algal populations, often by fish or sea urchins.

Holobiont – The host organism together with all associated microorganisms (bacteria, fungi, viruses, archaea).

Host–microbe interaction – Ecological and physiological relationship between a host organism and its microbial community.

Macroalgal forest – Dense stand of canopy-forming macroalgae that provides three-dimensional habitat for marine life.

Marine heatwave – Prolonged period of abnormally high sea surface temperature compared to typical temperatures for particular season and region.

Metatranscriptome – Complete set of RNA transcripts sequenced from an environmental community, reflecting functional activity.

Microbiome – Community of microorganisms associated with a host or habitat.

Morphotype – Distinct physical form or phenotype within a species, often related to environmental adaptation.

Multivariate analysis – Statistical approach analyzing multiple variables simultaneously, often used in community ecology.

Niche differentiation – Process by which competing species use the environment differently to coexist.

Ontogeny – Developmental history of an organism from zygote to adult stage.

Oogonium – Female reproductive structure in brown algae that produces egg cells.

Oogamy – Type of sexual reproduction involving large non-motile eggs and small motile sperm.

Overgrazing – Excessive consumption of algae by herbivores, leading to habitat degradation.

Phenotypic plasticity – Ability of an organism to alter its morphology or physiology in response to environmental conditions.

Photoinhibition – Reduction in photosynthesis due to damage from excess light.

Primary productivity – Rate at which photosynthetic organisms produce organic matter from inorganic carbon.

Recruitment – Addition of new individuals to a population through settlement, germination, or juvenile survival.

Resilience – Capacity of an ecosystem to recover after disturbance.

Restoration ecology – Scientific discipline focused on repairing and re-establishing degraded ecosystems.

Rhizoid – Root-like structure in algae that anchors the thallus to the substrate.

Symbiosis – Close and often long-term interaction between two or more different biological species.

Thallus – Undifferentiated vegetative body of algae, lacking true roots, stems, or leaves.

Transcriptome – Complete set of RNA molecules expressed in a cell, tissue, or organism.

Zygote – Diploid cell formed by fertilization of an egg cell by a sperm

Chapter 1
Introduction

In the coastal waters of the Mediterranean, forests not of trees but of seaweeds thrive — their golden-brown swaying canopies that move with the waves, offer beauty to the eye and provide shelter and nourishment to countless marine creatures.

This chapter provides an overview of the key topics relevant to this dissertation. It introduces the current knowledge on brown macroalgae from genus *Cystoseira sensu lato,* highlighting their ecological importance and the threats that endanger these underwater forests. It explains the historical distribution of macroalgae in the Adriatic Sea and reviews the approaches developed for their restoration. The study area is presented, followed by a detailed description of the biology of the study species *Gongolaria barbata* (Stackhouse) Kuntze and the reproduction of *Cystoseira* s.l. species.

1.1 Overview

In Mediterranean coastal environments, macroalgae play a foundational ecological role, representing the dominant autotrophic biomass and contributing significantly to habitat complexity, primary productivity and biodiversity (Mineur et al. 2015). Alongside seagrasses on sedimentary bottoms, the most important habitat-building species in the Mediterranean Sea are brown algae from the family Sargassaceae (Fucales, Phaeophyta) (Airoldi et al. 2014). Species from genus *Cystoseira sensu lato* C. Agardh are particularly important in the region, forming dense macroalgal forests and shaping benthic communities from the upper mediolittoral fringe to the deeper sublittoral zone (Ballesteros 2002; Ballesteros et al. 2009; Mangialajo et al. 2012; Piazzi et al. 2018).

The presence and health of the species from genus *Cystoseira sensu lato* are used as valuable bioindicators of ecosystem and water quality in the Mediterranean Sea. In European marine waters, these species are used in the assessment of the ecological status under the Water Framework Directive (2000/60/EC) and of the environmental status according to the Marine Strategy Framework Directive (2008/56/EC). Given their long lifespan and strong association with long-term ecological periodicity, the disappearance of *Cystoseira s.l.* from rocky substrata constitutes a clear and robust signal of environmental degradation and habitat loss.

Taxonomically, *Cystoseira* s.l. has undergone recent revision, resulting in its division into three genera: *Ericaria* Stackhouse, *Cystoseira* C.Agardh, nom. cons. and *Gongolaria* Boehmer (Molinari-Novoa & Guiry 2020). Despite this reclassification, these taxa share comparable ecological roles, life-history strategies, and morphological characteristics, and are therefore often considered collectively under *Cystoseira* s.l. The group currently

includes 49 species, with 14 species in the genus *Ericaria*, 15 in genus *Gongolaria*, and 20 species that remain assigned to the genus *Cystoseira* (Guiry 2024).

The most common species in Slovenian coastal waters are *Gongolaria barbata* and *Cystoseira compressa* (Esper) Gerloff & Nizamuddin. Other species, such as *Cystoseira foeniculacea* (Linnaeus) Greville and *Ericaria crinita* (Duby) Molinari & Guiry, are also present but do not form forests in this area (Orlando-Bonaca & Rotter 2018; Orlando-Bonaca et al. 2021b).

Gongolaria barbata, a study species and focus of this work, is widely distributed across the Mediterranean Sea (Báez et al. 2005; Boudouresque et al. 2022; Einav 2023; Falace & Bressan 2003; Iveša & Devescovi 2014; Orlando-Bonaca et al. 2021a; Ribera et al. 1992; Trikka et al. 2021) and also occurs in the Atlantic Ocean, in the Canary and Savage Islands (Blanfuné et al. 2022).

Historically, *G. barbata* was abundant in the northern Adriatic, forming extensive, dense underwater forests (Iveša & Devescovi 2014; Iveša et al. 2016; Munda 1979; Pignatti 1962; Sfriso 1987; Špan 1969). However, its populations, like those of other *Cystoseira* s.l. species, are now in sharp decline, driven by multiple pressures including overgrazing by herbivorous fish, increased sedimentation, and abnormal sea warming patterns (Airoldi & Cinelli 1997; Airoldi 2003; Blanfuné et al. 2025; Cáliz et al. 2019; Capdevila et al. 2019; Catra et al. 2019; Cimini et al. 2024; Dublin et al. 1990; Falace et al. 2021; Korpinen et al. 2007; Marletta et al. 2021; Monserrat et al. 2022; Monserrat et al. 2023; Orfanidis et al. 2021; Thibaut et al. 2005; Vergés et al. 2009; Verdura et al. 2021; Worm & Lotze 2006). Such brown algal forest regression is not limited to the northern Adriatic but is documented across the Mediterranean and is expected to intensify under ongoing climate change (Manca et al. 2024). The cumulative effects of direct and indirect anthropogenic stressors—habitat damage and loss, pollution, overfishing, climate change and extreme events, spreading of invasive species, and trophic-network alterations—often act additively or synergistically over short and long timescales, eroding the capacity of these ecosystems to provide essential goods and services (Airoldi 2003; Bianchelli et al. 2016; Claudet & Fraschetti 2010; Duggins et al. 1990; Monserrat et al. 2022; Piazzi et al. 2016; Scheffer et al. 2001; Worm & Lotze 2006). The loss of macroalgal biomass affects not only the algae themselves but also the ecosystems they structure and the ecosystem services they support (Eger et al. 2024).

Natural recovery after disturbance is typically slow and often unsuccessful, hindered by fragmented populations and limited dispersal (Airoldi et al. 2014; Buonomo et al. 2018; Cebrian et al. 2021; Clayton 1990; Gran et al. 2022; Iveša et al. 2016; Perkol-Finkel & Airoldi 2010; Scheffer et al. 2001; Smith et al. 2023a; Thrush et al. 2008). The emergence of alternative stable states (e.g., turf or barren grounds) can further impede recovery by locking degraded ecosystems into alternative, less desirable configurations that prevent the re-establishment of canopy-forming species (Airoldi et al. 1995; Airoldi & Virgilio 1998; Alestra et al. 2014; Gorgula & Connell 2004; Orlando-Bonaca et al. 2021a; Perkol-Finkel & Airoldi 2010).

Stressor mitigation and active restoration are therefore often needed in order to help preserve *Cystoseira* s.l. forests. Numerous conservation and restoration initiatives have been launched across the Mediterranean, alongside studies to better understand their biology and ecology. In recent years, ex situ and in situ cultivation and transplantation

have emerged as promising tools to support reforestation of these vulnerable habitats (De La Fuente et al. 2019b; Falace et al. 2006; Falace et al. 2018; Kaleb et al. 2023; Lardi et al. 2022; Lokovšek et al. 2023; Lokovšek et al. 2025; Orlando-Bonaca et al. 2021b; Orlando-Bonaca et al. 2022a).

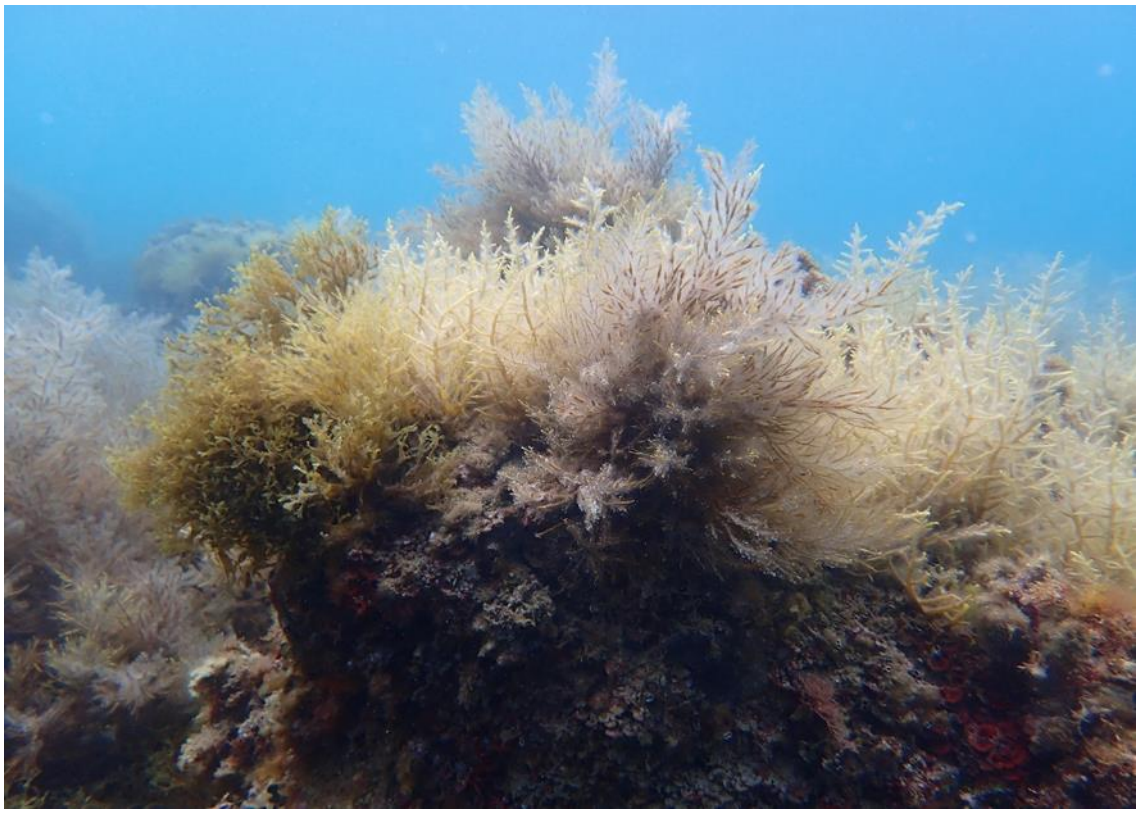


Figure 1: *Cystoseira* s.l forest near Izola, in Slovenian coastal waters - *Gongolaria barbata* and *Cystoseira compressa* in February 2021 (Photo: A. Lokovšek).

1.2 Ecological importance of *Cystoseira* s.l. forests

Brown seaweeds from genus *Cystoseira* s.l., represent a group of long-lived (Ballesteros 2002; Clayton 1990) slow-growing canopy-forming species that achieve high biomass (Ballesteros 1989). *Cystoseira* s.l. forests thrive on rocky substrates of the mediolittoral and infralittoral zones, typically growing on large limestone boulders or sandstone terraces, where they constitute a key component of the photophilic algal community. Some species grow even deeper, reaching into upper circalittoral. Their vertical distribution and community composition are shaped by environmental factors such as light availability, temperature, hydrodynamics, and grazing pressure (Irving et al. 2009; Mangialajo et al. 2012; Vergés et al. 2009).

Through photosynthesis, *Cystoseira* s.l. species absorb considerable amounts of carbon dioxide and thus act as natural carbon sinks, helping to regulate the carbon dioxide levels of the atmosphere and the oceans (Filbee-Dexter & Wernberg 2020; Krause-Jensen & Duarte 2016; Moreda et al. 2024; Peleg et al. 2020; Pessarrodona et al. 2023). Their dense

canopy provides additional ecosystem services by absorbing wave energy, stabilizing sediments and reducing coastal erosion (Papadimitriou et al. 2024). At the same time, their complex structure alters light penetration, temperature and hydrodynamics at the seafloor, creating a mosaic of benthic microhabitats (Hardison et al. 2013). The high productivity of these forests supports diverse trophic networks. Population dynamics are characterized by different regulatory mechanisms: sublittoral *Cystoseira* s.l. species are primarily influenced by top-down control, whereas subsurface species are more strongly controlled by bottom-up processes (Cardona et al. 2013; Hereu et al. 2008).

Compared to the average size of Mediterranean macroalgae, *Cystoseira* s.l. are relatively large species, growing between 10 cm and over 1 m in height (Ballesteros 2002; Ercegović 1952). They represent the highest level of morphological and functional complexity among Mediterranean algae and exhibit abundant three-dimensional growth. Their dense canopies provide shelter to numerous organisms (Cheminée et al. 2013; Cheminée et al. 2017; Lipej et al. 2023; Orlando-Bonaca et al. 2022b; Piazzi et al. 2018; Pitacco et al. 2014). Across the Mediterranean, a total of 597 taxa have been identified in *Cystoseira* s.l. beds, underscoring their exceptional biodiversity (Piazzi et al. 2018). The cauloids and branches serve as substrata for smaller epiphytic algae, including red taxa from the orders Gelidiales and Ceramiales, brown taxa from the Dictyotales, and green taxa such as Siphonales and Siphonocladales (Mačič & Sviržev 2014). These habitats also support a highly diverse assemblage of benthic invertebrates (Pitacco et al. 2014; Orlando-Bonaca et al. 2022), including crustaceans, molluscs, tunicates, bryozoans, and polychaetes (Orlando-Bonaca et al. 2022b; Pitacco et al. 2014). In the Black Sea, crustaceans were found to be the most abundant group related to *G. barbata*, with amphipods as the most represented taxa, followed by isopods, cumaceans, tanaids, and decapods (Adrian et al. 2016). Similar research from the Aegean Sea confirmed that amphipods are the dominant crustacean group in *Cystoseira* s.l. assemblages, where a total of 81 crustacean species were recorded (Kocatas et al. 2004).

Across the Mediterranean Sea, polychaetes rank among the most abundant macroinvertebrates in macroalgal habitats (Pinedo et al. 2024). Their diversity varies among host species, from 50 species on *E. crinita* to only six on *C. compressa* in the Aegean Sea (Çinar 2003) to 86 species on *Cystoseira mediterranea* Sauvageau in the western Mediterranean (Alós 1990). 58 polychaete species have been reported to inhabit *G. barbata*, with *Salvatoria clavata* (Claparède, 1863), *Platynereis dumerilii* (Audouin & Milne Edwards, 1833), *Nereis zonata* Malmgren, 1867, and *Polyophthalmus pictus* (Dujardin, 1839) among the most dominant and frequent (Ergen & Çinar 1994). Despite regional variation, *P. dumerilii*, *S. clavata*, and *Syllis prolifera* Krohn, 1852 are consistently dominant across *Cystoseira* s.l. assemblages.

In the northern Adriatic Sea, 69 mollusc species have been recorded within *Cystoseira* s.l. forests (Pitacco et al. 2014). Gastropods dominated both in species richness and abundance, followed by bivalves and placophora, and the frequent occurrence of juvenile individuals highlights the crucial role of these algal forests for molluscan recruitment (Pitacco et al. 2014). Among gastropods, *Jujubinus exasperatus* (Pennant, 1777), *Rissoa guerinii* Récluz, 1843, and *Bittium reticulatum* (da Costa, 1778) were the most common species (Orlando-Bonaca et al. 2022b; Pitacco et al. 2014). These micrograzer-detritivores

feed primarily on diatoms, epiphytic microalgae, and trapped detritus within the algal thalli (Ávila 2003; Paine 1984), migrating nightly toward branch apices, where epiphytes are most abundant (Gambi & Morri 2008; Rueda et al. 2008). Dominant bivalves include *Modiolus barbatus* (Linnaeus, 1758) and *Musculus costulatus* (Risso, 1826) (Pitacco et al. 2014), inhabiting hard substrata among algal holdfasts or attaching to algal fronds (Hrs-Brenko & Legac 2006). The sediment trapped among the thalli further increases habitat complexity, supporting soft-bottom species such as the bivalves *Kurtiella bidentata* (Montagu, 1803), *Gouldia minima* (Montagu, 1803), and the gastropod *Mangelia attenuata* (Montagu, 1803) (Antoniadou & Chintiroglou 2005). The prevalence of short-lived species with direct larval development inside egg capsules (Gambi & Morri 2008) confirms that *Cystoseira* s.l. canopies serve as key spawning and settlement sites (Antit 2013; Pérès 1967; Pitacco et al. 2014; Poulicek 1985).

Cystoseira s.l. stands also provide important breeding and nursery grounds for coastal fish (Lipej et al. 2023). Within these structurally complex canopies, numerous fish species find shelter, food, and nesting material (Bonaca & Lipej 2005; De La Fuente et al. 2019a; Duggins et al. 1990; Hinz et al. 2019; Lipej et al. 2023). Rich invertebrate community inhabiting macroalgal forests serve as a key food source (Lokovšek et al. 2022; Orlando-Bonaca et al. 2022b; Pitacco et al. 2014). Prey density is generally higher in structurally more complex algal stands such as *Cystoseira* spp. and *Halopteris* spp. than in simpler ones like Dictyotales (Bonaca & Lipej 2005; Cheminée et al. 2013; Cheminée et al. 2017; Lipej et al. 2023).

To date, 34 fish species have been recorded in *Cystoseira* forests of the northern Adriatic, including 8 species of wrasses (Labridae), 7 seabreams (Sparidae), 7 blennies (Blennidae), and 4 gobies (Gobiidae) (Lipej et al. 2023). The most frequently observed species is *Serranus scriba* (Linnaeus, 1758)), followed by *Gobius fallax* (Sarato, 1889), *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817)), *Symphodus cinereus* (Bonnaterre, 1788), and *Chromis chromis* (Linnaeus, 1758)). The most abundant among them are *C. chromis*, *G. fallax*, and *S. scriba* (Lipej et al. 2023). *Clinitrachus argentatus* (Risso, 1810)), is also closely associated with *Cystoseira* s.l. forests, where its brown stripes provide effective camouflage among brown algal stands (Orlando-Bonaca & Trkov 2016). Another frequent inhabitant is *Parablennius sanguinolentus* (Pallas, 1814) (Lipej et al. 2023; Orlando-Bonaca & Trkov 2016). *Tripterygion tripteronotus* (Risso, 1810)) also favors dense *Cystoseira* s.l. forests, unlike *Tripterygion delaisii* (Cadenat & Blache, 1970), which prefers rocky habitats at the coralligenous boundary (Lipej et al. 2023).

Cystoseira s.l. forests are of special importance for wrasses. Several species, including *Symphodus roissali* (Risso, 1810), *S. cinereus*, *S. ocellatus* (Linnaeus, 1758) and *S. tinca* Cantraine, 1833, build nests from algal fragments, sand, and shell debris (Lipej et al. 2009; Lipej et al. 2023; Sinopoli et al. 2015). Males selectively choose algal species that resist degradation and remain stable under wave exposure (Raventos 2004). In the northern Adriatic, over 26 macroalgae and seagrass species, including *Cystoseira* s.l., have been recorded in the nests of *S. cinereus*, *S. roisalli* and *S. ocellatus* (Lipej et al. 2009). The species most closely associated with *Cystoseira* s.l. forests is *S. roissali*, occurring predominantly in densely vegetated habitats dominated by *Gongolaria barbata* (Lipej et al. 2009). Multiple females spawn in a single nest, guarded and maintained by the territorial

male until hatching (Alonzo & Heckman 2010; Lejeune 1985), after which a new nest is often built nearby (Alonzo & Heckman 2010; Taborsky et al. 1987). Juveniles seek refuge within algal canopies (Cheminée et al. 2013; Cheminée et al. 2017; Lipej et al. 2023). Densities of juveniles — particularly *Symphodus* spp.— are 9-12-times higher in *Cystoseira* s.l. forests than in other erect or turf algal assemblages, underscoring their key role as nursery grounds for numerous coastal and commercially important fish species (Cheminée et al. 2013).

Habitat complexity and depth strongly influence species composition and behavior; declines in Labridae and Sparidae are often indicators of habitat degradation or loss of macroalgal cover (Cheminée et al. 2013; Cheminée et al. 2017; Lipej et al. 2023; Thiriet et al. 2014). The widespread decline of *Cystoseira* s.l. forests across the Mediterranean Sea has been accompanied by a marked reduction in the abundance and diversity of associated fish communities. In the northern Adriatic, species closely associated with canopy-forming algae, such as *Symphodus* spp. and *Diplodus* spp., have markedly decreased in density and frequency, while species less dependent on algal cover, such as gobiids on rocky substrates, remain largely unaffected (Lipej et al. 2023).

The loss of habitat complexity also affects benthic invertebrates. Reduced patch size and algal substrate modify the structure and composition of associated molluscan assemblages (Mancuso et al. 2021), suggesting that the decline or disappearance of *Cystoseira* s.l. forests may trigger bottom-up effects across trophic levels, ultimately weakening ecosystem functioning and the services these habitats provide (Mancuso et al. 2021).



Figure 2: Pair of *Symphodus ocellatus* above their nest next to the *Gongolaria barbata* forest near Izola (Photo: A. Lokovšek).

1.3 Historical distribution and regression of macroalgal communities in the coastal waters of northern Adriatic

Marine macroalgae are dominant primary producers on rocky substrata in the shallow waters of the northern Adriatic Sea. Historically, they were abundant, forming extensive vegetation stands that supported highly productive coastal ecosystems. Over the past century, however, macroalgal abundance and coverage have markedly declined, leading to profound alterations of benthic habitats compared with historical baselines (Airoldi & Beck 2007; Barmawidjaja et al. 1995; Falace et al. 2010; Hanel 2002; Iveša & Devescovi 2014; Lotze et al. 2011; Munda 1979; Munda 1980a, 1993b; Munda 1993a; Munda 2000; Newell & Ott 1999; Orlando-Bonaca et al. 2008).

Extensive *Cystoseira* s.l. forests dominated the northern Adriatic until the late 1960s, extending down to depths of 15 m (Newell & Ott 1999; Špan 1969). These populations collapsed between 1968 and 1972, mainly due to overgrazing by the black sea urchin *Paracentrotus lividus* (Lamarck, 1816), which transformed dense algal forests into barren grounds dominated by urchins (Ott 1992; Newell et al. 1999).

Along the Slovenian coastline, severe degradation macroalgal communities due to the sea urchin grazing was first recorded in the years 1972-1974 (Turk & Vukovič 1994; Vukovič 1976). Sea urchins consumed nearly all infralittoral vegetation, leaving only small refugia in the Strunjan Nature Reserve and in the area between Izola and Koper (Vukovič 1976). Consequently, algal belts formerly composed by communities of many *Cystoseira* s.l. species disappeared (Vukovič 1976; Vukovič 1982).

From 1970s through the mid-1980s, elevated nutrient inputs, coming mostly from Po River basin resulted in eutrophication across the northern Adriatic (Giani et al. 2012).This additional pressure further contributed to the decline of *Cystoseira* s.l. populations from 1970s through 1990s (Iveša et al. 2016; Munda 2000). Reports from the Istrian coast near Rovinj document the disappearance or decline of stands of sensitive species such as *Cystoseira* s.l. and *Fucus virsoides* J.Agardh (Iveša & Devescovi 2014; Munda 1980a, 1980b; Munda 1993a; Munda 2000). *F. virsoides* was replaced by ephemeral and turf-forming species, including *Gelidium pusillum* (Stackhouse) Le Jolis, while *Cystoseira* s.l. assemblages were replaced by more generalist taxa such as *Halopteris scoparia* (Linnaeus) Sauvageau, *Dictyota dichotoma* (Hudson) J.V.Lamouroux, *Halopithys incurva* (Hudson) Batters, and *Padina pavonica* (Linnaeus) Thivy, and under extreme eutrophic conditions by *Ulva* sp. (Munda 1993a). Similar transformations occurred along the Italian coast of the Gulf of Trieste, where sensitive taxa were replaced by tolerant species, community structure became simplified, and foundation species such as *Cystoseira* s.l. disappeared, giving way to fast-growing or turf-forming algae (Falace et al. 2010).

Since the late 1980s, reductions in phosphorus inputs—largely following bans on phosphates in detergents—and lower precipitation and river discharge have led to improved water quality (Giani 2011; Giani et al. 2012; Marini & Grilli 2023). By the 2000s, decreasing chlorophyll-a concentrations reflected a shift towards more oligotrophic conditions in the northern Adriatic (Mozetič et al. 2012). Improved water quality and reduced grazing pressure contributed to the partial recovery of *Cystoseira* s.l. populations, resulting in a higher ecological status of macroalgal assemblages (Sfriso & La Rocca 2005).

A pronounced recovery of *Cystoseira* s.l. taxa was documented in the northern Adriatic between 2000 and 2013 (Iveša & Devescovi 2014). Along the Croatian coastline, dense macroalgal forests persisted until the 1960s (Špan 1969), and *Cystoseira* s.l. abundance recorded between 2009 and 2013 was comparable to the richest assemblages (Iveša et al. 2016). However, since 2016 these forests have again declined sharply, with local extirpations reported in recent years along the Croatian Istrian coasts (Bilajac et al. 2024; Iveša et al. 2022).

In Slovenian coastal waters, macroalgal revitalization began around 1992, notably with the recovery of *Cystoseira* s.l. in the Strunjan Nature Reserve (Turk & Vukovič 1994). By 2006, canopy-forming *E. crinita* dominated rocky upper-infralittoral habitats in pristine environments, while *Gongolaria barbata* prevailed in areas with higher sedimentation (Orlando-Bonaca et al. 2008; Vukovič 1976). Yet, from 2006 to 2012, a decline in canopy-forming algae (*Cystoseira* spp., *Halopithys incurva*) was again observed in the area (Orlando-Bonaca & Rotter 2018). A decade-long study (2006–2016) showed strong spatial variation, with *Cystoseira* s.l. most abundant in marine protected areas (Cape Madona Nature Monument and Strunjan Nature Reserve) and the Habitat of *Posidonia* Nature Monument, whereas sites near urban areas were dominated by opportunistic species (*Cladophora* spp., *Dictyota* spp.) (Orlando-Bonaca & Rotter 2018). Long-term monitoring (2007–2019) further documented a significant decline in *Cystoseira* s.l. species —from around 66% to 25%— at two reference sites for macroalgae, with only a transient recovery in 2016 (Orlando-Bonaca et al. 2021a).

Macroalgal assemblages along the Italian coast of the Gulf of Trieste have also undergone profound transformations over the past six decades (Falace et al. 2010; Falace et al. 2024a). Between the 1960s and 2020s, 146 macroalgal species representing 38 genera, 14 families, and three orders dseaisappeared, with brown algae most affected. Species of Atlantic, Circumboreal, or Mediterranean affinity have been replaced by turf-forming and cosmopolitan taxa (Falace et al. 2024a). Within genera *Cystoseira* s.l. and *Sargassum*, a 25% reduction in species richness has occurred, with *Cystoseira amentacea* var. *stricta* and *Gongolaria sauvageauana* disappearing after the 1990s, and *Cystoseira foeniculacea* f. *foeniculacea* and *Sargassum hornschuchii* C.Agardh

no longer recorded in either Italian or Slovenian waters. Concurrently, several non-indigenous species have been introduced, including *Asparagopsis armata*, *Acrothamnion preissii*, *Aglaothamnion feldmanniae*, *Colaconema codicola*, *Colpomenia peregrina*, and *Codium fragile* (Falace et al. 2024a; Servello et al. 2019).

Synthesis of historical data from Italy, Slovenia, and Croatia reveals a consistent pattern: *Cystoseira* s.l. forests—once widespread and ecologically dominant—have undergone drastic regression since the mid-20th century. The combined effects of eutrophication, habitat alteration, sedimentation, and herbivory have driven their replacement by structurally impoverished communities with lower biodiversity. The increasing dominance of warm-affinity taxa reflects the growing influence of climate change (Falace et al. 2024a), while more recent shifts towards opportunistic assemblages are primarily driven by habitat degradation and sediment dynamics associated with maritime traffic and port activities rather than nutrient enrichment (Orlando-Bonaca et al. 2015; Orlando-Bonaca & Rotter 2018). Studies of phytoplankton and chlorophyll trends confirm

progressive oligotrophication across the northern Adriatic (Mentaschi et al. 2024; Mozetič et al. 2010; Mozetič et al. 2012; Solidoro et al. 2009).

At present, *Gongolaria barbata* is the dominant *Cystoseira* s.l. species along the Slovenian coast, while other taxa from this group have declined markedly (Orlando-Bonaca et al. 2021a), as observed on field (pers. obs.). Habitat degradation, sedimentation, overgrazing, and sea warming are increasingly favouring replacement by less structurally complex canopy-forming taxa such as *Halopithys incurva*, or by smaller annual species that are not indicators of good ecological status (Orlando-Bonaca & Rotter 2018; Orlando-Bonaca et al. 2021a).

Between March 2021 and September 2025, during the study period of this dissertation, *G. barbata* forests have experienced a pronounced decline in Slovenian coastal waters (pers. observ.). The once dense algal forest beneath the Piran cliffs has nearly vanished, while the forest at the Belvedere site (near Izola) has deteriorated markedly over the past three years, with only a few surviving cauloids. Although the forest at the Ruda site (near Izola) remains comparatively healthy, it is deteriorating rapidly due to *S. salpa* browsing, high sedimentation, and heavy epiphytic growth (pers. obs.). Regular monthly monitoring at Ruda since 2023 has revealed a consistent annual decline. The ongoing habitat deterioration across all three sites highlights the imminent risk of local extinction for *G. barbata* in Slovenian coastal waters, emphasising the urgent need for targeted protection and restoration measures and actions.

1.4 Recent threats and pressures

The Mediterranean Sea is undergoing profound environmental change, as much of its coastline is densely populated and subject to intense human activity (Benedetti-Cecchi et al. 2001; Bulleri & Chapman 2010; Claudet & Fraschetti 2010; Micheli et al. 2005; Munda 1993a; Orfanidis et al. 2021). Coastal development, maritime traffic, fishing, and tourism have all contributed to widespread alterations of natural habitats, leading to the decline of many important marine habitats including *Cystoseira* s.l. forests (Airoldi & Hawkins 2007; Barrientos et al. 2022; Blanfuné et al. 2025; Bologa & Sava 2006; Claudet & Fraschetti 2010; Manca et al. 2024; Straub et al. 2019; Thibaut et al. 2005). In the Adriatic Sea, major pressures driving their decline include direct habitat damage and loss from coastal urbanization and anchoring (Falace et al. 2010; Orlando-Bonaca & Rotter 2018; Orlando-Bonaca et al. 2021a), increased sedimentation that reduces light penetration, causes abrasions on the thalli and smothers recruits (Benedetti-Cecchi et al. 2001), and overgrazing by herbivorous organisms, which can prevent recovery and maintain barren grounds (Falace et al. 2018; Gianni et al. 2017; Gianni et al. 2018; Ling et al. 2015; Monserrat et al. 2023; Vergés et al. 2009). In addition, these stressors are increasingly compounded by climate-driven pressures, such as marine heatwaves and long-term warming trends, further threatening the persistence of *Cystoseira* s.l. forests across the basin (Bilajac et al. 2024; Cimini et al. 2024; De Bettignies et al. 2018; Eggert 2012; Falace et al. 2021; Giani et al. 2012; Irving et al. 2009; Lokovšek et al. 2024; Orfanidis 1991; Savva et al. 2018; Smale & Wernberg 2013). The cumulative effect of these pressures has led to a widespread regression of brown algal forests throughout the Mediterranean, with many

local populations disappearing entirely and others persisting only in spatial refuges where stressors are locally reduced (Iveša et al. 2022; Perkol-Finkel & Airoldi 2010; Thibaut et al. 2005; Vergés et al. 2009). The loss of these canopy-forming algae has profound ecological consequences, as it entails not only the decline of a key habitat but also the reduction of associated biodiversity and ecosystem functioning (Cheminée et al. 2017; Fukami & Nakajima 2011; Lipej et al. 2023; Mancuso et al. 2021). Addressing these declines requires not only mitigating the underlying drivers but also actively exploring options for restoration of the habitat. In this subchapter, I examine in detail the factors driving the decline of *Cystoseira* s.l. forests and explore the potential for their recovery.

1.4.1 Herbivory pressure

Among the aforementioned drivers of population decline, herbivory is one of the most important mechanisms determining the abundance and density of macrophytes (Lodge 1991) in sublittoral communities (Korpinen et al. 2007). In marine ecosystems, herbivores consume on average 40–48% of plant biomass, a rate that is 5–10 times higher than that observed in terrestrial ecosystems (Bakker et al. 2016). In coastal habitats, herbivore impacts on macrophytes are particularly strong (Pace et al. 1999).

In the Mediterranean, the two main native herbivores of macroalgal forests are the sea urchin *Paracentrotus lividus* and the herbivorous fish salema, *Sarpa salpa* (Linnaeus, 1758) (Guidetti 2006; Hereu 2006). Both herbivores act primarily as browsers, although in macroalgal forests sea urchins can also function as grazers, able to eliminate all erect macroalgae on site (Guidetti & Dulčić 2007; Privitera et al. 2008). In addition to these native herbivores, the non-indigenous rabbitfishes *Siganus luridus* (Rüppell, 1829) and *Siganus rivulatus* (Forsskål & Niebuhr, 1775) have established populations in eastern parts of the Mediterranean following Lessepsian migration through the Suez Canal and are now considered among the most catastrophic invasive species due to their severe grazing impacts on benthic macroalgal communities, including *Cystoseira* spp. forests (Nikolaou et al. 2023; Sala et al. 2011). In large parts of the eastern Mediterranean Sea, the dominance of *Siganus* spp. has coincided with a decline of *S. salpa* populations, as their feeding niches overlap considerably (Azzurro et al. 2007). Invasive rabbitfishes now represent the main herbivorous fish in eastern Mediterranean region (Bariche et al. 2004; Sala et al. 2011; Vergés et al. 2014), while in other regions of the Mediterranean where *Siganus* spp. are absent or occur at low abundances, the native *S. salpa* remains the dominant herbivorous fish.

The impacts of sea urchins on *Cystoseira* s.l. forests are also well documented. Numerous studies have shown that at high densities, sea urchin grazing can lead to formation of barren grounds dominated exclusively by crustose coralline algae. Such outbreaks can be linked to the overfishing of their natural predators - sparids of the genus *Diplodus* (*D. sargus sargus* (Linnaeus, 1758), *D. vulgaris* (Geoffroy Saint-Hilaire, 1817), as well as wrasses *Coris julis* (Linnaeus, 1758) and *Thallasoma pavo* (Linnaeus, 1758) – though other environmental factors may also play a role (Guidetti & Dulčić 2007). In the Mediterranean Sea, in addition to *P. lividus*, also *Arbacia lixula* (Linnaeus, 1758) is responsible for the formation of these barren grounds in areas once colonized by *Cystoseira* s.l. stands. The two species of sea urchins differ markedly in feeding strategies and ecological roles. *P. lividus* is a strict herbivore with a strong preference for erect fleshy algae (Privitera et al. 2008). It is capable of climbing onto arborescent *Cystoseira* s.l. thalli,

bending fronds downward and anchoring them with its tube feet in order to remove biomass directly from the canopy (Guidetti & Dulčić 2007). In contrast, *A. lixula* is omnivorous, with a preference for encrusting corallines (Privitera et al. 2008) and typically exhibits scraping behaviour at the base of *Cystoseira* s.l. thalli (Agnetta et al. 2015). Experimental studies confirm that *A. lixula* alone cannot overgraze arborescent *Cystoseira* s.l. to the extent of producing barren grounds, while *P. lividus* can directly destroy the canopy through grazing (Agnetta et al. 2015). These functional differences highlight their complementary roles: *P. lividus* acts as the principal driver of forest collapse, while *A. lixula* contributes to the persistence of barren states by targeting encrusting corallines and preventing macroalgal recolonization (Bonaviri et al. 2011; Privitera et al. 2008).

Overall sea urchin densities are higher in the southern Adriatic, where barren grounds are also more extensive (Guidetti & Dulčić 2007). In contrast, the northern Adriatic has recorded episodes of population explosions and subsequent collapses of *P. lividus*, which have led to the temporary formation of barren grounds followed by macroalgal recolonization (Boudouresque & Verlaque 2001; Hanel 2002; Vukovič 1982). These dynamics suggest that rocky reef communities in the region are highly variable over time, with cycles of forest loss and recovery shaped by fluctuations in *P. lividus* populations (Hereu et al. 2004; Sala et al. 1998).

Unlike sea urchins, fish do not graze continuously but feed by biting, which in principle allows algal growth to continue (Buñuel et al. 2020; Gianni et al. 2017; Havelange et al. 1997). This led to the long-standing assumption that herbivorous fish posed little threat to macroalgal forests, and as a result their role has historically received less attention. However, large schools of *S. salpa* can repeatedly visit the same macroalgal stands, removing new branches and reproductive structures from the apical parts of thalli (Buñuel et al. 2020; Jadot et al. 2006). Such recurrent feeding can have profound impacts on forest condition, and recent studies have demonstrated that schools of *S. salpa* can exert intense grazing pressure, resulting in severe overgrazing and, in some cases, the complete eradication of macroalgal forests (Buñuel et al. 2020; Gianni et al. 2017; Gianni et al. 2018; Santana-Garcon et al. 2023). Highest proportion of macroalgae is consumed by young individuals, while adults feed mainly on seagrass leaves (Buñuel et al. 2020; Havelange et al. 1997). In addition to *S. salpa*, some omnivorous sparid fishes such as *D. sargus*, *D. vulgaris*, and *D. puntazzo* (Walbaum, 1792) may also contribute to the grazing of *Cystoseira* s.l. forests; however, as key predators of sea urchins, they can simultaneously reduce urchin densities and thereby limit urchin-driven overgrazing (Mcclanahan & Sala 1997; Sala & Zabala 1996).

The overgrazing by *S. salpa* is most likely linked to trophic downgrading caused by the overfishing of large piscivores (Lotze et al. 2011; Marguin et al. 2025), which represent its main predators. Adult *S. salpa* has few predators in the Mediterranean, as large predatory fish primarily target juveniles, which are also the main consumers of macroalgae (Buñuel et al. 2020). Reduced predation is therefore expected to increase juvenile survival, resulting in more numerous cohorts of young individuals grazing on macroalgal stands (Rice et al. 1997). The species is usually not fished commercially or recreationally in the northern Adriatic, but it does get extracted as bycatch by artisanal and recreational fishers (Criscoli et al. 2006; Pallaoro et al. 2008). Due to few predators and negligible fishing pressure, it can possibly grow larger and forms more numerous schools (Jennings & Blanchard 2004).

The schools are highly length-assorted and there is a clear positive correlation between body length and school size, i.e. larger fish form larger schools (Buñuel et al. 2020).

Foraging strategies are also dependent on the size of school. While small *S. salpa* schools forage simultaneously, and scattered over large areas, larger schools (consisting of larger individuals) use a potentially cooperative strategy in which individuals fed rotationally and concentrate on smaller areas for longer periods of time (spot feeding) (Buñuel et al. 2020), which causes more damage to macroalgal forests. Overgrazing has been observed in all known forests of *G. barbata* in the Slovenian part of the Gulf of Trieste, as well as in numerous populations of *G. barbata* and other forest-forming *Cystoseira* s.l. species in the Croatian and Italian parts of the Gulf (Giani et al. 2012; Gianni et al. 2017; Gianni et al. 2018; Iveša et al. 2022).

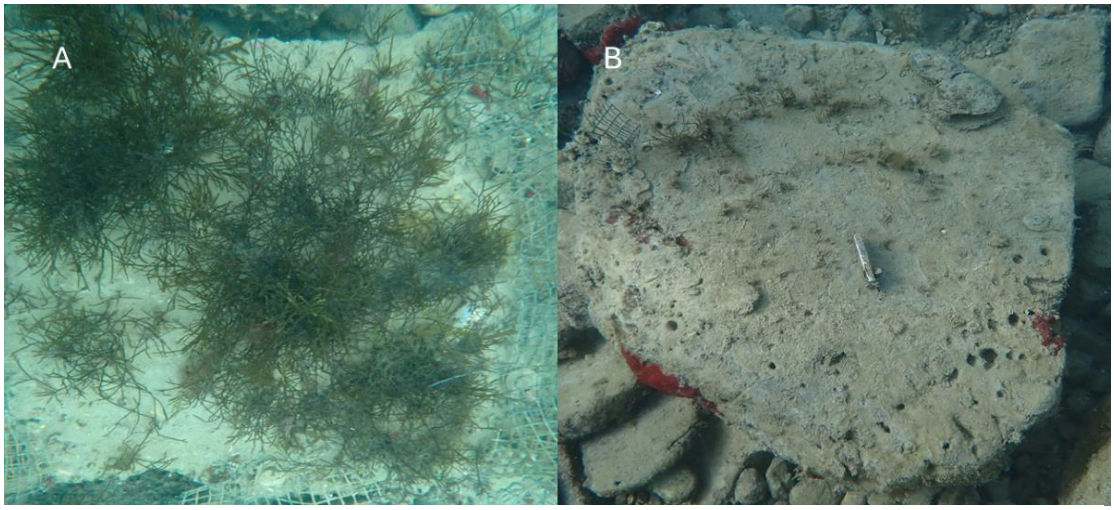


Figure 3: Grazing pressure (presumably by *Sarpa salpa*) on *Gongolaria barbata* in Slovenian coastal waters - July 2023. Photos A and B were taken 2 days apart and show the same boulder (Photo: A. Lokovšek).

On the Croatian coast of Istria, some healthy and ungrazed populations of *G. barbata* living in refugia with locally reduced herbivory was observed, such as in the Šćuza Lagoon and in rock pools at Cape Kamenjak and Bijela Uvala (Iveša et al. 2022). This aligns with finding from research conducted along the Spanish coast, where *Cystoseira* spp. are often confined to spatial refugia characterized by locally reduced herbivory (Vergés et al. 2009).

1.4.2 Sedimentation stress

In recent decades, increasing water turbidity and sediment loads have been documented worldwide as a result of various anthropogenic activities that pose a significant threat to marine biodiversity ({Unep} 1995). Sedimentation is one of the most important factors influencing the structure, biomass and metabolism of benthic communities (Airoldi et al. 1995; Airoldi & Cinelli 1997; Airoldi & Virgilio 1998; Airoldi 2003; Bach et al. 1993; Ceccherelli & Cinelli 1997; Gorgula & Connell 2004; Eriksson & Johansson 2005; Pinedo et al. 2000). The main sources of sediment input include river discharges, cliff erosion, resuspension and transport of particles and direct human activities (Airoldi 2003; Claudet & Fraschetti 2010; Connell 2005; Frignani & Langone 1991; Ogorelec et al. 1991). Along the densely populated Mediterranean coast, these pressures are particularly acute:

commercial and tourist ports, intense shipping traffic, coastal construction, beach erosion and nourishment, and bottom trawling all contribute to increased sediment resuspension and organic particle input (Benedetti-Cecchi et al. 2001; Furlan et al. 2019; Giani et al. 2012; Martín et al. 2014; Mineur et al. 2015; Perkol-Finkel & Airoldi 2010). Rivers bring large amounts of sediment from inland, while over-exploitation of land and alteration of the hydrological cycle exacerbate soil erosion and further increase sedimentation (Nearing 2004). Runoff from urban and agricultural areas not only increases sediment loads but also introduces pollutants such as organic contaminants (Glynn et al. 1984), heavy metals (Glynn et al. 1989) and excess nutrients, which often act synergistically to degrade marine habitats (Connell 2005, 2007; Gorgula & Connell 2004). Sediment particles can consist of clay, silt, detritus or sand and originate from both natural processes and human activities such as mining, road and harbor construction, dredging, beach replenishment and aquaculture (Airoldi & Cinelli 1997; Airoldi & Hawkins 2007).

The effects of sedimentation range from increased water turbidity, which reduces light penetration, to the accumulation of fine particles on the sea floor (Vitousek et al. 1997). Sediments deposited on the seabed are an important factor in stress and disturbance of benthic communities (Airoldi & Cinelli 1997; Airoldi & Virgilio 1998; Airoldi 2003; Airoldi & Hawkins 2007; Alestra et al. 2014; Ceccherelli & Cinelli 1997; Gorgula & Connell 2004; Eriksson & Johansson 2005; Irving et al. 2009). Their distribution is influenced by sediment properties, hydrodynamic conditions (Hiscock 1983) and the type of seabed. Larger particles tend to settle quickly near the source, while fine fractions remain suspended for longer periods due to turbulence and can be transported over long distances by the prevailing currents (Capuzzo et al. 1985; Bach et al. 1993; Moore 1972). The patterns of sediment accumulation are often highly heterogeneous, with marked differences observed even between sites located only 100 m apart, and with patchiness evident at meter scales (Airoldi & Virgilio 1998). The accumulation of the finest fractions is generally most pronounced in sheltered rocky habitats (Ogorelec et al. 1991; Pinedo et al. 2000) - exactly the type of environment in which *G. barbata* typically grows.

Sedimentation rates are determined not only by physical processes, but also by biological factors. In some systems, dense algal canopies promote sediment deposition by trapping particles and reducing resuspension (Airoldi & Cinelli 1997; Eckman et al. 1989; Moore 1972), whereas in others, the constant movement of fronds decreases accumulation through a “whiplash” effect (Kennelly 1989; Melville & Connell 2001). Macrophytes also stabilize sediments by attenuating waves and physically trapping particles, while their loss can lead to significantly higher sedimentation rates (Sfriso & La Rocca 2005). In the Venice lagoon, for example, unvegetated areas had sedimentation rates of over 4000 g m^{-2} per day, compared to only ~40–140 g m^{-2} per day in vegetated zones (Sfriso & La Rocca 2005). This highlights how sedimentation can act as both a driver and a consequence of macrophytes decline.

Sedimentation can profoundly alter the characteristics of the seabed, cause burial and scouring of macrophytes and ultimately reshape their distribution, abundance and diversity (Airoldi & Cinelli 1997; Airoldi 2003; Eriksson & Johansson 2005). Physical changes often lead to the replacement of stable hard substrate by unstable particles, resulting in the loss of suitable habitat for many species (Caretti et al. 2021; Cunning et al. 2019; Eriksson &

Johansson 2005; Martín et al. 2014; Österling et al. 2010; Saunders et al. 2017). The effects are not uniform for all taxa or habitats but vary in time and space, depending strongly on species morphology and reproductive strategies (Airoldi et al. 1995; Airoldi & Cinelli 1997; Airoldi & Virgilio 1998; Bach et al. 1993; Yallop et al. 1994). Direct effects include smothering, scouring and abrasion of thalli, while indirect effects manifest through altered competitive dynamics and reduced recruitment success (Airoldi et al. 1995; Airoldi & Cinelli 1997; Airoldi 2003; Eriksson & Johansson 2005). These mechanisms frequently interact, amplifying the overall stress on benthic communities.

The ability of macroalgae to tolerate sediment stress is closely linked to their morphological and life-history traits (Eriksson & Johansson 2005). Traits such as tough thalli, the capacity to regenerate upright axes from basal structures, opportunistic growth and reproduction, and vegetative propagation confer resilience in sediment-rich environments (Airoldi et al. 1995; Daly & Mathieson 1977; Littler et al. 1983; Stewart 1983). Species with extended reproductive periods—through continuous spore release (e.g., *Cladophora* sp.*, Ulva* spp.*)* or vegetative dispersal by fragmentation (e.g., *Caulerpa* spp., *Cladophora* spp., *Gelidium* spp.) are generally more tolerant of sedimentation (Eriksson & Johansson 2005). Algal turf (dense, low-growing assemblage of mainly filamentous and articulated algae that forms a mat-like layer on hard substrates in shallow coastal environments) may even benefit, with sediment deposition enhancing their growth (Airoldi & Cinelli 1997), whereas erect, long-lived canopy formers are negatively affected (Airoldi & Cinelli 1997; Airoldi 2003; Eriksson & Johansson 2005; Strain et al. 2014). Elevated sediment loads typically reduce macroalgal cover and diversity, shifting communities toward turf-dominated assemblages (Airoldi et al. 1995; Airoldi & Cinelli 1997; Alestra et al. 2014; Gorgula & Connell 2004; Munda 1993a; Orlando-Bonaca & Rotter 2018). Even fine-scale variability in sedimentation rates can strongly shape local community structure and diversity, particularly during recolonization of bare rock patches (Airoldi & Cinelli 1997).

Canopy-forming fucoids such as *Cystoseira* s.l. are among the most vulnerable to sediment stress, as their ecological success depends on sufficient light availability and the successful settlement of recruits on stable hard substrata (Irving et al. 2009; Orlando-Bonaca & Rotter 2018). Rocky coasts, where these forests typically occur, are particularly sensitive to increases in sediment load. Experimental studies have shown that reducing sediment input can markedly improve the survival and resilience of early life stages of *G. barbata* (Strain et al. 2015). Elevated sedimentation can cause up to 83% mortality among *G. barbata* seedlings (Strain et al. 2015). Sedimentation reduces light penetration, impairs photosynthesis, and smothers recruits, thereby preventing successful settlement and undermining forest regeneration. Over time, these pressures drive canopy fragmentation and the replacement of complex *Cystoseira* s.l. forests by simpler, turf-dominated assemblages, leading to reduced structural complexity and diminished ecosystem resilience (Irving et al. 2009; Orlando-Bonaca et al. 2022a). Observations further indicate that elevated sediment loads are consistently associated with declines in vertical complexity, fragmentation of forest patches, and a reduced capacity for recovery (Falace et al. 2010). Sea urchin grazing can therefore indirectly influence the local sedimentation regime by regulating canopy abundance (Airoldi 2003). Similar mechanisms have been observed in

mediolittoral systems, where the establishment of algal beds following the decline of grazing animals improved sediment retention (Branch et al. 1990), while herbivorous snails prevented sediment buildup either by bulldozing surfaces or by grazing on sediment-trapping algal films (Bertness 1984).

In Slovenian coastal waters, at least one healthy *G. barbata* forest was lost following increased sedimentation linked to beach construction in front of the Marine Biology Station Piran in 2016, while another forest, at Ruda site (Izola), is currently under severe pressure from sediment accumulation (pers. obs., see Fig. 4). In recent years, this population has also been heavily impacted by grazing from herbivorous fish. Consequently, the damaged thalli are increasingly overgrown by epiphytic algae, which trap additional sediment and further stress the thalli. As a result, most cauloids remain bare and unbranched for the majority of the year (pers. obs., see Appendix A).

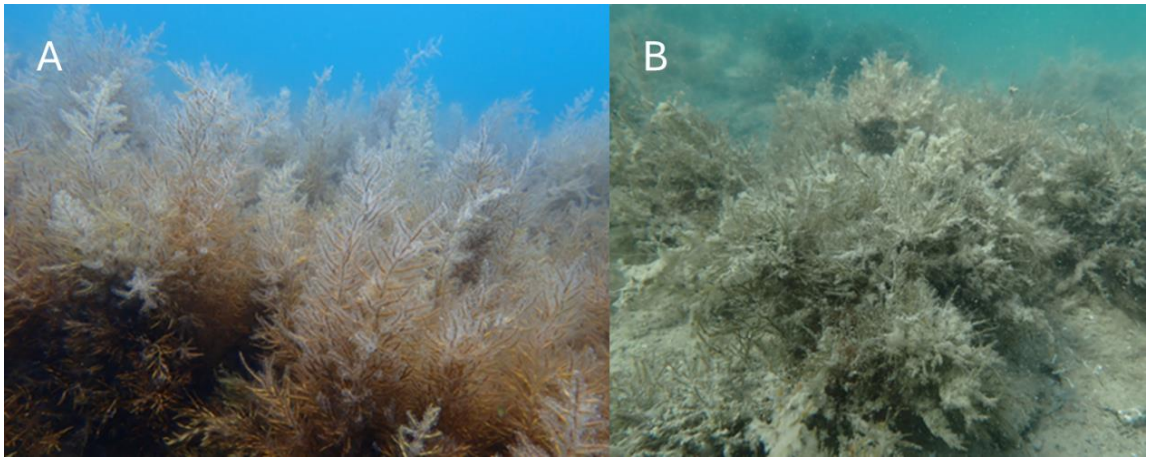


Figure 4: *Gongolaria barbata* forest in Izola (Ruda), two years apart; A - March 2022, B - March 2024 (Photo: A. Lokovšek).

1.4.3 Habitat damage and loss

Habitat damage and loss due to anthropogenic activities are among the most critical threats to biodiversity, structure and functioning of marine coastal ecosystems (Airoldi et al. 2008; Claudet & Fraschetti 2010; Lotze et al. 2006), with mediolittoral and upper-infralittoral belts particularly affected. The destruction of marine habitats has been documented for at least 150 years (Airoldi & Beck 2007) and represents one of the most pervasive pressures on coastal ecosystems worldwide (Novacek & Cleland 2001; Pimm et al. 1995). Occurring over extensive spatial scales, habitat loss compromises the integrity and functioning of ecological processes and diminishes the resilience of marine ecosystems (Airoldi & Beck 2007; Airoldi et al. 2008; Perkol-Finkel & Airoldi 2010; Scheffer et al. 2001; Thrush et al. 2008; Venkatesh & Vora 2024).

The Mediterranean coastline is among the most densely populated in the world, and the northern Adriatic has experienced intense coastal development (Munari 2013; Romano & Zullo 2014; Terzić et al. 2025; 2019). Construction of ports, seawalls, breakwaters, and tourist facilities has replaced natural rocky shores with artificial structures, reducing the

availability of suitable habitats for *Cystoseira* s.l. (Gianni et al. 2013; Tamburello et al. 2019; Thrush et al. 2006; Thrush et al. 2008; Verdura et al. 2018). Artificial substrates generally provide poorer conditions for recruitment, favoring fast-growing opportunistic and turf-forming algae over long-lived canopy species (Bulleri & Chapman 2010). The degradation and loss of habitats suitable for canopy-forming brown algae, particularly through the alteration or removal of the rocky substrata essential for fucoids attachment, reduce population stability, disrupt connectivity among stands, and isolate local populations, thereby making recovery increasingly unlikely (Gianni et al. 2013; Tamburello et al. 2019; Thrush et al. 2006; Thrush et al. 2008; Verdura et al. 2018). Such losses also intensify the effects of other stressors: fragmentation lowers habitat productivity, exacerbates overfishing by shrinking nursery and feeding grounds for fish, and reduces the capacity of forests to buffer the impacts of climate change, particularly by limiting dispersal and recruitment (Benedetti-Cecchi & Cinelli 1992; Duggins et al. 1990; Irving et al. 2009).

Coastal construction, dredging, and beach nourishment often increase sediment load and turbidity, while anchoring damage interacts with grazing and eutrophication to exacerbate canopy regression (Airoldi et al. 2014; Cebrian et al. 2021; Erftemeijer 2006; Furlan et al. 2019; Martín et al. 2014; Orfanidis et al. 2021; Orlando-Bonaca et al. 2021a; Strain et al. 2014; Thibaut et al. 2005). Human-induced damage to the seabed often leads to a shift from structurally complex habitats to less complex ones. For example, macroalgal canopies may be replaced by turf-forming seaweeds (Mangialajo et al. 2008) or by barren grounds (Filbee-Dexter & Scheibling 2014; Piazzi & Ceccherelli 2019).

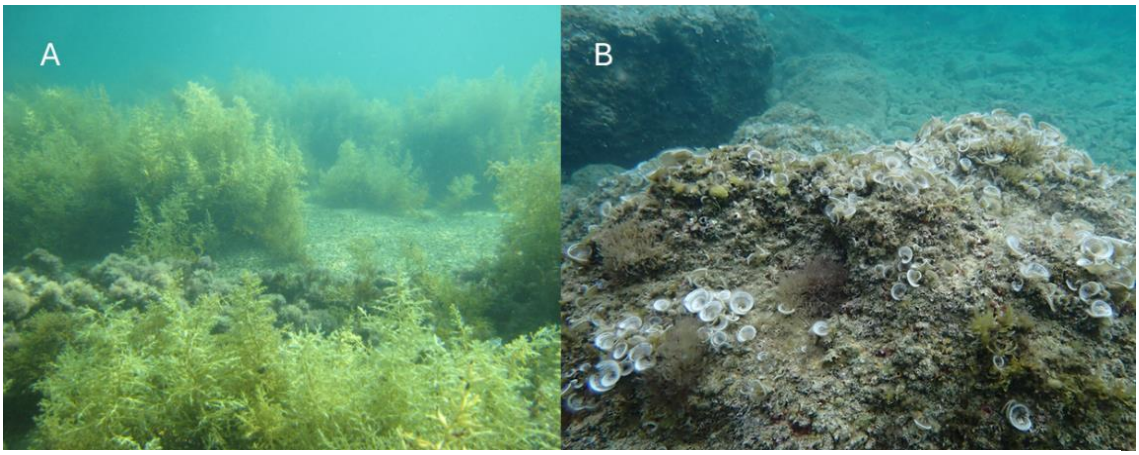


Figure 5: Following beach nourishment and coastal modification in front of the Marine Biology Station Piran, the *Gongolaria barbata* and *Cystoseira compressa* forest (A) - 2018 disappeared due to sedimentation and direct habitat damage (B) – 2024. The former *Cystoseira* s.l. assemblage has been replaced by *Padina pavonica*, *Dictyota dichotoma* and turf assemblage (Photo: A - A. Lokovšek, B – T. Makovec).

In the northern Adriatic, the combined pressures of habitat damage and loss and other anthropogenic stressors have contributed to the near disappearance of *Cystoseira* s.l. forests in many locations (Falace et al. 2010; Munda 1993a; Munda 2000; Munda 2008; Orlando-Bonaca et al. 2021a).

1.4.4 Marine water warming trends and thermal anomalies

Since the early 1980s, the Mediterranean Sea has warmed at an average rate of about 0.31 °C per decade, with an accelerated trend of nearly 0.45 °C per decade over the past two decades, making it one of the fastest-warming marine regions globally (Boero et al. 2008; Diffenbaugh et al. 2007; Guiot & Cramer 2016; Pastor et al. 2020; Skliris et al. 2025; Vargas-Yáñez et al. 2008; Vargas-Yáñez et al. 2010; Vitousek 1994). In the Adriatic Sea, observed trends indicate warming of roughly 0.04 °C per year (~0.40 °C per decade) in many areas, with especially strong increases during summer and significant warming also seen in near-bottom waters in the middle and northern basin (Da Costa et al. 2024; Pastor et al. 2020; Vilibić et al. 2019). The northern Adriatic is among the Mediterranean's most exposed subregions to climate warming. As the shallowest, northernmost, and one of the most productive parts of the basin (Bianchi & Morri 2000), it experiences pronounced seasonal extremes, with winter temperatures (now rarely) falling below 11 °C (Bianchi 2007) and summer peaks reaching (or exceeding) 30 °C. In the South Adriatic Pit, deep waters have warmed by 0.8 °C in just the last decade, with salinity increasing by 0.2 — changes that align with projections previously expected only by the end of the century (Terzić et al. 2025).

Therefore, sea warming has emerged as one of the major threats to marine ecosystems, driving sea level rise (Nerem et al. 2018), alterations of the hydrological cycle (Cheng et al. 2023; Cheng et al. 2024; Durack et al. 2012), modifications in ocean circulation (Caesar et al. 2018; Cheng et al. 2022), stratification, shifts in nutrient and dissolved gas distributions (Stramma & Schmidtko 2021; Venegas et al. 2023; Venegas et al. 2025), and an increasing frequency of extreme weather events. Altered temperature regimes are already reshaping species distributions, causing range shifts and local declines across a broad spectrum of marine taxa, including macroalgae (Sunday et al. 2012).

Among thermal anomalies, marine heatwaves (MHWs) are of particular concern. Defined as discrete, prolonged periods of anomalously high sea surface temperature, their frequency and intensity are rising globally (Hobday et al. 2018; Oliver et al. 2018). In the Mediterranean Sea, MHWs have caused massive declines in canopy-forming macroalgae and other foundation species, with cascading consequences for biodiversity and ecosystem functioning (Garrabou et al. 2022; Smale et al. 2019; Wernberg et al. 2016). These episodes not only disrupt reproductive processes and facilitate the establishment of non-native species but also drive biodiversity loss and range shifts (Smith et al. 2023b). Some of the most severe events were recorded in 1999 and 2003 in the northwestern Mediterranean, where prolonged thermal anomalies caused widespread mass mortality of benthic organisms, affecting many species across thousands of kilometers of coastline (Cerrano et al. 2000; Garrabou et al. 2009; Perez et al. 2000). More recently, between 2015 and 2019, basin-wide MHWs triggered massive declines in more than 50 taxa spanning eight phyla, extending from the surface to 45 m depth (Garrabou et al. 2022). MHWs in the Adriatic basin are also becoming more frequent and intense (Da Costa et al. 2024; Smith et al. 2023b).

Canopy-forming fucoids such as *Cystoseira* s.l. are particularly vulnerable to rising temperatures because they are long-lived, slow-growing (Ballesteros 2002), and

characterized by limited dispersal ability (Capdevila et al. 2018; Coleman & Brawley 2005a; Riquet et al. 2021). Thermal stress has been shown to block karyogamy (Nagasato et al. 1999), reduce photosynthetic efficiency in adults, and cause branch biomass loss (Verdura et al. 2021), and it has also been reported to disrupt reproduction. A recent study on *Cystoseira* s.l. populations in the Ligurian, Tyrrhenian and Ionian Seas demonstrated that high sea temperatures result in poorly developed conceptacles, reduced motility of male gametes, and low fertilization success (Cimini et al. 2024). Additionally, a MHW producing a ±2.65 °C anomaly lasting over one month in the Gulf of Trieste, induced *G. barbata* to form reproductive structures and mature receptacles on adventitious branches during the winter resting phase (Bevilacqua et al. 2019). Although this anomalous fertility was used for an ex situ restoration trial, subsequent performance was lower compared to reproduction during the regular season, with reduced zygote release and growth (Savonitto et al. 2021). These findings suggest that premature reproductive events can compromise later reproductive output.

Also early life stages of *Cystoseira* s.l. are very sensitive, with narrow thermal tolerances (Strain et al. 2015). Experimental work indicates that optimal temperatures for early development of most *Cystoseira* s.l. species range between 12–15 °C, while exposure above ~28 °C is lethal (Falace et al. 2021; Lokovšek et al. 2024). For *G. barbata*, seedlings grow optimally at 15 °C and more slowly at 18 °C, while temperatures above 24 °C result in deformities and low survival (Lokovšek et al. 2024). Adults can survive short-term exposure to ~32 °C, but thalli exhibit visible thermal damage; beyond this threshold, at 34 °C, cauloid disintegration and branch necrosis occur, resulting in irreversible decay (Bilajac et al. 2024). Similarly, *Ericaria giacconei* D.Serio & G.Furnari seedlings develop best at 12–15 °C, experience high mortality at 18–24 °C, and do not survive exposure to 28 °C (Falace et al. 2021).

Taken together, these observations demonstrate that high temperatures affect both vegetative development and reproductive success in *Cystoseira* s.l., reducing fertility, recruitment potential, and ultimately canopy complexity (Andrews et al. 2014; Bartsch et al. 2013). The consequences are not only ecological but also evolutionary. Population genetic studies of *Ericaria selaginoides* (Linnaeus) Molinari & Guiry*, Ericaria amentacea* (C.Agardh) Molinari & Guiry*, and C. compressa* across their Atlantic–Mediterranean ranges revealed highly differentiated populations with limited connectivity and distinct genetic diversity (Buonomo et al. 2018). Climate projections predict dramatic habitat contraction for all three species, with *E. amentacea* suffering habitat losses of up to 90% under extreme scenarios (Buonomo et al. 2018). These declines disproportionately affect unique evolutionary lineages, threatening genetic erosion and reducing adaptive potential (Buonomo et al. 2018). The semi-enclosed nature of the Mediterranean further limits opportunities for poleward dispersal, rendering regional fucoid populations particularly vulnerable to extinction compared with Atlantic populations (Bianchi 2007; Boero et al. 2008; Chust et al. 2024; Diffenbaugh et al. 2007; Vargas-Yáñez et al. 2010).

1.4.5 Eutrophication and oligotrophication

Eutrophication is one of the most widespread human-induced alterations of marine ecosystems (Vitousek 1994). In marine, coastal and estuarine habitats, it results primarily from nutrient enrichment, particularly nitrogen, phosphorus and organic matter, originating from land-based sources such as agriculture, deforestation, urbanization, with domestic and industrial wastewater discharges, as well as marine activities such as aquaculture and from atmospheric deposition related to road traffic and shipping emissions. Increased nutrient inputs can also alter the ratio between dissolved nitrogen and phosphorus (DIN:DIP). The Redfield ratio of 16:1 represents the optimal balance for phytoplankton growth, while significant deviations indicate nutrient limitation: a lower ratio results in nitrogen limitation, while a higher ratio leads to phosphorus limitation of primary production. Species that are less sensitive to such nutrient imbalances can outcompete those with more specific requirements (Ptacnik et al. 2010).

Eutrophication affects canopy-forming algae in multiple, often interacting ways. Increased concentrations of inorganic nutrients usually lead to an increase in the total biomass of algae, but to a decrease in species diversity. Excessive nutrient loading leads to phytoplankton blooms that reduce water transparency and restrict light penetration (Cloern 1999; Domingues et al. 2017; Gómez et al. 2000), limiting the depth distribution of seagrasses and macroalgae, including fucoids. When phytoplankton blooms collapse, the decomposition of organic matter depletes oxygen in the bottom water, leading to hypoxia or anoxia (Boesch & Rabalais 1991; Djakovac et al. 2015; Justić 1991; Kralj et al. 2019). In closed or semi-enclosed basins, such conditions can cause mass mortality of benthic organisms and simultaneously stress canopy-forming algae by increasing the sulfide concentration in the sediments, weakening holdfasts, and reducing the energy available for reproduction (Boesch & Rabalais 1991; Djakovac et al. 2015; Justić 1991; Kralj et al. 2019; Newell & Ott 1999).

In many Mediterranean regions, nutrient and chemical pollution have favored opportunistic taxa, resulting in the replacement of specialized and more susceptible fucoids by more tolerant species such as *Halopithys incurva, Dictyota* spp., *Halopteris scoparia* (Linnaeus) Sauvageau, *Padina pavonica* and turf-forming red algae (Arévalo et al. 2007; Luigi & Giulia 2017). The rapid growth of ephemeral species, including filamentous and epiphytic algae, shades the substrate, restricts the depth distribution of macrophytes and smothers canopy-forming species (Barmawidjaja et al. 1995; Newell & Ott 1999). Heavy epiphytic overgrowth impairs photosynthesis, gas exchange and reproductive success and ultimately threatens survival of erect macroalgae (Belegratis et al. 1999; D'antonio 1985; Karez et al. 2004; Mačič & Svirčev 2014). Over time, nutrient enrichment alters competitive dynamics, resulting in the replacement of perennial canopy-forming algae by ephemeral algal turfs (Karez et al. 2004; Rodriguez-Prieto & Polo 1996). This shift leads to a simplification of habitats, a loss of species diversity and biotic homogenization, with opportunistic species reaching high biomass and displacing non-nitrophilous taxa (Piazzi et al. 2011; Worm & Lotze 2006). Increased nutrient loads also facilitate the settlement of non-native macroalgae, which often displace slow-growing fucoids by colonizing readily available substrates and preventing their recruitment (Alestra et al. 2014; Bellgrove et al.

2010; Ceccherelli & Cinelli 1997; Steen & Scrosati 2004). However, in some cases, dense *Cystoseira* s.l. beds can act as effective barriers against invasion by e.g. *Caulerpa cylindracea* Sonder highlighting their role in buffering ecosystems against bioinvasion (Bulleri 2016; Piazzi et al. 2016).

Fluctuations in *Cystoseira* populations have been associated with patterns of nutrient enrichment also in the northern Adriatic between the 1970s and early 2000s (Iveša & Devescovi 2014); see also Chapter 1.3). The ecological consequences include reduced structural complexity, the loss of associated biodiversity and a reduction in ecosystem services (Giani et al. 2012; Gouvêa et al. 2017; Iveša et al. 2016; Karez et al. 2004; Luigi & Giulia 2017; Marini & Grilli 2023; Newell & Ott 1999; Piazzi et al. 2011; Worm & Lotze 2006).

A trend towards oligotrophication has been observed in the northern Adriatic Sea since 1990s, as evidenced by a long-term decline in chlorophyll-*a* concentrations (Mozetič et al. 2010) and a significant decrease in phosphorus inputs (De Wit & Bendoricchio 2001). Early signs of this shift appeared in the early 1980s through long-term time data on nutrients, chlorophyll-*a,* and phytoplankton biomass (Marić et al. 2012; Marini & Grilli 2023), becoming more pronounced in the 2000s in the northeastern Adriatic (Djakovac et al. 2012) and in the Gulf of Trieste (Cozzi et al. 2020). Recent studies attribute the ongoing oligotrophication of the northern Adriatic Sea primarily to a significant decline in river discharge from the Po Plain and the resulting drop in nutrient inputs (Mentaschi et al. 2024; Verri et al. 2024).

Oligotrophication has mitigated many of the negative effects of eutrophication and is often considered a sign of good environmental health and a desirable outcome of management efforts aimed at mitigating past nutrient enrichment and eutrophication (Fiori et al. 2016; Justić 1987; Le Fur et al. 2019; Tsiamis et al. 2013). It created favorable conditions for the recovery of *Cystoseira* s.l. forests, since lower nutrient concentrations reduce the competitive advantage of fast-growing ephemeral algae and reopen substrates to recolonization by perennial fucoids (Blanfuné et al. 2019; Dublin et al. 1990; Hanel 2002; Iveša & Devescovi 2014; Iveša et al. 2016; Orlando-Bonaca & Rotter 2018; Sales et al. 2011). However, excessive oligotrophication can lead to reduced productivity and a decline in biological resources (Stockner et al. 2000). This trend is expected to be most pronounced in the northernmost Adriatic, particularly in the northeastern sector, where annual net primary production, phytoplankton biomass, and chlorophyll-a concentrations are projected to decrease by 37.8%, 46.3%, and 54.5%, respectively, between 2030 and 2050 (Mentaschi et al. 2024).

1.5 Protection and restoration of *Cystoseira* s.l. forests in the Mediterranean Sea

In response to the widespread decline of *Cystoseira* s.l. forests in the Mediterranean Sea, considerable effort has been devoted to developing and testing innovative restoration tools. Several techniques have shown promise in promoting the re-establishment of self-sustaining populations (Cebrian et al. 2021; De La Fuente et al. 2019b; Falace et al. 2006; Falace et al. 2018; Gianni et al. 2018; Gianni et al. 2020; Kaleb et al. 2023; Lardi et al.

2022; Lokovšek et al. 2023; Lokovšek et al. 2025; Orlando-Bonaca et al. 2021b; Orlando-Bonaca et al. 2022a; Piazzi & Ceccherelli 2019; Smith et al. 2023a; Tamburello et al. 2019; Verdura et al. 2018). However, since the disappearance of these forests usually results from a combination of stressors, restoration cannot rely on transplantation alone but requires a broad suite of complementary measures. The priority is always to mitigate ongoing anthropogenic pressures, such as eutrophication, overgrazing, and sedimentation (Airoldi & Cinelli 1997; Cebrian et al. 2021; Gianni et al. 2018; Gianni et al. 2020; Piazzi & Ceccherelli 2019; Smith et al. 2023a). While certain drivers, such as climate change, cannot be directly mitigated, habitat restoration can be undertaken once other primary stressors are reduced or eliminated. Restoration efforts should therefore focus on areas historically inhabited by *Cystoseira* s.l., where pressures have been mitigated. It is also essential to select species appropriate to the site and to identify suitable donor populations from which to source restoration material (Airoldi et al. 2014; Cebrian et al. 2021; Gianni et al. 2013; Smith et al. 2023a).

In this subchapter, particular attention is devoted to identifying best practices for restoration, describing the cultivation techniques developed for *Cystoseira* s.l. species, and reviewing restoration efforts carried out in the Mediterranean to date.

1.5.1 Conservation legislation and protection status of *Cystoseira* s.l.

Over the last decades, several international frameworks have included algal forests within their scope (UNEP/MAP 1995, Water Framework Directive 2000 (2000/60/EC), Habitat Directive (92/43/EEC), Marine Strategy Framework Directive (2008/56/EC)). The first regional protection measure for the Mediterranean Sea was introduced in 1976 with the adoption of the Barcelona Convention (*Convention for the Protection of the Mediterranean Sea against Pollution*), which came into force in 1978 (Directive 76/464/EEC). Its primary objective was to prevent, combat, and eliminate pollution of the Mediterranean, while safeguarding the marine environment in support of sustainable development. The Convention, together with its seven Protocols under the framework of the Mediterranean Action Plan (MAP), remains the principal legally binding multilateral environmental agreement (MEA) for the region (UNEP/MAP 2000). In 1995, the Convention was amended and renamed the *Convention for the Protection of the Marine Environment and the Coastal Region of the Mediterranean.* A key development was the adoption of the Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean (SPA/BD Protocol), which was signed in 1996 and entered into force in 1999 (UNEP/MAP 1995). Initially, only five *Cystoseira* species (today renamed in *E. amentacea, E. mediterranea, Ericaria sedoides* Neiva & Serrão*, G. montagnei,* and *Ericaria zosteroides* (C.Agardh) Molinari & Guiry) were included in Annex II: List of Endangered or Threatened Species (UNEP/MAP 2018). In 2004, Annex II was expanded to include all species of the genus *Cystoseira* s.l., except for the widespread and more tolerant *C. compressa.* The Annexes to the SPA/BD Protocol also include the common criteria for the establishment of Specially Protected Areas of Mediterranean Importance (SPAMIs), and the list of species whose exploitation is regulated under the Protocol.

Cystoseira s.l. species are considered a conservation priority in SPAMIs (UNEP/MAP 2018).

At the European level, *Cystoseira* s.l. forests are included as habitat types in Annex I of the Habitats Directive (92/43/EEC) since 1992. Annex I identifies "natural habitat types of community interest whose conservation requires the designation of special areas of conservation," thereby recognizing *Cystoseira* s.l. belts as habitats of priority concern. All species of *Cystoseira* s.l are listed as Habitat reef under code 1170. Different species are used for the definiton of the NATURA2000 Habitat1170 on the Mediterranean coast (92/43/EEC).

Under the Bern Convention, *Convention on the Conservation of European Wildlife and Natural Habitats* (Directive 79/409/EEC) which was adopted in 1979 and came into force in 1982, some *Cystoseira* s.l. species are listed in Appendix I (*Strictly protected flora species*), where their conservation is identified as a regional priority. These species are *E. amentacea, E. mediterranea, E. sedoides, G. montagnei, E. zosteroides.*

Although all these different treaties and frameworks together appear to form a robust system of legal protection, the degree of actual, enforceable protection remains limited. Neither the Habitats Directive (Directive 92/43/EEC), nor the Bern (Directive 79/409/EEC) or Barcelona Convention (Directive 76/464/EEC) provide direct legal protection tools for macroalgae. For instance, Annex II of the Barcelona Convention lists *Cystoseira* s.l. species as "endangered or threatened" and obliges Parties to "accord protected status," but does not itself enforce conservation measures. Likewise, the Bern Convention designates 5 *Cystoseira* s.l. species as "strictly protected," yet its implementation and enforcement mechanisms remain weak. Moreover, Annex IV of the Habitats Directive (92/43/EEC) which would require strict protection of listed species, does not include any macroalgae.

Nevertheless, benthic macroalgae, including *Cystoseira* s.l. forests, are incorporated into regular national monitoring programmes under both the Water Framework Directive (Directive 2000/60/EC) and the Marine Strategy Framework Directive (Directive 2008/56/EC). Within these frameworks, macroalgae are used as biological quality elements to assess the ecological status and environmental quality of coastal and transitional waters. Several indices have been developed for this purpose, including the CARLIT index (Ballesteros et al. 2007) and the Ecological Evaluation Index continuous formula (EEI-c) (Orfanidis et al. 2011), both of which use the composition and coverage of macroalgal assemblages as indicators of environmental conditions and anthropogenic pressure. Monitoring of macroalgal communities contributes to the evaluation of multiple MSFD descriptors (Decision 2017/848), particularly Descriptor 1 (Biodiversity), Descriptor 5 (Eutrophication), and Descriptor 6 (Seafloor integrity), providing essential information on ecosystem structure and function. Consequently, macroalgae play a key role in assessing eutrophication levels and other anthropogenic pressures, habitat condition, and overall ecosystem health (Ballesteros et al. 2007; Directive 2008; Gerakaris et al. 2022; Nikolić et al. 2013; Orfanidis et al. 2011; Orlando-Bonaca et al. 2008; Orlando-Bonaca et al. 2021a; European Union 2008; WFD 2003)

1.5.2 Restoration approaches and methodologies

Conservation strategies based solely on habitat protection and the reduction of anthropogenic stressors may be effective when *Cystoseira* s.l. forests are still relatively intact (Cebrian et al. 2021; Gianni et al. 2013; Smith et al. 2023a; Tamburello et al. 2019; Verdura et al. 2018). However, in cases where they are already heavily degraded, fragmented, and undergoing significant losses, passive protection alone is insufficient to halt or reverse their decline (Cebrian et al. 2021; Smith et al. 2023a). While mitigation of stressors is essential for creating favorable environmental conditions, it must be followed by active restoration interventions, that accelerate recovery of *Cystoseira* forests and promote the re-establishment of self-sustaining populations (Cebrian et al. 2021; De La Fuente et al. 2019b; Fabbrizzi et al. 2023; Falace et al. 2006; Falace et al. 2018; Gianni et al. 2013; Gianni et al. 2018; Lardi et al. 2022; Orlando-Bonaca et al. 2021b; Orlando-Bonaca et al. 2022a; Perkol-Finkel & Airoldi 2010; Rindi et al. 2023; Tamburello et al. 2019; Verdura et al. 2018). The rationale for restoration therefore lies in bridging this gap: combining passive approaches (*e.g.*, stressor mitigation and habitat protection) with active measures (*e.g.*, transplantation and recruitment enhancement) to restore functional canopy forests and their associated ecological roles.

Several restoration approaches have been tested in the Mediterranean region, aiming to introduce new individuals into degraded habitats or to provide fertile material to obtain new recruits (Cebrian et al. 2021; Eger et al. 2022; Gianni et al. 2013). The method initially proposed consisted of transplanting adult individuals of *Cystoseira* s.l. from donor sites in good condition to recipient sites where forests had declined (Falace et al. 2006; Perkol-Finkel et al. 2012; Robvieux 2013; Susini et al. 2007). Transplanting juveniles was a next step in developing better restoration methods, as it reduces the impact on long-lived adult individuals whose larger canopies contribute disproportionately to habitat structure and reproductive output (Perkol-Finkel & Airoldi 2010; Perkol-Finkel et al. 2012).

However, given the widespread regression of *Cystoseira* s.l. forests and the threatened or endangered status of most canopy-forming species, harvesting individuals from donor populations is both undesirable and unsustainable. Consequently, alternative and less destructive methods have been developed, focusing on the collection of fertile branches from adults individuals at donor sites and cultivation of seedlings (Clausing et al. 2022; De La Fuente et al. 2019b; Falace et al. 2006; Falace et al. 2018; Falace et al. 2021; Lardi et al. 2022; Medrano et al. 2020; Orlando-Bonaca et al. 2021b; Orlando-Bonaca et al. 2022a; Tamburello et al. 2019; Verdura et al. 2018).

Non-destructive in situ and ex situ techniques are best suited for the recovery of endangered species to avoid depletion of donor populations (De La Fuente et al. 2019b). To maintain genetic integrity and enhance restoration success, reproductive material should ideally be collected from populations located as close as possible to the recipient site and from habitats with comparable environmental conditions (Buonomo et al. 2018; Clark et al. 2020; Coleman & Brawley 2005a; Riquet et al. 2021). *Cystoseira* s.l. species are monoecious, producing both male and female gametes on the same individual, within the same conceptacle (Guern 1962; Rindi et al. 2023; Savonitto et al. 2019), therefore this approach is particularly effective.

During the reproductive season of *Cystoseira* s.l. species, the collection of fertile material from donor populations should be done very carefully. To minimize impacts on reproductive capacity and maintain genetic variability, no more than three fertile apices or less than 5% should be harvested from each algal thallus (Cebrian et al. 2021). Gamete release and fertilization can then be induced by exposing receptacles to a thermal shock, typically by keeping them at 4 °C in darkness for 12–24 hours, which stimulates gamete release (De La Fuente et al. 2019b; Falace et al. 2018; Gerard 1982; Lokovšek et al. 2023; Lokovšek et al. 2025; Orlando-Bonaca et al. 2022a).

After this preparation period, the material collected can be used with different techniques:

1. **in situ cutivation**, by placing fertile branches directly at the restoration site. Success rates of in situ seeding are limited in exposed intertidal and shallow subtidal habitats with strong wave action or currents, because zygotes are easily swept away into the water column (Cebrian et al. 2021; Verdura et al. 2018). In sites with low hydrodynamic conditions, where zygotes can successfully settle and attach, in situ cultivation methods have been tested as a cost- and labor-efficient method for restoring *Cystoseira* s.l. populations, since once settled, seedlings require only periodic monitoring (Cebrian et al. 2021; Lokovšek et al. 2023; Verdura et al. 2018). Different approaches to in situ seeding have been developed:

a. to put fertile apices in mesh bags.

The mesh should be fine enough that the receptacles will not escape through the hole (recommended mesh size 1.20 x 1.28mm, grocery mesh bag for vegetables are good option). This bag will serve as a dispersal bag. Previous studies suggest placing 10 – 20 fertile receptacles in each bag (Cebrian et al. 2021; Verdura et al. 2018), but more receptacles mean better chance of successful seeding and recruitment (author's unpublished data). Right balance between the availability of material at donor population and promoting success at recipient population should be found, not to deplete the donor site. The dispersal mesh bags can be:

- fixed to vertical picks, that are hammered into the substratum, with the bag positioned about 25 cm above the seafloor. Picks are spaced 2–3 m apart (Verdura et al. 2018). The surrounding area should provide suitable settlement substrate, cleared of turf algae. This can be achieved by deploying large, flat stones pre-cleaned of organisms or by brushing the natural rocky surface. Bags are typically removed after ~4 days (pers. obs.);
- tied directly to rocks (See Chapter 7). Mesh bags are secured horizontally onto the substrate so that zygotes fall directly onto the target surface. This reduces the risk of reproductive material being lost to the water column. Bags are left in place for 4–7 days before removal.

b. to seed reproductive material with bottomless containers (author's personal technique, See Chapter 7). A bottomless container is placed on the substrate, into which a mesh bag with fertile apices is briefly shaken (≈2 min) to release gametes and/or zygotes directly onto the settlement surface. This approach is highly time-efficient, as divers do not need to return to remove the bag, but its effectiveness is reduced under medium to high hydrodynamic conditions (pers. obs.).

2. **ex situ cultivation** of seedlings in controlled conditions before transplantation to the field (see Fig. 6). Ex situ cultivation is generally more suitable for environments with high hydrodynamics and grazing pressure (Cebrian et al. 2021; Lokovšek et al. 2023). The relatively simple life cycle of *Cystoseira* s.l. species enables successful cultivation under controlled conditions with appropriate temperature, light and nutrient regimes. With this technique, a large numbers of healthy individuals are grown (Cebrian et al. 2021; Falace et al. 2006; Verdura et al. 2018), since the survival, fitness and growth rates during early life stages can be enhanced (De La Fuente et al. 2019b; Falace et al. 2018; Lokovšek et al. 2023; Orlando-Bonaca et al. 2022a).

The protocol for collecting fertile branches of *Cystoseira* s.l. and preparing them in the laboratory for ex situ cultivation follows the same procedure as that used for in situ cultivation. Additionally, if epiphytic algae or epizoans are present on the fertile branches, they should be carefully removed using a soft brush to avoid damaging the receptacles. Several seeding methods have been tested:

a. One approach involves placing fertile branches in mesh bags, which are then floated on the surface of aerated aquaria containing filtered natural seawater in a closed-circuit system (Cebrian et al. 2021).
b. Alternatively, branches can be placed directly onto cleaned substrata (e.g., natural rocks, clay tiles, or concrete surfaces) using tweezers. This is performed in a shallow layer of seawater, just sufficient to cover the material and facilitate the release of gametes and the settlement of zygotes (Falace et al. 2018; Lokovšek et al. 2023; Lokovšek et al. 2024; Lokovšek et al. 2025; Orlando-Bonaca et al. 2021b).

Substrata must be thoroughly prepared prior to use: natural rocks should be scrubbed with a hard brush, while clay tiles should be cleaned and, if previously used, disinfected with a bleach solution (Falace et al. 2018; Lokovšek et al. 2023; Lokovšek et al. 2024; Lokovšek et al. 2025; Orlando-Bonaca et al. 2021b). Aquaria or containers should be disinfected prior use. After 2–4 days, once gametes have been released and zygotes have attached to the substrate, adult branches are removed to prevent contamination (Lokovšek et al. 2023; Lokovšek et al. 2024; Lokovšek et al. 2025; Orlando-Bonaca et al. 2021b).

During the initial days of cultivation, water movement in the aquaria should be minimized to facilitate zygote settlement (Lokovšek et al. 2023; Lokovšek et al. 2024; Lokovšek et al. 2025; Orlando-Bonaca et al. 2021b). Once settlement has occurred, gentle water circulation is recommended to improve mixing and nutrient exchange. Settled zygotes can be observed with the naked eye as brownish spots (<1 mm in size). Throughout cultivation, environmental parameters—including photoperiod, temperature, and irradiance—should be adjusted to reflect the natural conditions of the donor population (Verdura et al. 2015). The water must be filtered or sterilized and partially renewed at least once or twice per week (Lokovšek et al. 2023). To promote growth, Von Stosch medium or species-specific biostimulants may be added (Kaleb et al. 2023; Lokovšek et al. 2025).



Figure 6: Starting ex situ cultivation in the temperature-controlled room - fertile apices are placed on clay tiles to release gametes and zygotes (Photo: A. Lokovšek).

As an alternative to closed systems, open systems can also be employed (Lokovšek et al. 2023), in which seawater is continuously pumped directly from the sea into the cultivation tanks and subsequently discharged back, maintaining constant circulation. To minimize the risk of contamination, it is advisable to sterilize the incoming water with a UVC sterilizer (Lokovšek et al. 2023). The main advantage of an open system is that temperature and other water parameters remain naturally stable, and regular water changes are unnecessary, making the method more time- and labor-efficient. However, a significant drawback is the potential introduction of epiphytic algae and other organisms such as polychaetes (Lokovšek et al. 2023), since UVC sterilization is only effective against unicellular organisms and does not eliminate multicellular ones.

Seedlings are typically maintained in aquaria or containers for at least 2–3 weeks, regardless of whether they are grown in closed systems/mesocosms or open systems (De La Fuente et al. 2019b; Falace et al. 2018; Gerard 1982; Kaleb et al. 2023; Lokovšek et al. 2023; Lokovšek et al. 2024; Lokovšek et al. 2025). During this period, they should reach a minimum size of 1–1.5 mm before being transplanted (Lokovšek et al. 2023). At this stage, seedlings can either be further cultivated under controlled conditions or transferred directly to the field (Lokovšek et al. 2023). Many studies demonstrated successful outcomes with the tiles with germlings placed on suspended lantern nets (hybrid method) in the water column, which were later (after 2-3 months) transferred to the seabed with protective cages (Lokovšek et al. 2025).

Despite the application of various cultivation techniques, protecting transplanted germlings from grazers and browsers remains a considerable challenge, particularly in areas with abundant schools of herbivorous fish (Gianni et al. 2017; Gianni et al. 2018; Gianni

et al. 2020), as also observed in the field (pers. obs.). Protective cages, although widely used in the last 5 years in the northern Adriatic, have significant limitations: they require regular maintenance to control the overgrowth of epiphytic algae and are prone to damage from fishing activities, heavy thunderstorms, and storm surges (Orlando-Bonaca et al., 2021, 2022). Furthermore, once cages are removed, adult specimens once again become highly vulnerable to grazing (pers. obs.). This highlights the need to test and develop new deterrent devices.

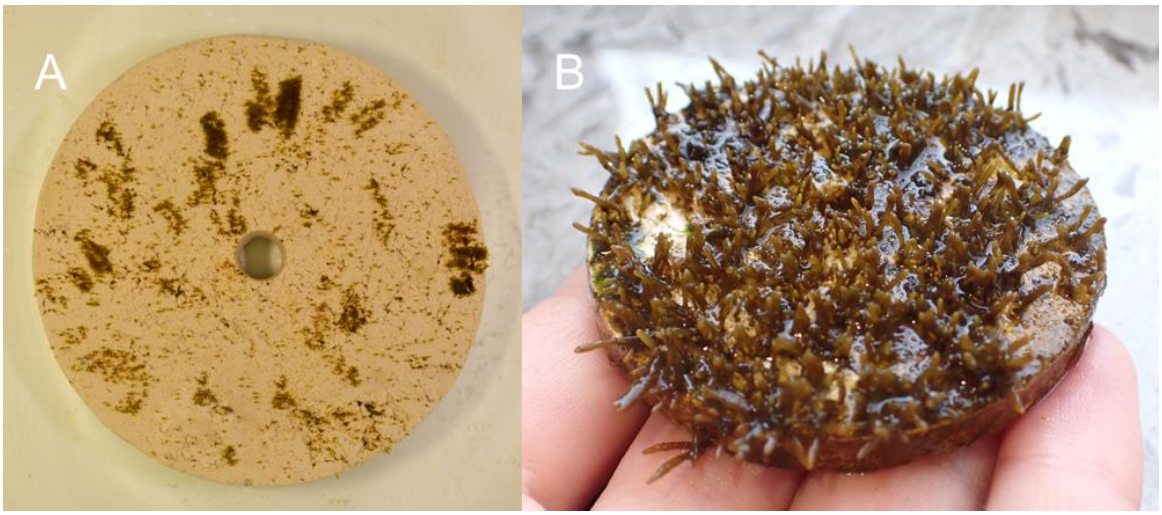


Figure 7: Seedlings of *G. barbata* on clay tiles; A - 2 weeks, B – 3 months old (Photo: A. Lokovšek).

1.6 Life cycle and reproduction of *Cystoseira* s.l.

In brown algae from the order Fucales, the gametophyte stage is highly reduced. The dominant life phase is the sporophyte (diploid), with the reduced gametophyte (haploid) confined to superficial cavities located on the apical branches of the sporophyte (Ballesteros 2002).

The apical parts of the branches of *Cystoseira* s.l species develop into specialized structures called receptacles. Each receptacle bears numerous small cavities known as conceptacles. All *Cystoseira* s.l. species are monoecious, producing both female and male gametes on the same thallus, and they are self-compatible hermaphrodites capable of reproducing via self-fecundation (Bologa & Sava 2006; Coleman & Brawley 2005b; Coleman & Brawley 2005a). Typically, hermaphroditic, bisexual conceptacles occur, while unisexual thalli or conceptacles can develop in some species, but are rather an exception (Rindi et al. 2023).

When a conceptacle is mature, it contains several oogonia, typically between 5–6 and 12, although the exact number varies from species to species (Guern 1962). These oogonia are usually at different stages of development and are formed in the part of the conceptacle opposite the ostiole. The ostiole is a small pore that connects the inner chamber where gametes develop, to the surrounding seawater and appears externally as a tiny opening on

the surface of the receptacle. Each oogonium produces a single egg cell, which can be spherical (90–130 µm in diameter) or ovoid (110–130 × 140–180 µm) (Rindi et al. 2023).

In the conceptacle, near the ostiole, a ring of antheridial branches develops (Guern 1962). These slender, hair-like structures each contain 64 male gametes, known as antherozoids. In some species, such as *G. barbata*, each antherozoid bears a pigmented dot or "stigma" rich in carotenoids; these colored spots give the antheridial layer a distinct brownish-orange appearance (Rindi et al. 2023). Other species like *C. compressa* lack a stigma, resulting in the antheridia forming a greyish layer that may be hard to distinguish from the surrounding paraphyses (Guern 1962).

Once ripe, the gametes are released through the ostiole. Fertilization is oogamous and takes place externally (Guern 1962; Savonitto et al. 2019). Thousands of biflagellate antherozoids are released and encounter the larger, rapidly sinking egg cell near the conceptacle. The fertilized egg cell develops into a zygote that settles on the seafloor near the parent thallus (Riquet et al. 2021). The zygote attaches to the substrate via a sticky fertilization membrane that facilitates adhesion. Polarization then takes place: the initially homogeneous cytoplasm differentiates metabolically and forms the vertical growth axis that defines the poles of the rhizoid and the thallus (Falace et al. 2018; Guern 1962; Savonitto et al. 2019). Within the first 12–24 hours, the spherical zygote undergoes its first equatorial division (De La Fuente et al. 2019b; Falace et al. 2018; Lardi et al. 2022; Savonitto et al. 2019). Within 20-42 hours after fertilization, a second division follows, which is usually perpendicular to the first, but in some cases also parallel. The third division is also perpendicular to the first and usually takes place in the upper cell (Falace et al. 2018). During the first two days, several successive divisions take place without increasing the volume of the embryo (as observed in *E. amentacea*) and four primary rhizoids develop (Falace et al. 2018). In the following days, these rhizoids elongate into long filaments that attach the embryo to the substrate, strengthening the initial adhesion. As the embryo elongates, it gradually loses its spherical form. About a week after fertilization, the fertilization membrane begins to detach. Growth continues through subsequent cell divisions, accompanied by the development of secondary rhizoids (Falace et al. 2018; Lardi et al. 2022). An invagination forms in the apical region, from which hyaline (apical) hairs emerge between days 7 and 12 (Falace et al. 2018; Lardi et al. 2022), after which the embryo assumes an upright position. By the second week, the germlings with numerous rhizoids continue to elongate and small lateral branches with early cryptostomata appear. By the third week, several cryptostomata are fully developed. The germling gradually transitions into a juvenile sporophyte with primary branches (Lardi et al. 2022; Falace et al. 2018). The rhizoids differentiate into a multicellular, discoid holdfast that provides permanent attachment, while species-specific branching patterns develop (Guern 1962).

The juvenile sporophyte stage can last from several months to few years before sexual maturity is reached (Capdevila et al. 2018), as also observed in in situ cultures (pers. obs.). Adult sporophytes have a perennial cauloid and a highly branched thallus, can live for several years, and produce receptacles seasonally (Ballesteros 2002; Ercegović 1952).

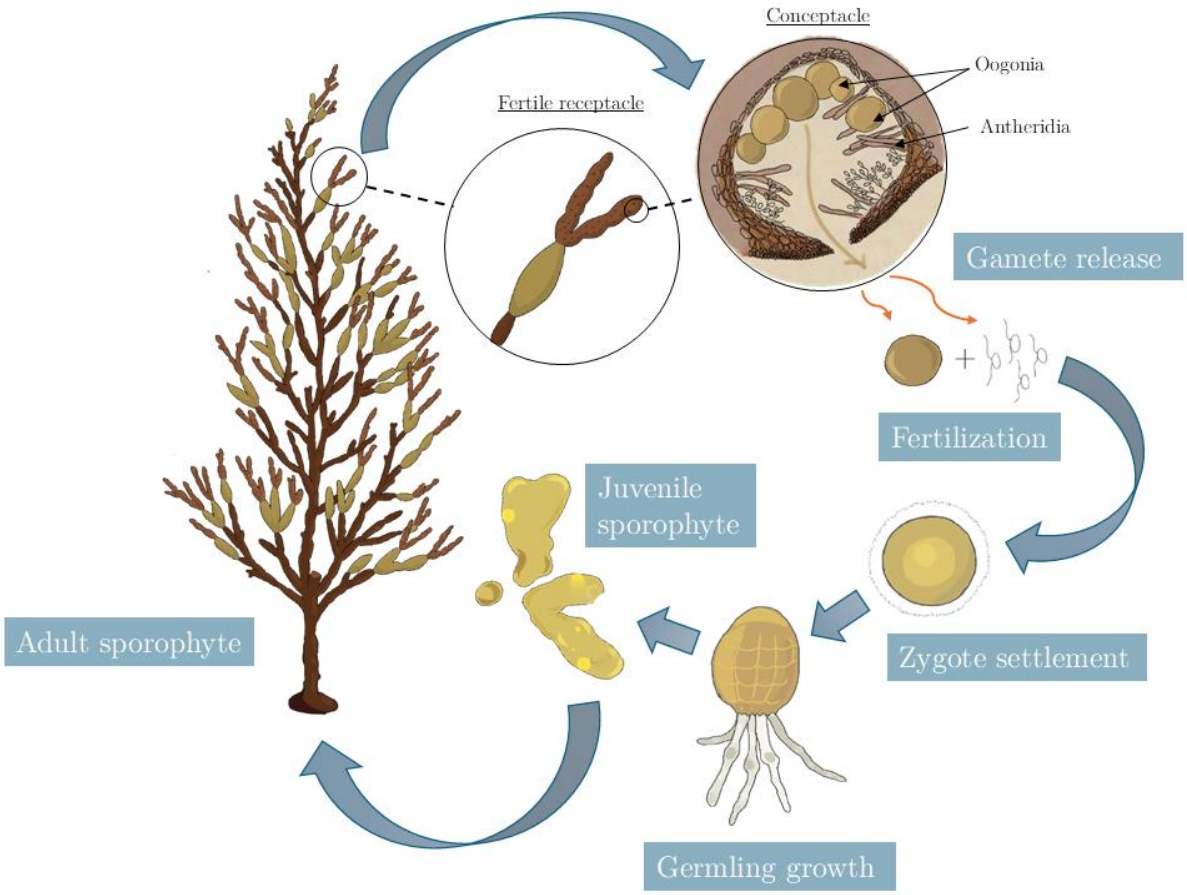


Figure 8: Life cycle of *Gongolaria barbata* (Illustration: A. Benedejčič).

Growth and development follow broadly similar patterns in all *Cystoseira* s.l. species, but the timing of each stage varies according to the species and environmental conditions (Falace & Bressan 2006; Irving et al. 2009; Kaleb et al. 2023; Lardi et al. 2022; Lokovšek et al. 2023; Lokovšek et al. 2024; Lokovšek et al. 2025). Based on embryological traits such as the egg cell shape, the number of primary rhizoids, and the sequence of segmentation of zygote—three groups of *Cystoseira* s.l. have been described (Guern 1962). The first group is characterized by spherical eggs (90–130 µm), antherozoids with pigmented stigma, early appearance of the rhizoidal cell, and embryos with four primary rhizoids. It includes also our study species, *G. barbata*. The second group has ovoid eggs, antherozoids without stigma, and embryos that develop eight primary rhizoids (Guern 1962). The third group has spherical eggs that elongate before segmentation, antherozoids with stigma, and segmentation of zygote beginning with a protrusion at the rhizoidal pole before division. The embryos form four primary rhizoids (Guern 1962).

Most *Cystoseira* s.l. species in the Mediterranean reproduce from late winter to late spring, when sea temperatures are the lowest (De La Fuente et al. 2019b; Falace et al. 2018; Garreta 2000; Lokovšek et al. 2024; Orlando-Bonaca et al. 2021b; Rindi et al. 2023; Rodríguez-Prieto et al. 2013). The timing can vary depending on species and location and is controlled by day length, light availability and temperature (Liu et al. 2017;

Papadimitriou et al. 2022). Temperatures outside the tolerance range for early life stages, generally between 10 and 18 °C for most species, can constrain reproductive development or reduce survival (see Chapter 4; (Marletta et al. 2021)). Furthermore, thermal anomalies may shift or shorten the reproductive window, ultimately decreasing reproductive success (Cimini et al. 2024).

Because egg cells are relatively large and heavy, dispersal capacity of *Cystoseira* s.l. species is limited, typically not exceeding a few meters from the parent sporophyte (Buonomo et al. 2018; Capdevila et al. 2018; Riquet et al. 2021). The estimated dispersal distance of *G. barbata* is less than 600 m (Riquet et al. 2021). This means that gene flow is restricted, and populations are genetically disconnected even at small spatial scales (<10km). Within isolated populations, this can result in occurrence of genetic drift (Riquet et al. 2021).

1.7 Study area

The northern Adriatic Sea is the shallowest, most enclosed and northernmost part of the Mediterranean Sea. It is highly dynamic system, strongly influenced by freshwater inflow, surface heat fluxes and meteorological conditions (Artegiani et al. 1997; Boicourt et al. 2021; Buljan 1976). More recent studies confirm and refine this characterization, highlighting the high spatial and temporal variability of its physical and biogeochemical properties (Grilli et al. 2020; Vilibić et al. 2019). It has cyclonic water circulation, with surface waters flowing north-eastward along the Croatian and Slovenian coast and returning south-westward along the Italian coast (Artegiani et al. 1997; Ursella et al. 2018). Discharges from the Po and other rivers generate a strong southward coastal current along the Italian shore (Western Adriatic Current, WAC), which sustains the cyclonic circulation, while the weaker, warmer, and saltier Eastern Adriatic Current (EAC) flows northward along the eastern coast (Poulain et al. 2001). At depth, colder and denser waters move southward, and a bathymetrically constrained transverse current near the 50 m isobath connects the EAC and WAC (Poulain et al. 2001). Stratification develops from spring to autumn due to surface heating and freshwater buoyancy, whereas winter cooling and strong north-easterly Bora winds induce deep mixing and the formation of dense waters (Boicourt et al. 1999; Poulain et al. 2001; Terzić et al. 2025). Riverine nutrient loads, particularly from the Po, strongly influence the western and northern coasts, supporting high primary production during stratified periods (Cozzi & Giani 2011; Degobbis et al. 2000). In contrast, the eastern part of the basin remains more oligotrophic, where internal remineralization plays a larger role in nutrient cycling (Mentaschi et al. 2024).

The Gulf of Trieste, extending from Cape Savudrija in Croatia to Grado in Italy, represents the northernmost extension of both the Adriatic and the Mediterranean Sea (see Fig. 9). Stretching across three countries and encompassing the entire Slovenian coastline, it covers an area of about 600 km^2, with a volume of approximately 9.5 km^3 and a maximum depth of 37 m. Oceanographically, it is a shallow and semi-enclosed basin, with an average depth of around 18 m and limited water exchange through its open western boundary (Cosoli et al. 2013; Malačič & Petelin 2001).

Its hydrological and physical characteristics are shaped by the interplay of meteorological factors, freshwater inflow from the rivers Soča (Isonzo), Timava, Rižana and Dragonja, and exchanges with the northern Adriatic (Malačič & Petelin 2001). Hydrodynamics are dominated by a northward-flowing eastern current along the Istrian coast. Substantial water exchange also occurs between the Grado–Marano Lagoon and the open sea through six tidal channels, while rivers along other parts of the Gulf contribute less than 10 % to the freshwater balance (Cozzi et al. 2012). Circulation is generally counter-clockwise in deeper layers and clockwise near the surface, and the latter is strongly modulated by prevailing easterly winds, particularly the Bora (Stravisi 1983). The semi-enclosed shape of the Gulf, together with dominant offshore winds from the northeast and its shallow depth, results in relatively sheltered conditions (Boicourt et al. 1999).

The Soča River is the major source of land-derived nutrients, particularly nitrate, and also the principal driver of salinity fluctuations. Average salinity in the area is 37 but can vary widely depending on river discharge (Mozetič et al. 1998). Gulf's hydrography is characterized by seasonal variability. Temperatures typically range between 6 and 28 °C (Malačič et al. 2006), although a trend towards increasing temperatures has been observed in recent decades. With winter temperatures falling below 10 °C, the Gulf of Trieste is the coldest region of the Mediterranean Sea during the winter months (Boicourt et al. 1999). During summer, solar heating and freshwater inflow create a typical thermal stratification of the water column, while in winter the column becomes vertically homogeneous due to autumnal cooling and wind-induced mixing (Boicourt et al. 1999; Mozetič et al. 1998).

Geologically, the Gulf of Trieste lies on a carbonate platform overlain by Holocene sediments (Trobec et al., 2018), with Cretaceous–Paleogene carbonates locally covered by Eocene flysch (Carulli, 2011). The Friulian plain to the north consists of Quaternary glacial–fluvial deposits. Western sectors contain up to 70 % biogenic carbonate, while inner bays (Koper, Piran, Muggia, Panzano) are dominated by clayey silt with ~30 % carbonate (Ogorelec et al., 1991). The carbonate fraction derives from in situ production, and terrigenous minerals originate mainly from flysch erosion, largely via the Soča River. Sedimentation rates are highest in the inner bays (1–5 mm yr^{-1}) and lowest offshore, where Holocene deposits reach 1–5 m in thickness (Trobec et al., 2018).

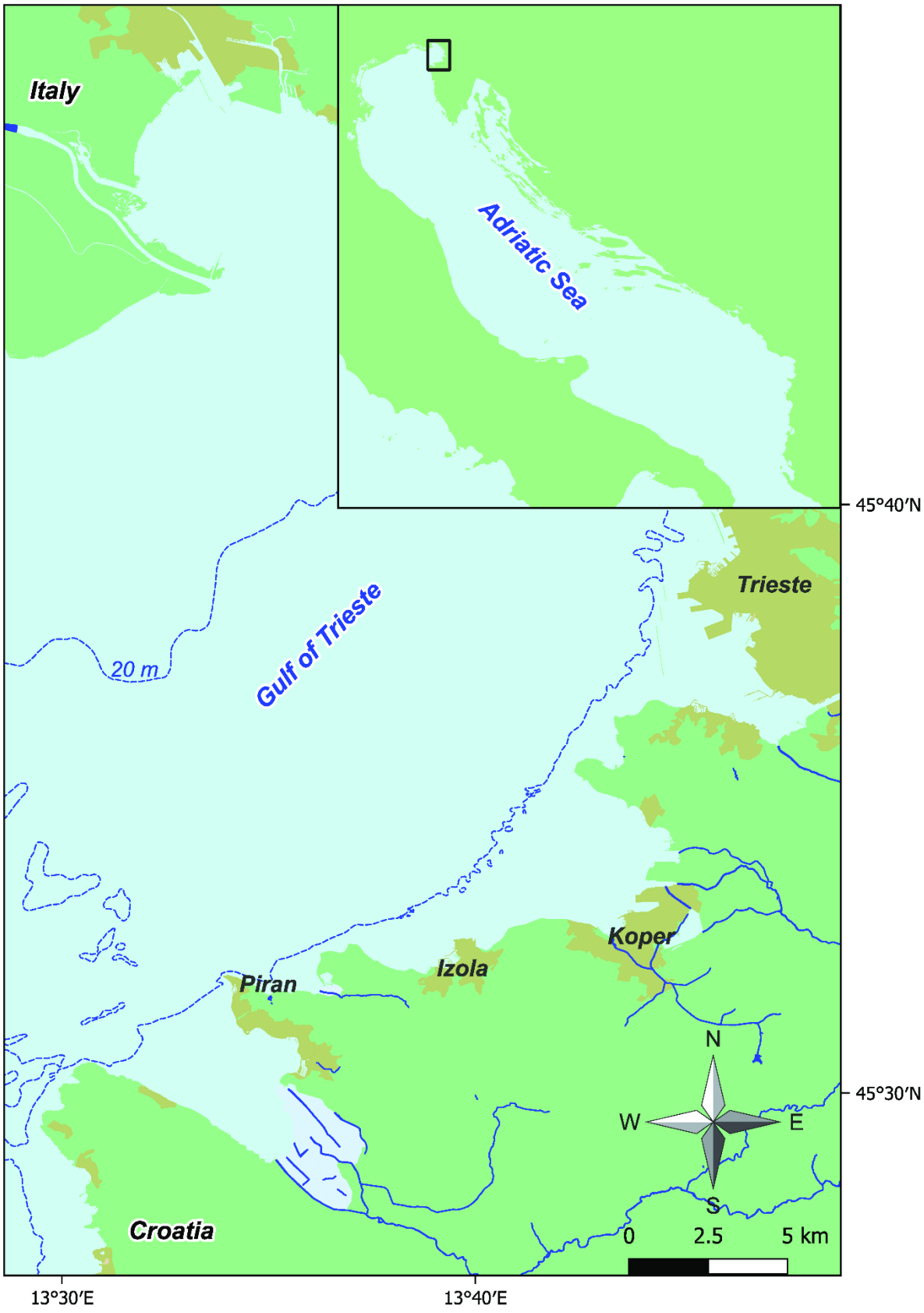


Figure 9: Adriatic Sea and Gulf of Trieste (Map: M. Šiško).

1.8 Species under study: *Gongolaria barbata*

Gongolaria barbata is a shallow-water *Cystoseira* s.l. species that thrives in sheltered, well-lit marine habitats, rock pools and coastal lagoons (Ballesteros 2002; Barcelo Mc 2000; Ercegović 1952; Falace et al. 2024b; Iveša et al. 2022). It is widespread across the Mediterranean Sea, extending to the Atlantic coasts near the Strait of Gibraltar in the west and east into the Levantine Basin (Garreta 2000). Occurrences have also been reported from the Canary and Savage Islands (Blanfuné et al. 2022), and along the Romanian coast of the Black Sea (Marin & Spinu 2023, 2024). In the Mediterranean it typically occupies the upper sublittoral (about 0.5–2 m depth, occasionally deeper in clear waters) (Garreta 2000; Rodríguez-Prieto et al. 2013).

Its morphology is notably variable among sites, reflecting strong phenotypic plasticity. In coastal lagoons, it can develop a free-floating form, previously known as the separate species *Cystoseira aurantia*, now renamed *Gongolaria barbata* f. *aurantia* (Kützing) Falace, Alongi & Kaleb (Falace et al. 2024b) In the Šćuza Lagoon in Croatian Istria, however, the local *G. barbata* population shows yet another distinct morphology that does not correspond to *G. barbata* f. *aurantia* described from Slovenian lagoons (Falace et al. 2024b; Iveša et al. 2022). A detached, free-floating form has been observed, in which the holdfast is absent or non-functional and deformed, cauloid is heavily ramified and lose its' arborescent shape and thalli drift among seagrass fronds and detritus (Iveša et al. 2022; Smith et al. 2025). This form is probably adaptation to lagoonal conditions (Iveša et al. 2022; Smith et al. 2025). In coastal waters, *G. barbata* grows in an erect, non-caespitose form, firmly attached to hard substrata (Ballesteros 2002). The attached thalli occur on rocky seafloors of the upper infralittoral zone all year-round and represent the sporophyte generation. The colour of the thalli varies from golden-brown to dark brown or almost black (Ercegović 1952; Garreta 2000; Rodríguez-Prieto et al. 2013). Younger branches are lighter in colour. The thallus attaches to the substrate with a discoid holdfast called the basal plate, which is compact, thin, usually 5-7 mm, occasionally up to 10 mm or more in width, round-oval shaped with lobed margins (Ercegović 1952; Garreta 2000). From the basal plate grows the cauloid, a perennial, thick, stem-like structure, and the most compact part of the alga. *G. barbata* is a monopodial species, therefore it has a single main axis that is cylindrical, smooth, and uniform along its entire length, measuring 3-5 mm in diameter and up to 50 cm in height (Garreta 2000). The axis is usually simple and slightly curved or twisted at the top. Tophules are absent (Garreta 2000). The apex is prominent, elevated above the insertion point of the mature primary branches, especially during the active growth period in winter and spring, when it may rise 1 to 2 cm above the base of the branches (Garreta 2000). It is smooth, completely bare, without spines and appears flat and truncated at the very tip, although in rare cases, it may be slightly thickened. A prominently raised and bare apical tip is the most distinct and reliable morphological feature of this species (Ercegović, 1952). In favourable conditions, *G. barbata* grows rapidly, as indicated by wide spacing between the still undeveloped primary branches at the tip of the axis (Ercegović 1952). It can grow to 1 m in height (Garreta 2000).

The apical vegetative part with primary branches of *G. barbata* grows from the cauloid (See Fig. 10). Primary branches further divide into secondary branches. Primary branches are well developed, especially in spring. Secondary and higher order branches are filiform (Garreta 2000). Adventitious branches emerge from the scars left by fallen primary branches or from other points along the axis. Typically, one or two adventitious branches grow from each scar, with more than two being uncommon (Ercegović 1952). These branches do not differ significantly from the normal primary branches at the apex of the plant, except that they are usually shorter and less frequently bear aerocysts and receptacles (Garreta 2000). They can develop at any time of year but are usually formed during the summer and autumn. Aerocysts are usually present at the ends of the branches (See Fig. 10) but are sometimes absent (Garreta 2000). They are arranged in chains or occur isolated. All branches have visible cryptostomata. Receptacles develop at the ends of secondary branches in spring and early summer (Ercegović 1952). They are simple, 1 mm wide, 0.5 – 2 cm long, usually cylindrical or spindle-shaped, pedicellate, and terminate in a mucron (Ercegović 1952).

Morphological characteristics of the thallus of *G. barbata* exhibit season- and temperature-related variability (Iveša et al. 2022; Orlando-Bonaca et al. 2021b). In general, the most active vegetative period begins in the second half of autumn, typically in early or mid-November (Ercegović 1952). Primary branches that develop during this time are shorter, with evenly sized and shorter secondary branches, giving them a lanceolate shape (Ercegović 1952). They are darker in colour and have fewer aerocysts and receptacles, which are also short (Ercegović 1952). In contrast, primary branches that develop during winter and the first half of spring show much more vigorous growth, evident in their greater length and the density of all structural elements (Ercegović 1952). In the Slovenian coastal sea, *G. barbata* lose their lighter-coloured secondary branches in winter, and in some cases also primary ones, often leaving only the bare cauloid, which may become overgrown with epiphytes (pers. obs.). New primary branches start growing at the end of winter or in early spring. The reproductive period of *G. barbata* typically begins between February and May in Slovenian coastal waters (pers. obs.), when seawater temperatures range between 8 and 23°C. During the peak of the vegetative period—usually in April or early May—primary branches reach their maximum length, sometimes up to 40 to 45 cm (pers. obs.).

At this time, the entire thallus can measure up to 80 centimeters or even 1 m in length (Ercegović 1952; Garreta 2000).

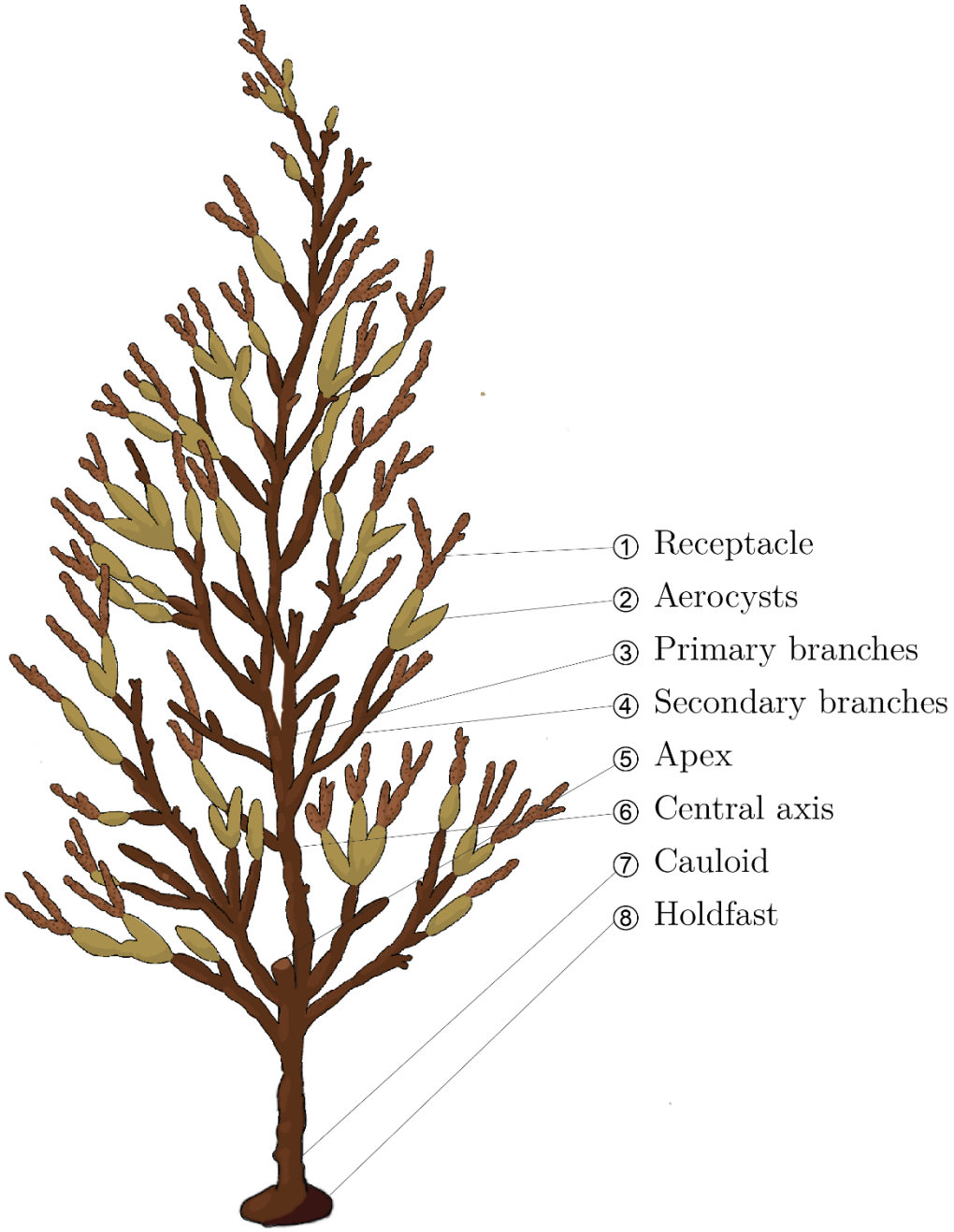


Figure 10: Morphology of *G. barbata* (Illustration: A. Benedejčič).

1.9. Objectives of the Dissertation

The main objective of this dissertation is to develop and optimize integrative, evidence-based approaches for the restoration of *Gongolaria barbata* forests in the northern Adriatic Sea, based on knowledge of the species' biology and life cycle, alongside an understanding of the stress factors that threaten its persistence. The work addresses the urgent need for scalable, ecologically informed restoration strategies for canopy-forming brown algae, which

are undergoing widespread decline due to cumulative anthropogenic pressures and climate-related stressors.

Building on recent advances in macroalgal cultivation, this research aims to improve the survival and growth of early-stage *G. barbata* seedlings destined for restoration purposes. Laboratory and field experiments are used to refine and simplify cultivation protocols, address key knowledge gaps in ex situ cultivation, assess temperature tolerance, explore host-microbiome interactions, and test the use of microalgal extracts. Collectively, this work provides new insights into the biology of *G. barbata* and generates applied knowledge that supports the successful restoration of functional, canopy-forming macroalgal communities in degraded Mediterranean coastal systems.

The specific objectives of the dissertation, aligned with the published research outputs, are:

1. **Improvement and simplification of ex situ cultivation protocols:**
   Development of a cost-effective, reproducible laboratory method for the cultivation of *Gongolaria barbata* seedlings, comparison of different seawater treatments (e.g. UV sterilization vs. filtration) and evaluation of seedling survival and growth in both mesocosms (ex situ) and hybrid systems (in situ).
2. **Evaluation of thermal stress on early life stages:**
   Experimental evaluation of thermal sensitivity of *G. barbata* seedlings under controlled conditions, focusing on growth, deformity and survival across temperature regimes reflecting current and predicted climatic conditions in the northern Adriatic Sea.
3. **Evaluation of the potential of algal extracts as biostimulants on early life stages growth***:*
   Evaluate the effects of microalgal and macroalgal extracts (e.g. *Desmodesmus* sp., *Trichormus variabilis*, *Cylindrotheca closterium* and AlgatronCifo®, a commercial *Macrocystis pyrifera* extract) on seedling survival and development and determine the species- and concentration-specific responses to promote early stages growth in ex situ cultivation systems.
4. **Characterization of the microbiome of *G. barbata*:**
   Investigate the composition, diversity, and host specificity of microbial communities associated with different parts of the thallus of *G. barbata* and explore environmental drivers of microbiome structure as well as variation across morphotypes and habitats.
5. **Assessment of intraspecific genetic diversity:**
   Use molecular markers (cox1 mitochondrial gene) to assess the genetic homogeneity among morphologically and ecologically distinct *G. barbata* populations in the northern Adriatic and western Mediterranean, testing whether the observed phenotypic plasticity reflects true infraspecific diversity or environmentally induced variation.

Chapter 2
Improvement of ex situ cultivation protocols

2.1 Paper: Keep It Simple: Improving the Ex Situ Culture of *Cystoseira* s.l. to Restore Macroalgal Forests

This chapter focuses on refining ex situ cultivation protocols for the habitat-forming brown alga *Gongolaria barbata*, with the aim of enhancing the effectiveness and scalability of restoration practices in the northern Adriatic. Restoration of *Cystoseira* s.l. forests is often constrained by limited dispersal capacity, high sensitivity of early life stages, and strong grazing pressure. These challenges underscore the importance of developing cost-efficient and reliable methods. Building on previous work (De La Fuente et al. 2019b; Falace et al. 2006; Falace et al. 2018; Orlando-Bonaca et al. 2021b) this study aimed to streamline ex situ protocols, reducing costs and labor demands of while assessing the survival and growth of juvenile thalli under mesocosm and open-system conditions. In addition, the study tested whether early transfer of juveniles to the marine environment could improve restoration success.

Specific objectives:
I. Test whether UV-sterilized seawater provides a reliable and less resource-demanding alternative to ultra-filtration in ex situ mesocosm cultivation.
II. Compare mesocosm and open-system approaches for cultivating *G. barbata* germlings from two donor populations in the Gulf of Trieste.
III. Assess the performance of young thalli during the critical transition from laboratory to field conditions, using lantern net baskets as an intermediate protective phase.
IV. Identify cost- and time-saving practices that maintain restoration success while facilitating more frequent and scalable cultivation.

Hypothesis:
Replacing ultra-filtration with UV sterilization and shortening the laboratory phase through early transfer to lantern net baskets will simplify the cultivation of *G. barbata* without compromising survival and growth. This hybrid approach is expected to reduce costs and labor while providing protection from biofouling and herbivory, thus offering a practical pathway to upscale restoration efforts in the Adriatic.

Key findings:

- A low-cost hybrid ex situ–in situ cultivation system for *Gongolaria barbata* was successfully improved and tested.
- Seedlings grown under this system achieved high survival and growth rates compared to traditional methods.
- The approach demonstrated that ex situ rearing followed by in situ acclimatization can produce healthy, transplantable juveniles.
- The method offers a scalable, reproducible, and cost-efficient technique for restoring canopy-forming brown algae.
- Findings highlight the feasibility of restoring degraded macroalgal forests through integrative cultivation and outplanting strategies.

The objectives outlined above were achieved, and the hypothesis was supported by the results presented in this chapter.

The research work is presented in the following publication and is listed below:
Lokovšek, A., Pitacco, V., Trkov, D., Zamuda, L. L., Falace, A., & Orlando-Bonaca, M. (2023). Keep it Simple: Improving the Ex Situ Culture of *Cystoseira* s.l. to Restore Macroalgal Forests. Plants, 12(14), 2615. https://doi.org/10.3390/plants12142615

Ana Lokovšek contributed to this paper as follows: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review & editing.

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Article

Keep It Simple: Improving the Ex Situ Culture of *Cystoseira s.l.* to Restore Macroalgal Forests

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Abstract: Brown algae from genus *Cystoseira s.l.* form dense underwater forests that represent the most productive areas in the Mediterranean Sea. Due to the combined effects of global and local stressors such as climate change, urbanization, and herbivore outbreaks, there has been a severe decline in brown algal forests in the Mediterranean Sea. Natural recovery of depleted sites is unlikely due to the low dispersal capacity of these species, and efficient techniques to restore such habitats are needed. In this context, the aims of our study were (1) to improve and simplify the current ex situ laboratory protocol for the cultivation of *Gongolaria barbata* by testing the feasibility of some cost-effective and time-efficient techniques on two donor sites of *G. barbata* and (2) to evaluate the survival and growth of young thalli during the laboratory phase and during the most critical five months after out-planting. Specifically, the following ex situ cultivation methods were tested: (A) cultivation on clay tiles in mesocosms with culture water prepared by three different procedures (a) filtered seawater with a 0.22 µm filter membrane, (b) filtered seawater with a 0.7 µm filter membrane (GF), and (c) UV-sterilized water, and (B) cultivation on clay tiles in open laboratory systems. After two weeks, all thalli were fixed to plastic lantern net baskets suspended at a depth of 2 m in the coastal sea (hybrid method), and the algal success was monitored in relation to the different donor sites and cultivation protocol. The satisfactory results of this study indicate that UV-sterilized water is suitable for the cultivation of *G. barbata* in mesocosm, which significantly reduces the cost of the laboratory phase. This opens the possibility of numerous and frequent algal cultures during the reproductive period of the species. Additionally, if the young thalli remain in the lantern net baskets for an extended period of several months, they can grow significantly in the marine environment without being exposed to pressure from herbivorous fish.

Keywords: *Gongolaria barbata*; ex situ cultivation; mesocosm; open system; method improvement; brown algal forests restoration

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Citation: Lokovšek, A.; Pitacco, V.; Trkov, D.; Zamuda, L.L.; Falace, A.; Orlando-Bonaca, M. Keep It Simple: Improving the Ex Situ Culture of *Cystoseira s.l.* to Restore Macroalgal Forests. *Plants* **2023**, *12*, 2615. https://doi.org/10.3390/plants12142615

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1. Introduction

Canopy-forming fucoids (Fucales, Phaeophyta) are the dominant habitat builders along the rocky shores of the Mediterranean [1,2]. In particular, *Cystoseira sensu lato* and *Sargassum* species form dense forests that are among the most productive Mediterranean coastal communities [3,4]. These habitats thrive from intertidal to circalittoral bottoms, with diverse species replacing each other along a bathymetric gradient [5]. *Cystoseira sensu lato* species (hereafter referred to as *Cystoseira*) have recently been subdivided into three genera: *Cystoseira*, *Ericaria*, and *Gongolaria* [6]. They are long-lived species [7,8] with relatively large size and high biomass [9]. Important ecosystem services [10] provided by *Cystoseira* algal forests include high primary production [11], rich communities of algae and invertebrates

in the understory [12–15], high fish densities and diversity [16,17], and long-term carbon sink and nutrient cycling [18–20]. Other human interests in *Cystoseira* include the production of numerous bioactive metabolites with therapeutic properties [21]. *Cystoseira* spp. species have been declared as "species of Community interest" in the European Habitats Directive (92/43/EEC) [22]. With the exception of *Cystoseira compressa*, all *Cystoseira* spp. are also listed in Annex II (List of Threatened Marine Species of the Mediterranean) of the Barcelona Protocol (UNEP/MAP, 2009) on Specially Protected Areas and Biological Diversity [23]. They are also monitored according to IUCN, RAC/SPA (Regional Activity Centre for Specially Protected Areas, established under the Barcelona Convention), and MedPan (Mediterranean Network of Marine Protected Areas) requirements. Moreover, due to their sensitivity to environmental changes, *Cystoseira* spp. are used as relevant bioindicators for assessing the ecological and environmental status of coastal waters under the Water Framework Directive (WFD, 2000/60/EC) [24] and the Marine Strategy Framework Directive (MSFD, 2008/56/EC) [25].

As a result of anthropogenic stressors, such as coastal urbanization [26,27], marine pollution/eutrophication [28–33], and turbidity/sedimentation [29,34], *Cystoseira* populations have gradually disappeared in many coastal areas. They have often been replaced by so-called algal turf [4,27,35,36], sea urchin barrens with coralline algae [37,38], or other upright macroalgae (e.g., Dictyotales and Sphacelaries), where an alternative stable state is established [39]. The low turf mat then prevents the growth of more complex algae [40,41] and even restricts grazing due to the large amount of sediment trapped in the turf.

This process is called biotic homogenization and results in a loss of biodiversity [42]. Human activities in the marine environment also negatively affect connectivity, leading to habitat fragmentation and loss and genetic disjunction even at small spatial scales [34,43]. In addition, the zygotes of *Cystoseira* sink rapidly [7] so that they stick to the substrate near the parent algae. Therefore, due to their low dispersal capacity, colonization of new or damaged areas is difficult, so habitat fragmentation has a major impact on these species. In addition, herbivorous fishes have been shown to be capable of permanently damaging brown algal forests [44] and have been defined as important ecosystem modifiers. In particular, salema (*Sarpa salpa* (L., 1758)), whose abundance has increased, probably due to the overfishing of large piscivorous predators [44,45], is responsible for the lower success of *Cystoseira* restoration efforts in the Adriatic Sea [46–48].

Many researchers have also recently reported changes in the distribution and abundance of macroalgal forests due to increasing water temperatures and thermal stresses such as marine heat waves [46,49,50]. Such anomalies can affect the phenology and physiology of canopy-forming species, reduce their performance, increase their susceptibility to other pressures, and lead to population declines and local extinctions [51,52]. Thermal anomalies can also lead to changes in associated species and their interactions [53,54], which can affect ecosystem functions and associated ecosystem services [55,56]. Therefore, the loss of brown algal forests also leads to a reduction in the ability of the oceans to sequester carbon dioxide and contribute to climate change mitigation [57].

The first strategy to address the decline of *Cystoseira* species and their habitat was their protection through international agreements (e.g., Bern Convention [58], Barcelona Convention, Directive 92/43/EEC [23], European Red List of Habitats [59]). Nevertheless, there is little evidence of the natural recovery of degraded *Cystoseira* forests [29,60,61]. For these reasons, restoration actions are needed, also in the context of the UN Decade on Ecosystem Restoration (2021–2030) [62] and the EU Biodiversity Strategy to 2030 [62] (with the proposal of the nature restoration law being its key element). However, implementing successful restoration efforts requires detailed knowledge of the current and past distribution of lost habitats and species, the pressures that have led to their decline, and accurate characterization of donor populations [47,63].

In recent decades, restoration attempts have been made in the Mediterranean to prevent further loss of fucoids, and several methods have been tested, from transplanting adult thalli [64–66] or juveniles [29,67], in situ seeding [61,68] and out-planting ex situ

grown germlings [10,46–48,64,68–72]. Non-destructive in situ and ex situ techniques are best suited for the recovery of endangered species to avoid the depletion of donor populations [10]. In particular, several efforts have been made to develop cost- and time-efficient methods for the ex situ cultivation of *Cystoseira s.l.* species. However, several factors must be considered when cultivating germlings for reforestation, such as water parameters, potential infestation by epiphytic algae, and the development of microbial biofilms on culture surfaces. While epilithic microbial biofilms are an important component of the coastal ecosystem and have numerous beneficial effects, such as serving as primary producers, food resources for herbivorous animals [73–75], and promotors of larval settlement and macroalgal recruitment [76], they have also been shown to affect the growth and success of neighboring macroalgae by releasing allelopathic compounds [77] and potentially suppressing their growth.

On the other hand, open laboratory system cultures of *Cystoseira s.l.* can also be negatively affected by grazing pressure, especially at early-life stages [78]. Herbivorous gastropods, crustaceans, and polychaetes can also play a role in consuming various parts of the thalli, potentially affecting its biomass and the overall health of the algae [78]. Grazing plays a crucial role in regulating the density of canopy-forming fucoids and represents a major challenge in marine forest restoration efforts.

The present research focused on the restoration of *G. barbata.* The main objective of the study was to improve and simplify the current ex situ laboratory protocol for the cultivation of *Gongolaria barbata* [46] by testing the feasibility of some cost-effective and time-saving techniques on two populations (donor sites) of *G. barbata* and assessing germling survival and growth during mesocosm culture and during the most critical five months after outplanting (as germlings need to adapt to the coastal sea conditions and start developing articulated thalli). Specifically, the following ex situ cultivation methods were tested: (A) cultivation on clay tiles in mesocosms with culture water prepared with three different procedures and (B) cultivation on clay tiles in open laboratory systems. After the cultivation phase, all thalli were fixed to plastic lantern net baskets suspended at a depth of 2 m in the coastal sea, and the restoration success was monitored in relation to the different donor sites and the cultivation protocol.

2. Materials and Methods

2.1. Study Area and Material Collection

The study area is located in the Gulf of Trieste, a shallow, semi-enclosed bay in the northernmost part of the Adriatic Sea, with an average depth of only 18.7 m and a maximum depth of 37 m. The area is known for the highest tidal amplitudes (average = 88 cm; [79]) and the lowest temperatures in winter [80]. Water temperature and salinity are strongly influenced by river discharge. The Slovenian part of the Gulf of Trieste accounts for one-third of the total surface area of the Gulf [81]. The coastal relief varies from steep rocky cliffs to gradual sloping beaches. The lower part of the coast, in particular, has been heavily modified by anthropogenic development and urbanization. Today, only one-fifth of the coastline remains in its natural state [79,81]. The bottom along the coast is mainly rocky and consists of alternating layers of flysch, sandstone, and soft marl [79].

A sharp decline in fucoids has been reported in the northern Adriatic Sea [35,36,82], leading to displacement by turf-forming taxa in shallow waters. The increasing abundance of these low-lying algae is likely related to human-induced hydro morphological changes to the coastline and high sediment resuspension rates [82]. In addition, negative impacts by native herbivorous fish have also been documented [46,48]. Currently, *Gongolaria barbata* (Stackhouse) Kuntze and *Cystoseira compressa* (Esper) Gerloff and Nizamuddin are quite common in the Gulf of Trieste on the Slovenian coast, while they have almost disappeared on the Italian coast. Other species of *Cystoseira s.l.* are already rare in Slovenian waters and extinct in the Italian waters of the Gulf of Trieste.

Fertile apices of *G. barbata* were collected from donor populations at two sites:

1. Izola Merkur, which is characterized by healthy and dense populations of *G. barbata* and *C. compressa*; coordinates: 45°32.653, 13°40.554.
2. Izola Belvedere, which is characterized by healthy and dense populations of *G. barbata;* coordinates: 45°31.979, 13°38.488.

The two donor sites (Figure 1) for the collection of apical fronds were selected according to previous studies [48,82] and SCUBA diving surveys in spring 2022.

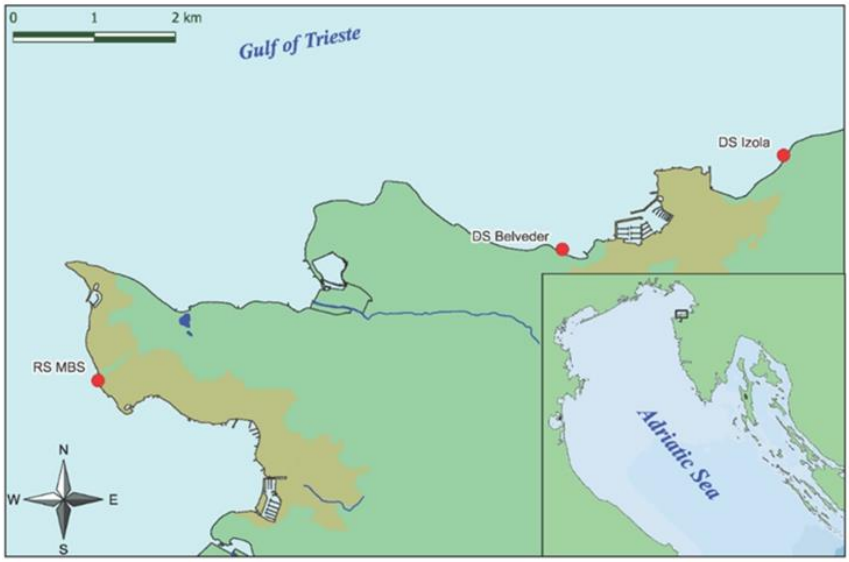


Figure 1. Map of the study area showing donor sites (DS) of fertile apices of *G. barbata* and receiving site (RS).

In early April, the fertile apical fronds of *G. barbata* were collected by SCUBA divers at approximately 2 m depth. We collected up to 10% of fertile apices per individual thalli to avoid a negative effect on the population. The apices were cut with scissors and kept in mesh bags that were stored on the boat in buckets with seawater and immediately transported (within 30 min) to the laboratory of the Marine Biology Station Piran (MBSP).

2.2. Laboratory Work

The current protocol for preparing water for the ex situ culture of *G. barbata* [46] requires large volumes of ultra-filtered seawater (0.22 µm filter membrane), which is time-consuming and costly. Therefore, three different water treatments were tested for the present study: (a) filtered seawater with a 0.22 µm filter membrane, (b) filtered seawater with a 0.7 µm filter membrane (GF), and (c) UV-sterilized water.

2.2.1. Preliminary Testing of the Effectiveness of UV Sterilizers

Before using UVC-sterilized water for ex situ cultivation experiments, the efficiency of an Eheim Clear UVC 11 W sterilizer was tested.

1. Test for bacterial growth on agar plates. Cultures from unsterilized, 1× UVC-sterilized, and 5× UVC-sterilized water (water flowed through sterilizer 5 times at water flow 150 L/h) were inoculated onto growth media plates by (a) adding 100 µL of each water sample to separate agar plates, (b) filtering 50 mL of each water sample through a 0.45 µm filter that was then put on separate agar plates. The agar plates were monitored, and CFU (=colony forming units) were counted daily for 3 days.
2. Test for bacterial and microalgal growth on the clay tiles in the growth chamber. Three 60 cm × 30 cm × 30 cm aquaria were prepared, each containing three clay tiles. Unfiltered seawater (control) was added to the first aquarium, 1× UVC-sterilized water

to the second, and 5× UVC-sterilized water to the third. Under established culture conditions (T = 17 °C, photoperiod 15:9 h light:dark cycle, light intensity = 125 µmol photons m^{-2} s^{-1}), the clay tiles were monitored daily for 23 days.

2.2.2. Ex situ Cultivation in the Mesocosm

In the laboratory, apices with mature receptacles from two donor populations were cleaned of epibionts with a soft brush, rinsed with filtered seawater, wrapped in aluminum foil, and stored overnight (24 h, approximately 5 °C). This thermal shock triggered the release of gametes from the conceptacles, allowing fertilization [69]. *G. barbata* is a monoecious species; therefore, self-fecundation can occur. In environmentally controlled rooms, the next day, fertile apices from the two populations were placed on clay tiles in aquaria (previously disinfected with 5% bleach solution) with differently treated seawater (Figure 2).

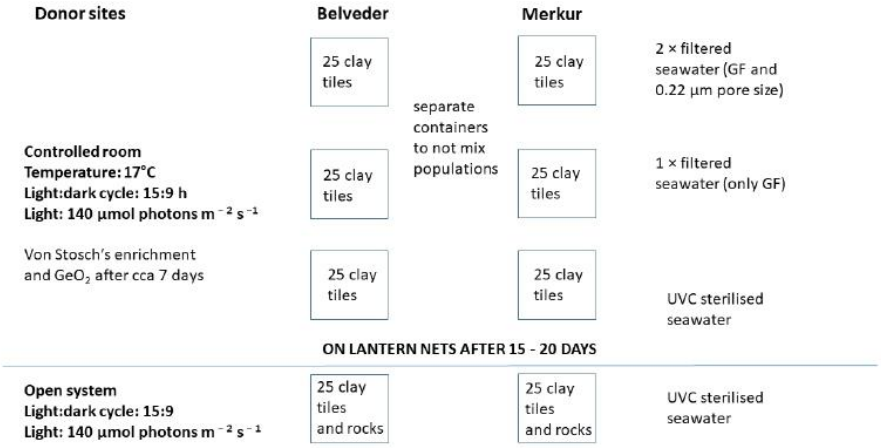


Figure 2. Ex situ cultivation scheme for *G. barbata* at Marine Biology Station Piran in spring 2022.

A total of 6 aquaria (40 cm × 34 cm × 9 cm) were prepared, 3 for each donor site; the first with 2× filtered seawater (through GF filter and 0.22 µm pore size filter), the second with only GF filtered seawater, and the third with seawater filtered through a UVC sterilizer (Figure 2). Before the experiment, all aquaria were disinfected with a 5% bleach solution. All aquaria were covered with plexiglass lids to prevent water evaporation. The temperature in the growth chamber was set to 17 °C, and the photoperiod was set to a 15:9 h light:dark cycle. The light intensity was set to approximately 140 µmol photons m^{-2} s^{-1}. For illumination, 4 Osram Fluora Florescent tubes 36 W with a length of 120 cm and a luminous flux of 1400 lumens per container were used.

Twenty-five clay tiles (diameter = 6 cm, with a central hole of 0.6 cm) were placed in each aquarium, and 5–6 apical fronds were carefully placed on each clay tile. On the first day, the aquaria were filled with water to a maximum height of 1 cm (2 mm above the apices) to prevent the apices from floating. Three additional glass slides with apices were added to each aquarium to monitor, measure, and photograph the development of zygotes every 24 h using an Olympus SZX16 stereomicroscope. The apices were left on the tiles for 2 days and then carefully removed with forceps. On day 3, 1 L of treated seawater (GF + 0.22, GF or UVC) was added. From day 6, the water was changed every 2–3 days, and Von Stosch's solution and germanium dioxide (to prevent diatom contamination) were added [10,69]. On day 6, air pumps and bubblers were also installed in the aquaria. Ten randomly selected tiles from each aquarium were photographed vertically with a Nikon D850 camera after the first and second weeks to assess the coverage of *G. barbata* germlings.

During the first 16 days, the juvenile *G. barbata* thalli were measured and photographed every 24 h under the Olympus SZX16 stereo microscope.

2.2.3. Ex Situ Cultivation in Open System

Two 60-liter aquaria were prepared and supplied with UV-sterilized seawater, one for each donor site. A Kessil H160 Tuna Flora LED grow lamp with a spectrum of 380–780 nm was installed above each aquarium. The photoperiod was set to a 15:9 h light:dark cycle. The light intensity was set to approximately 140 µmol photons m^{-2} s^{-1}. To prevent sediment from settling on the clay tiles and on young algal thalli, the tiles were placed on a net 5 cm above the bottom. A total of 48 clay tiles were placed in each aquarium, and 5–6 apical fronds of *G. barbata* were carefully placed on each clay tile. On the first day, the aquaria were filled with water to a maximum height of 5.5 cm (2 mm above the apices) to prevent the apices from floating. After 48 h, the apical fronds were carefully removed, and aquaria were filled with water to a height of 25 cm. Seawater, pumped directly from the sea in front of the MBSP, flowed directly into an Eheim Clear UVC 11 W sterilizer and then into the aquaria. Sediment accumulating under the net was vacuumed once a week. For the first 16 days, the young *G. barbata* thalli were measured and photographed every 24 h under a stereomicroscope, then once per week with a caliper. After three months, the lighting was changed to the blue spectrum to accelerate the growth of *G. barbata* thalli, and a stream pump (Sicce Voyager nano, Q max 1000 L/h) was added to the aquaria to improve circulation in the system.

2.3. Out-Planting in the Marine Environment

After 16 days, the labeled clay tiles from the different experimental conditions in the mesocosm were transferred to lantern net baskets in front of the MBSP (Figure 3). The tiles were secured to the bottom of the lantern net baskets with zip ties. The lantern net baskets were placed at a depth of approximately 2 m and tied to a concrete weight (placed at a depth of 4 m on a sediment bottom), with buoys attached above them. *G. barbata* development was monitored photographically from day 28 by SCUBA diving, initially every two weeks until day 71. From day 71, development was monitored approximately once a month until day 159 (23 weeks). Nutrient levels, water temperature, pH, oxygen saturation, and salinity were measured every 2–3 weeks at a depth of 3 m at the oceanographic buoy Vida (https://www.nib.si/mbp/en/; accessed on 21 February 2023).

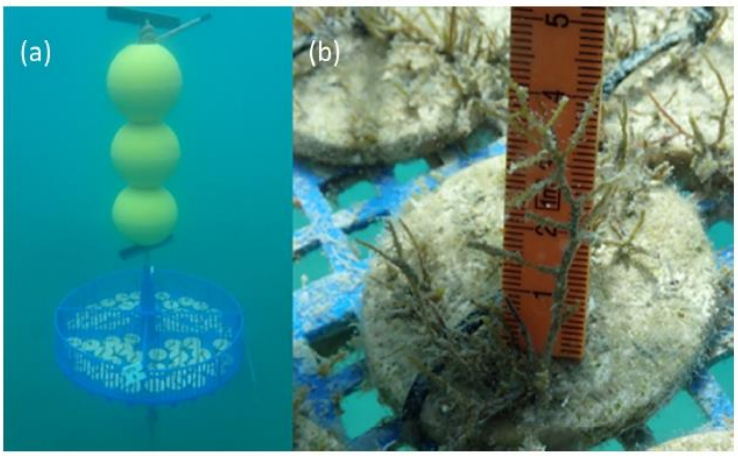


Figure 3. (**a**) A lantern net basket with clay tiles on day 16, and (**b**) measuring the length of *G. barbata* thalli on clay tiles during routine photographic monitoring (M 0.22 on day 122).

2.4. Photo-Processing and Statistical Analyses

Photos from the ex situ culture in thermostatic chambers were processed using the ACDSee program and then analyzed using the ImageJ program to calculate the coverage of *G. barbata* germlings on each tile in the first two weeks. Percent cover was determined manually by thresholding and selecting pixels in the color spectrum that corresponded

to areas with *G. barbata* germlings [72]. Statistical analyses were performed using R 4.2.2 (R Core Team, 2020). A two-way robust ANOVA based on trimmed means (20% trimming level) [83] was performed for the main effect and interaction to check for differences in thalli length and cover of *G. barbata* germlings between two fixed factors: donor site (with 2 levels: B and M) and treatment (with 4 levels: UV, 0.22, GF, and open system UV). The test was chosen because it is more robust to deviation from normality and homogeneity of variance than the classic two-way ANOVA. A trimmed mean discards a defined percentage at both ends of the distribution, achieving nearly the same amount of power as the mean in the case of a normal distribution and reducing substantially standard error in the presence of outliers [83]. The statistical outcome measure of this test, Q, is interpreted in the same fashion as the traditional F statistic [83]. Tukey's multiple comparisons of means were used to compare the treatments and the donor sites independently. Analyses were performed using WRS2 packages [83]. Data were then graphically represented with boxplots using the ggplot2 package [84].

For the evaluation of germlings' length and coverage in the laboratory and thalli length in lantern net baskets, the tiles were considered replicates, but we faced a problem of spatial pseudoreplication, as some of the tiles belonged to the same aquarium. For logistical reasons, it was not possible to have completely independent replicates in the laboratory phase, but careful interspersion and randomization allowed us to reduce the risk of an aquarium effect during the second (in situ) phase. This aspect was taken into account for statistical analyses and in the interpretation of the results, keeping in mind the risk of inflated Type I errors in the case of a simple pseudoreplication [85]. For this reason, two-way ANOVA was not applied to the results of the laboratory phase.

3. Results

3.1. Preliminary Test of the Efficiency of UV Sterilizers

3.1.1. Evaluation of Bacterial Growth on Agar Plates

Six agar plates were examined on days 2 and 3, and colony-forming units (CFU) were counted on each plate (Table 1). Samples 1, 3, and 5 were prepared by applying 100 µL of each water treatment sample to the culture medium plates (1× UV-sterilized water, 5× UV-sterilized water, and untreated seawater). Samples 2, 4, and 6 were inoculated by filtering 50 mL of each water treatment sample through a 0.45 µm filter using vacuum filtration. No colonies formed in 1× UV-sterilized water on days 2 and 3 (Table 1). The calculated CFU/L showed that 1× UV-sterilized water prevented the growth of bacterial colonies and could be tested for the cultivation of *G. barbata* germlings.

Table 1. Results of tests with differently treated seawater on agar plates (SW—untreated seawater, TNTC—too numerous to count, CFU—number of colony forming units).

Day	Sample_ID	Sample Treatment	Counting Method	Volume of Seawater [µL]	CFU	CFU/L
2	1	SW	On agar plate	100	22	220,000
2	2	SW	On filter	10,000	>250	TNTC
2	3	1× UVC	On agar plate	100	0	0
2	4	1× UVC	On filter	50,000	11	220
2	5	5× UVC	On agar plate	100	0	0
2	6	5× UVC	On filter	50,000	1	20
3	1	SW	On agar plate	100	26	260,000
3	2	SW	On filter	10,000	>250	TNTC
3	3	1× UVC	On agar plate	100	0	0
3	4	1× UVC	On filter	50,000	12	240
3	5	5× UV	On agar plate	100	1	10,000
3	6	5× UV	On filter	50,000	2	40

3.1.2. Evaluation of Bacterial and Microalgal Growth on Clay Tiles in the Growth Chamber

The first growth of biofilm on clay tiles in unsterilized seawater was observed on day 15. A brownish biofilm formed on 2 of 3 clay tiles in this aquarium. No growth was detectable on the clay tiles in the aquaria with 1× and 5× UV-sterilized water. On day 18, a brownish biofilm formed on all 3 clay tiles in the aquarium with non-sterilized seawater, while the clay tiles in the sterilized water were still clear. On the 23rd day, the brownish biofilm on the clay tiles in the non-sterilized seawater spread and covered most of the surface of the tiles, while the tiles in the other 2 aquaria still showed no signs of growth. This result proves that the sterilizer works well to prevent the growth of a microbial biofilm, which could suppress the recruitment and growth of *G. barbata* germlings in the first few weeks.

3.2. Growth and Survival of Germlings in the First and Second Week in Mesocosm and Open System

On the fourth day, germlings had an average size ranging from 90.41 ± 13.90 µm (Belveder 0.22 µm) to 137.08 ± 45.90 µm (Merkur GF). The first development of diatoms on the tiles in the mesocosms was observed on the sixth day; therefore, GeO_2 was added to prevent further growth. The average size of the germlings in the mesocosm ranged from 92.09 ± 20.35 µm (Belveder GF) to 119.87 ± 10.10 µm (Merkur UV) and 98.51 ± 12.00 µm in the open systems (Figure 4).

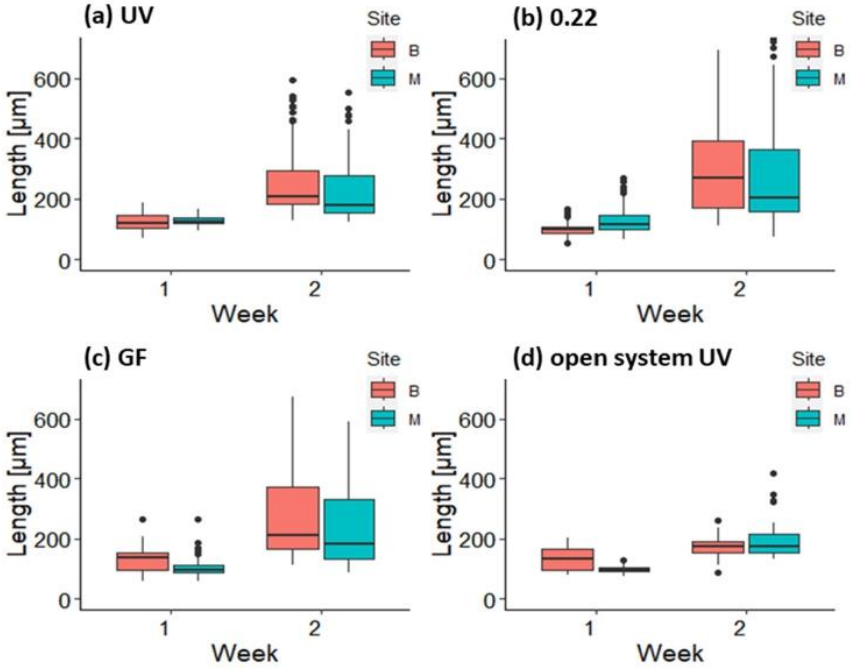


Figure 4. Length (µm) of germlings of *G. barbata* on clay tiles from donor sites B (Belveder) and M (Merkur) during the first two weeks in the three mesocosm treatments: (**a**) UV-sterilized water, (**b**) filtered through 0.22 µm filter, (**c**) filtered through 0.7 µm filter, and (**d**) in the open system.

In the first week, the average size of germlings ranged from 156.71 ± 21.09 µm (Belveder open system) to 258.05 ± 20.71 µm (Merkur GF).

In the second week, germlings in the open system were smaller, with the largest individuals up to 400 µm tall, while the longest germlings in the mesocosms were up to 600 µm tall (Figure 4). The average length of the germlings in the open system was 182.02 ± 29.70 µm for the thalli from the Belveder donor site and 299.39 ± 75.05 µm for the thalli from the Merkur donor site.

Coverage of *G. barbata* germlings on clay tiles did not show any clear pattern related to donor sites or water treatments (Figure 5). After 2 weeks, the coverage on most tiles was between 2 and 3% (Figure 5). In the GF treatment, higher coverage was observed on tiles from the Belveder donor site (Belveder GF = 2.96 ± 1.5, Merkur GF = 0.70 ± 0.19). In the treatments UV and 0.22, the opposite pattern was observed, with higher coverage on tiles from Merkur in the second week (Belveder UV = 1.28 ± 1.09, Merkur UV = 1.75 ± 0.85, Belveder 0.22 = 1.67 ± 1.02, Merkur 0.22 = 2.05 ± 0.94). Diatoms were not more abundant in the aquaria with UV-treated seawater than in the other aquaria with filtered water (pers. obs.).

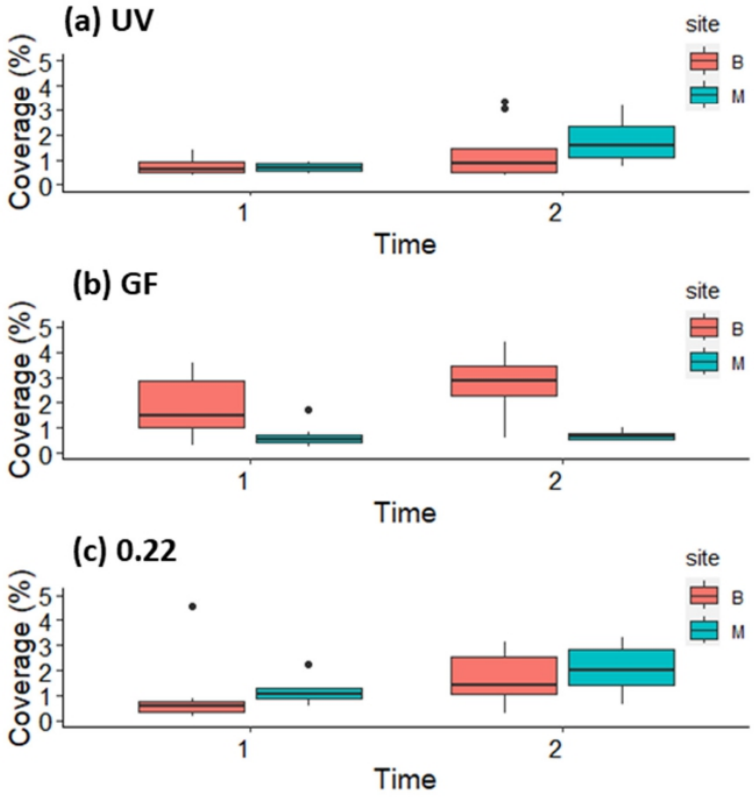


Figure 5. Coverage (%) of *G. barbata* germlings on clay tiles with germlings from donor sites B (Belveder) and M (Merkur) during the first two weeks in the three mesocosm treatments: (**a**) UV-sterilized water, (**b**) filtered through 0.7 µm filter, and (**c**) filtered through 0.22 µm filter.

3.3. Growth of Germlings in the Lantern Net Baskets

After outplanting the germlings from the mesocosm in the lantern net baskets in the sea (on day 16), their growth accelerated. After two weeks in the lantern net baskets, the germlings quadrupled their average size from 529.14 ± 61.33 µm (about 0.5 mm) to 2291.52 ± 702.30 µm (2.3 mm). After about one month in the lantern net baskets, the average size of the recruits was approx. 6.2 ± 1.9 mm, and after 2 months, they reached an average length of 21 ± 2.6 mm (Figure 6).

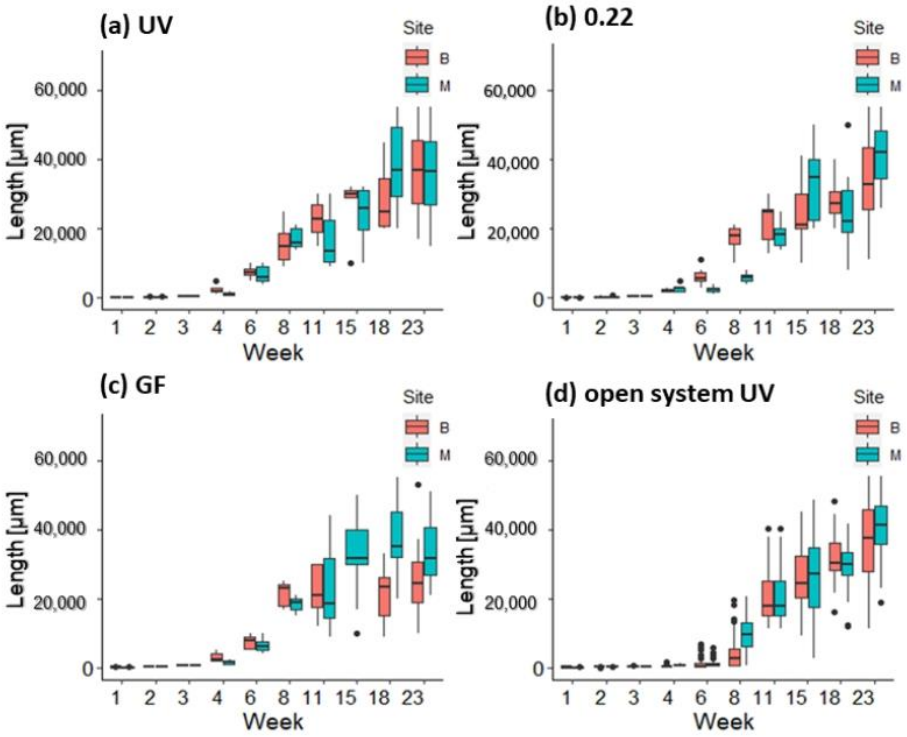


Figure 6. Length (µm) of *G. barbata* germlings on clay tiles from donor sites B (Belveder) and M (Merkur) from different treatments: (**a**) UV = sterilized water, (**b**) 0.22 = water filtered through 0.22 µm filter, (**c**) GF = water filtered through 0.7 µm filter, and (**d**) in the open system, in the laboratory (first 15 days) and on lantern net baskets after out-planting (from day 16).

At week 15, germlings from the UV-treated open system were the same size as the thalli transferred from the mesocosm to the lantern net baskets (Figure 6; Belveder open system = 25.6 ± 8.9 mm, Merkur open system = 28.9 ± 18.4 mm, Belveder UV = 26.4 ± 9.2 mm, Belveder GF = 23.6 ± 6.1 mm, Belveder 0.22 = 24.7 ± 9 mm, Merkur UV = 24.5 ± 7.8 mm, Merkur GF = 33.2 ± 11.2 mm, Merkur 0.22 = 25.4 ± 12.1 mm). The germlings on the lantern net baskets reached an average length of 32 mm after 122 days (17 weeks), and an average length of 34 mm, and a maximum length of 43 mm after 164 days. There was a difference (2-way ANOVA, $p < 0.01$) only between germlings Belveder GF and Merkur open system and Belveder GF and Merkur 0.22 (Tukey's test, $p < 0.01$). There was no difference between germlings' length from UV treatment and the other methods (Tukey's test, $p > 0.01$).

The young thalli were also exposed to some minor grazing. Some hermit crabs, juvenile fish from the family Blennidae, and a specimen of *Maya crispata* were found in lantern net baskets.

During the experimental period (early March–late September), the temperature was highest in late July (Table 2). Salinity and pH were stable, while O_2 saturation was highest in June and lowest in late September. In general, nutrient levels were highest in April and May, consistent with the period of accelerated algal growth (see Figure 6, weeks 2–8).

Table 2. Seawater parameters in Slovenian coastal waters from March to September 2022 (salinity, pH, O_2 saturation, and nutrients). Data were collected at a depth of 3 m at the oceanographic buoy Vida (https://www.nib.si/mbp/en/, accessed on 6 July 2023).

Date and Time	Depth [m]	T [°C]	Salinity	pH	O_2 Saturation [%]	PO_4 [mg/L]	NO_2 [mg/L]	NO_3 [mg/L]	NH_4 [mg/L]	SiO_3 [mg/L]
15 March 2022 09:40	3	10.31	38.86	8.18	102.6	0.01	0.098	0.12	0.03	2.06
4 April 2022 09:00	3	11.09	38.46	8.21	100.89	0.01	0.006	0.36	0.03	2.68
12 April 2022 10:20	3	12.05	38.31	8.19	103.08	0.01	0.028	0.72	0.03	2.85
26 April 2022 08:50	3	13.02	38.46	8.19	104.78	0.02	0.006	0.03	0.03	1.8
17 May 2022 09:10	3	20.61	38.36	8.16	108.97	0.02	0.006	0.03	0.03	2.07
31 May 2022 09:50	3	19.3	38.47	8.16	104.37	0.03	0.006	0.08	0.03	1.37
14 June 2022 13:40	3	21.43	38.24	8.15	109.91	0.01	0.014	0.12	0.03	0.56
28 June 2022 08:45	3	25.96	38.09	8.14	104.41	0.01	0.016	0.17	0.03	0.78
12 July 2022 09:50	3	24.64	38.16	8.02	104.42	0.01	0.01	0.18	0.04	1.11
27 July 2022 11:40	3	26.22	38.1	8.15	104.01	0.01	0.006	0.07	0.03	0.5
17 August 2022 09:30	3	24.97	38.42	8.15	102.35	0.01	0.006	0.11	0.03	0.94
30 August 2022 10:00	3	25.22	38.42	8.15	102.16	0.01	0.006	0.18	0.21	0.84
13 September 2022 09:13	3	24.79	38.36	8.15	99.9	0.01	0.022	0.26	0.67	1.08
27 September 2022 10:43	3	21.78	38.27	8.12	95.59	0.01	0.07	0.55	0.84	3.48

3.4. Growth of Germlings in the Open System

After one month in the open system, the biofilm began to grow on the tiles. The seawater temperature was 13 °C at the end of week 4 (early May). The epiphytic brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngbye was detected in the aquaria. The biofilm was removed from the tiles with a soft brush, taking care not to damage the *G. barbata* germlings. To reduce the growth of the biofilm, the lighting was adjusted to a blue spectrum on day 55 (week 7, early June). A decrease in epiphyte and biofilm growth was soon observed, while there was no difference in the *G. barbata* growth. The increased water turbulence after the installation of the stream pump had a positive effect on algal growth, as the thalli became visibly thicker after about 1 week. On day 71 (week 10, mid-June, seawater temperature = 21 °C), some polychaetes (subcl. Errantia, fam. Neredidae, mainly the species *Platynereis dumerilii* (Audouin and Milne Edwards, 1833)) were found in both aquaria (Figure 7). The polychaetes that entered the water through the inflow water pipes grazed on *G. barbata* germlings and incorporated parts of the algae into the outer layer of their tube. Tubes of polychaetes were observed on many clay tiles. Polychaetes were removed manually by brushing each tile and searching for hidden worms in the algal canopies and under the tiles. Almost every week, we also found *Patella* sp. on the tiles. They entered the system in the larval stage and went unnoticed until they attached themselves to the clay tile. After 16 weeks (end of July, seawater temperature = 26 °C), the average length of germlings was 23 ± 5.1 mm for the Belveder and 28 ± 13.6 mm for the Merkur donor population. The maximum length of the Belveder germlings was 48 mm, while the Merkur recruits reached 57 mm. After 23 weeks (middle September, seawater temperature = 24 °C), the average length of the Merkur germlings was 42 ± 9.6 mm, while the Belveder germlings were slightly smaller with an average length of 39 ± 13.8 mm. No significant difference was found between the growth values of the two sites after 23 weeks (2-way ANOVA, $p > 0.01$). The maximum length of Merkur germlings was 62 mm, while the tallest Belveder germlings measured 50 mm.



Figure 7. Factors that affected the growth of *G. barbata* thalli in the open system: (**a**) the presence of the polychaete *Platynereis dumerilii*, (**b**) large tube of *P. dumerilii*, which was also made with fragments of *G. barbata*, (**c**) *Patella* sp. grazing on *G. barbata*, (**d**) undetermined gelatinous biofilm, and (**e**,**f**) *Ectocarpus* spp. and undetermined biofilm growing on clay tiles.

4. Discussion

Restoration is increasingly recognized as an appropriate strategy to actively initiate the recovery of degraded coastal habitats, including brown algal forests. In our study, we tested whether it is possible to grow *G. barbata* with seawater treated only with a UVC sterilizer. This procedure provided promising results, as after 164 days, there was no significant difference in the growth or survival of germlings between UV-treated water and two types of filtered seawater (see Figure 6).

Although the results of the laboratory phase do not provide statistical confidence due to the problem of pseudoreplication, the observational data did not show a pattern suggesting different growth and survival of germlings when UV-sterilized water was used compared to filtered water for cultivation of *G. barbata* in mesocosms. The use of UV sterilizers significantly reduces the cost and labor of the laboratory phase. This opens the possibility of numerous and frequent algal cultures, reducing costs and labor during the reproductive period of *Cystoseira s.l.* species. However, it should be noted that UVC sterilizers do not destroy multicellular organisms or their eggs and larvae and do not prevent outbreaks of epiphytic algae and polychaete invasions in the open system (*pers. obs.*). This was not a problem in the mesocosms because the water was changed every 2–3 days, and therefore, the polychaete larvae and epiphytic algae did not have enough time to settle and develop. Cultivation of *G. barbata* in the open system was rather tricky. The growth of germlings was slower than in the mesocosm (see Figure 4), probably due to the lower temperature of the water (which was pumped directly from the coastal sea), the lack of additional nutrients (Von Stosch medium), and the massive growth of epiphytes (see Figure 7). Switching the lighting to a blue spectrum on day 55 helped reduce the amount of biofilm on the tiles, as this spectrum has been shown to inhibit the growth of certain types of bacteria and cyanobacteria [86,87]. This is due to the fact that blue light has shorter wavelengths (between 400 and 470 nm) and higher energy, which can disrupt cellular processes and metabolic activities, thereby inhibiting their growth [87,88]. The germlings transferred from the UV-treated open system to the lantern net baskets grew rapidly during the first weeks in the sea and were as large as the thalli from the mesocosm by week 15 (late July; see Figure 6). Transferring algal germlings to floating structures reduces contamination with biofilm and filamentous algae that compete with *G. barbata* for available resources

and interfere with the growth and development of its germlings [48,71,72], as the constant movement and turbulence of water around floating structures help to disrupt the biofilm and prevent the accumulation of new microorganisms [89].

In addition, lantern net baskets also serve as protection against fish grazers of the species *Sarpa salpa*. Fish perceive floating baskets as unfamiliar objects and do not immediately associate them with food; therefore, germlings in the basket have more chances to survive (*pers. obs.*). A hybrid method involving a carefully controlled laboratory phase and early out-planting to the sea provided satisfactory results in the Slovenian coastal area [47]. The shortened times in the mesocosm resulted in lower culture maintenance and overall costs, which is essential for expanding restoration efforts beyond scientific interest and is now required by international policies and standards [63].

Larval stages of grazing invertebrates were able to enter the open system with pumped seawater, despite UV treatment. The most problematic were the polychaete worms, which appeared in large numbers and were difficult to detect, as they incorporated parts of algae into the outer layer of their tubes. Some tube-building polychaetes have a unique association with algae, which they utilize for tube construction. Tubes are formed by mucus released from specialized glands in polychaete bodies and mixed with sand, sediment particles, or algal parts and filaments to form a structure around the worm's body [90] and provide additional structural support and camouflage [91]. The polychaete species *P. dumerilii*, identified in the open system, has glandular masses on the parapodia [92]. These glands excrete layers of fine filaments that form an elastic tubular coating into which algae and other particles are incorporated [92]. As the worm grows, it continuously adds new segments to the tube, increasing its length and adding new layers from the inside, making the tube wall thicker [91]. In most cases, when the larva of a tube-dwelling polychaetes settles, it builds the initial tube. The time required for this initial tube formation may vary depending on the species. For some species, the tube can be produced in a matter of minutes by secretions from the body surface, while for others, it may take hours to complete the tube-building process [93,94].

While the removal of *Patella* sp. and other gastropods was quick, since we could easily detach them from the clay tile with a scalpel blade, the removal of polychaetes was more difficult because their tubes were located between germlings, and we had to carefully brush each tile without damaging the thalli. Another disadvantage of the open system was the constant influx of sediment that settled on the algae and the surface of the clay tiles. The amount of sediment and dissolved nutrients increased after stormy and windy weather, and this increased nutrient concentration led to the proliferation of filamentous algae such as *E. siliculosus.* A dieback of *G. barbata* germlings was observed after they were overgrown by these filamentous algae. Epiphytes are known to negatively affect the germlings by limiting light and nutrient availability and, consequently, reducing host fitness and photosynthesis [95–97].

Algal growth in the open system and on the lantern net baskets was affected by rising seawater temperatures and nutrient concentrations (see Table 2 and Figure 6). Between the 6th and 8th week of the experiment, when the temperature reached 20 °C (mid–late May), algal growth increased significantly (2-way ANOVA, $p < 0.01$; see Figure 6). Higher temperatures within the species' optimal range had a positive effect on the growth of *G. barbata*, as the maximum net photosynthesis of the species is between 20 °C and 30 °C [98]. At temperatures above 28 °C, photosynthetic activity begins to decrease, and the synthesis of phenolic compounds such as phlorotannins may be inhibited [99]. Phlorotannins are important anti-grazing, antibacterial, and UV-protecting agents [100–103]. Nutrients were present in higher concentrations at the beginning of summer and end of summer (see Table 2). The installation of a stream pump (Sicce Voyager nano, Q max 1000 L/h) in the open system at week 13 had an additional positive effect on the growth of *G. barbata* since the thalli became thicker. Exposing young thalli to mild mechanical disturbances such as increased water movement is, therefore, very important to strengthen young *G. barbata* and prepare them for the conditions in the sea. Water movement is vital for nutrient uptake by

macroalgae for several reasons. Firstly, it facilitates the distribution of dissolved nutrients, ensuring a continuous supply for algal growth [104]. Secondly, it enhances the mass transfer processes, promoting the exchange of gases and nutrients between the water and algal tissues [105,106]. Lastly, water movement reduces the thickness of the boundary layer (a thin layer of water adjacent to the algal surface that can act as a barrier to nutrient uptake by creating a concentration gradient that hinders nutrient diffusion) and provides mechanical stimulation, improving nutrient contact with algal surfaces and optimizing nutrient uptake rates [107]. These results are also supported by the study of Clausing et al. [72], who found that germlings exposed to mild but repeated stress were more resistant and grew better.

Thalli were kept in lantern net baskets for more than 5 months and were not noticed by the *S. salpa* schools even when they were larger than 5 cm. This result is very important because the main problem for the successful restoration of *G. barbata* populations in the northern Adriatic Sea is the survival of the thalli at the time of their out-planting. *S. sarpa* populations in the Adriatic Sea have increased dramatically over the last decade, probably due to overfishing of large predatory fish. Therefore, the current priority is to develop an efficient method to reduce grazing pressure on *G. barbata* donor and receiving sites. Various fish deterrent devices [44], anti-grazing devices [46], and protective cages [47,48] have been tested, but these methods have many drawbacks (e.g., visual pollution, frequent cleaning of cages to maintain light intensity required for algal growth, damage by fishing lines or net baskets, and swimmers), and are not suitable for use in large areas. Therefore, in parallel with ex situ algal cultivation combined with the hybrid lantern net phase, the development of innovative deterrent devices should be accelerated to reduce this pressure and allow the ecosystem to rebalance. In addition, countermeasures would be useful to regulate herbivore populations, such as motivating fishermen to target species such as *S. salpa*. *Cystoseira s.l.* species will have no choice but to try to adapt to the multiple impacts of climate change that are already affecting most marine ecosystems [108], but regulation of herbivorous fish populations must be considered an extremely urgent step to be implemented in areas of the Mediterranean where they have been shown to play a major role in the regression of brown algal forests.

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Chapter 3
Thermal Stress and Early Life Stages of *Gongolaria barbata*

3.1 Paper: Too Hot to Handle: Effects of Water Temperature on Early Life Stages of *Gongolaria barbata* (Fucales)

This chapter examines how elevated seawater temperatures affect the early life stages of *Gongolaria barbata*, a key habitat-forming species in the Mediterranean. In the northern Adriatic, summer seawater temperatures often rise above 28 °C, exceeding the species' general thermal optimum of 10–25 °C. While adult plants can tolerate a wider thermal range, the earliest stages of development are far more sensitive, with optimal growth occurring around 15 °C. Unusually warm winters, where temperatures remain above historical norms, further threaten germling survival during recruitment. Since the persistence of *Cystoseira* s.l. forests depend on the successful establishment of these vulnerable early stages, understanding their thermal tolerance is essential for predicting the resilience of coastal algal forests under future climate warming.

The study was conducted using reproductive material collected from natural populations in the Gulf of Trieste. Germlings were cultivated under controlled laboratory conditions at four temperatures (15, 18, 24, and 28 °C), spanning typical spring values to extreme late-spring and summer conditions already recorded in the region. Over a 16-day period, survival, growth, and deformities were monitored to identify thresholds of thermal tolerance and to assess how warming influences recruitment success.

Specific objectives:
I. Assess survival and growth rates of *G. barbata* germlings across a gradient of seawater temperatures.
II. Determine the onset of temperature-induced deformities and mortality.
III. Identify the upper thermal limits of germling development in relation to present and projected warming trends in the northern Adriatic.
IV. Provide insights into how future marine heatwaves could constrain the recruitment and persistence of *Cystoseira* s.l. forests.

Hypothesis:
Early life stages of *G. barbata* are highly sensitive to thermal stress. Temperatures above 24 °C were expected to cause significant mortality and deformities, creating a recruitment bottleneck that threatens long-term population survival.

Key findings:

- Germlings developed normally at 15 and 18 °C, showing high survival and healthy growth.
- At 24 °C, germlings experienced severe stress, with deformities and substantial mortality within two weeks.

- At 28 °C, complete mortality occurred within the first week, confirming the extreme vulnerability of early stages to high temperatures.
- The results identify 24 °C as a critical threshold, beyond which recruitment success is drastically reduced.
- Findings highlight that increasing frequency and intensity of marine heatwaves could severely limit the recovery and persistence of *Cystoseira* s.l. forests in the northern Adriatic.

The objectives were achieved, and the hypothesis was supported by the results presented in this chapter.

The work presented in this chapter has been published as:
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Article

Too Hot to Handle: Effects of Water Temperature on the Early Life Stages of *Gongolaria barbata* (Fucales)

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Abstract: *Gongolaria barbata* plays a crucial role as a habitat-forming Fucales species in the Mediterranean Sea, thriving in shallow, sheltered coastal regions, where it exhibits optimal growth in a temperature range of 10 to 25 °C. In the northern Adriatic Sea, a semi-enclosed part of the Mediterranean, there has been a remarkable increase in seawater temperatures in recent decades, often exceeding 28 °C in summer. These high temperatures pose a significant threat to the vulnerable early life stages of *G. barbata*. This study delves into the effects of four temperatures (15, 18, 24, and 28 °C) on the growth of *G. barbata* over its first 16 days, closely monitoring mortality, deformities, and overall survival. Our experiments reveal that higher temperatures can result in deformities and increased mortality of germlings. Notably, a temperature of 28 °C resulted in the death of all germlings within the first week, whereas those exposed to 24 °C survived until the second week, albeit with significant deformities prior to death. In contrast, germlings cultivated at 15 and 18 °C exhibited normal development with minimal deformities. These results highlight the susceptibility of the early life stages of *G. barbata* to temperature-induced stress and provide valuable insights into the potential consequences of rising seawater temperatures in the Mediterranean.

Keywords: *Gongolaria barbata*; increased temperature; early stages; deformities; mortality

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1. Introduction

The Mediterranean Sea, with its rich tapestry of habitats, hosts a diverse array of species. Notably, the brown algae of the order Fucales are foundational organisms that shape shallow rocky reef ecosystems. These seaweeds form complex three-dimensional canopies, akin to marine forests, with the *Cystoseira s.l.* species (comprising genera *Ericaria*, *Gongolaria* and *Cystoseira*) playing a pivotal role [1,2]. These marine forests make an important contribution to coastal ecosystem services [3], supporting primary production, promoting nutrient cycling, attenuating waves, sequestering carbon, and fostering biodiversity [4–7].

Cystoseira s.l. species exhibit a wide distribution throughout the Mediterranean Sea and Eastern Atlantic, with several species endemic to the Mediterranean region. Among the 16 accepted species of the genus *Gongolaria* [8], *Gongolaria barbata* (Stackhouse) Kuntze inhabits the Levantine Sea in the east [9], as well as areas along the African coasts at the south (Algeria [10], Libya [11], Morocco [12], Tunisia [13]), and extends to the northern coastlines encompassing Croatia [14–16], France [17], Greece [18], Italy [19,20], Slovenia [21], and Spain [22]. Along the Romanian Black Sea coast, *G. barbata* is the only remaining representative of *Cystoseira s.l.* [23]. Additionally, it is found around the islands of Malta [24], Corsica [25], Sardinia, and Sicily, as well as the Balearic Islands [26]. In Menorca, the restocked population of *G. barbata* has expanded significantly over the past 10 years, with recruits and juveniles dominating the population and some individuals reaching large size classes [26]. Its distribution also reaches into the Atlantic, including the Canary and

Salvage Islands. In the Mediterranean region, it is classified as threatened by the Barcelona Convention (Annex II; UNEP/MAP, 2013; [27]), together with other species of *Cystoseira s.l.* (except *Cystoseira compressa)* [28].

The extensive distribution of *G. barbata* underscores its adaptability, phenotypic plasticity, and ecological resilience across diverse marine environments. However, this species, that was previously common in the northern Adriatic, is now found only at few sites with scattered populations [21,29]. In the Gulf of Trieste, which represents the species' northernmost point of distribution, three distinct forms are nowadays observed, whether attached or detached [30]. The new proposed nomenclature for these forms comprises *Gongolaria barbata* f. *aurantia* (Kützing) Falace, Alongi, et Kaleb for the detached, free-living form in a lagoon environment, and *G. barbata* f. *hoppei* (C. Agardh) Falace, Alongi, et Kaleb and *G. barbata* f. *turneri* (J. Agardh) Falace, Alongi, et Kaleb for two morphologically different attached forms living in coastal waters [30].

Regrettably, there has been a decline in and local extinction of almost all *Cystoseira s.l.* species in recent decades due to a combination of natural and human impacts [21,31–36] such as climate change, overfishing, coastal development, etc.

In the Mediterranean, changing sea temperatures, alongside other abiotic and biotic factors, are altering the distribution and abundance of algal species [16,37,38]. These shifts have the potential to reshape local biodiversity and community structures, affecting ecosystem resilience. Changes in seawater temperatures directly affect the reproduction of macroalgae by affecting gametogenesis, fertilization, and growth during early life stages [39–42]. These changes can have far-reaching effects on population dynamics and long-term viability. The maintenance of populations of foundation species depends on the persistence of adults and the successful recruitment of early life stages. While adults generally exhibit a wider range of thermal resistance, early life stages are more sensitive [40,43–45] and often represent a 'bottleneck' in the development and maintenance of algal populations [46–48]. Unusual temperature patterns, including thermal anomalies and ocean warming, also pose significant threats to *Cystoseira s.l.*, impacting reproduction, growth, survival, and ecological dynamics [29,44,49–54]. Consequently, temperature-induced changes may trigger cascading effects on organisms that rely on *Cystoseira s.l.* for habitat and food [55].

The effects of increased temperatures surpass the direct impacts on brown seaweeds [56]. Observations of *G. barbata* in the northern Adriatic Sea in the last 5 years show that the main threat to the remaining populations is the high grazing pressure by the herbivorous fish *Sarpa salpa* (pers. obs., [57]). Ocean warming, a prominent facet of global climate change, disrupts ecosystem dynamics by influencing metabolic processes, nutrient availability, and the behavior of key herbivorous grazers, including sea urchins and herbivorous fish [21,57–59]. As these grazers become more active in response to higher temperatures, they may consume more biomass, potentially leading to overgrazing and the degradation of macroalgal forests [60]. In addition to higher activity of grazers, chemical defenses are reduced under climate stressors such as elevated pCO_2 and reduced salinity. *Gongolaria rayssiae* (Ramon) Molinari & Guiry, an endemic species along the Levantine coast, has shown a decline in carbon sequestration rates under high temperatures [61], while *Cystoseira compressa* (Esper) Gerloff & Nizamuddin, growing along the Italian coastline, showed a decrease in photosynthesis when exposed to air temperatures above 28 °C [53].

In the Mediterranean Sea, where accelerated warming and more frequent thermal anomalies are occurring [62–64], understanding the response of *Cystoseira s.l.* species to temperature changes is crucial. Although the adult algal stages are more extensively studied, it is important to study temperature effects also on early life stages [56]. Studies on *Ericaria giacconei* D. Serio & G. Furnari showed sensitivity to elevated temperatures that lead to massive mortality of zygotes and embryos [56]. This is particularly important, as recruitment plays a crucial role in maintaining healthy brown algal forests.

This study aimed to assess the effect of elevated temperature on germination and early thallus ontogeny of *G. barbata* in the northern Adriatic Sea, where the species still forms

some algal forests in the shallow infralittoral zone [15,16,21,29,65–67]. In this area, the species reaches its maximum vegetative and reproductive development from late winter to late spring/beginning of summer (February–May [29]), when sea temperatures range between 8 and 23 °C. The study deals with the development of zygotes and germlings at four different temperature treatments ranging from 15 to 28 °C, corresponding to the currently possible temperatures in the northern Adriatic Sea. Growth and survival of germlings were monitored for 16 days in mesocosms to assess the critical temperature threshold for the early stages of this species. The study provides evidence for the likely response of this species to projected climate change and highlights the detrimental effects of increased seawater temperatures on the early life stages of *G. barbata*.

2. Methods and Materials

2.1. Study Area

The study area is located in Slovenia, in the northernmost part of the Adriatic Sea. The Slovenian coastal sea encompasses the southern part of the Gulf of Trieste, with a coastline of about 46 km. This region is characterized by a shallow coastal sea, reaching a maximum depth of 37 m and an average depth of 18.7 m, making it one of the shallower areas in the northern Adriatic Sea. The seabed along the coastline is predominantly rocky and consists of alternating layers of Eocene flysch, sandstone, and soft marl, which contribute to the ecological complexity of the region [68,69]. Due to its shallowness, the Gulf of Trieste undergoes significant fluctuations in water temperature and salinity throughout the year. These fluctuations are mainly influenced by the inflow of freshwater, primarily from the Isonzo River and by the bora wind blowing offshore. The average surface water temperature is around 25 °C in summer, occasionally reaching up to 28 °C, while, in winter, it can drop below 8 °C [70]. The average salinity in this area is 37–38 °C [71,72]. Temperature stratification occurs in the water column between April and September, with a seasonal thermocline in spring [73]. The northern Adriatic is additionally influenced by intense surface and lateral fluxes, and has highest amplitudes between high and low tide (average = 88 cm [69]) in the Adriatic Sea. In the surface layer, the water circulation is clockwise, while, in the lower layer, circulation is anticlockwise [74].

2.2. Reproductive Material Collection

The selection of the donor site was based on criteria established in previous studies [65,66] and SCUBA diving surveys in spring (February–May) 2023. Only healthy and dense underwater forests of *G. barbata* were selected as suitable donor populations. For this experiment, apices with mature receptacles of *G. barbata* were harvested from the donor population in Izola (coordinates: 45°32.653, 13°40.554; Figure 1), which is known for dense populations of *G. barbata* and *Cystoseira compressa* (Esper) Gerloff & Nizamuddin.

To verify fertility, a few receptacles were collected on a preliminary dive. A dissection of a few receptacles was performed to examine them under a stereomicroscope. Once the fertility of receptacles was confirmed, a dive was planned to collect the reproductive material needed for the experiment. On 15 May 2023, the receptacles were collected at an average depth of 2 m by SCUBA divers. The fertile apical parts with the receptacles were carefully cut off with scissors and placed in mesh bags, which were stored on the boat in plastic buckets filled with seawater. Within 30 min, they were taken to the laboratory of the Marine Biology Station (NIB).

In order to avoid negative effects on the donor population, up to 10% of the fertile apices per algal thalli were collected. Notably, the collection of receptacles posed a challenge due to the reduced availability caused by the observed overgrazing by herbivorous fish [66].



Figure 1. Study area, with the location of the donor site (DS Izola) for *Gongolaria barbata* and the laboratory of the Marine Biology Station Piran (RS MBS). Light blue represents sea, dark blue represents river/lake and brown represents urban areas.

2.3. Laboratory Work

In the laboratory, apices with mature receptacles from the donor population were cleaned of epibionts with a soft brush, rinsed with filtered seawater, wrapped in aluminum foil, and stored overnight (24 h at approximately 4 °C). This thermal shock triggered the release of gametes from the conceptacles, allowing fertilization [42]. Twelve experimental aquaria (Figure 2), each measuring 50 cm × 20 cm × 20 cm, were set up in an environmentally controlled room, with three aquaria assigned to each temperature condition (15, 18, 24, 28 °C). Temperatures were selected according to the normal spring temperatures in the Gulf of Trieste (between 15 and 18 °C) and the possible highest temperatures at the end of spring or beginning of summer (24 and 28 °C). All aquaria and equipment were previously cleaned with 5% solution of sodium hypochlorite (bleach). All aquaria were covered with plexiglass lids to prevent water evaporation and changes in salinity. The temperature of the environmentally controlled room was set at 15 °C, resulting in three aquaria without water heater. In the remaining nine aquaria, NEWA Therm Next 50W water heaters were installed and adjusted to specific temperatures (18, 24, or 28 °C). These water heaters were positioned beneath a zinc wire net stand, on which 10 clay tiles (diameter = 6 cm, with a central hole of 0.6 cm) and 3 Petri dishes (of the same diameter) per aquarium were arranged. Five to six apical parts (approx. 3 cm in length) with, altogether, ten receptacles were placed on each clay tile and Petri dish. Petri dishes were used for measurements and monitoring of germlings' development during the first 5 days because the germlings were too small to be visible on the granulated surface of clay tiles. On the first day, aquaria were filled with UVC-sterilized water to a maximum height of 2 mm above the apices. The photoperiod was set to 15:9 light/dark cycle, which mimics day length during gamete release, fertilization, and early life growth stages of *G. barbata* in the northern Adriatic Sea. The light intensity was set to approximately 140 µmol photons m^{-2} s^{-1}. For illumination, 4 Osram Fluora Florescent tubes with power of 36 W, length of 120 cm, and luminous flux of 1400 lumens per container were used.

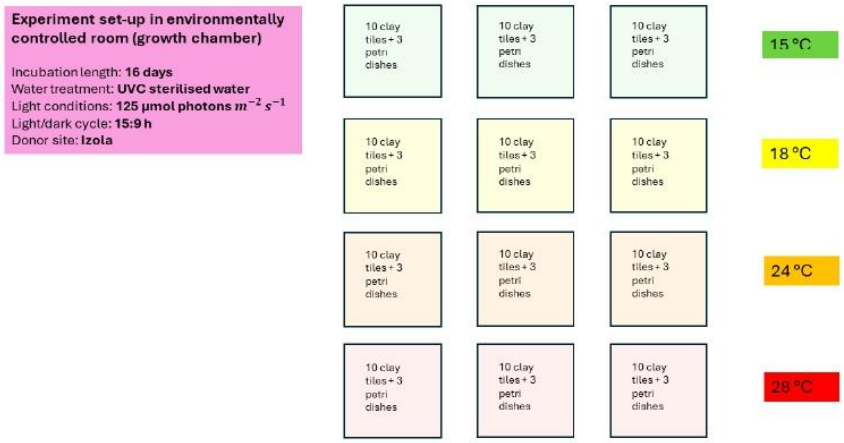


Figure 2. Ex situ cultivation scheme for *G. barbata* at the Marine Biology Station Piran. Each box represents one aquaria with clay tiles and petri dishes which are used as a substrate for *G. barbata* germlings. Three replicate aquaria per each temperature regime were used (green = 15°C, yellow = 18°C, orange = 24°C, red = 28°C).

Receptacles were left on clay tiles for two days to allow gamete release. Following this period, the receptacles were removed, and 1 L of UVC-sterilized seawater per aquarium was added (maintaining a level of approximately 2 cm above the clay tiles). Air pumps and bubblers were then installed in each aquarium and GeO_2 was added to prevent diatom growth. After removal of the receptacles, an area of 0.2 cm^2 was photographed on one randomly selected tile per replicate aquaria of each temperature regime at 24 h intervals. Measurements were taken over a period of 16 days, which was adequate to monitor the mortality of germlings at different temperatures. Water change was performed every 3 days. Length of randomly chosen 25 germlings per each aquarium (75 altogether per each temperature regime) was measured using a stereomicroscope (Olympus SZX16). Average size and percentage of alive, dead, and deformed germlings were calculated. The criterion for determining mortality was the absence of pigmentation, indicating that the germling had entered a state of decomposition. Germlings exhibiting abnormalities such as irregular cell divisions or concave and convex parts were classified as deformed rather than dead (and were not included in either the alive or dead category, as we were unable to detect this). In the calculation of average size, all randomly chosen germlings were included, dead or alive. Percentages of dead, alive, and deformed germlings were calculated on the randomly chosen area of 0.2 cm^2. In the first 5 days, the lengths of the germlings were measured on Petri dishes, while, from the 6th day onward, the measurements were taken on germlings grown on clay tiles.

2.4. Photo-Processing and Statistical Analyses

Photographs from the culture in thermostatic chambers were processed using the ACDSee program. The number of alive, deformed, and dead germlings was counted in the photographed area of 0.2 cm^2, and the percentage of alive, dead, and deformed germlings was calculated for each day and for 4 different temperatures.

Statistical analyses were performed using R 4.2.2 (R Core Team, 2020, Vienna, Austria). A two-way robust ANOVA [75] was performed for the main effect and interaction to check for differences in thalli length of *G. barbata* germlings between two fixed factors: day (with 14 levels) and temperature (with 4 levels: 15, 18, 24, 28 °C). The test was chosen because it is an extension of the one-way ANOVA that examines the influence of two different categorical independent variables on one continuous dependent variable. The two-way ANOVA aims to assess not only the main effect of each independent variable, but also whether there is an interaction between them. Tukey's multiple comparisons of means

were used to compare temperatures and days independently. One-way robust ANOVA and subsequent post hoc tests [76] were performed to check for differences in thalli length of *G. barbata* germlings between different treatment at specific time intervals. Analyses were performed using the WRS2 and AICcmodavg packages [77]. Data were then graphically represented with boxplots using the ggplot2 package [78]

3. Results

Survival and Growth of Germlings in Mesocosm

A significant difference in germlings' growth was observed in the four different temperature regimes (ANOVA $F_{(3,1742)} = 2274.1$, $p < 0.0001$; Table 1), with significant interaction between the factors 'day' and 'temperature' (ANOVA $F_{(36,1742)} = 164.95$, $p < 0.0001$; Table 1). Notably, on the second day less than half germlings growing at 24 °C and 28 °C survived (Figure 3), and the longest germlings were measured at 18 °C and 24 °C (Figure 4, Tukey's Test, $p < 0.05$, Table 1). By the third day, the germlings growing at 15 °C had outgrown the germlings cultured at 24 °C and 18 °C (Figure 4). The maximum length reached by the germlings was 523 µm, measured on the 16th day at 15 °C. In comparison, the germlings at 18 °C reached a maximum length of 340 µm on the 16th day. Germlings at 24 °C survived only until the 13th day and reached a maximum length of 187 µm. The germlings at 28 °C reached their maximum length of 222 µm on the fourth day, and no living germlings were found after the sixth day (Table 2).

Table 1. Results of two-way robust ANOVA, and Tukey's pairwise comparison of different treatments on selected days. Df = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean of the sum of squares. Significance codes: '***': <0.0001; '**': <0.001; '*': <0.01; '.': <0.05; NS: not significant.

2-Way ANOVA							
Factor		**Df**	**Sum Sq**	**Mean Sq**	**F Value**	***p*-Value**	**Significance**
Temperature		3	6,041,970	2,013,990	2274.1	<0.0001	***
Day		12	490,429	40,869	46.15	<0.0001	***
Temperature × Day		36	5,258,925	146,081	164.95	<0.0001	***
Residuals		1742	1,542,751	886			
Pairwise comparison							
Day 2				Day 5			
	15 °C	18 °C	24 °C		15 °C	18 °C	24 °C
18 °C	.			18 °C	***		
24 °C	.	NS		24 °C	***	***	
28 °C	NS	***	**	28 °C	***	***	.
Day 7				Day 12			
	15 °C	18 °C	24 °C		15 °C	18 °C	24 °C
18 °C	.			18 °C	**		
24 °C	***	***		24 °C	***	***	
28 °C	***	***	***	28 °C	***	***	.
Day 14				Day 16			
	15 °C	18 °C	24 °C		15 °C	18 °C	24 °C
18 °C	*			18 °C	***		
24 °C	***	***		24 °C	***	***	
28 °C	***	***	NS	28 °C	***	***	NS

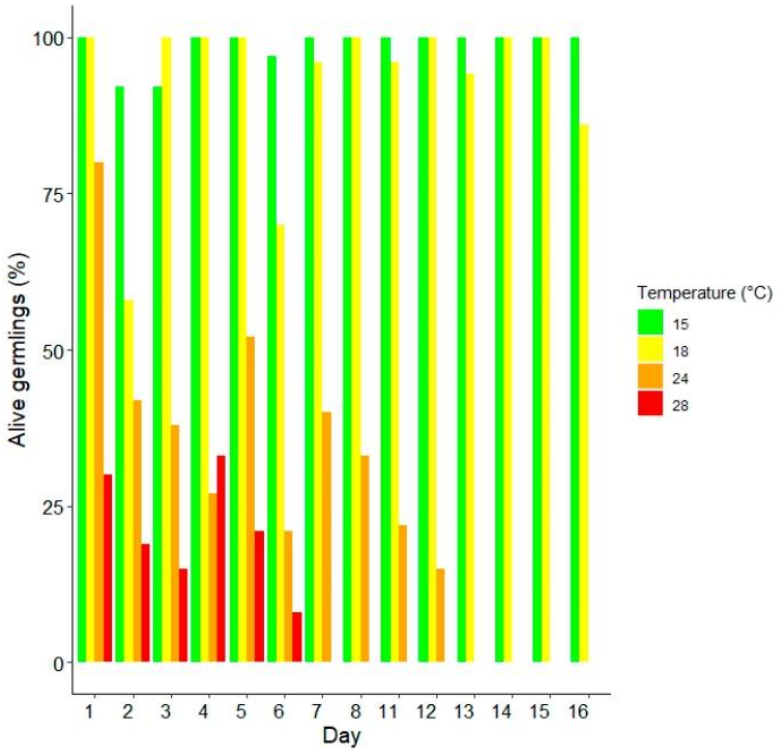


Figure 3. Percentage of alive germlings of *G. barbata* at 15, 18, 24, and 28 °C. The number of living, deformed, and dead germlings was counted daily in a randomly photographed area of 0.2 cm^2.

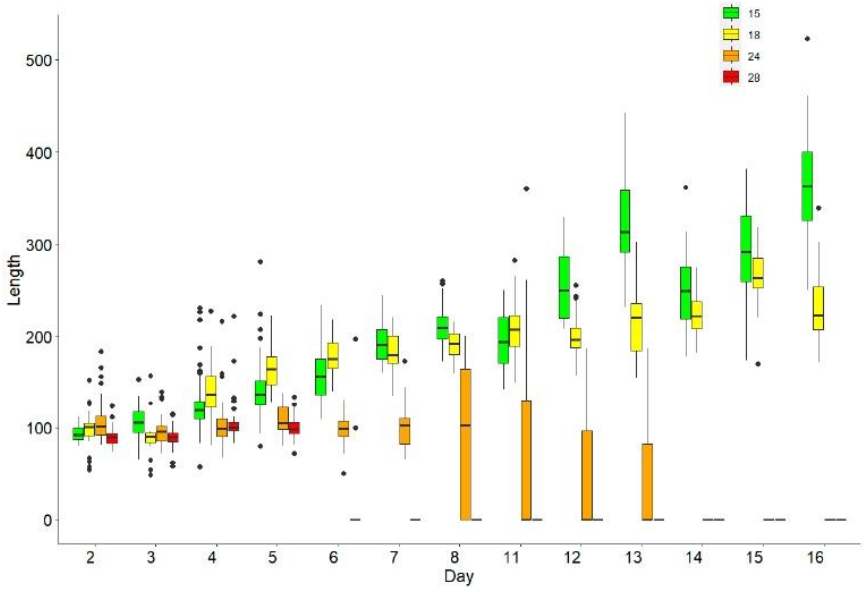


Figure 4. Growth of *G. barbata* germlings (length in µm) in the mesocosms at 15, 18, 24, and 28 °C. The length of living germlings was measured daily in a randomly photographed area of 0.2 cm^2. Boxplots display median, first and third quartiles (boxes), the largest value no further than 1.5× inter-quartile range from the third quartile (upper whisker), the smallest value at most 1.5× inter-quartile range below the first quartile (lower whisker), outliers (dots).

Table 2. Gongolaria barbata germlings' growth at 15, 18, 24, and 28 °C. Average size ± SD and percentage of alive, deformed, and dead germlings on a specific day, calculated from measurements of 25 randomly chosen germlings at each temperature.

Day	Temperature	Average Size [μm] ± SD	%Alive	%Deformed	%Dead
2	15	93.4 ± 8.6	92	8	8
	18	99.1 ± 15.4	58	0	42
	24	106.6 ± 22. 8	42	20	58
	28	90.5 ± 10.0	19	45	81
5	15	130.0 ± 33.8	100	0	0
	18	120.2 ± 35.8	100	0	0
	24	107.7 ± 20.2	52	38	48
	28	96.2 ± 11.0	21	10	79
7	15	196.7 ± 24.9	100	0	0
	18	182.6 ± 19.4	96	0	4
	24	102.4 ± 24.2	40	13	60
	28	98.2 ± 45.4	0	0	100
12	15	252.5 ± 36.7	100	0	0
	18	212.9 ± 35.3	100	0	0
	24	198.6 ± 58.8	15	18	75
	28	0	0	0	100
14	15	367.9 ± 62.5	100	0	0
	18	235.6 ± 42.7	100	0	0
	24	0	0	0	100
	28	0	0	0	100
16	15	367.9 ± 62.5	100	0	0
	18	235.6 ± 42.7	86	9	14
	24	0	0	0	100
	28	0	0	0	100

On the fifth day, germlings growing at temperatures below 20 °C were developing faster and healthier than those at higher temperatures (Table 1, Tukey's Test, $p < 0.05$, Table 2). All germlings growing at 15 and 18 °C were alive and no deformed germlings were found (Figure 3). The percentage of alive germlings was lower at higher temperatures (Figure 3, Table 2); a mere 15% of the counted germlings were alive at 28 °C, with others either dead or severely deformed (Figure 5).

Also, on the seventh day, all germlings observed at 15 and 18 °C were still alive and healthy. In contrast, all germlings growing at 28 °C were already dead, while some of germlings were still alive at 24 °C, with half of them severely deformed (Table 2, Figure 5).

On the 12th day, the average size of germlings reached over 200 μm at 15 °C; those growing at 18 °C were slightly smaller, while those growing at 24 °C were, on average, half of that size (Tukey's Test, $p < 0.05$, Table 1).

On the 14th day, all seedlings growing at temperatures below 20 °C were still alive and healthy, while the seedlings growing at temperatures above 20 °C (at 28 °C and 24 °C) were dead.

On the 16th day, all the germlings at 15 °C were alive. At 18 °C, the vast majority of the germlings were alive, and nearly one-tenth were deformed. Overall, the rate of deformities

was higher in germlings grown at 24 and 28 °C, and the same was true for the mortality rate (Figures 4 and 5).

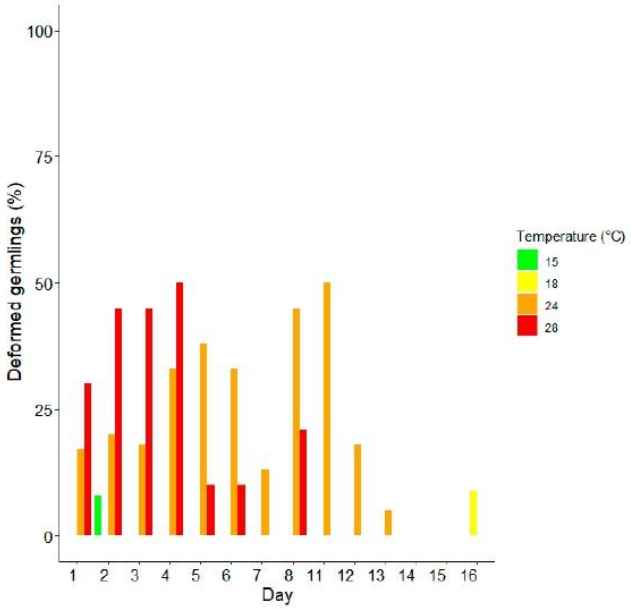


Figure 5. Percentage of deformed *G. barbata* germlings at 15, 18, 24, and 28 °C. The number of deformed germlings was counted daily in a randomly photographed area of 0.2 cm^2.

The percentage of deformed germlings was highest at 24 and 28 °C, 1–2 days before the critical point at which all germlings died (Figures 3 and 5). Germling mortality was highest at 28 °C, followed by germlings grown at 24 °C. Deformations and deaths also occurred occasionally at 15 and 18 °C (Figures 3 and 5).

4. Discussion

The algal populations most negatively affected by water warming and most vulnerable to future marine heatwaves are likely to be populations of temperate canopy-forming kelps and fucoids [14,79–81]. Conversely, as temperatures rise, turf-forming seaweeds, especially invasive species, may increase in abundance and act as competitors, which can lead to replacement of long-lived, complex habitat-forming seaweeds with smaller ephemeral species [81]. In general, high mortality of shallow water fucalean species (such as *Ericaria crinita* (Duby) Molinari & Guiry) has been observed in the northwestern Mediterranean Sea due to rising summer temperatures [14,54]. Several studies have shown that temperatures above 28 °C are detrimental to Fucales [37,82,83]. Regional extinctions of kelps and fucoids following marine heatwave events are likely to have long-term consequences for the ecological structure and functioning, reducing habitat structure and effective services that seaweed-dominated reefs provide [81]. Marine heatwaves are linked to many other potential stressors such as increased herbivory [84–86], altered current patterns [87,88], changes in water clarity, solar radiation [89], and desiccation stress [90] in the intertidal zone, which makes it difficult to study temperature effects in situ.

Gongolaria barbata is a geographically widespread species that belongs to the warm-temperate Mediterranean-Atlantic group [59,63] that requires temperatures of at least 10 °C for reproduction and development of germlings. It is a monoecious species, with oogonia and antheridia that are fertile in the conceptacles from the end of winter to the end of spring [91]. After the fertilization, heavy and large zygotes exhibit highly restricted dispersal; therefore, they fall on the substrate a few centimeters to meters away from the parental thallus. A day or two after fertilization, a typical Sargassous germling with four

primary rhizoids attaches to the rocky surface where it starts growing [42]. Rhizoids are later transformed into discoid holdfast. An earlier study from the Gulf of Trieste reported that the vegetative development of *G. barbata* is maximal at 14 °C [77], which is aligned with the findings on *G. barbata* in the Aegan Sea [59]. Optimal temperatures for adult germlings of *G. barbata* are known to vary between 15 and 25 °C [59,73], while its upper temperature limit was reported to be at 30 °C [37].

The reproductive season of *G. barbata* is becoming increasingly unpredictable, as it can be altered by the general increase in water temperatures and/or marine heatwaves, as recently observed in the northern Adriatic [44,92]. Targeted laboratory experiments, such as those conducted in our study, are useful to provide new evidence of the negative effects of increased temperatures without possible interactions with other factors. The temperatures tested in our study (15, 18, 24, and 28 °C) were chosen according to the possible temperatures in the months of *G. barbata* reproduction in the northern Adriatic (March–May [29,66,93]), considering the 10-year data series of the oceanographic buoy Vida (in Piran, Slovenia data from the oceanographic buoy Vida; https://www.nib.si/mbp/en/; accessed on 8 December 2023) and the predicted temperatures in the Mediterranean in the context of climate change.

While global air and sea surface temperatures have already risen by 0.4–0.8 °C in the past century and are predicted to rise by a further 2–3 °C in the coming century [76–78], the temperature of the Mediterranean Sea is predicted to rise even more than the average, i.e., between 1.3 and 6.3 °C from 1900 to 2100 [94]. In recent decades, higher seawater temperatures have been observed in the northern Adriatic Sea (see Figures S1 and S2). In the Gulf of Trieste, the shallowest part of the northern Adriatic, summer temperatures have already increased by 2.6 °C from 1991 to 2003 [95]. The average winter temperature (December–February) in the last decade (2013–2023) recorded by the oceanographic buoy Vida was 11.5 °C. This stands in stark contrast to the period before 1970 when average winter temperatures in the Gulf of Trieste rarely exceeded 10 °C. Notably, in the last decade, the average annual winter temperatures have consistently surpassed the 10 °C mark (see Figure S1; [96]). Additionally, the Gulf of Trieste experienced several marine heatwaves in the summer and autumn of 2023. In October 2023, the lowest temperature in this area was 25 °C, exceeding the normal temperatures for this time of year, which range between 16 and 22 °C (data from the oceanographic buoy Vida; https://www.nib.si/mbp/en/; accessed on 8 December 2023).

While increased temperatures can initially benefit seaweeds [97] by accelerating their metabolic processes [98–100], affecting nutrient uptake and biochemical pathways and increasing photosynthetic rates [99,101], their beneficial effects are present only until temperature optimum is reached and then rapidly decline at temperatures above optimum [99]. Prolonged exposure to temperatures above optimum can lead to decreased photosynthetic efficiency and photoinhibition [98], posing a challenge for sustainable primary production. Our results are consistent with this statement, as, at the beginning of germling growth (second day), germlings growing at 24 °C reached the largest length and were the most developed, followed by germlings growing at 18 °C (Figure 4). But, from the third day onward, the germlings cultured at 15 °C grew faster than those at 18, 24, or 28 °C. The reason for this could be related to metabolic stress, which disrupts essential physiological functions and results in reduced growth, deformations, and mortality [102].

A recent study on the cultivation of *G. barbata* even reported that, for thalli collected in the Gulf of Trieste, the development of fertile receptacles followed by the zygote release occurred exclusively at 14 °C [91]. Our experiments have shown that a temperature of 18 °C is still suitable for normal germlings' development, but germlings at 15 °C developed faster and reached larger lengths than others (see Figures 3 and 4). Prolonged exposure to temperatures above 18 °C during the reproductive period may also lead to widespread mortality of zygotes/embryos and reduce the reproductive success of the species. Results of our study confirm that temperatures above 24 °C inhibit the development of *G. barbata* germlings. All germlings growing at temperatures of 24 °C and above were dead after two

weeks. This finding aligns with similar observations on other *Cystoseira s.l.* species. For example, *Ericaria zosteroides* (C. Agardh) Molinari & Guiry, identified as a deep-sea species, showed a tolerance threshold of 24 °C [41], while *Ericaria selaginoides* (Linnaeus) Molinari & Guiry (previously *Cystoseira tamariscifolia*) [52] and *E. crinite* [54], species from shallower waters, exhibited a threshold of 28 °C. The settlement efficiency of *E. zosteroides* zygotes decreased at 20 °C and mortality significantly increased at 24 °C [41], while settlement efficiency of *Ericaria giacconei* zygotes increased from 12 to 18 °C, started to decrease at 24 °C, and dropped significantly at 28 °C, where most of the eggs and zygotes had undergone cell lysis [56]. The embryos of *E. giacconei* fully developed only at 12 and 15 °C, while mortality increased sharply at 28 °C [56]. Survival rate and growth of *Ericaria amentacea* (C. Agardh) Molinari & Guiry (previously *Cystoseira amentacea*) embryos was significantly higher when growing at 20 °C than at 24 °C [42]. However, the mortality was quite high, averaging 50% under all conditions (20 and 24 °C, low and high light), which can be attributed to the high stochastic gamete and zygote mortality observed in the natural environment of *Cystoseira*, but also to the fact that temperatures above 20 °C are at the upper temperature limit for germlings [42].

During our experiment, there was also a significantly higher degree of deformations observed among the germlings growing at 24 and 28 °C compared to those at 15 and 18 °C (see Figure 5). On the fourth day (Figure 6), the germlings developed normally at 15 and 18 °C, whereas many at higher temperatures were either deceased or deformed. The first sign that the seedling is not developing normally is usually bleaching, an indication that the alga is losing its photosynthetic pigments from the tissue [53,103]. If the bleached tissue cannot recover, this represents a disruptive stress that can lead to death [49]. In our experiment, loss of pigments was observed in germlings growing at 24 and 28 °C (Figures 6 and 7), as also seen in *E. giacconei* [56]. Consistently with other studies, our results show that higher temperatures can significantly impact the settlement and survival of recruits. Reduced recruitment of new individuals due to temperature anomalies or other pressures is therefore a critical bottleneck for the population dynamics of *G. barbata* and other *Cystoseira s.l.* species. Prolonged recruitment failures over several years can lead to reduced population densities that ultimately threaten long-term survival.

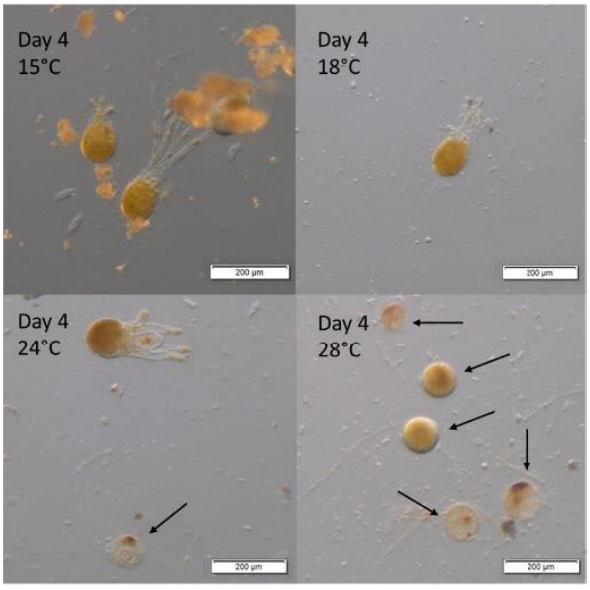


Figure 6. Germlings grown on Petri dishes at 4 different temperatures (15, 18, 24, and 28 °C) on day 4 of the experiment. Arrows point at dead germlings.

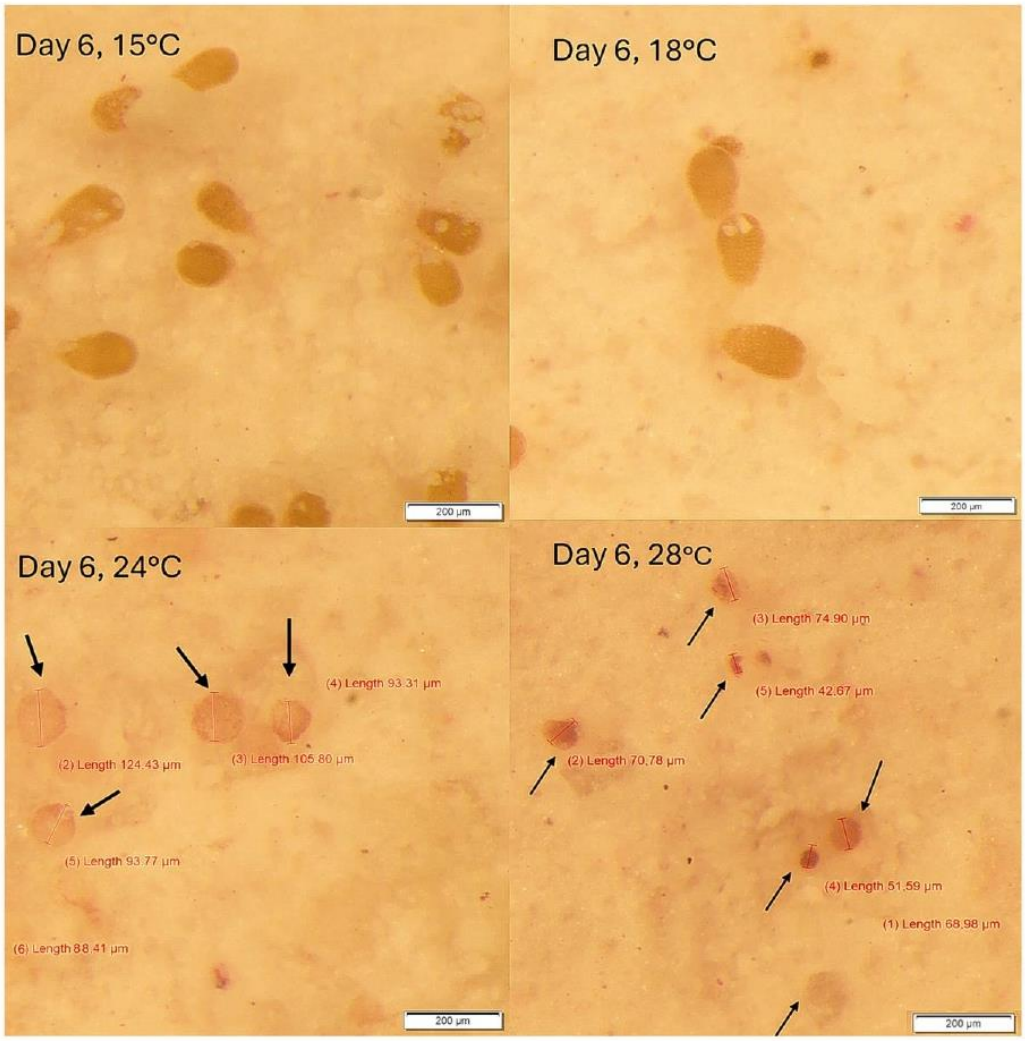


Figure 7. Germlings grown on clay tiles at different temperatures (15, 18, 24, and 28 °C) on day 6 of the experiment. Arrows point at dead germlings.

While each species has its specific temperature niche, temperature tolerance is also influenced by the species' thermal history, namely, the temperatures to which the population has been acclimated [51]. For instance, certain *G. barbata* populations flourish in the extremely shallow lagoons of the Adriatic Sea, where summer temperatures can easily exceed 28 °C and may even heat up to 34 °C for several days [14]. *Gongolaria barbata* growing in the lagoon exhibits a broader temperature tolerance compared to other populations, capable of withstanding temperatures even below 0 °C [14]. However, temperatures below 10 °C and above 28 °C have been shown to be detrimental, especially over longer periods [14,37,82,83]. Freezing temperatures may lead to the loss of lateral branches and tissue damage that is indicated by darkening of the cauloid [37], while high temperatures may result in cauloids overgrown by epiphytes and also loss of lateral branches [14]. When conditions return to the optimal temperature range of 10–25 °C, the damage on *G. barbata* thalli recovers. Even under normal thermal conditions, *G. barbata* loses phylloids and branches every autumn and epiphytes grow on its cauloid [93]. New growth begins in early spring and the frond reaches its largest size in spring or summer [93], which shows that the species is phenotypically acclimated to temperature variations. Seaweeds exhibit three

types of temperature responses: (1) genetic adaptation, which means that algae undergo long-term evolutionary changes to adapt to local conditions over thousands to millions of years; (2) phenotypic acclimation, which means that the phenotype adjusts in response to environmental temperature variations over hours to days; and (3) physiological regulation, which involves immediate responses of algae to temperature changes that occur within seconds to minutes [101]. The ability of adult plants to regenerate after prolonged exposure to very low or high temperatures suggests genetic adaptation to the conditions of the northern Adriatic Sea [14].

Our findings indicate that early life stages of *G. barbata* are particularly vulnerable and cannot survive at temperatures exceeding 24 °C. This vulnerability may be due to such high temperatures being abnormal during the period of zygote settlement and germling development of *G. barbata* in the Mediterranean (see Figures S1 and S2, based on [96]).

Our experiment highlights that *G. barbata* requires temperatures around 15 °C for successful reproduction and the healthy development of germlings. However, temperatures surpassing 24 °C during germling development drastically decrease survival rates, with the majority perishing within the first 1–2 weeks. At temperatures of 24 °C and above, germlings experience bleaching, deformation, and, ultimately, death. Further research is needed to understand the complexity of these responses. This knowledge is crucial for predicting and managing the impacts of climate change on marine ecosystems, where canopy-forming brown algae play an indispensable role in maintaining ecological balance and resilience. In the context of continued warming in the northern Adriatic Sea, *G. barbata* may face long-term survival challenges. While adult algal populations are more adaptable and can withstand shorter periods of higher temperatures [14,37], we demonstrated that the early life stages of this species are very sensitive to thermal anomalies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse12030514/s1, Figure S1: Average winter temperatures (December–February) in the Gulf of Trieste in the period 1934–2022 (data from SEANOE database [96]), Figure S2: Average late spring (May) temperatures in the Gulf of Trieste in the period 1934–2022 (data from SEANOE database [96]).

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Chapter 4
Biostimulant Effects on Early Life Stages of Mediterranean Fucales

4.1 Paper: Enhancing Ex Situ Cultivation of Mediterranean Fucales: Species-Specific Responses of *Gongolaria barbata* and *Ericaria crinita* Seedlings to Algal extracts

This chapter explores the potential of algal biostimulants to enhance the growth and resilience of early life stages of Mediterranean Fucales. The widespread decline of *Cystoseira* s.l. forests has made ex situ cultivation and seedling outplanting essential tools for restoration. However, the survival and growth of germlings are often constrained by physiological stress, limited nutrient availability, and microbial contamination, creating a bottleneck for restoration success. Inspired by the established use of algal biostimulants in agriculture and aquaculture, this study tested whether microalgal and macroalgal extracts could improve seedling performance in *Gongolaria barbata* and *Ericaria crinita.*

The experiments were carried out in controlled mesocosm trials, using extracts from the cyanobacterium *Trichormus variabilis*, the green alga *Desmodesmus* sp., the diatom *Cylindrotheca closterium*, and a commercial kelp-based formulation (AlgatronCifo®). Seedling survival, growth, and deformities were monitored across different extract types and concentrations, with particular focus on dose-dependent and species-specific responses.

Specific objectives:
I. Test the effects of different microalgal and macroalgal extracts on the survival and development of *G. barbata* and *E. crinita* seedlings.
II. Identify optimal extract types and concentrations that enhance early-stage growth without inducing cytotoxicity or microbial overgrowth.
III. Compare the performance of a commercial kelp-derived formulation with microalgal extracts to evaluate practical applications for large-scale restoration.
IV. Assess species-specific responses and the potential of biostimulants as a scalable tool to improve ex situ cultivation protocols.

Hypothesis:
Microalgal and macroalgal extracts, when applied at appropriate concentrations, enhance the survival and growth of Mediterranean Fucales seedlings, thereby increasing their resilience and suitability for restoration.

Key findings
- Low concentrations of *Desmodesmus* sp. extract significantly enhanced growth and survival of seedlings, confirming its potential as a biostimulant.
- High concentrations of all extracts produced cytotoxic effects, reducing survival and increasing deformities.
- Responses were species-specific.

- *G. barbata* performed best in control conditions, showing limited benefit from biostimulant addition.
- *E. crinita* showed clear positive responses to several extract treatments, highlighting its greater sensitivity to supplementation.
- The commercial kelp-based formulation (AlgatronCifo®) performed comparably to microalgal extracts but also showed dose-dependent risks, stressing the need for careful concentration control.
- Overall, the results demonstrate that biostimulants can enhance early-stage performance in some Fucales, but their use requires optimization to avoid harmful side effects.

The objectives were achieved, and the hypothesis was partly supported by the results presented in this chapter. While *Desmodesmus* sp. extract at low concentrations proved effective, species-specific differences emphasize the need for tailored approaches in restoration programs.

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Enhancing ex situ cultivation of Mediterranean Fucales: species-specific responses of *Gongolaria barbata* and *Ericaria crinita* seedlings to algal extracts

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ABSTRACT

Mediterranean brown algal forests, dominated by *Cystoseira sensu lato* species, are undergoing widespread decline due to the cumulative effects of anthropogenic pressure and climate-related stressors. Restoration efforts increasingly rely on ex situ cultivation and outplanting of seedlings, yet early developmental stages often suffer from low survival and growth rates. This study investigated the potential of algal extracts to enhance the seedling development and survival in two canopy-forming species, *Gongolaria barbata* and *Ericaria crinita*. We tested extracts from a cyanobacterium (*Trichormus variabilis*), two microalgae (*Desmodesmus* sp. and *Cylindrotheca closterium*), and a commercial macroalgal formulation (AlgatronCifo®) at varying concentrations under controlled mesocosm conditions. Seedling performance was significantly influenced by extract type, and target species identity. Notably, a low-concentration *Desmodesmus* sp. extract (0.07 mg mL^{-1}) improved survival and growth, whereas *T. variabilis* exerted an inhibitory effect on *G. barbata*. AlgatronCifo® did not outperform *Desmodesmus* sp. extract in promoting seedling development. These findings suggest that specific extracts from green microalgae could improve protocols for the early stages of restoration, offering a scalable tool for rehabilitating degraded marine forests. However, the results underscore the importance of species-specific optimization and the need for in situ validation of biostimulant-based restoration approaches.

1. Introduction

Mediterranean macroalgal forests, dominated by canopy forming brown algae of the order Fucales (primarily the *Cystoseira sensu lato* complex), form structurally complex habitats that are functionally analogous to kelp forests in temperate and cold oceans. These foundational ecosystems support high biodiversity, providing food, shelter and critical ecosystem services, while acting as significant carbon sinks (Cheminée et al., 2017; Piazzi et al., 2018; De La Fuente et al., 2019a; Peleg et al., 2020; Sant and Ballesteros, 2021; Steneck et al., 2002). However, these vital ecosystems have undergone severe decline, with several populations becoming fragmented or locally extinct, often replaced by ephemeral and filamentous taxa (Thibaut et al., 2015; Mariani et al., 2019; Bernal-Ibáñez et al., 2021; Orlando-Bonaca et al., 2021a). This degradation stems from multiple anthropogenic pressures including habitat destruction, increased sediment resuspension, chemical pollution, and overgrazing by herbivores (Mangialajo et al., 2008; Orlando-Bonaca and Rotter, 2018; Piazzi and Ceccherelli, 2019; Orfanidis et al., 2021). Climate change has exacerbated these impacts, particularly through the increasing frequency and intensity of marine

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heatwaves (Bevilacqua et al., 2019; Verdura et al., 2021; Martínez et al., 2023).

The natural recovery potential of these damaged ecosystems is severely limited even if the primary stressors have been removed, with recolonization being rare due to propagule dispersal limitations and lack of source populations (Clayton, 1990; Galobart et al., 2023), with only isolated cases of recovery documented (Perkol-Finkel and Airoldi, 2010; Iveša et al., 2016; Medrano et al., 2020). Consequently, human-assisted restoration has proven to be essential (Cebrian et al., 2021), as recognized by international policies like the EU Nature Restoration Act (EC, 2024).

Significant progress has been made in developing restoration techniques for *Cystoseira s.l.* species over the past fifteen years. Collaborative research efforts have yielded diverse protocols encompassing both in situ and ex situ approaches (Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019b; Medrano et al., 2020; Orlando-Bonaca et al., 2022; Lokovšek et al., 2023). Although still implemented at small to medium scales, these restoration measures currently represent the most viable strategy to reverse the loss of *Cystoseira s.l.* forests in the Mediterranean (Smith et al., 2023).

In the northern Adriatic basin, where populations from the order Fucales have declined sharply (Falace et al., 2010, 2024; Orlando-Bonaca and Rotter, 2018; Iveša et al., 2022) ex situ cultivation approaches have been used in various restoration initiatives (Savonitto et al., 2021; Orlando-Bonaca et al., 2021b, 2022; Lokovšek et al., 2023, 2024). Kaleb et al. (2023) demonstrated that the commercial algal biostimulant AlgatronCifo® (from *Macrocystis pyrifera* (Linnaeus) C. Agardh (Phaeophyceae) can improve physiological performance and induce fertility in adult thalli of *Gongolaria barbata* (Stackhouse) Kuntze. This finding aligns with the well-established use of algal biostimulants in terrestrial agriculture and emerging applications in seaweed farming (Nanda et al., 2022; Jiksing et al., 2022).

Algal biostimulants (as defined by EU Regulation, 2019/1009 (see European Union, 2019)) are known to promote nutrient uptake and assimilation, increase chlorophyll content and photosynthetic activity, and improve resilience to abiotic stressors (Calvo et al., 2014). Microalgae and cyanobacteria offer particular advantages as biostimulants sources, due to their efficient cultivation and bioactive compound production (Sánchez-Quintero et al., 2023), including phytohormones, amino acids, antimicrobial metabolites (Parwani et al., 2021; Rossi and De Philippis, 2015) and ROS-scavenging antioxidants (Abinandan et al., 2019). Extracts have enhanced stress tolerance of terrestrial plants (Puglisi et al., 2020), but marine applications remain untapped.

Notably, microalgae exhibit substantial biocontrol potential against phytopathogens through bioactive metabolites (Grabowski et al., 2024), thus addressing a critical constraint in ex situ restoration: microbial contamination during early developmental stages (e.g., Savonitto et al., 2021; Malfatti et al., 2023). The ability of algal compounds to suppress common pathogens while potentially enhancing beneficial microbial associations addresses a critical bottleneck in seedling cultivation (Malfatti et al., 2023).

Despite these advances, the application of microalgal- and cyanobacterial-derived biostimulants in marine habitat restoration remains largely unexplored. Their demonstrated capacity to enhance growth (e.g., frond elongation, rhizoid development), stress resistance (e.g., to temperature fluctuations, oxidative stress), and microbial symbiosis, combined with cost-effective scalability in photobioreactor systems, positions them as transformative tools for optimizing ex situ cultivation protocols in macroalgal restoration programs.

The present study addresses this critical knowledge gap through two primary objectives:

1. Quantifying the species-specific responses of *Gongolaria barbata* and *Ericaria crinita* (Duby) Molinari & Guiry to microalgal and macroalgal extracts, measuring the effects on embryo development, seedling growth, and survival;
2. Evaluating practical applications for enhancing ex situ restoration of Mediterranean brown algal forests.

By investigating these novel biostimulant approaches, this work aims to develop more effective and scalable methods to restore these vital but threatened marine ecosystems.

2. Material and methods

2.1. Microalgae extracts production and characterization

The study utilized three microalgal strains selected for their documented biostimulant properties and ecological relevance: the cyanobacterium *Trichormus variabilis* (VRUC168), the green microalga *Desmodesmus* sp. (VRUC281), and the diatom *Cylindrotheca closterium* (VRUC291). These strains originate from the Vergata Rome University Culture Collection (VRUC), where they are maintained in batch stock cultures under controlled conditions (18 °C, 30 µmol photons m^{-2} s^{-1}, 12:12 light:dark cycle) using BG11.0 medium for *T. variabilis* and BG11 medium for *Desmodesmus* sp. and *C. closterium*.

The selected strains offer complementary biostimulant properties rooted in their distinct biochemical profiles. *Trichormus variabilis* enhances plant growth and antioxidant defenses under abiotic stress through the production of exopolysaccharides, phytohormones, and nitrogen-fixing metabolites, as demonstrated in crops like rice and maize (Bauenova et al., 2024). *Desmodesmus* species show robust biostimulant activity across plant systems, promoting germination, seedling vigor and nutrient uptake via their high content of cytokinins (particularly zeatin), amino acids and antioxidant compounds (Pereira et al., 2021; Perkol-Finkel and Airoldi, 2010; Araújo et al., 2023). *Cylindrotheca closterium* has antioxidant and anti-inflammatory potential, with transcriptomic studies confirming its biosynthetic capacity for bioactive metabolites (Lauritano et al., 2019; 2020). This phylogenetic and functional diversity provides a comprehensive biochemical foundation for developing nature-based solutions to improve the success of macroalgal restoration and ecosystem resilience.

The strains were mass cultivated indoors in photobioreactors of different geometry and capacity at 24 °C, with an irradiance of 30 µmol photons m^{-2} s^{-1} and an L/D cycle of 12:12 in semi-continuous mode. Harvesting of biomass involved settling and centrifugation (4500×*g* for 10 min) after the stationary phase, approximately after 4 weeks of growth. Specifically, *T. variabilis* was intensively cultured in an 8 L column photobioreactor, *Desmodesmus* sp. in a 2 L Erlenmeyer flask and *C. closterium* in a 6 L spherical system, with all cultures exposed to air insufflation for mixing. The harvested biomass was finally lyophilised (Telstar LyoQuest) for further experiments.

For biomass extraction, 3 g of the freeze-dried biomass samples were grinded and placed in a 1 L Erlenmeyer flask and 300 mL bidistilled water was added. Then the biomass was resuspended and treated for 20 min under high pressure (1 atm) and at high temperature (121 °C). After cooling, the biomass was centrifuged at 4500 g for 10 min and the supernatants, referred to as 'green extracts', were collected in 50 mL Falcon tubes, stored at −20 °C and freeze-dried.

2.1.1. Biochemical characterization of the extracts

The total nitrogen (TN) and phosphorus (TP) content of the extracts was quantified using persulfate digestion (Langner and Hendrix, 1982). The digestion reagent consisted of 50 g L^{-1} potassium persulfate ($K_2S_2O_8$), 30 g L^{-1} boric acid (H_3BO_3), and 15 g L^{-1} sodium hydroxide (NaOH). 10 mL of the digestion reagent was added to 5 mg of extracts in 100 mL Erlenmeyer flasks, which were then sealed with aluminum foil and subjected to autoclave digestion (121 °C for 30 min at 1 atm). During digestion, nitrogen- and phosphorus-containing compounds were oxidized to nitrate (NO_3^-) and orthophosphate (PO_4^{3-}), respectively, due to the alkaline environment created by the reagent (pH ≈ 9.7). The subsequent decomposition of potassium persulfate led to a

drop in the pH value (≈1.5), promoting phosphorus oxidation.

After cooling to room temperature, the TN concentration was determined spectrophotometrically by recording the absorbance at wavelength (λ) of 220 nm using quartz cuvettes. The concentration of orthophosphates in the digested sample (corresponding to TP) was determined using the colorimetric molybdenum blue method (Murphy and Riley, 1962). The reagent was freshly prepared before each analysis and added in the following sequence:

- 100 mL sulfuric acid (136 mL L^{-1}),
- 250 mL ammonium molybdate (40 g L^{-1}),
- 100 mL potassium antimonyl tartrate (3 g L^{-1})
- 50 mL ascorbic acid (9 g L^{-1})

The reaction between orthophosphates, ammonium molybdate, and potassium antimonyl tartrate resulted in the formation of a phosphomolybdate complex, which was subsequently reduced by L-ascorbic acid to form the molybdenum blue complex, which has a maximum absorbance at a wavelength of 882 nm. A volume of 2 mL of the reagent was added to the samples and calibration standards. After the colorimetric reaction, the TP concentration was measured spectrophotometrically at 882 nm using plastic cuvettes. The calibration curves were prepared with known concentrations of KNO_3 and KH_2PO_4. Absorbance readings were made using an ONDA UV-30 Scan spectrophotometer.

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method reagent, as described in Di Marco et al. (2013). Briefly, 10 mg of each sample was extracted in 4 mL of absolute methanol (CH_3OH) overnight at room temperature with continuous stirring (orbital shaker; 110 rpm). The suspensions were then centrifuged (5000×*g*, 20 min), and 20 μL of each supernatant was transferred to a Greiner Multiwell plate, followed by the addition of 80 μL Folin reagent diluted in ultra-pure H_2O (1:10 *v/v*). The mixture was incubated for 8 min before 20 μL of 20 % sodium carbonate solution was added. After 2-h of incubation, the absorbance was measured at 765 nm using a Tecan Spark fluorescence microplate reader (Tecan, Switzerland). The results were expressed as μg gallic acid equivalents (GAE) per mg fresh weight (mg FW).

2.2. Study sites and sample collection

Fertile apices of *Gongolaria barbata* were collected by SCUBA divers in April–May 2024 in Izola, Slovenia (45.5436°N, 13.6764°E) at 1–2 m depth, harvesting ≤10 % of reproductive tissue per individual to minimize population impact. *Ericaria crinita* samples were collected in June 2024 from Bijela Uvala, Croatia (45.1868°N, 13.5890°E) at 0.5–2 m depth. All specimens were transported to the Marine Biology Station Piran (MBSP, Slovenia) and the Center for Marine Research Rovinj (CMRR, Croatia) within 4 h of collection in a dark, temperature-controlled environment (4 °C). Upon arrival at the MBSP and CMRR nursery facilities, the apical parts of the thalli of *G. barbata* and *E. crinita* were rinsed with filtered seawater to remove epibionts and then stored at 4 °C for 24 h to promote gamete release. The fertile material was then cultured in a thermostatic chamber in three different experimental setups. The cultivation process followed the protocol of Falace et al. (2018) and De La Fuente et al. (2019b), which was further improved in Lokovšek et al. (2023).

2.3. Experimental designs

The sampling sites, brown algae species and experimental purposes are summarized in Table 1. The three experimental setups, and the concentrations of the algal extracts tested are explained in detail in subsections 2.3.1-2.3.3.

Table 1
Overview of sampling sites, species and time periods, experimental purposes, nursery facilities, types of algal extracts tested, and concentrations used in ex situ experiments conducted in 2024.

Sampling site	Sampled species	Sampling period	Experimental purposes	Nursery facility	Algal extracts tested	Concentrations
Izola (Slovenia)	*Gongolaria barbata*	early April 2024	1st experiment - Preliminary screening of microalgal extracts on early developmental stages	MBSP	*Trichormus variabilis, Cylindrotheca closterium, Desmodesmus* sp.	for all: 2.25 mg mL^{-1} and 4.50 mg mL^{-1}
Izola (Slovenia)	*Gongolaria barbata*	mid-April 2024	2nd experiment - Selection of the most effective microalgal extract and concentration on early developmental stages	MBSP	*Trichormus variabilis, Cylindrotheca closterium, Desmodesmus* sp.	for all: 1.2 mg mL^{-1}, 0.6 mg mL^{-1}, 0.3 mg mL^{-1}, 0.15 mg mL^{-1} and 0.07 mg mL^{-1}
Izola (Slovenia)	*Gongolaria barbata*	mid-May 2024	3rd experiment - Comparative assessment of microalgal vs. macroalgal extracts on early developmental stages	MBSP	*Desmodesmus* sp. (D), AlgatronCifo® (A)	0.07 mg mL^{-1} (D) and 4.5 mL L^{-1} (A)
Rovinj (Croatia)	*Ericaria crinita*	early June 2024	3rd experiment - Comparative assessment of microalgal vs. macroalgal extracts on early developmental stages	CMRR	*Desmodesmus* sp. (D), AlgatronCifo® (A)	0.07 mg mL^{-1} (D) and 4.5 mL L^{-1} (A)

2.3.1. First screenings of microalgal extracts and concentrations on G. barbata germlings

The first experimental trial was designed to evaluate the effects of three algal extracts (*Trichormus variabilis*, *Desmodesmus* sp., and *Cylindrotheca closterium*) on early developmental stages of *G. barbata* germlings. Based on previous studies of macroalgal cultivation and biostimulant applications (Guzmán-Murillo et al., 2013; Amer et al., 2019; Santini et al., 2021), we tested each extract at two concentrations (2.25 mg mL^{-1} and 4.50 mg mL^{-1}).

The experiment was performed in a temperature- and light-controlled room using 40 mL Petri dishes. Ambient conditions were maintained at 17 °C with a 15:9 h photoperiod (L:D) according to established protocols (Orlando-Bonaca et al., 2021b). Illumination was provided by four 36W Osram Fluora fluorescent tubes (120 cm in length) delivering an irradiance of approximately 140 μmol photons m^{-2} s^{-1} to the culture surface.

The experimental setup (Fig. 1) comprised triplicate dishes for each extract-concentration combination (18 treatment dishes in total) plus three control dishes containing Von Stosch enriched medium prepared with UVC-sterilized seawater (VS). The VS control followed methodologies from multiple previous studies of *G. barbata* cultivation (Falace et al., 2018; De La Fuente et al., 2019b; Savonitto et al., 2021; Orlando-Bonaca et al., 2021b, 2022; Lokovšek et al., 2023).

Five apices of *G. barbata* (approx. 3 cm long), each bearing mature receptacles, were placed in each Petri dish and left undisturbed for 48 h to allow gamete release and fertilization. The apical segments were then carefully removed so as not to disturb zygote development. Zygote development and growth, and survival rate was monitored by photographing a fixed area of 0.12 cm^2 in each dish at 24–48 h intervals using a stereomicroscope equipped with a high-resolution digital camera. This photographic documentation continued throughout the early developmental phase to track treatment effects on germination success and early growth.

2.3.2. Evaluation of the concentration effects of the different microalgal extracts on G. barbata seedlings

Based on the results of the preliminary screening, we conducted a second experiment to identify the most effective algal extract and determine its optimal concentration for promoting the development of *G. barbata* seedlings. This refined experimental approach utilized smaller culture volumes (10 mL Petri dishes) and a series of dilutions prepared from a 4.5 mg mL^{-1} stock solution (Fig. 2).

The experimental setup comprised duplicate treatments (n = 2) for each extract-concentration combination, with UVC-sterilized seawater serving as a control medium. For each replicate, we placed three fertile apical segments in petri dishes containing the control medium and waited 48 h for gametes to be released under controlled conditions. After the gametes were released, we carefully removed the apical segments and added the algal extracts at predetermined concentrations.

The development of the zygotes and survival rate was systematically monitored during the 14-day experimental period using standardised photo documentation (see 2.3.1). To maintain optimal culture conditions and prevent accumulation of metabolites, we changed the medium after seven days of incubation. The environmental parameters remained consistent with those of the first screening experiment to ensure comparability. This methodological continuity allowed a direct comparison of the results in both experimental stages, while the second phase focused specifically on concentration optimization.

2.3.3. Comparative effects of microalgal (Desmodesmus sp.) and macroalgal (AlgatronCifo®) extracts on G. barbata and E. crinita development

Following the concentration optimization phase (Section 2.3.2), we carried out comparative trials between the selected microalgal extract (*Desmodesmus* sp. at 0.07 mg mL^{-1}) and the commercial macroalgal biostimulant AlgatronCifo® (4.5 mL L^{-1}, Cifo S. p.A.), with Von Stosch (VS) serving as a control. The macroalgal product was selected due to its documented efficacy in enhancing fertility of adult *G. barbata* (Kaleb et al., 2023) and promoting seedling development of *Ericaria amentacea* (Malfatti et al., 2023). Parallel experiments with *G. barbata* and *E. crinita* allowed a direct comparison of the species-specific responses to these different biostimulant sources. The nutrient concentrations of AlgatronCifo® and VS medium are shown in Table 2, as reported in Kaleb et al. (2023) and Malfatti et al. (2023).

The experimental setup was standardised for both species (Fig. 3). For each treatment, we prepared three 1-L aquaria with twelve clay tiles with a diameter of 6 cm as substrate. Each tile received five fertile apical segments and was allowed to release gametes for four days before the biostimulant was applied. Seedling development and survival rate was photographically monitored (see 2.3.1), with *G. barbata* observed for 16

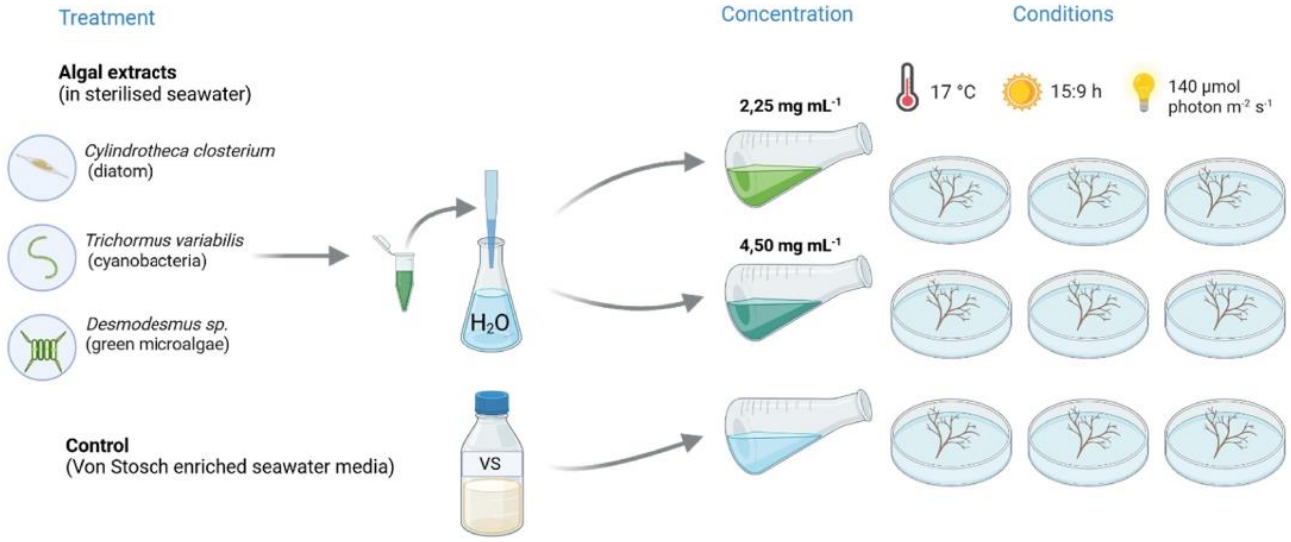


Fig. 1. Experimental setup for the preliminary screening of algal extracts on *Gongolaria barbata* germlings. Three microalgal extracts were tested at two concentrations (2.25 and 4.50 mg mL^{-1}), alongside a VS medium control. Each treatment was replicated in three 40 mL Petri dishes, with five fertile apices placed in each dish.

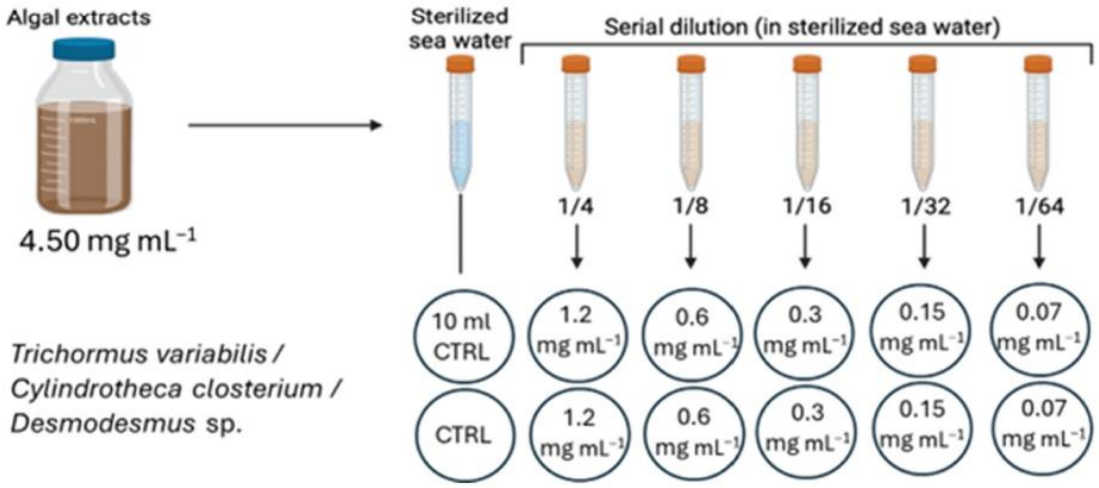


Fig. 2. Concentration optimization of microalgal extracts on *Gongolaria barbata* development. Experimental setup to test dose-dependent effects using duplicate 10 mL Petri dishes per treatment (three fertile apices each) with UVC-sterilized seawater control. Evaluated serial dilutions (0.07–1.2 mg mL^{-1}) of selected extracts identified in preliminary screening.

Table 2
Nutrients values of AlgatronCifo® and Von Stosch (VS) were reported by Kaleb et al. (2023) and Malfatti et al. (2023).

µg/L	Algatron pure product	Algatron at 4.5 mL L^{-1}	VS
TN	87466.70	530.60	
Urea-NO_2	6.10	0.03	
N-NO_2		1.30	1.27
N-NH_4	81546.67	373.50	0.75
N-NO_3	167.80	53.30	52.52
TP	200.10	7.03	
PO_4	214.70	1.97	
P-PO_4		1.17	5.3
N:P		324.58	10.29

days and *E. crinita* for 20 days to account for developmental differences. All cultures were maintained at 17 °C under a 15:9 light:dark cycle with an irradiance of 125–140 µmol photons $m^{-2}s^{-1}$, with media changes and continuous aeration beginning on day 10 of each experiment.

2.4. Statistical analyses and image processing

For the initial assessment of treatment effects (see 3.1), statistical analyses were performed using GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed to assess differences between treatments. Significance thresholds were set as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; and **** = $p < 0.0001$. Results are presented as mean

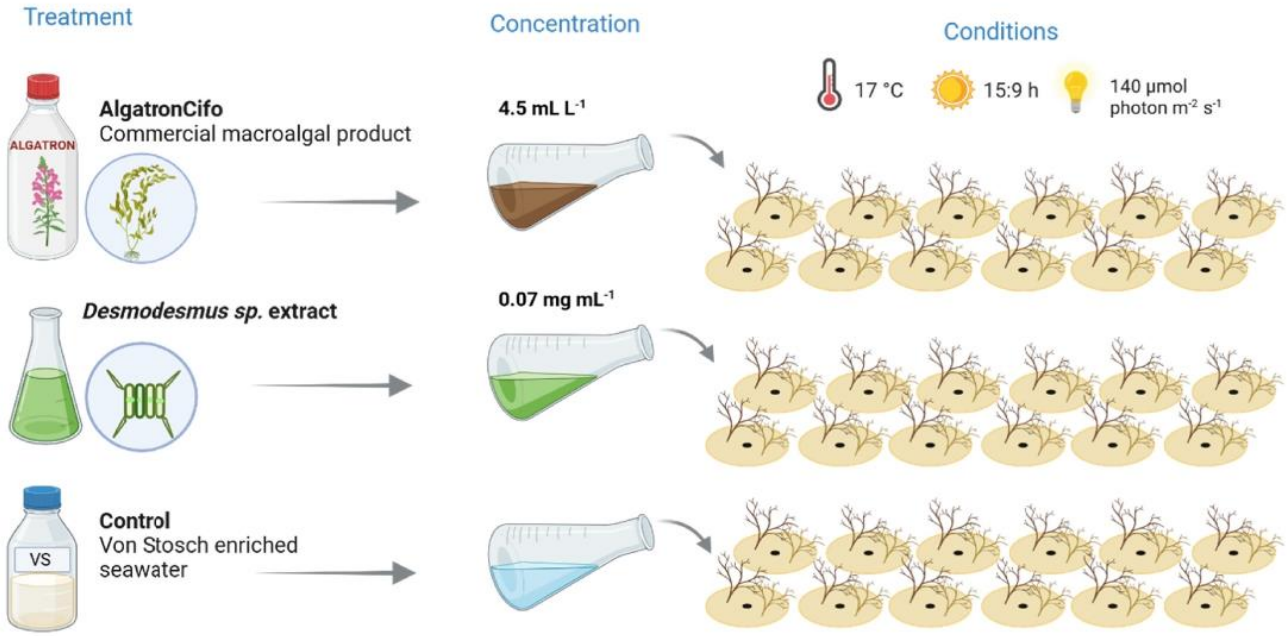


Fig. 3. Experimental setup for testing microalgal vs. macroalgal algal extracts on *Gongolaria barbata* and *Ericaria crinita* germlings development. Treatments included *Desmodesmus* sp. extract (0.07 mg mL^{-1}), the commercial macroalgal product AlgatronCifo® (4.5 mL L^{-1}), and a VS medium control. Each treatment was replicated in three 1 L aquaria, each containing twelve clay tiles (6 cm diameter).

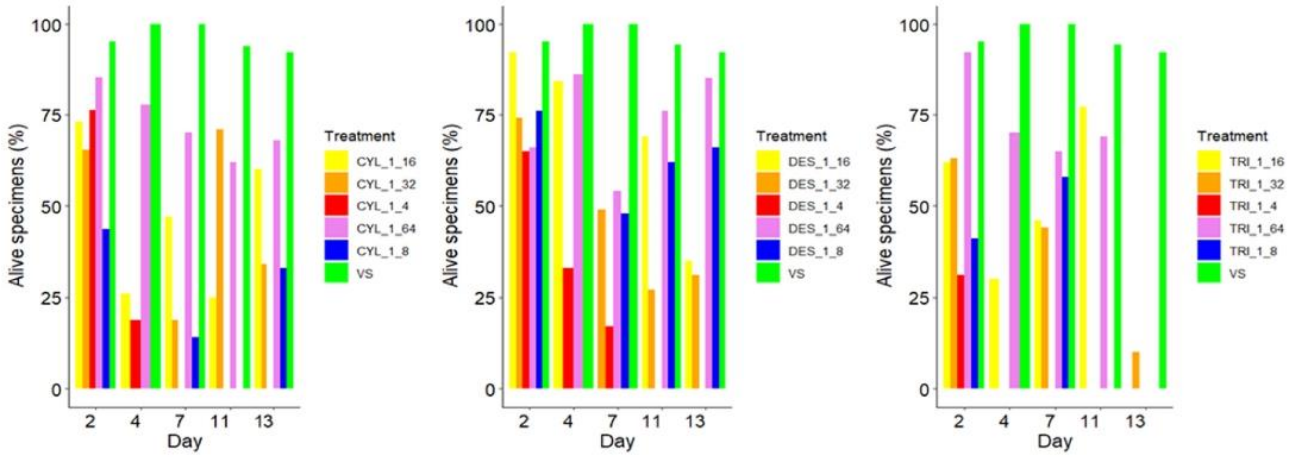


Fig. 6. Percentage of surviving *G. barbata* germlings in serial dilutions of the three algal extracts (see 2.3.2). The number of living germlings was counted in randomly photographed areas of 0.12 cm^2. CYL= *Cylindrotheca closterium*; DES = *Desmodesmus* sp.; TRI = *Trichormus variabilis;* VS =Von Stosch enriched seawater. Dilutions: 1/4 = 1.2 mg mL^{-1}, 1/16 = 0.3 mg mL^{-1}, 1/32 = 0.15 mg mL^{-1}, 1/64 = 0.07 mg mL^{-1}.

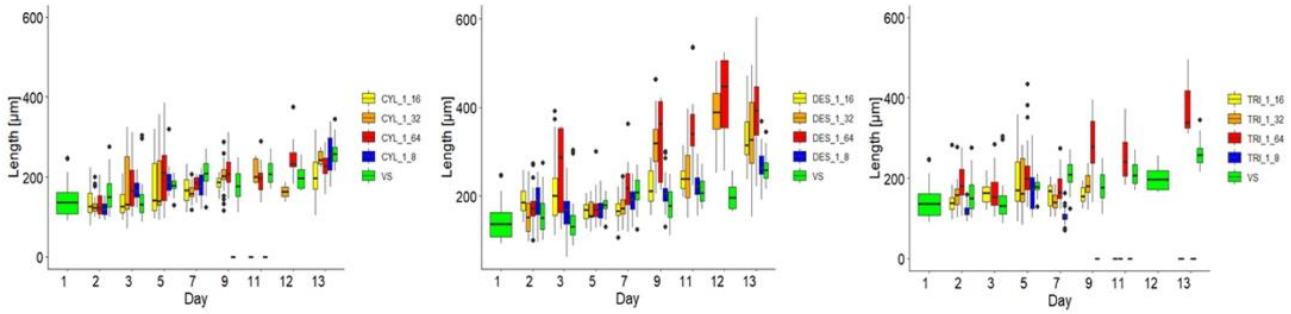


Fig. 7. Growth of *G. barbata* germlings (length in µm) in serial dilutions of the three extracts (see 2.3.2). Length measurements were made on living germlings in randomly photographed areas of 0.12 cm^2. The boxplots show the median, the first and third quartiles (boxes), the largest value no further than 1.5 × interquartile distance from the third quartile (upper whisker), the smallest value at most 1.5 × interquartile distance below the first quartile (lower whisker), outliers (dots). CYL= *Cylindrotheca closterium*; DES = *Desmodesmus* sp.; TRI = *Trichormus variabilis;* VS = Von Stosch enriched seawater. Dilutions: 1/4 = 1.2 mg mL^{-1}, 1/16 = 0.3 mg mL^{-1}, 1/32 = 0.15 mg mL^{-1}, 1/64 = 0.07 mg mL^{-1}.

The ANOVA results showed highly significant main effects on germling survival for both Treatment ($F = 124.25$, $p < 0.001$) and Day ($F = 123.97$, $p < 0.001$). Remarkably, the Treatment × Day interaction was not significant ($F = 14.64$, $p = 0.205$), suggesting that the effects of treatment on survival remained consistent throughout the trial. Post-hoc comparisons showed specific significant differences: the VS control outperformed both CYL_1_16 ($p < 0.05$) and CYL_1_32 ($p < 0.05$) treatments, while additional significant contrasts occurred between CYL_1_64 and CYL_1_4 ($p < 0.05$), and DES_1_64 versus CYL_1_8 ($p < 0.05$).

When measuring seedling length, the PERMANOVA results showed even more pronounced effects. Day accounted for most of the variation (59.4 %, $F = 195.13$, $p = 0.001$), while Treatment explained a smaller but still significant proportion (9.3 %, $F = 10.17$, $p = 0.001$). Importantly, we found a significant Treatment × Day interaction ($F = 5.34$, $p = 0.003$), indicating that the magnitude and direction of the effects of treatment on growth changed over time.

3.3. Species-specific responses to optimized biostimulant treatments

In *G. barbata* trials (see 2.3.3), survival rates after 16 days of culture were comparable for all treatments, though VS and *Desmodesmus* sp. (0.07 mg mL^{-1}) showed slightly higher values than AlgatronCifo® (Fig. 8). Growth patterns showed more marked differences, with VS

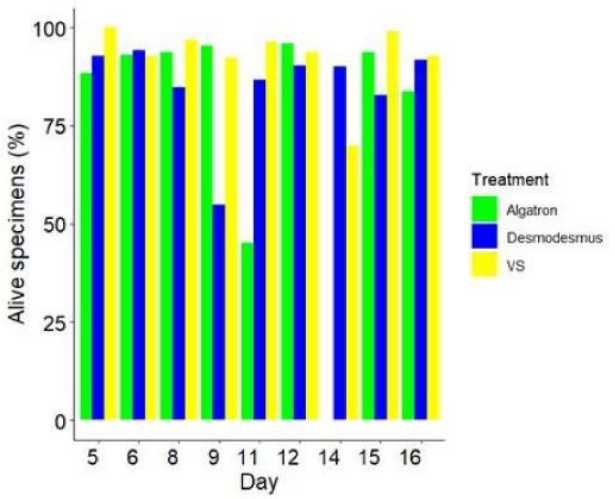


Fig. 8. Percentage of surviving *G. barbata* germlings after 16 days of cultivation in three treatments: *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and VS= Von Stosch enriched seawater (see 2.3.3).

control cultures reaching 983.95 μm, significantly higher than *Desmodesmus* sp. (395.26 μm) and AlgatronCifo® (339.4 μm) (Fig. 9).

Statistical models confirmed the absence of treatment effects on survival (ANOVA, $F = 1.512$, $p = 0.250$), while growth variation was significantly explained by both treatment (16.23 % of variation, PERMANOVA, $F = 254.62$, $p = 0.001$) and temporal factors (37.79 %). The significant Treatment × Day interaction (PERMANOVA, $F = 44.55$, $p = 0.003$) indicates dynamic growth responses over time. Pairwise tests confirmed statistically significant differences between treatments (see Supplementary Table S1).

Parallel experiments with *E. crinita* showed contrasting response patterns (Figs. 10 and 11). After 20 days of ex situ cultivation on clay tiles, survival rates were generally comparable for all three treatments, though subtle differences were apparent. The treatments containing *Desmodesmus* sp. extract (0.07 mg mL^{-1}) and AlgatronCifo® (4.5 mL L^{-1}) showed slightly higher survival rates compared to the VS control, as illustrated in Fig. 10.

The growth measurements showed clearer treatment effects. Treatment with *Desmodesmus* sp. extract resulted in a maximum seedling length of 773 μm on day 18 of cultivation. In comparison, the VS control reached a maximum length of 710 μm on day 20, while the AlgatronCifo® treatment showed more limited growth, reaching a maximum of 593 μm at the end of the experiment (Fig. 11). Despite these differences in final size, all treatments showed broadly similar growth trajectories throughout the duration of the experiment, as can be seen in Figs. 11 and 12.

The statistical analysis of the survival data yielded significant results. Treatment had a significant effect on survival percentage (ANOVA, $F = 6.000$, $p = 0.007$), while Day showed no significant effect (ANOVA, $F = 1.641$, $p = 0.1367$). Post-hoc Tukey HSD tests revealed specific significant differences between VS and the two treatments with AlgatronCifo® ($p = 0.008$) and Desmodesmus sp. extract ($p = 0.037$).

For seedling length measurements, PERMANOVA results showed highly significant effects. Day explained most of the variation (83.4 %, $F = 448.59$, $p = 0.001$), while Treatment explained a smaller but still significant proportion of the variation (0.21 %, $F = 7.92$, $p = 0.001$). The analysis also revealed a significant Treatment × Day interaction ($F = 6.34$, $p = 0.001$), which accounted for an additional 2.36 % of the variation. This interaction effect suggests that the influence of treatments on seedling length changed systematically over the course of the experiment. These patterns were confirmed by pairwise comparisons between treatments. The detailed results can be found in Supplementary Table S2.

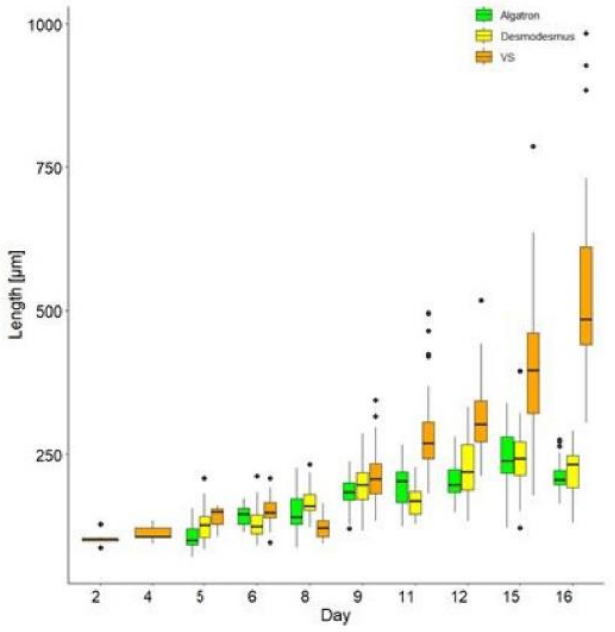


Fig. 9. Growth of *G. barbata* seedlings (length in μm) after 16 days of cultivation in *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and VS= Von Stosch enriched seawater (VS, control) (see 2.3.3). The boxplots show the median, the first and third quartiles (boxes), the largest value that is no further than 1.5 times the interquartile range from the third quartile (upper whisker), the smallest value that is no more than 1.5 times the interquartile range below the first quartile (lower whisker), outliers (dots).

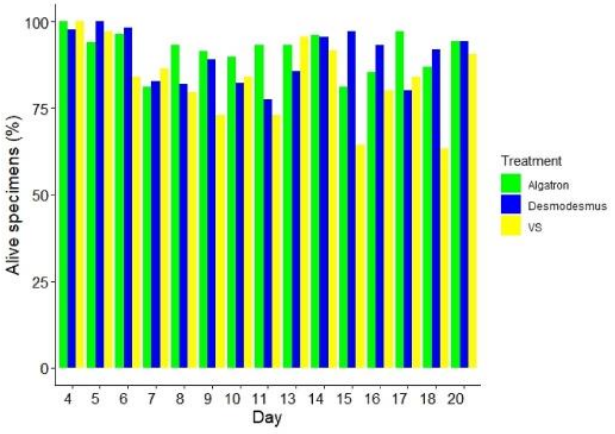


Fig. 10. Percentage of surviving E. *crinita* germlings after 20 days of cultivation in three treatments: *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and von Stosch enriched seawater (VS, control) (see 2.3.3).

4. Discussion

This study provides evidence that algal-derived biostimulants can significantly affect the early development of *G. barbata* and *C. crinita*, though their effects are highly dependent on the composition, concentration and target species of the extract. Our findings reveal several critical patterns that further the understanding of the application of biostimulants in macroalgal restoration.

The pronounced negative effects observed at higher extract concentrations (2.25–4.50 mg mL^{-1}) are consistent with previous reports of organic overload disrupting microbial balance in culture systems (Godlewska et al., 2016). The microbial bloom that accompanied these treatments likely created unfavorable conditions for germling development through multiple mechanisms: (1) competition for space and resources, (2) altered water chemistry via microbial respiration, and (3) potential proliferation of pathogens. This phenomenon illustrates the delicate balance required in ex situ culture systems, where organic input must be carefully calibrated to avoid destabilising the microbiome.

The superior performance of the *Desmodesmus* sp. extract at lower concentrations (especially 0.07 mg mL^{-1}) can be attributed to its distinct biochemical profile. With almost twice the nitrogen and phosphorus content of other extracts tested, this green algal extract likely delivers key nutrients in a more bioavailable form than conventional media. The organic nitrogen compounds in microalgal extracts could be particularly beneficial as they bypass the energy-intensive nitrate reduction pathway required for the assimilation of inorganic nitrogen in marine macrophytes. This metabolic shortcut could explain the initial growth advantage observed in *Desmodesmus*-treated seedlings compared to VS controls.

At lower concentrations, *Desmodesmus* sp. extract showed the highest biostimulatory effect on *G. barbata*, achieving growth and survival rates comparable to or even exceeding those of the control medium (VS). This effect is probably due to its high nitrogen and phosphorus content,

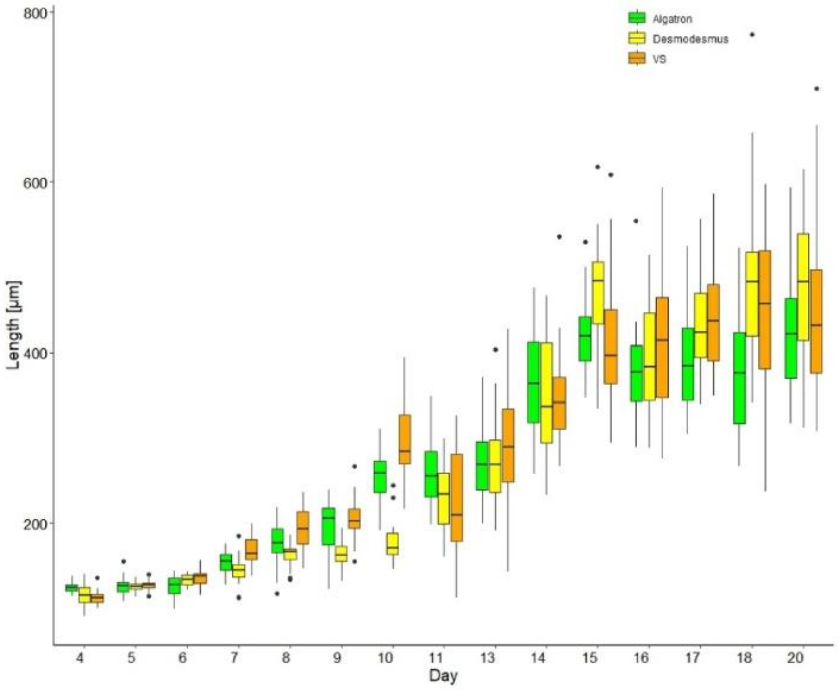


Fig. 11. Growth of *E. crinita* germlings (length in µm) after 20 days of cultivation in *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and Stosch's enriched seawater (VS, control) (see 2.3.3). The boxplots show the median, the first and third quartiles (boxes), the largest value that is no further than 1.5 times the interquartile range from the third quartile (upper whisker), the smallest value that is no more than 1.5 times the interquartile range below the first quartile (lower whisker), outliers (dots).

which is almost twice that of *Cylindrotheca closterium*, increasing its fertilizing capacity. In contrast, *Trichormus variabilis* performed consistently worse, possibly due to inhibitory secondary metabolites. The different performance of *C. closterium* could be related to its phenolic content, which is potentially beneficial at low concentrations but can be inhibitory at higher concentrations (Godlewska et al., 2016).

The comparative analysis between the two target species (i.e. *G. barbata* and *E. crinita*) revealed both shared trends and species-specific responses. In the 16-day cultivation of *G. barbata*, germling survival was high (~80–90 %) in all treatments. However, the VS medium yielded the longest thalli (~984 µm), and neither the extract of *Desmodesmus* sp. (0.07 mg mL^{-1}) nor the commercial biostimulant AlgatronCifo® performed significantly better than the control. While both extracts initially showed a growth-stimulating effect, this advantage diminished over time. This is particularly noteworthy considering that previous studies have reported positive effects of AlgatronCifo® on other species from the order Fucales, including seedling *Ericaria amentacea* (Malfatti et al., 2023) and adult *G. barbata* (Kaleb et al., 2023). In our case, neither the kelp-derived AlgatronCifo® nor the extract of *Desmodesmus* sp. outperformed the VS medium for *G. barbata* germlings. In contrast, *E. crinita* showed a clearer positive response to treatment with biostimulants. After 20 days of culture, the survival rate was significantly higher for the *Desmodesmus* sp. and AlgatronCifo® treatments compared to the control (VS). The seedlings treated with *Desmodesmus* sp. also attained the largest size (~773 µm). Though the absolute differences were modest, they were statistically significant. At the end of the experiment, the survival rate had decreased more in the VS and was about 10–15 % higher in the treated groups (see Fig. 10). These results suggest that algal extracts are particularly effective in supporting the early development of *E. crinita*, likely by mitigating physiological stress and compensating for nutrient limitations in the culture medium.

Interestingly, the extract of *Desmodesmus* sp. showed a slight advantage over AlgatronCifo® in promoting the growth of *G. barbata*, although both extracts proved to be similarly effective on *E. crinita*. This suggests that AlgatronCifo derived from *Macrocystis pyrifera*, which is rich in complex polysaccharides and phytohormones, may not fully fulfil the physiological requirements of early-stage *G. barbata* germlings. In contrast, the simpler biochemical composition of *Desmodesmus* sp. extract appears to be more compatible with the metabolic needs of the young thalli of both species.

Overall, these results underline the need for species-specific optimization in the application of algal biostimulants in macroalgal culture and restoration protocols. While both *G. barbata* and *E. crinita* responded positively to algal extracts, the magnitude and type of response differed, demonstrating that even closely related taxa can exhibit different physiological sensitivities to the same treatment.

These different results can be attributed to diverse physiological and molecular mechanisms by which algal extracts exert their effects. One of the most important mechanisms of action is the improved availability of nutrients. The extracts tested, especially that of *Desmodesmus* sp. (see section 2.1.4), contained significant amounts of organic nitrogen and phosphorus. These organic forms are more readily assimilated than their inorganic counterparts, bypassing enzymatic conversion steps and directly supporting important metabolic functions such as protein synthesis and cell division. Comparable fertilizing effects of microalgae biostimulants have been widely documented in agricultural systems (Chabili et al., 2024).

Additionally, algal extracts often contain essential micronutrients (e.g. Fe, Zn, Mn, Cu) and vitamins that act as cofactors in critical metabolic pathways (Godlewska et al., 2016). Brown algae in particular require exogenous vitamin B12 for their growth, which is usually supplied by symbiotic bacteria or enriched culture media (e.g. VS medium). Therefore, extracts from microalgae or seaweed enriched with B vitamins can support the metabolism of macroalgae more effectively than synthetic media alone (Godlewska et al., 2016).

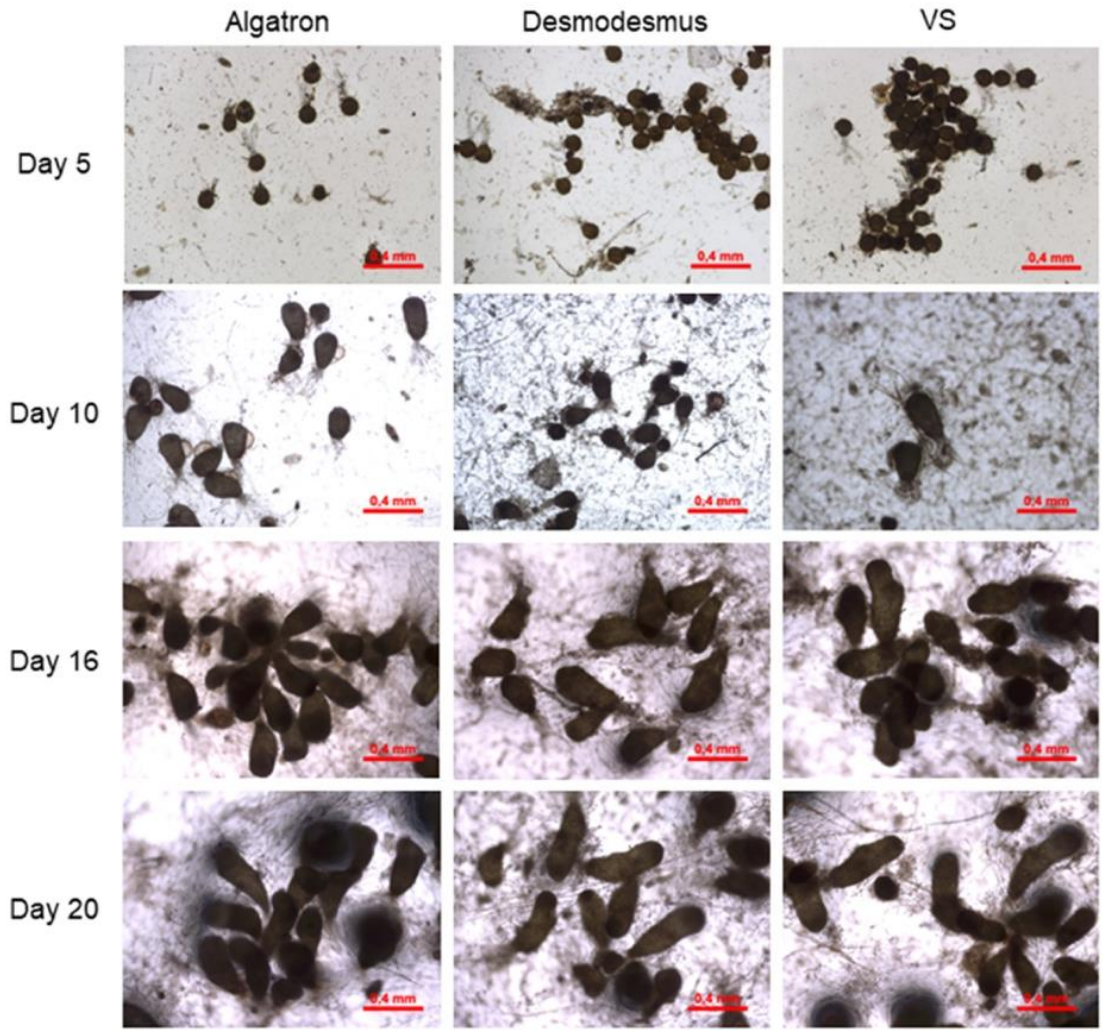


Fig. 12. Status of *Ericaria crinita* germlings under three treatments on Day 5, Day 10, Day 16 and Day 20 of the experiment in Rovinj.

Beyond their contribution to nutrition, algal biostimulants can provide phytohormone-like compounds, including auxins, cytokinins, gibberellins and abscisic acid. While these compounds are widely known for their regulatory role in terrestrial plant growth and stress responses, their functional importance is also increasingly recognized in macroalgal systems (Godlewska et al., 2016). Several studies suggest that the growth-promoting effect of algal extracts is not solely due to their mineral content, but that the presence of signalling molecules plays a crucial role (Ghaderiardakani et al., 2019).

In our study, the use of *Desmodesmus* sp. and *Macrocystis* (AlgatronCifo®) extracts probably led to such hormonal signalling. For example, cytokinins contained in green algal extracts are known to stimulate cell division and expansion in higher plants (Mazepa et al., 2021) and could similarly promote mitotic activity and thallus elongation in macroalgal germlings. Likewise, auxin-like compounds could influence the polarity and differentiation of zygotes — processes that are well documented in fucoid algae (Falace et al., 2018). While brown algae have endogenous hormonal pathways, the application of exogenous auxin analogues could enhance or modulate these mechanisms, potentially promoting rhizoid development and early thallus growth.

In addition, the presence of sulfated polysaccharides in AlgatronCifo®, such as laminarin, alginate and fucose-rich compounds, could influence cell wall architecture (Beuder and Braybrook, 2023) and signal transduction (Chi et al., 2018; Zhang et al., 2020). Although the exact hormonal composition of such extracts is still largely uncharacterised, these polysaccharides could interact with developmental signalling pathways.

In terrestrial systems, cytokinin-enriched extracts of *Desmodesmus* sp. have been shown to increase chlorophyll content and biomass by about 30 % (Mazepa et al., 2021), further supporting the potential of these compounds to promote macroalgal growth.

Another relevant mechanism is the stimulation of photosynthesis and overall metabolic activity. Algal biostimulants can increase pigment concentration and improve photosynthetic efficiency (Ali et al., 2022; Blunden et al., 1996; Yao et al., 2020), which ultimately increases energy production and carbon sequestration. In our experiments, such effects may have contributed to the greater growth and survival observed in the treated germlings. As chlorophyll biosynthesis depends on key nutrients such as nitrogen, magnesium and iron — all of which are commonly found in algal extracts (Godlewska et al., 2016) — these treatments likely supported the improved photophysiological performance.

The stress-protective properties of algal extracts deserve particular attention in restoration contexts. Early developmental stages of macroalgae are particularly vulnerable to abiotic stressors (e.g. temperature fluctuations, light intensity, osmotic shifts) and biotic pressures (e.g.

microbial colonisation, herbivory) (Ladah and Zertuche-González, 2007; Edwards, 2022). The *Desmodesmus* sp. extract contained increased amounts of polyphenols and antioxidant compounds, which may have helped to protect germling cells from oxidative damage. Although these effects do not directly stimulate growth, they contribute to improved overall viability.

Another aspect is the antimicrobial potential of algal biostimulants. Many algae and microalgae extracts have antimicrobial properties and have been shown to inhibit harmful microorganisms (Godlewska et al., 2016). The modest survival advantage observed in biostimulant-treated *E. crinita* seedlings could be due to reduced microbial pressure or selective promotion of beneficial microbial taxa. Malfatti et al. (2023) showed that kelp extracts altered the microbiome of *Ericaria amentacea* seedlings in a way that correlated with improved growth. Although we did not characterise the microbial assemblages in this study, it is plausible that similar probiotic effects occurred. Conversely, the application of high extract concentrations in the first experiment triggered a microbial bloom that overwhelmed the germlings, emphasising the critical importance of appropriate dosing.

The temporal dynamics of the responses to the treatments show that the positive effects of the algal extracts are particularly pronounced at certain stages of development. This pattern is especially evident during the crucial early post-settlement phase when the germlings undergo polarity and perform the first cell division. Our data show that the efficacy of treatment varies significantly over time, suggesting that the timing of application should be considered when designing the protocol as well as the composition of the extract. These results support the potential benefit of pulsed applications timed to coincide with critical developmental transitions, rather than continuous dosing, to maximise treatment efficacy while minimising potential adverse effects.

Within a restoration context, algal extracts represent a promising biotechnological tool to improve nursery performance. Their integration into growing protocols can improve vigour prior to outplanting while avoiding the ecological risks of direct nutrient enrichment (Eger et al., 2020; Earp et al., 2022; MacDonnell et al., 2022).

Taken together, these results strengthen the emerging view that algal extracts can significantly improve the fitness of early-stage macroalgal seedlings intended for restoration. The inclusion of *Desmodesmus* sp. extracts in rearing protocols could contribute to the development of more resilient seedlings for outplanting and ultimately improve restoration success.

Nonetheless, some limitations must be acknowledged. All experiments were conducted under controlled laboratory and nursery conditions. Field trials are essential to validate whether the benefits in the nursery phase also translate into better performance after outplanting under natural conditions. While Malfatti et al. (2023) reported that seedlings treated with biostimulants maintained their advantage post-transplantation, further validation across species, habitats and environmental conditions is essential.

Furthermore, our study focussed on early life stages; the responses of older seedlings or adults are still largely unexplored. Life stage–specific optimization may be necessary, as mature tissue may respond differently to nutrient or hormone supplementation (Kaleb et al., 2023). Future research should build on these findings along several key directions: (1) field validation of biostimulant-primed germlings in different environments and seasonal conditions; (2) transcriptomic or proteomic studies to elucidate mechanisms of action such as upregulation of nitrogen assimilation genes or stress-related genes; (3) detailed assessment of biostimulant-induced microbiome shifts and their functional consequences; and (4) broader exploration of biostimulant applications in benthic macroalgal restoration.

As marine ecosystems are exposed to increasing degradation, biotechnological tools orientated towards ecological processes are urgently needed. Algal extracts represent a scalable, cost-effective and ecologically sound strategy by utilising natural metabolic and signalling compounds. With further refinement, this approach can increase the success of restoration efforts not only for *Cystoseira* s.l. forests, but also for other important coastal habitats such as kelp forests and seagrass beds.

CRediT authorship contribution statement

Ana Lokovšek: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Martina Orlando-Bonaca:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Edi Gljušćić:** Writing – review & editing, Methodology, Investigation, Data curation. **Andrea Bilajac:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ljiljana Iveša:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Alberta Di Cave:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Saverio Savio:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Federico Ortenzi:** Writing – review & editing, Methodology, Investigation. **Domen Trkov:** Writing – review & editing, Investigation, Conceptualization. **Roberta Congestri:** Writing – review & editing, Methodology, Conceptualization. **Annalisa Falace:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Data availability

Most data presented in this study are contained within the article and Supplementary Materials (Tables S1 and S2). The rest are available on request from the corresponding author.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Chatgpt 4.0 in order to improve language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Chapter 5
Genetic Diversity of *Gongolaria barbata*

5.1 Paper: Contribution to the Knowledge of *Gongolaria barbata* (Sargassaceae, Fucales) from the Mediterranean: Insights into Intraspecific diversity

This chapter investigates the infraspecific diversity of *Gongolaria barbata,* a canopy-forming species characterized by remarkable morphological variability. Historically, distinct morphotypes have been described as separate infraspecific taxa, but their interpretation has varied, with some considered seasonal stages, ecotypes, or independent taxa. Such polymorphism complicates taxonomy, conservation planning, and restoration strategies, underscoring the need to clarify the relationship between morphological traits, ecological conditions, and genetic identity.

To address this, we applied an integrative approach combining molecular, morphological, and ecological analyses. Attached (haptophytic) and free-floating (pleustophytic) specimens were collected from several sites in the Gulf of Trieste and from Sardinia. Vegetative and reproductive traits were quantified and compared across populations, while mitochondrial cox1 sequences were analyzed to test for genetic divergence between morphotypes. This integrative approach aimed to clarify whether morphological variability reflects genetic differentiation or environmentally driven phenotypic plasticity.

Specific objectives:
I. Characterize vegetative and reproductive morphology of *G. barbata* populations across contrasting ecological conditions.
II. Assess genetic differentiation between free-floating and attached forms using mitochondrial cox1 sequences.
III. Clarify the taxonomic status of historically described infraspecific taxa such as *hoppei*, *turneri*, and *aurantia*.
IV. Provide insights into the ecological and evolutionary significance of infraspecific diversity in *G. barbata.*

Hypothesis:
The morphological variability observed among populations of *G. barbata* represents site-specific phenotypic plasticity rather than true genetic divergence, with distinct morphotypes reflecting adaptation to local ecological conditions.

Key findings
- Stable morphological differences were documented between northern Adriatic populations.

- Izola thalli matched f. *hoppei* (long fronds, spindle-shaped receptacles, abundant aerocysts).
- Strunjan and Piran thalli corresponded to *f. turneri* (short fronds, cylindrical receptacles, few or no aerocysts).
- Molecular analyses (cox1) revealed no genetic divergence among morphotypes, supporting the role of phenotypic plasticity rather than genetic isolation.
- Free-floating lagoon forms were genetically indistinguishable from attached thalli, confirming they are detached but viable branches that persist and occasionally reproduce in calm environments.
- The historically described *C. aurantia* was clarified as a lagoon-associated ecoform, formally recombined as *Gongolaria barbata* f. *aurantia*.
- The study formally recognized three ecologically defined infraspecific taxa: *G. barbata* f. *hoppei*, *G. barbata* f. *turneri* and *G. barbata* f. *aurantia*.

The objectives were achieved, and the hypothesis is confirmed by the results presented in this chapter.

The research work is presented in the following publication and is listed below:
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Contribution to the knowledge of *Gongolaria barbata* (Sargassaceae, Fucales) from the Mediterranean: insights into infraspecific diversity

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Abstract

Gongolaria barbata (Sargassaceae, Fucales) is a widespread species for which several infraspecific taxa have been described, indicating its polymorphism. This study contributes to the understanding of the molecular, nomenclatural, morphological and ecological aspects of *G. barbata* in the Mediterranean and sheds light on the infraspecific diversity and its implications for the taxonomy of this species. Molecular analyses were performed using sequencing of the mitochondrial cox1 gene on both haptophytic and pleustophytic forms from different sites in the Adriatic and Tyrrhenian Seas. Vegetative and reproductive morphology was studied on thalli samples from the Adriatic. Our results showed that there are different morphotypes within *G. barbata* populations related to specific environmental conditions, suggesting infraspecific variation. In contrast, molecular analyses showed no differences between samples, regardless of whether individuals are growing "attached" to a substrate or "unattached". We also discussed the taxonomic status and nomenclatural issues related to certain infraspecific taxa previously proposed for *G. barbata*. In particular, the confusion surrounding *Cystoseira aurantia* is clarified.

Key words: ecotypes, taxonomy, Northern Adriatic, *Cystoseira s.l.*

Introduction

In the Mediterranean, with the exception of the endemic *Fucus virsoides* J. Agardh, which is restricted to the Adriatic Sea (Orlando-Bonaca *et al.* 2013), the Fucales are characterized by the widely distributed *Sargassum* genus and the polyphyletic *Cystoseira sensu lato* (*s.l.*). The latter, following phylogenetic analysis made by Draisma *et al.* (2010), Bruno de Sousa *et al.* (2019) and Orellana *et al.* (2019), has been formally divided by Orellana *et al.* (2019) into three genera: *Cystoseira sensu stricto* (*s.s.*), *Carpodesmia* Greville and *Treptacantha* Kützing. More recently, Molinari & Guiry (2020) re-instated *Gongolaria* Boehmer and *Ericaria* Stackhouse for *Carpodesmia* and *Treptacantha*, respectively, due to the priority principle. That, resulted in numerous new nomenclatural combinations.

Many studies have investigated the phenology of *Cystoseira s.l.* species and shown that they undergo both morphological and reproductive changes throughout the year (Gómez-Garreta *et al.* 1982, Hoffmann *et al.* 1992, Serio 1996a, Serio 1996b, Pizzuto 1998a, Pizzuto 1998b, Alongi *et al.* 1999, Marzocchi *et al.* 2003, Falace *et al.* 2005, Falace

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& Bressan 2006, Savonitto *et al.* 2019, Medrano *et al.* 2020). Nevertheless, few studies have focused on morphological variation among populations of fucoid species and have also reported significant differences in reproductive traits (De Paula & De Oliveira 1982, Silva *et al.* 2004, Savonitto *et al.* 2019, Sadogurska *et al.* 2021, Orlando-Bonaca *et al.* 2022).

In the Mediterranean Sea as well as in the Black Sea and the Sea of Azov *Gongolaria barbata* (Stackhouse) Kuntze [formerly *Cystoseira barbata* (Stackhouse) C. Agardh] is widespread and inhabits areas with different climatic and ecological conditions (Guiry & Guiry, 2023). Within its geographic range, *G. barbata* exhibits great morphological variability (Falace & Bressan 2006, Sadogurska *et al.* 2021). For this reason, numerous infraspecific taxa of this species are known in literature like:

Cystoseira barbata var. *hoppei* "*hoppii*" (C. Agardh) J. Agardh [afterwards reduced to the rank of a form by Woronichin (1908: 117) as *C. barbata* f. *hoppei* "*hoppii*" (C.Agardh) Woronichin] and *C. barbata* var. *turneri* J. Agardh, both reported by Sauvageau (1912) from Banyuls-sur-Mer (France) who considered them as seasonal morphologies of *G. barbata* (as *C. barbata*); *C. barbata* f. *insularum* Ercegović, *C. barbata* f. *punctata* Ercegović and *C. barbata* subsp. *tophuloidea* Ercegović [the latest later reduced to the rank of variety by Giaccone (in Amico *et al.* 1986: 906) as *C. barbata* var. *tophuloidea* (Ercegović) Giaccone], considered by Cormaci *et al.* (2012) as *Taxa Inquirenda* (TI) because they were based on morphological traits of low taxonomic value; *C. barbata* var. *flaccida* (Kützing) Woronichin whose basionym *C. flaccida* Kützing [considered by Woronichin (1908) as the basionym of a variety of *C. barbata*], was considered as the basionym of a variety of *C. crinita* Duby [= *Ericaria crinita* (Duby) Molinari *et* Guiry] by Schiffner (1933) who proposed the new combination *C. crinita* var. *flaccida* (Kützing) Schiffner. Both *C. barbata* var. *flaccida* and *C. crinita* var. *flaccida* were considered by Cormaci *et al.* (2012) as TI, while recently, Sadogurska *et al*. (2021) considered *C. barbata* var. *flaccida* and C. *barbata* f. *hoppei* × *flaccida* (sic!) as synonyms of *E. crinita* f. *bosphorica* (Sauvageau) Sadogurska, Neiva *et* Israel (= *E. bosphorica* (Sauvageau) Serio *et* Furnari). It should be noted that erroneously Sadogurska *et al.* (2021) considered "*C. barbata* f. *hoppei* x *flaccida* Woronichin (1908: 118) as a synonym of *E. bosphorica* (as *E. crinita* f. *bosphorica*) not realizing that because it is not a validly published name with no status under the art. 12 of ICN (Turland *et al.* 2018), it can't be a synonym of any validly published name. However, they probably meant that the above specimen described by Woronichin shows the same features of *E. bosphorica* (as *E. crinita* f. *bosphorica*). Finally, more recently, Giaccone (in Amico *et al.* 1986: 906) proposed the new combination *C. barbata* f. *aurantia* (Kützing) Giaccone, based on *C. aurantia* Kützing, a species described by Kützing (1843) from the Gulf of Trieste, for specimens that, as reported by Giaccone & Bruni (1973: 65) with reference to ecotypes of *C. barbata*, are pleustophytic and live in low-hydrodynamic, low-light and sediment-rich environments. It should be noted that the above form was considered by Gómez Garreta *et al.* (2000: 115) as a synonym of *C. barbata* f. *repens* Zinova *et* Kalugina [= *G. barbata* f. *repens* (Zinova *et* Kalugina) Sadogurska], a taxon described on specimens from the Black Sea. *G. barbata* f. *repens* is currently the only recognised infraspecific taxon according to Guiry and Guiry (2023). Finally, it should be noted that the molecular sequences of a free-swimming specimens from Cádiz (Atlantic Ocean, Spain) and from Tenerife (Atlantic Ocean) were referred to as *C. barbata* f. *aurantia* by Bruno de Sousa *et al.* (2019) and *C. aurantia* by Orellana *et al.* (2019), respectively. However, Tenerife's specimen belongs to the clade of *Cystoseira s.s.* and not to that of *Treptacantha* (= *Gongolaria*) to which *C. aurantia* from the Adriatic Sea belongs (see discussion). On this based, *C. aurantia sensu* Orellana *et al.* (2019) was listed as a synonym of *'C. barbata* f. *aurantia'* in AlgaeBase (Guiry & Guiry 2023) as well in following reports (e.g., Battelli & Catra 2021, Ramdani *et al.* 2021).

The recognition of such a large number of intraspecific taxa within *C. barbata* (= *G. barbata*) shows that researchers have considered *C. barbata* from the Mediterranean as a highly polymorphic taxon. Therefore, this research aims to investigate infraspecific variation in that species in the Adriatic Sea by combining molecular and morphological approaches and ascertain whether formal recognition of infraspecific taxa is justified. Additionally, we seek to determine the appropriate taxonomic rank that should be applied given the observed variation.

Materials & methods

Specimen collections

Haptophytic (= fixed to substrata) specimens were collected during the 2021 and 2022 reproductive seasons at three sites in the Gulf of Trieste (northern Adriatic Sea):

1) near Strunjan Landscape Park (45.53298, 13.64146), characterized by natural populations of *G. barbata* associated with *Cystoseira compressa* (Esper) Gerloff *et* Nizamuddin at depths of 1 to 3 m;

2) near Izola (45.543567, 13.676371), characterised by healthy and dense populations of *G. barbata* and *C. compressa* at depths of 1 to 3 m;

3) near Piran (45.5284, 13.5754), where belts of *G. barbata* occur in association with *C. compressa* and *Ericaria crinita* (Duby) Molinari *et* Guiry at depths of 1 to 3 m (Orlando-Bonaca *et al.* 2021b).

All sites have a shallow rocky seabed and are moderately exposed to wave action (Orlando-Bonaca *et al.* 2008).

Pleustophytic (= free-floating) specimens were collected in the Gulf of Trieste (Northern Adriatic, Eastern Mediterranean):

1) near the lagoon of Grado (45.681471, 13.434312). The area is connected to the lagoon by several flow-through channels and is characterised by a sedimentary bottom and shallow water depth (0–0.5 m)

2) in the Stjuža lagoon (Strunjan Stjuža Nature Reserve, 45.530556, 13.606389). The Stjuža lagoon is connected to the sea only by a flow-through channel, the water flow depends exclusively on the ebb and flow of the tide, and due to the shallow water depth (0.5 m) the water is subject to rapid heating and cooling.

For molecular comparison, both fixed and free-floating specimens of *G. barbata* were also collected in Sardinia (Tyrrhenian Sea, Western Mediterranean):

1) near Santa Giusta (39.864178, 8.57486), a lagoon area where pleustophytic specimens were collected;

2) near Portoscuso (39.205277, 8.37568), haptophytic specimens were collected in rock pools.

For molecular analysis, the specimens (TABLE 1) were kept in separate clean bags, dried and stored in silica gel and delivered to the Applied and Comparative Genomics Laboratory of the University of Trieste for DNA extraction.

TABLE 1. List of samples used for the molecular analyses.

ID sample	GenBank ID (first and second half of the Cox-1 gene portion)	Locations	Geographical coordinates	Morphotype
POZ	OR335716 OR343920	Portoscuso Sardinia, Italy	39.205277, 8.37568	haptophytic
LAG1	OR335717 OR343921	Santa Giusta, Sardinia, Italy	39.864178, 8.57486	pleustophytic
LAG2	OR335718 OR343922	Santa Giusta, Sardinia, Italy	39.864178, 8.57486	pleustophytic
LAG3	OR335719 OR343923	Santa Giusta, Sardinia, Italy	39.864178, 8.57486	pleustophytic
RAD	OR335720 OR343924	Grado, Italy	45.681471, 13.434312	haptophytic
F23	OR335721 OR343925	Grado, Italy	45.681471, 13.434312	pleustophytic
F24	OR343926	Grado, Italy	45.681471, 13.434312	pleustophytic, used only for "2nd half alignment"
B3	OR335722 OR343927	Strunjan Landscape Park, Slovenia	45.681471, 13.434312	haptophytic
B6	OR335723 OR343928	Strunjan Landscape Park, Slovenia	45.681471, 13.434312	haptophytic
M2	OR335724 OR343929	Izola, Slovenia	45.543567, 13.676371	haptophytic
M7	OR335725 OR343930	Izola, Slovenia	45.543567, 13.676371	haptophytic
M8	OR335726 OR343931	Izola, Slovenia	45.543567, 13.676371	haptophytic
P1	OR335727 OR343932	Piran, Slovenia	45.5284, 13.5754	haptophytic
P2	OR335728 OR343933	Piran, Slovenia	45.5284, 13.5754	haptophytic
P4	OR335729 OR343934	Piran, Slovenia	45.5284, 13.5754	haptophytic
Str1	OR335730 OR343935	Strunjan Stjuža Nature Reserve, Slovenia	45.530556, 13.606389	pleustophytic
Str2	OR335731 OR343936	Strunjan Stjuža Nature Reserve, Slovenia	45.530556, 13.606389	pleustophytic

Reproductive morphology of Adriatic haptophytic samples

The length and width of randomly selected receptacles (N= 100) for each of the three haptophytic populations of the Gulf of Trieste (i.e., Strunjan, Piran, Izola) were measured under a stereomicroscope (Leica MZ 6, Leica Microsystems, Wetzlar, Germany) and photographed with a Nikon Coolpix 4500 camera (Nikon Corporation, Tokyo, Japan). Measurements were made on receptacles by randomly collecting 4 receptacles from 25 individuals in each population. The number of conceptacles was determined by counting the total number of ostioles on the outer surface of each receptacle using a stereomicroscope (Savonitto *et al*. 2019, Orlando-Bonaca *et al*. 2022).

Molecular analyses

Genomic DNA (gDNA) was extracted from 17 samples using the E.Z.N.A.® Plant DNA Kit (Omega Bio-tek, USA) and following the manufacturer's protocol for dried plant samples, with minor modification as a supplemental wash of ethanol 70% before DNA elution. DNA was checked for quality and concentration on Nanodrop 2000 (Thermo Fisher Scientific, USA).

All samples were amplified for two overlapping fragments of the mitochondrial gene cox1 (Neiva *et al.* 2022) giving a sequence of 1255 bp (overlap of 34 nucleotides), primers are listed in TABLE 2. The amplification mix was prepared as follows: 1X AccuStart II PCR SuperMix (Quantabio, USA), 0.3 µM both forward and revers primers and 20 ng gDNA in a final volume of 20 µL. PCRs were performed in a thermal cycler XT96 (VWR International) with the following parameters: 94°C for 5', 35 cycles: 94°C for 30", 49°C for 45", 72°C for 1' and finally 72°C for 5'.

TABLE 2. Sequences of primers used to amplify gDNA, alongside their annealing temperature and reference.

Locus [primers]	Primers 5' → 3'	Ta (°C)	Source	Note
cox1 [Gaz2]	F: 5'-CCAACCAYAAAGATATWGGTAC -3' R: 5'-GGATGACCAAARAACCAAAA-3'	49	Lane *et al.* 2007	1st half of cox 1 gene portion
cox1 [cox1-789F /cox1-1378R]	F: 5'-TNTAYCARCATTTATTTTGGTT-3' R: 5'-TCYGGNATACGNCGNGGCATACC-3'	49	Silberfeld *et al.* 2010	2nd half of cox 1 gene portion

PCR products were checked on TAE 1,5 % agarose gel to verify DNA amplification and then purified with 0,65X Mag-Bind® TotalPure NGS (Omega Bio-tek, USA) according to the manufacturer's instructions. The purified PCR products were sent to an external service to be Sanger sequenced (Eurofins Genomics, Ebersberg, Germany).

Phylogenetic analysis

The chromatograms representing each of our samples were manually cleaned at the 5' and 3' edges and the primer sequences were removed, resulting in two groups of sequences, one representing the first and the other the second half of the cox1 sequence. Although the group of the second half consisted of a smaller number of sequences, we considered this comparison especially for the inclusion of the sequences of *C. compressa*, voucher TFC:15262, and *C.* sp., voucher TFC:15276, both collected in Tenerife (Spain) (GenBank MH513832 and MH513833).

Sequences were aligned with MUSCLE (Edgar, 2004) within MEGA-X (version 10.1.1, Kumar *et al.* 2018), using as outgroup for the first tree *Himanthalia elongata* voucher GWS039908, GenBank MN184240, and for the second tree *Himanthalia elongata*, GenBank EU681409.

The phylogeny of our specimens was inferred together with other selected sequences (full list in Supp. Inf, Table S1) The analysis were run based on the Hasegawa-Kishino-Yano substitution model (Hasegawa *et al*. 1985) +G, which was evaluated as the best-fitting model for both the dataset with MEGA-X (version 10.1.1, Kumar *et al*. 2018). The trees were built using the software MrBayes 3.2.7_0 (Ronquist *et al*. 2012) on the web service of NGphylogeny.fr (Lemoine *et al*. 2019) with 100,000 generations. The trees of the two halves of the cox1 gene portions were visualized and embellished by iTOL: Interactive Tree Of Life (Letunic & Bork 2021)

Results

Vegetative and reproductive morphology

Our haptophytic specimens from the northern Adriatic show the characters considered as diagnostic for the identification of the *G. barbata* (Gómez Garreta *et al.* 2000, Cormaci *et al.* 2012) viz.: a non-caespitose habit with a single cauloid, simple or branched, attached to the substrate by a basal disc 5–10 mm in diameter; the apex of the cauloid smooth and very pronounced in relation to the base of the primary branches. However, thalli from Izola had long fronds (with main branches up to 50 cm), while those from Piran and Strunjan were smaller and had shorter primary branches (up to 20 cm) and showed also reproductive structures with significant morphological differences between the three populations studied. The fertile specimens collected from both Strunjan and Piran had short, simple and cylindrical receptacles that were sparsely mucronated and had rare or no aerocysts (never in chain) (FIGURE 1, TABLE 3). The receptacles were predominantly smooth. In contrast, fertile specimens from Izola had long, terminal, fusiform, mucronate or pedicellate receptacles, numerous single or concatenated aerocysts with chains of up to 5 aerocysts. The conceptacles were prominent. In addition, a higher number of conceptacles was found in the receptacles of Izola specimens than in those of Strunjan (30.5 ± 1.23 conceptacles receptacle^{-1} in Izola versus 25.33 ± 0.73 conceptacles receptacle^{-1} in Strunjan) (TABLE 3).

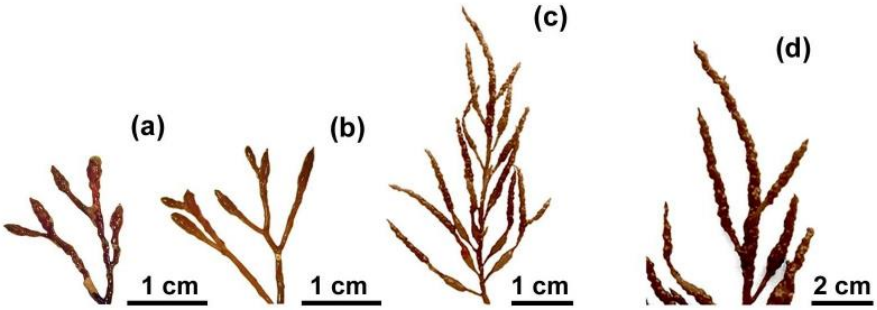


FIGURE 1. Fertile apical fronds of *G. barbata* populations from Piran (a), Strunjian (b) and (c, d) Izola

TABLE 3. Reproductive morphology of the haptophytic *Gongolaria barbata* specimens from the Gulf of Trieste.

	Izola	Strunjan	Piran
Cryptostomata	numerous	numerous	numerous
Aerocysts	abundant, voluminous, oval to spindle shaped, isolated or in chains (up to 5 aerocysts)	absent or very rare (isolated)	absent or very rare (isolated)
Receptacle shape	simple, fusiform mucronate (long), peduncolate	simple or bifid, cylindrical, rarely mucronate (short)	simple or bifid, cylindrical, rarely mucronate (short)
Receptacle length	6.33 ± 0.33 mm, up to 19.79 mm	4.71 ± 0.18 mm, up to 9.97	4.55 ± 0.17, up to 10.89 mm
Receptacle width	0.96 ± 0.02 mm	0.77 ± 0.02 mm	0.84 ± 0.02 mm
Conceptacle	prominent	smooth or slightly prominent	smooth or slightly prominent
Conceptacles receptacle^{-1}	30.45 ± 0.45 conceptacles receptacle^{-1} (up to 93 conceptacles receptacle^{-1})	24.96 ± 0.25 conceptacles receptacle^{-1} (up to 65 conceptacles receptacle^{-1})	

The length of the receptacles differed significantly between the sites (Kruskal-Wallis, chi-square = 22.14, $P < 0.001$) with a significant difference between the population of Izola and Piran (pairwise Wilcoxon test, $P < 0.001$) and between the population of Izola and Strunjan (pairwise Wilcoxon test, $P < 0.001$) (FIGURE 2).

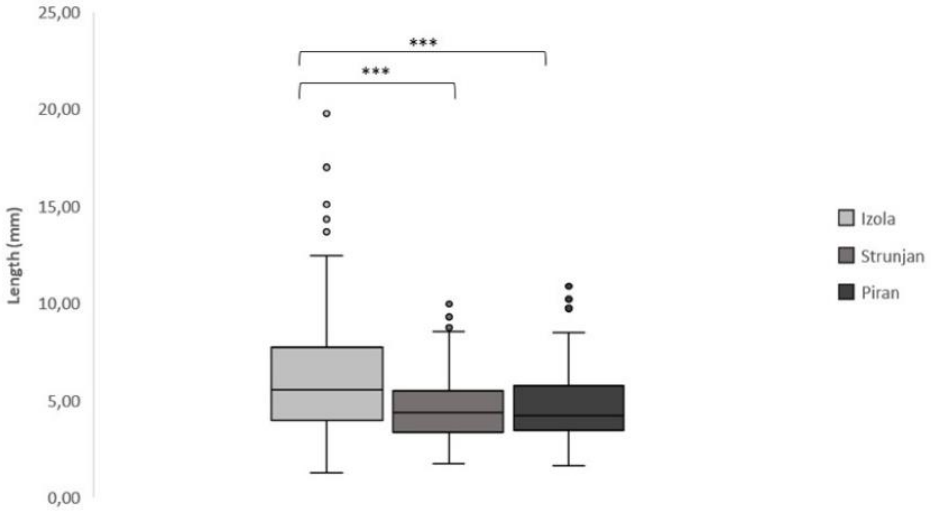


FIGURE 2. Boxplot of the length (mm) of the receptacles from Izola (light grey), Strunjan (dark grey) and Piran (black) sampling sites. The dark horizontal line is the median value (Q2), the extreme values of the box are the first (Q1) and third quartile (Q3), the distance between the two vertical segments is the range of the data and the dots are the extreme values. The stars represent the result of the Wilcoxon Pairwise test.

Overall, the thalli of the Izola population well-matched with *C. barbata* f. *hoppei* viz.: long fronds and long spindle-shaped receptacles with aerocysts ending in a mucron, while those of the Strunjan and Piran populations showed traits of *C. barbata* var. *turneri*, viz.: squat fronds, short cylindrical receptacles without aerocysts and not always mucronate.

The free-living thalli collected in the Gulf of Trieste lagoons are 20–30 cm long, always without anchorage and cauloid, floating on the bottom and on the surface. The primary branches show diffuse growth and can no longer be distinguished from the secondary ones; the branches are cylindrical, slender and oriented in all directions. Aerocysts may or may not be present (usually absent). The thalli collected were sterile (FIGURE 3).



FIGURE 3. Morphology of *Gongolaria barbata* f. *aurantia comb. nov.*

Phylogenetic analyses

The set of sequences representing the first half of the cox-1 gene portion consisted of 81 sequences, including one sequence as outgroup (full list of sequences in TABLE S1).

Our samples, collected in Adriatic (Italy and Slovenia) and Tyrrhenian (Sardinia, Italy), were clustered with those of *Treptacantha barbata* (Stackhouse) Orellana *et* Sansón (MT978057-58), *Gongolaria barbata* (OK480438-39) and *Cystoseira barbata* (KY682970), regardless of their "attached" or "unattached" morphology (FIGURE 4). The group comprising *Cystoseira compressa* and *Cystoseira pustulata* (Ercegovič) Neiva *et* Serrão has been condensed, but the tree with all visible sequences is available in FIGURE S1.

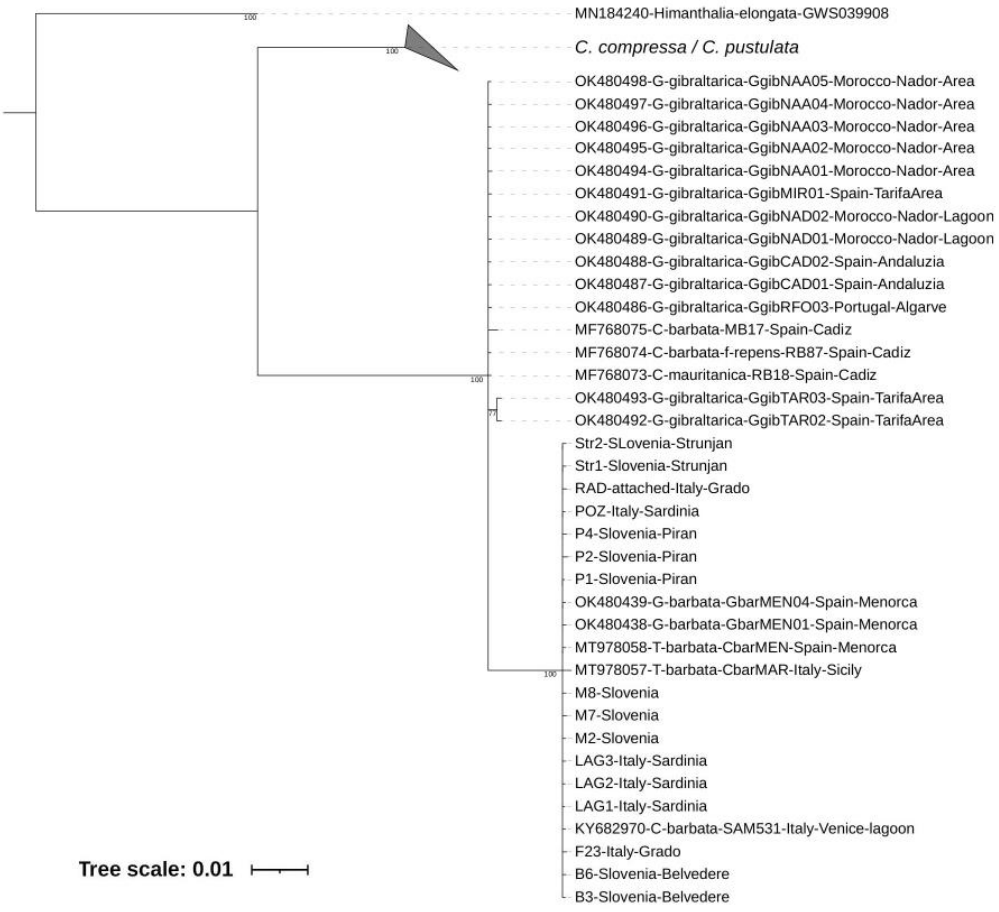


FIGURE 4. Phylogenetic tree of the 1st half of the cox 1 gene portion. The cluster including all *C. compressa* and *C. pustulata* sequences were condensed. Numbers on branches represent the node support and the bar represents the scale for branches length.

The samples identified by the submitters as *C. barbata* (MF768074-75), both collected in Cádiz (Spain), were clustered with *Gongolaria gibraltarica* (Sauvageau) Neiva, Bermejo *et* Serrão.

The set of sequences representing the second half of the cox 1 gene consisted of 67 sequences, including one sequence as outgroup (full list of sequences in TABLE S1).

Also considering the second half of the cox 1 gene, the specimens collected in the Northern Adriatic and Sardinia (Italy) clustered with *G. barbata* voucher GbarMEN04 collected in Menorca (Spain) (GenBank OK480439) and among them with no regards of the "attached" or "unattached" morphology (FIGURE 5 with condensed branches and FIGURE S2 with all visible sequences). The sequence of the specimen *C. aurantia sensu* Orellana *et al*. (2019) (deposited in GenBank as *Cystoseira* sp., COI sequence: MH513833) were clustered with *Cystoseira compressa* (Ercegovič) Neiva et Serrão.

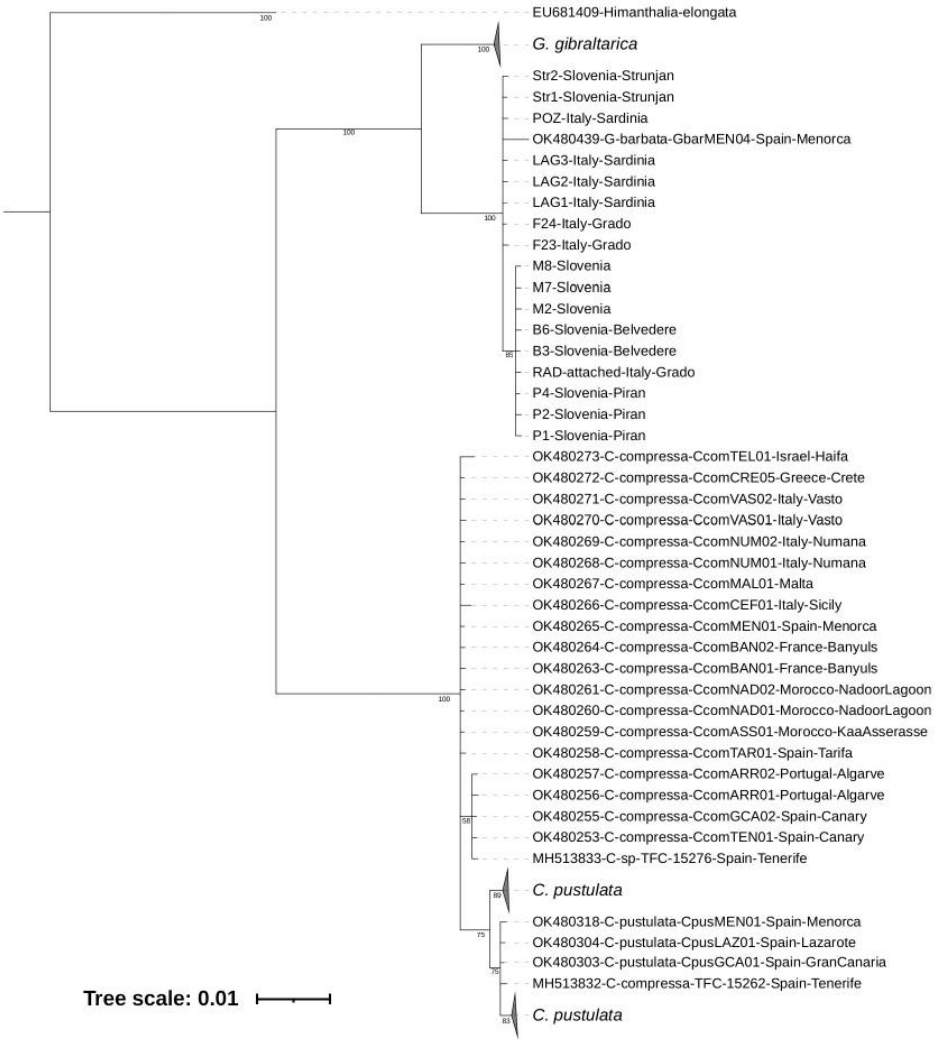


FIGURE 5. Phylogenetic tree of the 2nd half of the cox 1 gene portion. The cluster including all *G. gibraltarica* sequences were condensed. Numbers on branches represent the node support and the bar represents the scale for branches length.

Discussion

For *Gongolaria barbata*, a great morphological plasticity has been documented along the northern Adriatic Sea (Falace & Bressan 2006, Savonitto *et al.* 2019, Sadogurska *et al.* 2021, Orlando-Bonaca *et al.* 2022). It has been reported that *G. barbata* does not undergo a true dormancy period, as receptacles are present throughout the year, although their maximum development occurs in spring and early summer (Sauvageau 1912, Ercegović 1952, Gómez-Garreta *et al.* 1982). In contrast, for the Gulf of Trieste, Falace & Bressan (2006) indicated that *G. barbata* undergoes seasonal morphological changes in which fronds are lost, with a peak of fertility between March and May. On the other hand, Marzocchi *et al.* (2003) reported for the Venice Lagoon (northern Adriatic) that the fronds of *G. barbata* develop in autumn and winter and fall off in summer, although the cauloid is never completely bare.

Over the years, populations in the Gulf of Trieste (i.e., Izola, Strunjan and Piran) have been studied (Falace & Bressan 2006, Falace & Zanelli 2006, Savonitto *et al.* 2021, Orlando-Bonaca *et al.* 2021a, Orlando-Bonaca *et al.* 2021b, Orlando-Bonaca *et al.* 2022). The same observations for the same sites made in this work show the persistence of these morphological traits in the different populations, ascribable to *C. barbata* f. *hoppei* (i.e. Izola population) and

C. barbata var. *turneri* (i.e. Strunjian and Piran populations). This means that the differences between populations are not due to seasonal fluctuations, as Sauvageau (1912) and Giaccone & Bruni (1973) claim, but are rather related to different ecological conditions. The consistency of the morphological traits of these populations seems to argue for the assignment to separate intra-specific taxa, i.e., the occurrence of diverse visible traits due to minor genetic differences (Boudouresque, pers. comm. in Robvieux 2013) and especially for *hoppei* (i.e., Izola populations) and *turneri* (i.e., Piran and Strunjan) as described by J. Agardh (1842). Nevertheless, molecular analyses showed no differences between the Slovenian samples of *G. barbata* (both pleustophytic and haptophytic forms) and the Italian samples collected in both the Adriatic and in Sardinia, based on the mitochondrial cox 1 gene sequencing data.

Although the three sampling sites on the Slovenian coast are very close, several studies have reported divergence over short distances between contrasting environments (Russell 1978, Sideman & Mathieson 1985, Kilar & Hanisak 1989). Izola is subject to greater riverine inputs from the Rižana and Badaševica rivers (Cozzi *et al.* 2012). In addition, wastewater discharge from the municipality of Izola increases nutrient levels (Cozzi *et al.* 2008). These factors lead to increased sedimentation and turbidity of the water. In contrast, the Strunjan and Piran sites are exposed to higher hydrodinamism but lower anthropogenic pressure (Orlando-Bonaca *et al.* 2015), as they are located on a coastline that is still in its natural state. In high turbidity environments, the development of aerocysts allows algae to stretch to the surface and improve access to light (Lüning 1991). The elongated shape of the receptacles could be another adaptation to increase the surface area exposed to light and thus improve photosynthesis. To further confirm the site-specific phenotypic plasticity of *G. barbata*, recruits from the Strunjan population (i.e., lower sedimentation, higher hydrodinamism), grown ex-situ and transplanted to the Miramare Marine Protected Area (Gulf of Trieste), which is exposed to a high sedimentation rate and lower hydrodinamism as in Izola (Ogorelec *et al.* 1991), developed long receptacles with concatenated aerocysts (Savonitto *et al.* 2019).

In summary, among the haptophytic forms, the morphological traits of the two described varieties *hoppei* [characterized by flexuous and elongated fronds; fusiform (up to 20 mm), simple, with long mucrons, pedunculate receptacles; numerous, voluminous, oval to fusiform aerocysts, solitary or more often in chains up to 5) and *turneri* [characterized by squat and short fronds; cylindrical receptacles (up to 10 mm), simple or bifid, not always mucronate (the mucron, when present, is short) and aerocysts absent or very rare, never in chains] are well recognizable as the extremes of a morphological variability due to well-identified, site-dependent ecological traits and that these variations remain constant throughout the year and in successive years (TABLE 5). In the absence of genetic differences, but in order to retain the ecological information associated with the two ecoforms, we retain the two infraspecific taxa mentioned above as distinct, for which we propose the following two new combinations under the genus *Gongolaria*: *Gongolaria barbata* f. *hoppei* and *G. barbata* f. *turneri*. In contrast, the descriptions of *G. barbata* f. *barbata* (Gómez Garreta *et al*. 2000, Cormaci *et al.* 2012), put into evidence a noticeable variability in reproductive characters (i.e. terminal, cylindrical or fusiform, slightly rough or smooth, pedunculate when exceeding an aerocyst, with variable length and a terminal mucron) and in thallus morphology (i.e. well-developed primary branches, especially in spring; secondary order branches bearing single aerocysts or arranged in chains) given as seasonal phenological aspects (TABLE 5).

As for the free-living form collected in lagoons, all analysed specimens from the Adriatic and Sardinia are morphologically similar and genetically correspond to *G. barbata*, with no variability between bases. This confirms the hypothesis that they are branches detached from the cauloid that continue to grow in calm environments (i.e., shallow and stagnant waters), such as lagoons. Loss of branches occurs in most *Cystoseira s.l.* and is related to seasonal periodism. In shallow and calm waters, these fronds can remain trapped in seagrasses or on the bottom and continue their growth. By remaining buoyant, they lose the apicality of the growth of the main branches, the spacing between the nodes also increases and they appear looser, so that it is no longer possible to distinguish between branches of different orders. It has even been shown that detached, sterile main branches of *G. barbata* can continue to grow and become fertile under favorable temperature and nutrient conditions (Kaleb-Sanchez-Pedro *et al.* 2023). Detached main branches of *G. barbata* were also observed by Iveša *et al.* (2022) in the Šćuza lagoon on the southern coast of Istria (northern Adriatic, Croatia).

Bruno de Sousa *et al.* (2019) placed C. *barbata* f. *aurantia* (COI sequence deposited in GenBank: MF768074, as *C. barbata* f. *repens*) in clade 2 by analyzing a free-living specimen from the Atlantic (Cádiz, Spain) that also showed high genetic affinity to *C. mauritanica* Sauvageau (COI sequence deposited in GenBank: MF768073) (Figures 3, 4 in Bruno de Sousa *et al.* 2019). For these two sequences, Neiva *et al.* (2022) proposed the reassignment under the names *Gongolaria gibraltarica* f. *lacunarum* Neiva *et* Serrão and *Gongolaria gibraltarica*, respectively, based on the comparison with Atlantic specimens (e.g., Nadar in Morocco, Algarve in Portugal).

A short time before, Orellana *et al.* (2019) include four taxa from the Canary Islands in the clade *Cystoseira s.s.* (= *Cystoseira* 1): *C. foeniculacea* (L.) Greville, *C. compressa*, *C. humilis* Schousboe *ex* Kützing and *Cystoseira aurantia*, all of which described as arborescent and epilithic cespitose species, although *C. aurantia* is shown in their figures as free-living branches (Figures 3 and 4 in Orellana *et al.* 2019). This taxon is characterized by cylindrical, smooth main branches and large aerocysts (up to 7 mm long) arranged mainly along the branches of the last order (Orellana *et al.* 2019). However, although it was renamed *C. aurantia* by Orellana *et al.* (2019), the molecular sequences of this taxon are curiously deposited in GenBank as *Cystoseira sp.* (COI sequence: MH513833) and were later genetically identified as *C. compressa* by Neiva *et al.* (2022). Orellana *et al.* (2019) also deposited in GenBank the COI sequence of a sample of *C. compressa* (MH513832), which was later described as *C. pustulata* by Neiva *et al.* (2022). It should be noted that Neiva *et al.* (2022: Table 4) incorrectly inverted the original ID of the two COI sequences deposited by Orellana *et al.* (2019), MH513832 and MH513833, and referred to the former as *Cystoseira aurantia* and the latter as *Cystoseira compressa*.

So far, free-living forms have been reported not only for *G. barbata*, but also for *C. foeniculacea* (Linnaeus) Greville f. *dubia* (Ercegovič) Bouafif *et* Verlaque (Bouafif *et al.* 2016), *G. gibraltarica* f. *lacunarum* (Neiva *et al.* 2022), *C. foeniculacea* f. *formosensis* Neiva *et* Serrão (Neiva *et al.* 2022). The ability to cut off branches that can continue to grow may be more widespread than previously documented and could be an adaptive growth form associated with a dispersal strategy that deserves further investigation.

Based on the above and our genetic analyses, the floating forms in the Atlantic do not seem to belong to *G. barbata*, so that the assignment of *C. aurantia* [= *C. barbata* f. *aurantia* (Kützing) Giaccone)] by Ramdani *et al.* (2021) based on morphology alone should be reconsidered, also because Neiva *et al.* (2022) found *G. gibraltarica* f. *lacunarum* at the same location. Considering that the floating forms are morphologically convergent and poorly typified, their recognition based only on morphological traits alone is questionable, since the identification of *Cystoseira s.l.* is based mainly on diacritical features related to cauloid (e.g., caespitose or not, with smooth or spiny apex, prominent or not) or branching, which are absent or highly modified in the unattached forms. Finally, it should be noted that the identification keys of *Cystoseira s.l.* (e.g., Cormaci *et al.* 2012, Gómez-Garreta 2000) list *C. barbata* f. *aurantia* (or f. *repens*) as the only possible free-living ecotype, which has also led to misidentifications.

Therefore, we retain the form '*aurantia*' for the free-living form, which refers to detached branches in lagoon environments that continue their biological cycle and also attain fertility. Such a form is here combined under the genus *Gongolaria* as *Gongolaria barbata* f. *aurantia*

Taxonomy

Gongolaria barbata* f. *aurantia (Kützing) Falace, Alongi *et* Kaleb *comb. nov.*

Basionym: *Cystoseira aurantia* Kützing, 1843: 357. Phycologia generalis……Leipzig XXXII+458 pp, 80 pls.

Homotypic synonym:

Cystoseira barbata f. *aurantia* (Kützing) Giaccone in Amico *et al.* (1986: 906). Un nuovo approccio allo studio della sistematica del genere *Cystoseira* C. Agardh (Phaeophyta, Fucales). Bollettino dell'Accademia Gioenia di Scienze Naturali di Catania, 18(326): 887–988.

Note: in absence of molecular evidences, we here consider *G. barbata* f. *aurantia* from Mediterranean as a distinct taxon from *C. barbata* f. *repens* Zinova *et* Kalugina [= *G. barbata* f. *repens* (Zinova *et* Kalugina) Sadogurska] described for the Black Sea, due to its morphological characters rather different from those of Zinova *et* Kalugina's taxon as described and illustrated by Sadogurska (2021: 301, Fig 3), particularly due to the presence of short primary axis in f. *repens* (not present in f. *aurantia*) and both lateral and ultimate branches in f. *repens* (neither lateral nor ultimate branches distinguishable in f. *aurantia*) (see Sadogurska, 2021: 301, Fig 3a and Cormaci *et al.* 2012 tav 93 fig 1, respectively).

Gongolaria barbata* f. *hoppei (C. Agardh) Falace, Alongi *et* Kaleb *comb. nov.*

Basionym: *Cystoseira hoppei* "*hoppii*" C. Agardh, 1820: 59, Species algarum……Volumen primum. Pars prima. pp. [i–iv], [1]–168. Lundae [Lund]: *ex* officina Berlingiana.

Homotypic synonyms: *Cystoseira barbata* var. *hoppei* "*hoppii*" (C. Agardh) J. Agardh 1842: 51, Algae maris Mediterranei et Adriatici, …… pp. [i]–x, 1–164. Parisiis [Paris]: Apud Fortin, Masson et Cie.

Cystoseira barbata f. *hoppei* "*hoppii*" (C. Agardh) Woronichin 1908: 117, Buryya vodorosli (Phaeophyceae) Chernago Morya [The brown algae of the Black Sea]. Botanicheskii Zhurnal (1–2): 19–46, 113–138, 11 figures, 2 tables. [in Russian with German summary]

Gongolaria barbata* f. *turneri (J. Agardh) Falace, Alongi *et* Kaleb *comb. et stat. nov*.

Basionym: *Cystoseira barbata* v. *turneri* J. Agardh 1842: 50, Algae maris Mediterranei et Adriatici, …… pp. [i]–x, 1–164. Parisiis [Paris]: Apud Fortin, Masson et Cie.

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Chapter 6
Microbiome Diversity and Host Specificity in *Gongolaria barbata*

6.1 Paper: Diversity of the Surface Microbiome of Canopy-Forming Brown Macroalgae (Fucales) in Northern Adriatic

This chapter explores the diversity and host specificity of the microbiome associated with the habitat forming brown alga *Gongolaria barbata*. According to the meta-organism concept, macroalgae and their associated microbiota form a functional unit in which microorganisms contribute to growth, defense, and resilience. Despite their importance, the composition and ecological role of the microbiome in *Cystoseira* s.l. species remain poorly studied, creating a major gap in knowledge relevant to restoration and long-term ecosystem stability.

Understanding the structure and variability of algal microbiomes is particularly important for restoration efforts, since microbial communities may differ among algal species, populations exposed to different environmental conditions, and even across algal body parts. Such differences may strongly influence seedling survival, growth, and stress resistance during cultivation. Identifying population- or tissue-specific microbial associations can therefore help refine cultivation protocols and improve restoration outcomes.

In this study, two morphologically distinct but genetically identical populations of *G. barbata* from the Slovenian coastal sea (Belvedere and Merkur) were analyzed using 16S rRNA amplicon sequencing. Microbial assemblages were characterized across different algal parts (holdfast, axis, branches, receptacles, and aerocysts) and compared with those in surrounding seawater and sediments. This design allowed the assessment of both host-part specificity and population-level variation in microbiome composition.

Specific objectives:
I. Characterize the taxonomic diversity of microbiomes associated with *G. barbata* compared to surrounding seawater and sediments.
II. Test whether microbiomes differ among algal body parts and between morphologically distinct populations.
III. Assess the degree of host specificity and the potential role of horizontal transmission from sediment communities.
IV. Provide baseline knowledge for future hypothesis-driven research on algal–microbiome interactions, with direct implications for restoration.

Hypothesis:
The microbiome of *G. barbata* exhibits low host specificity, with a large proportion of its bacterial community overlapping with surrounding environmental microbiota. However, basal algal body parts such as the holdfast may host distinct microbial assemblages, partly recruited from adjacent sediments.

Key findings

- Around 75% of algae-associated Amplicon Sequence Variants overlapped with the core coastal ecosystem microbiome, confirming generally low host specificity.
- Despite this overlap, microbiomes differed significantly between algal parts, seawater, and sediment, with basal structures (holdfast and axis) being most similar to sediment, suggesting horizontal transmission from sediments.
- Deciduous parts (branches, receptacles, aerocysts) showed distinct communities shaped by selective pressures in the water column.
- Morphologically distinct but genetically identical populations (Belvedere and Merkur) harbored similar microbiomes overall, although holdfast and axis showed greater between-site variation than upper thallus parts.
- The study establishes a baseline framework for integrating microbiome knowledge into restoration, highlighting the need to consider microbial associations when cultivating seedlings for outplanting.

The objectives outlined above were achieved, and the hypothesis was supported by the results presented in this chapter.

The research work is presented in the following publication and is listed below:
Orel, N., Lokovšek, A., Orlando-Bonaca, M., & Tinta, T. (2025). Diversity of the surface microbiome of canopy-forming brown macroalgae (Fucales) in the northern Adriatic. Microbiology Spectrum, 13(4), e02204-24. https://doi.org/10.1128/spectrum.02204-24

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Diversity of the surface microbiome of canopy-forming brown macroalgae (Fucales) in the northern Adriatic

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ABSTRACT Canopy-forming brown macroalgae (Fucales) offer numerous key ecosystem services in Mediterranean coastal areas. However, anthropogenic pressures and climate change have significantly impacted their habitats, leading to an extensive population decline. Interactions between algae and microbiota are a major ecological aspect, yet they represent a significant knowledge gap. In our baseline study, we describe the diversity and host specificity of the microbiome of two genetically identical but morphologically distinct populations of *Gongolaria barbata* from anthropogenically impacted northern Adriatic Sea. Our preliminary results showed that the microbiomes of *G. barbata* exhibited low host specificity, with 75% of the algae-associated amplicon sequence variants (ASVs) being part of the core coastal ecosystem microbiome. However, microbiomes of specific algal parts, ambient seawater, and sediment differed significantly in terms of alpha diversity and composition. In contrast, the holdfast and axis show higher similarity with sediment microbiomes, indicating potential horizontal transmission pathways. Microbiomes associated with deciduous parts of morphologically distinct *G. barbata* populations showed no difference in alpha diversity and composition. In contrast, higher variation in alpha diversity and lower sequence proportion of shared ASVs were observed in the holdfast and axis of the two distinct populations. Our observational study provides valuable new insights and baseline for future hypothesis-driven research on the interactions between algae and associated microbiota—a knowledge gap that needs to be addressed in the future for better understanding of the ecological and evolutionary dynamics of coastal ecosystems.

IMPORTANCE Our study focuses on the microbiomes of canopy-forming brown macroalgae from the Fucales order, essential habitat builders in Mediterranean coastal areas. These habitats, offering key ecosystem services, face significant declines due to anthropogenic pressures and climate change. We used next-generation 16S rRNA amplicon sequencing to reveal novel insights into the diversity and host specificity of *Gongolaria barbata* populations in impacted ecosystems. Our findings suggest environmental factors influence the structure of the algae microbiome, with potential recruitment from adjacent sediment communities. This research enhances the understanding of marine ecosystems' ecological and evolutionary dynamics, providing valuable insights for conservation and management efforts.

KEYWORDS *Gongolaria barbata*, Fucales, macroalgae-associated microbiomes, host-microbiome, 16S rRNA amplicon sequencing, anthropogenically impacted coastal ecosystems

Canopy-forming Fucales (Phaeophyta) are dominant habitat builders along rocky shores of the Mediterranean Sea (1, 2). In particular, species of the genus *Cystoseira sensu lato*, lately subdivided into the three genera *Cystoseira*, *Ericaria*, and *Gongolaria* (3), which can grow from intertidal to upper-circalittoral bottoms, are building dense forests

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that are among the most productive communities in the Mediterranean coastal area (4), providing several key ecosystem services (5–10).

Due to interplaying anthropogenic stressors (e.g., coastal constructions, local pollution sources, and sediment resuspension) and climate change (e.g., increase of seawater temperature), *Cystoseira s.l.* populations have gradually disappeared in many coastal areas in the Mediterranean Sea (4, 11, 12). A sharp decline of fucoids has also been reported for the anthropogenically impacted coastal zone of the Gulf of Trieste, northern Adriatic Sea (11–13), where genetically identical yet morphologically different populations can be found at different locations along the Slovenian coast (14). When studying the effects of climate change and/or anthropogenic pressures on eukaryotic organisms, we often overlook a major ecological aspect: the interactions with associated microbiota. Yet, according to the "meta-organism" concept, any multicellular organism is composed of the macroscopic host in a synergistic interaction with microorganisms (15, 16). This concept postulates that microbes are an essential part of the host's phenotype and hence should be considered when addressing evolutionary and ecological aspects of the host (17, 18). The importance of the microbiota for the host's fitness, health, reproduction, behavior, population dynamics, interactions with the environment, survival, and decay are increasingly recognized for a wide diversity of meta-organisms (16). The microbiome of diverse algae is quite well studied, yet being host-species-specific (19, 20), we are far from capturing the entire algae diversity (e.g., less abundant, endangered, difficult to access) and functional potential of algae-associated microbiota. The algal microbiome can have beneficial and detrimental effects on the host; it has been shown that it modulates algal growth, morphogenesis, reproduction, and chemical defense against other micro- and macro-fouler (21, 22). Yet, the underlying mechanisms of algae-microbes associations are largely unknown.

Despite numerous studies on the rich macrofauna and flora associated with *Cystoseira s.l.*, there is little knowledge about its associated microbiota (23–25). These studies collectively underline the significance of *Cystoseira s.l.*-associated microbiomes in shaping host health, stress resilience, and potential restoration strategies, while also pointing to gaps in understanding their functional roles and interactions. Among these studies, only Mancuso et al. (23) provided some insights into host specificity toward individual algae parts, while none of these studies explicitly addressed host specificity at the level of different *Cystoseria s.l.* populations. To preserve their populations and the ecosystem services they provide in the ocean of change, the associated microbiota as crucial components of *Cystoseira s.l.* metaorganism need to be further explored. Building up on previous studies, our objectives were to (i) further characterize the composition of the *Gongolaria barbata* microbiome, (ii) determine host specificity, specifically toward individual algae part and host population, and (iii) to study differences in the microbiome of two genetically identical populations of *G. barbata* in the northern Adriatic, which show considerable morphological differences and could therefore also harbor significantly different microbiomes. We employed a high-throughput next-generation sequencing approach to provide insights into microbial diversity at unprecedented detail, allowing for the comprehensive analysis of microbiota composition and variation among different algal parts and populations.

MATERIALS AND METHODS

Study area and sampling campaign

The Gulf of Trieste is a shallow, semi-enclosed bay in the northernmost part of the Adriatic Sea. For many decades, the coastal area has been affected by various anthropogenic impacts such as construction, intensive fishing, sewage discharges, and mariculture (26). Thalli of *G. barbata* were collected by SCUBA divers in April 2022 from two sampling sites (Fig. 1), selected according to Lokovšek et al. (27). The first site, Belvedere, is characterized by a dense population of *G. barbata* (Fig. 2B). The apical fronds of these thalli had short, simple, and cylindrical receptacles that were sparsely mucronate and

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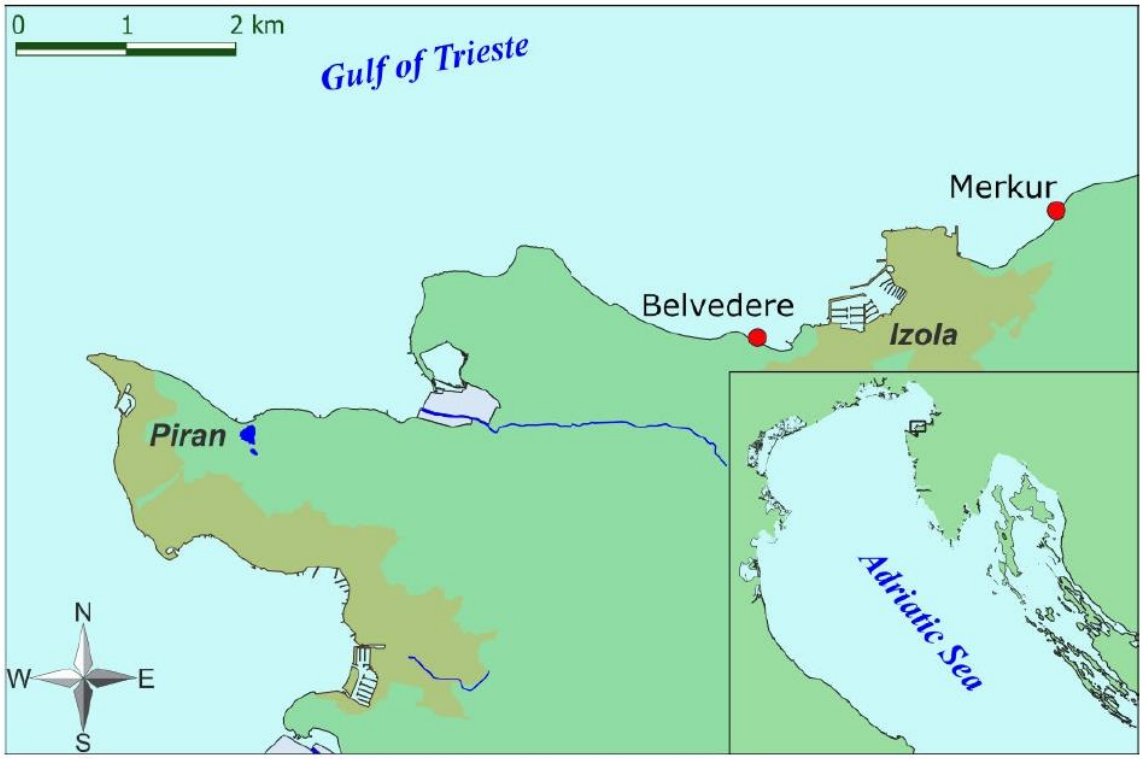


FIG 1 Map of the study area showing the two sampling sites (Belvedere and Merkur) of *G. barbata* populations, near Izola.

had few or no aerocysts. The algae grow on sandstone boulders that are surrounded by sandy substrate. The second site, Merkur, is characterized by dense populations of *G. barbata* and *Cystoseira compressa* (Fig. 2D). The apical fronds of these thalli had long fusiform or mucronate receptacles, with numerous single or concatenated aerocysts. The algae grow on flysch terraces positioned between areas with bare sand. Terraces are often covered by sediments from flysch crumbling. The area is exposed to freshwater inputs from the Rižana and Badaševica rivers (28); therefore, a high resuspension of sediments is present, with consequent lower water transparency.

The Merkur site was sampled on 4 April 2022, and the Belvedere site on 5 April 2022. At each site, three whole thalli of *G. barbata* were collected by scraping beneath their holdfast on boulders, at approximately 2 m depth. The samples were stored in zip-lock plastic bags to protect them from external contaminants and preserve the integrity of the associated microbiome. Additionally, seawater samples were collected ~0.5 m above the thalli (one 1.5 L sample per location), in pre-acid washed, Milli-Q rinsed plastic bottles, and sediment samples were taken with small sterile corers (one 200 mL corer per location), very close to the algal holdfast (approximately 2 cm–5 cm). All samples were immediately transported (within 30 min) to the laboratory, where they were further processed. During transportation, the sampled material was stored in a portable refrigerator.

In the laboratory, all thalli individual body parts were divided into subsamples (~10 g for each part in 50 mL falcon tube): (i) holdfast and lower part of the cauloid (primary axis) (P), (ii) lower part of primary branches (V), (iii) aerocysts (A), and (iv) receptacles (R) (Fig. 2A and C). For analysis of ambient microbiomes, 1 L of ambient seawater samples were filtered onto 0.2 µm polyethersulfone filters (PALL Life Sciences), and ambient sediment samples were stored in sterile 50 mL tubes. All the samples were then stored in sterile cryo tubes at −80°C until DNA extraction. For chemical analysis, inorganic

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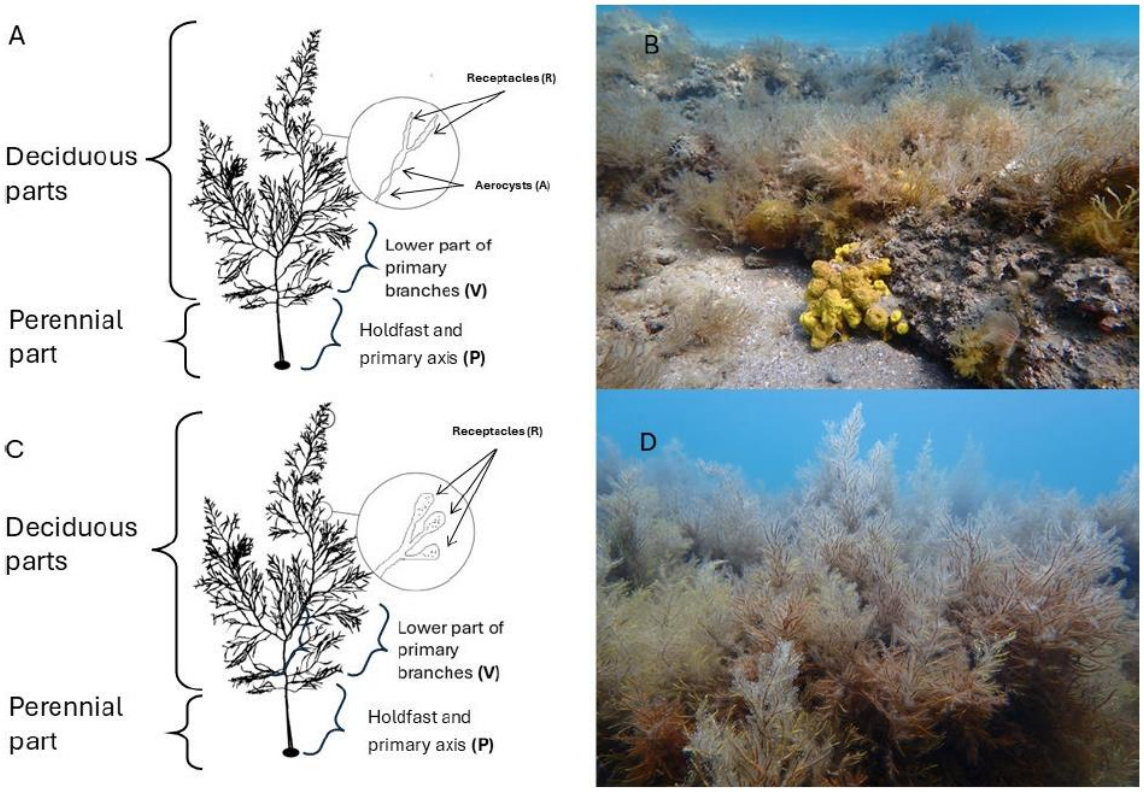


FIG 2 Phenotypically distinct populations of *Gongolaria barbata* from the two sampling sites. (A) Schematic drawing of a thallus from the Belvedere population, characterized by the absence of aerocysts and smaller receptacles. (B) Photography of the Belvedere population, characterized by lower growth. (C) Schematic drawing of a thallus from the Merkur population, featuring several aerocysts and longer receptacles. (D) Photography of the Merkur population, with taller thalli reaching up to 1 m.

nutrients, and dissolved organic matter analysis, samples were processed as described under "Chemistry," below.

Analysis of algae-associated and ambient microbiome using 16S rRNA amplicon sequencing

DNA was extracted from the filters with the DNeasy PowerWater Kit (Qiagen). Two hundred fifty milligrams of sediment was used for DNA extraction with a DNeasy PowerSoil Kit (Qiagen). One hundred milligrams of algae was used for DNA extraction with the DNeasy Plant Pro Kit (Qiagen). Amplification and sequencing were performed by Microsynth AG (Balgach, Switzerland). Primers 515F (5′-GTG YCA GCM GCC GCG GTA A-3′) (29) and 805R (5′-GGA CTA CNV GGG TWT CTA AT-3′) (30) were used for amplification of the V4 region of bacterial 16S rRNA gene. The amplicons were sequenced on the Illumina Miseq platform (2 × 250 bp paired-end). Sequencing generated a data set of 25 samples with 9,309,075 read pairs (Table S2).

Bioinformatic and statistical analyses were conducted in R (v.4.2.2). Paired-end reads were trimmed, denoised, and merged using the "DADA2" package (31). Forward and reverse reads were quality trimmed to 150 bp at a maximum expected error rate of 2. Sample inference was done in "pool" mode to resolve also rare amplicon sequence variants (ASVs). After merging paired-end reads, chimeras were removed with the method "pooled". Only ASVs occurring at least twice and with a length between 252 and 256 bp were kept for further analyses, resulting in 347,887 ± 135,941 merged sequences per sample (Table S2). Taxonomy was assigned using the SILVA database (SSU 138.1) (32).

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Further taxonomic filtration and analysis of the microbial community described in this paragraph were performed using "phyloseq" (33). ASVs assigned to Eukaryota, chloroplast, and Mitochondria or not assigned at the phylum level were excluded. The "iNEXT" (34) package was used to make rarefaction curves for each sample. The number of observed ASVs, Chao1, Shannon, and inverse Simpson indices were calculated through the "phyloseq" package to describe alpha diversity. A test for a significant difference in alpha diversity indexes was performed by one-way analysis of variance (ANOVA). The test was selected based on a previous analysis of normality using the Shapiro-Wilk method. The presence of a significant difference between sample groups is indicated with a significance level of at least $P < 0.05$. *Post hoc* analyses were performed using the Tukey test. Phyla with a total abundance of less than 35 were removed for further analyses. Bacterial community composition was analyzed using weighted principal coordinates analysis (PCoA) based on Bray-Curtis distances calculated from relative abundance data. Permutational analysis of variance (PERMANOVA) was performed using the adonis2 function in the R package "vegan" (35) to test for significant differences between groups of samples, with 999 permutations. *Post hoc* pairwise PERMANOVA comparisons were conducted for algal body parts and ambient samples using the "pairwiseAdonis" package (36). Shared ASVs were visualized with "venn" (v.1.1) (37).

Chemistry

Dissolved organic carbon (DOC) and nitrogen

Samples for DOC and total dissolved nitrogen (TDN) analyses were filtered through combusted Whatman GF/F (~0.8 µm pore size) filters using acid-, Milli-Q water rinsed and a combusted glass filtration system. DOC and TDN analyses were performed by a high-temperature catalytic method using a Shimadzu TOC-L analyzer equipped with a total nitrogen unit (38). The calibration for non-purgeable organic carbon was done with potassium phthalate, and for TDN, potassium nitrate was used. The results were validated with Deep-Sea Reference water for DOC (CRM Program, Hansell Lab). The precision of the method, expressed as relative standard deviation, was <2%.

Dissolved inorganic nutrients

Dissolved inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-) and dissolved inorganic phosphorus (PO_4^{3-}) concentrations were determined spectrophotometrically by segmented flow analysis (QuAAtro, Seal Analytical) following standard methods (39). The validation and accuracy of the results were checked with reference material (KANSO CO., LTD.) before and after sample analyses. The quality control is performed annually by participating in an inter-calibration program (QUASIMEME Laboratory Performance Study).

Graphic design

Graphs were plotted in R Studio (40) with the "ggplot2" package (41). A graphical abstract was created with BioRender.com. The layout was adapted from "Ocean Layout," by BioRender.com (2024), retrieved from https://app.biorender.com/biorender-templates. All final figures were edited using the vector graphics editor Inkscape v.1.1 (42). The map in Fig. 1 was made in ArcGIS and Fig. 2 in Adobe Illustrator and Microsoft Office PowerPoint.

RESULTS

Diversity differences between algal-associated and ambient microbial communities

We observed richness from 308 to 9,742 bacterial ASVs per sample (Table S2). Rarefaction curves reached a plateau in all of the examined communities (Fig. S1). Thus, our sequencing depth represents most of the bacterioplankton diversity (34). However, two samples of R microbiome from the Merkur sampling site (A1_R_IZ and A3_R_IZ) were

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removed from further analysis since only a low number of reads were obtained for these two samples (1,622 and 2,404, respectively).

There was significant variation among samples from distinct sources (different algal parts, seawater, sediment) for all alpha diversity indices (ANOVA test, Chao1: $F_{5,16}$ = 20.69, P < 0.0001; Shannon: $F_{5,16}$ = 35.64, P < 0.0001; Evenness: $F_{5,16}$ = 35.54, P < 0.0001). Total species richness (Chao1) and Shannon diversity index of ambient sediment communities were significantly higher than that of ambient seawater assemblage (Tukey's honestly significant difference [HSD], adjusted P < 0.05; Table S4, Fig. 3A). Bacterial communities associated with algae were overall characterized with significantly lower richness (Tukey's HSD for V, A, and R, adjusted P < 0.0001), Shannon diversity index (Tukey's HSD for V, A, and R, adjusted P < 0.0001; for P, adjusted P < 0.001), and evenness (Tukey's HSD for V, A, and R, adjusted P < 0.0001; for P, adjusted P < 0.001) than that of the adjacent sediment, except for communities associated with holdfast and axis (P), which resembled species richness of sediment assemblages (Tukey's HSD; Table S4, Fig. 3A). Algae-associated communities also exhibited significantly lower Shannon diversity index and evenness than ambient seawater assemblage (Tukey's HSD for R, adjusted P < 0.001; for V and R, adjusted P < 0.01), except for communities associated with holdfast and axis (P) (Tukey's HSD; Table S4, Fig. 3A). At the same time, there was no statistical difference in total species richness between ambient seawater- and algae-associated communities (Tukey's HSD; Table S4, Fig. 3A). Within the algae microbiome, alpha diversity indices revealed no significant difference among communities associated with V, R, and A (Tukey's HSD; Table S4, Fig. 3A). On the other hand, these communities exhibited significantly lower richness, Shannon diversity index, and evenness than communities associated with holdfast and axis (P) (Tukey's HSD for all diversity indices: for R, adjusted P < 0.001; for V and A, adjusted P < 0.01) (Table S4, Fig. 3A). Alpha diversity indices revealed no differences between bacterial communities sampled at the two locations, except for species richness (ANOVA test, $F_{5,16}$ = 20.69, P < 0.05; Table S3). On the other hand, after grouping by different algal parts, ANOVA showed that communities associated with holdfast and axis (P) were significantly different at the two locations in terms of species richness, Shannon diversity index, and evenness (Fig. 3A, Table S5).

Weighted PCoA based on Bray-Curtis distance revealed significant variation in bacterial community composition based on isolation source (algal body part and different ambient samples; PERMANOVA test, R^2 = 0.55, P < 0.001) and spatial variation by sampling location (PERMANOVA test, R^2 = 0.09, P < 0.001) (Fig. 3B). *Post hoc* pairwise PERMANOVA tests showed that communities associated with the holdfast and axis (P) significantly differed from all other algae-associated and seawater communities (R^2 = 0.47 for A, R^2 = 0.29 for R, adjusted P < 0.01; R^2 = 0.38 for V, adjusted P < 0.001). These communities also differed from sediment and seawater communities, but with a lower significance level (R^2 = 0.33 for sediment and R^2 = 0.46 for seawater, adjusted P < 0.05) (Table S6).

Taxonomic composition of algae-associated microbial communities differed from adjacent sediment and seawater communities. The most profound difference between algae-associated and ambient microbial communities was a higher relative abundance of Acidimicrobiia (within which 88.1%–100% were affiliated with the *Microtrichaceae* family) in algae-associated communities, representing 12.3%–28.6% of sequence proportions in A-, R-, and V-associated communities, while less than ~2% in ambient microbial communities (Fig. 4A). Sequence proportion represents the proportion of sequences belonging to a specific taxon (e.g. species, family, order, etc.) relative to the total number of sequences in the sample. Gammaproteobacteria were detected in all samples in our study, representing 26.8 to 43.9% of sequence proportions in ambient assemblage and 2.7%–44.8% in algae-associated communities (Fig. 4A). However, while the sediment and holdfast and axis (P) Gammaproteobacterial population was very diverse, the V, R, and A were predominated by the Granulosicoccales (Fig. 4B), more precisely *Granulosicoccaceae* family (56.3%–97.1% of Gammaproteobacteria population, Table S8). On the other hand, we could not detect *Granulosicoccaceae* in sediment or seawater assemblage (<0.1% of

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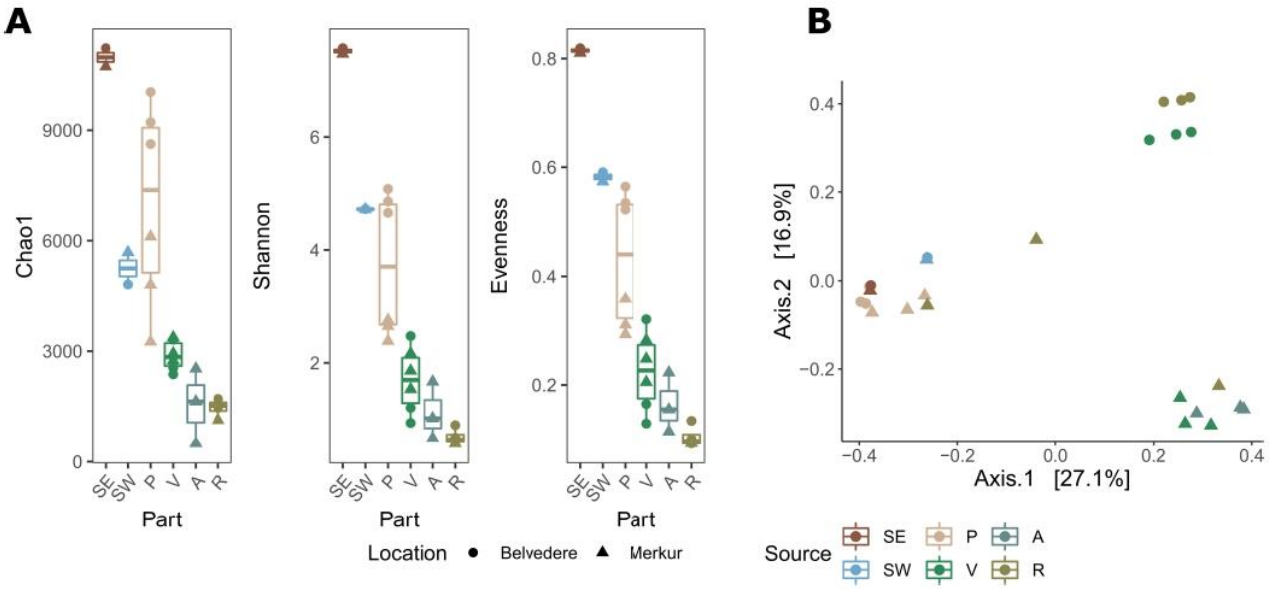


FIG 3 (A) Alpha diversity indices (Chao1 richness estimator, Shannon diversity index, and species evenness) of samples from distinct sources (different algal parts [P, V, A, R], seawater [SW], sediment). (B) Weighted PCoA based on Bray-Curtis distances of bacterial community composition. Each point represents a sample, colored by algal part or ambient source and shaped by sampling location.

sequence proportion, Table S8). Bacteroidia were present in ambient and algae-associated communities, where they represented between 12.7 and 56.6% of sequence proportions, respectively, and were predominated by the *Flavobacteriaceae* family (51.7%–97.8% of the Bacteroidia population, Table S8), while Cytophagales and Bacteroidales were detected mainly in sediment and holdfast and axis (P) samples. Likewise, Desulfobacteria, Desulfobulbia, and Planctomycetes were more abundant in sediment and holdfast and axis (P) samples as compared to other algae parts and seawater, in particular at the Merkur site. *Nitrososphaera* was predominantly detected in holdfast and axis (P) samples (0.5–6.6 of sequence proportion), while Polyangia was mainly in aerocysts (2.6%–7% of sequence proportion). Ambient seawater communities were dominated by Alphaproteobacteria (44.6%–43.3% of sequence proportion, predominated by SAR11, followed by Rhodobacterales and Puniceispirillales) (Fig. 4C). On the other hand, this bacterial class represented only a minor part of alga-associated microbiomes (<24.3% of sequence proportion, predominantly affiliated with Rhodobacterales), except for the holdfast and axis (P), where Alphaproteobacteria represented 39.7–25.8 of the community (within which Rhodobacterales and Rhizobiales contributed equal or larger share compared to other algal parts) (Fig. 4C). Within Alphaproteobacterial populations Puniceispirillales, Kiloniellales, and Parvibaculales were unique to holdfast and axis (P), however representing only a minor percentage of total P-associated microbiome (Fig. 4C). Cyanobacteria were almost exclusively found in ambient seawater, where they represented around 10% (9.7%–8.9%) of the sequence proportion (Fig. 4A) and were dominated by Synechococcales, while they represented less than 6% of algae-associated and sediment communities, predominantly by Cyanobacteriales.

Microbiome of *G. barbata*

Determination of the similarity of microbial communities associated with different algal parts, ambient seawater, and sediment was performed based on analysis of the presence/absence of ASV (Fig. S2). Core microbiome, i.e., ASVs detected in all samples of our study set, comprised 545 ASVs out of 13,723 ASVs recruited from all samples in total (Fig. S2A). Nevertheless, these core microbiome ASVs represented a major part of the bacterial community (74.2%–93.9% of sequence proportion) on deciduous parts (i.e. V, A, and R)

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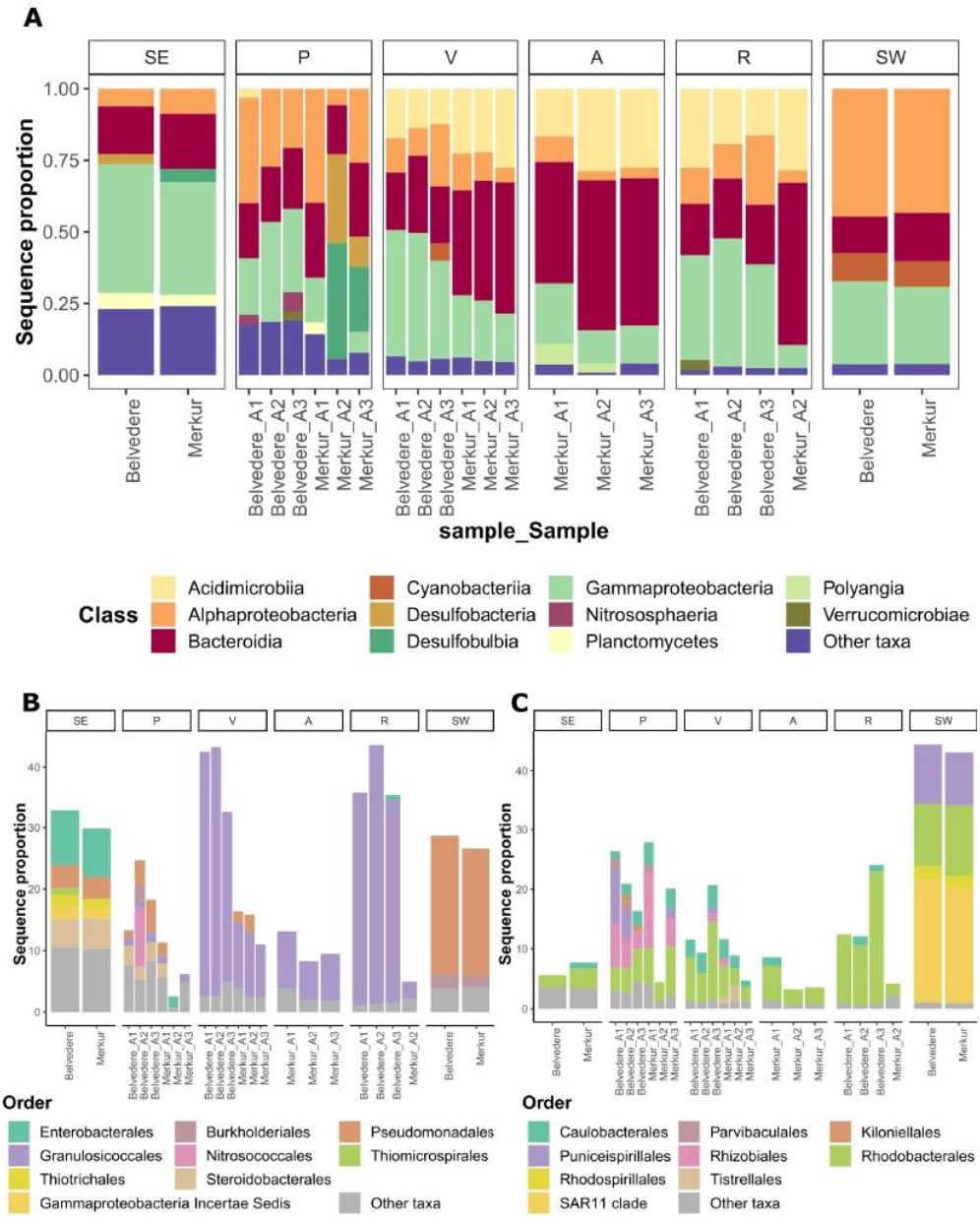


FIG 4 Sequence proportion of dominant bacterial genera associated with different algal parts (P, V, A, R), ambient seawater (SW), and sediment (SE) microbiomes in two different locations (Belvedere, Merkur). (A) Class-level bacterial community structure (classes showing a sequence proportion <3% are grouped as "other taxa"). (B) Order-level composition of Gammaproteobacterial populations (orders showing a sequence proportion <1% are grouped as "other taxa"). (C) Order-level composition of Alphaproteobacterial populations (orders showing a sequence proportion <1% are grouped as "other taxa").

implying a low level of specificity of the microbiota associated with algae (Fig. S3). Holdfast and axis (P) microbiomes comprise a lesser extent of the core ASVs (22.0%–58.6%, Fig. S3). The number of unique ASVs to the algae microbiome (i.e., not found in seawater or sediment) and shared among all parts of the algae was low (43) for the whole algae and was even lower for deciduous parts (6) (Fig. S2A). There were 1,082 ASVs unique to holdfast and axis (P) (mainly belonged to classes Bacteroidia, Alphaproteobacteria, Gammaproteobacteria and Planctomycetes), but only 17 ASVs to V (mainly Alphaproteobacteria, Bacteroidia, Gracilibacteria), 9 for A (mainly Bacteroidia) and 1 for R (Gammaproteobacteria) (Fig. 2A, Fig. S4). The holdfast and axis (P) and sediment samples

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shared the highest number of unique ASVs (3,487) (Fig. S2A), representing 14.3%–16.5% and 1.7%–8.9% of sequence proportion for sediment and holdfast and axis (P), respectively (Fig. S5). Among sediment and holdfast and axis (P)-specific ASVs, there were representatives of Gammaproteobacteria, Bacteroidia, Alphaproteobacteria, and Planctomycetes (Fig. S4).

When examining the two sampling locations individually, more differences become apparent. The Belvedere sampling site was characterized by a smaller number of ASVs unique to deciduous parts (76 for A, V, and R combined out of 13,007 ASVs in the Belvedere sampling site), as compared to the Merkur location (290 unique for A, V, and R combined out of 12,230 ASVs in Merkur sampling site) (Fig. S2B). About 5% of ASVs were shared between deciduous parts (A, V, and R combined) and holdfast and axis (P) at each sampling location, while there were more ASVs unique to holdfast and axis (P) at location Belvedere (1,940) as compared to Merkur (822) (Fig. S2B). There was a high number of ASVs shared between holdfast and axis (P) and sediment at both sampling locations; 3,848 and 1,696 at Belvedere and Merkur, respectively. (Fig. S2B).

When comparing the two sampling locations, 72.3% of sediment ASVs and 50.3% of holdfast and axis (P) ASVs were shared between both locations (Table S7), representing 59.6%–86.1% of sequence proportion in P samples (Fig. S6). Among microbial groups associated with holdfast and axis (P) at both sampling locations were *Desulfocapsaceae, Desulfosarcinaceae, Flavobacteriaceae, Rhodobacteraceae*, etc. (Fig. S6). At the same time, only 43.34% of deciduous parts ASVs were shared between the two locations (Table S7), representing 86.7%–97.6% of sequence proportion in deciduous parts samples (Fig. S6). Among microbial groups associated with algal deciduous parts at both sampling locations were *Flavobacteriaceae*, *Granulosicoccaceae*, *Microtrichaceae*, etc. (Fig. S7). When comparing specific deciduous parts separately, 40.9% and 11.1% of ASVs were shared between locations for primary branches and receptacles, respectively (Table S7).

DISCUSSION

Morphological plasticity in algal hosts is not dictating microbiome composition

The present study represents the first attempt to characterize in detail the microbiome associated with the two phenotypically distinct populations of the canopy-forming brown alga *G. barbata* in the anthropogenically impacted coastal zone of the northern Adriatic Sea. In this area, the species exhibits considerable morphological plasticity, with the peak of fertility between March and May (14, 44, 45). After molecular analyses, which revealed no genetic differences (14), the population at the Merkur site was attributed to the morphotype of *G. barbata* f. *hoppei* (elongated fronds and numerous oval to fusiform aerocysts), while the population from the Belvedere site can be considered as *G. barbata* f. *turneri* (short fronds and absent or very rare aerocysts) (Fig. 2). Although the two sites along the Slovenian coast are close to each other, our preliminary analysis suggests that they differ in the chemical characteristics of ambient seawater (Table S1). However, the sample size would need to be increased in the future to support our preliminary findings. Nevertheless, the measured concentration of organic and inorganic nutrients was within the range previously reported for the Gulf of Trieste (46, 47) (with slightly higher inorganic nutrient concentrations [in particular, NH_4^+ and NO_3^-], but lower DOC and TDN measured at the Belvedere site). The Merkur site is characterized by high sedimentation and suspension rates, resulting in increased water turbidity (48). In contrast, the Belvedere site is exposed to less anthropogenic pressure as it is located at the beginning of a still pristine coastline. Consequently, we can speculate that the two populations might be adapted to different sedimentation rates, salinity, and turbidity, which could potentially affect the length of the branches and the presence of aerocysts. However, additional analysis of environmental parameters at the two sites would need to be conducted in the future. Nevertheless, alpha diversity indices revealed no significant differences between bacterial communities associated with algae or ambient samples at the two locations, except for holdfast and axis, which

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differed in terms of species richness, Shannon diversity, and evenness (Fig. 3A, Table S5), suggesting a low degree of host specificity. Hence, based on our preliminary results, the two genetically identical populations of *G. barbata* in the northern Adriatic, which show considerable morphological differences, harbor similar microbiomes. Nevertheless, the deciduous parts from two locations shared 43.3% of ASVs, which accounted for the majority of sequence read proportions in these samples (86.7%–97.6%). In contrast, the holdfast and axis shared 50.3% of ASVs, representing a slightly smaller portion of their respective communities (59.6%–86.1%). This suggests that sediment may have a greater influence on the structure of the algal microbiome compared to the surrounding seawater. However, note that our study lacks chemical analysis of the sediment, and we did not conduct specific experiments to further investigate this aspect. Therefore, we cannot draw any conclusions about the impact of sediment chemistry on the microbiomes associated with algal holdfast and axis parts. On the other hand, when inspecting the deciduous part separately, we observed that primary branches from two sampling locations shared ~40% of ASVs, while receptacles shared only 11%, suggesting increasing environmental selective pressure, such as exposure to light, nutrient availability, or hydrodynamic forces, as algae grow higher up in the water column. At the same time, we acknowledge that our current study cannot separate environmental selective pressure from intrinsic factors of algae (e.g., tissue biochemical composition or physiology of different algal parts) as the drivers of observed microbial differences. Additional chemical, microbiological, and environmental analyses are required to investigate the influence of environmental selective pressures and algal intrinsic factors on microbiome composition. Indeed, it has been shown that macroalgae surfaces are rapidly colonized with bacteria, potentially recruited via horizontal transmission from surrounding seawater (49), forming dense surface biofilms (20, 43, 50), probably representing the first interface interacting with the surrounding system. It has been previously suggested that epiphytic bacteria colonizing algal tissues respond rapidly to fluctuations in environmental factors (24, 51).

Microbiomes of different algal parts exhibit different levels of host specificity

Our preliminary results imply a low level of host specificity for the microbiota associated with *G. barbata,* as 75% of algae-associated ASVs were also detected in the core microbiome in our entire data set (i.e., ASVs detected in all sequenced samples from ambient seawater, sediment, and all algae thalli parts) (Fig. S2A and S3). Nevertheless, microbiomes associated with deciduous algae parts (i.e., primary branches, receptacles, and aerocysts) were characterized by significantly lower alpha diversity measures than ambient seawater and sediment microbiomes (Fig. 3A). These results are in line with the findings of previous studies, which suggested that epiphytic bacteria, particularly growing on Fucales, differ significantly from the microbiome on abiotic surfaces and in the surrounding water (50, 52). Compared to the algal thalli surfaces, the sediment and seawater generally had a slightly higher bacterial diversity, confirming earlier research postulating that algae actively select their associated microbiota (53).

On the other hand, there was no significant difference between species richness of microbiomes associated with holdfast and axis and ambient microbiomes. Additionally, our results show a high number of ASVs shared between holdfast and axis and sediment microbiota, at both sampling locations (Fig. S2A and S5). Based on this, one could speculate a horizontal mode of microbiome transmission (i.e., acquisition of microbiome from the environment and not from the parent–progeny relationship) from the sediment community (acting as seed source) to the algae holdfast and axis body parts. Even more so, when comparing the two locations, the potential source of microbial seeds was highly similar, with ~70% shared ASVs between sediment microbiomes, resulting in ~50% similarities between holdfast and axis microbiomes between the two locations. Furthermore, among sampled algal body parts, we observed the highest number of unique ASVs in holdfast and axis samples (1,082 out of 10,754 ASVs, representing 0.5%–4.5% of sequence proportions). Hence, this does suggest a certain level of host specificity

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of microbiota associated with algae holdfast and axis parts. We hypothesize that the algal-associated microbiota is acquired (e.g., via horizontal transmission) from sediment communities during early developmental stages, such as the holdfast and primary axis, which serve as anchors and are in direct contact with the sediment. This hypothesis will be addressed in future studies. Later, during the growth of the algae in the water column toward the light, the associated microbiota is subjected to greater selection pressure, resulting in significantly different microbial communities being associated with deciduous algal parts, characterized by lower alpha diversity (Fig. 3A). This again implies that this part of the algal thalli/developmental stage of the host represents pressure for the associated microbiome, selecting only specific microbial groups associated with primary branches, receptacles, and aerocysts.

Algae deciduous and holdfast and axis parts harbor different microbiomes

The number of ASVs unique to the algae microbiome and at the same time shared among all parts of algal thalli was low (50 ASVs). The number of unique ASVs shared among deciduous algae parts was even lower (6), with 17 ASVs unique to primary branches, 9 for aerocysts, and 1 for receptacles, suggesting some level of body part specificity. The microbial group that seems to exhibit the most specificity toward algae deciduous parts was Acidimicrobiia (12.3%–28.6%), which represents only a minor part of holdfast-associated communities (less than 3.1%) and less than 2% of ambient microbiota (Fig. 4A). Accordingly, Acidimicrobiia were previously associated with another genus of brown algae from the *Sargassaceae* family (23, 54). Moreover, in our data set, basically the entire community of Acidimicrobiia was affiliated with the *Microtrichaceae* family, which was previously shown to constitute the core microbiome of green, brown, and red macroalgal phycosphere (55). Another important member of specific algae deciduous parts microbiota seems to be the *Granulosicoccaceae* family, which was present in very low proportion (<0.1% of sequence proportion) in ambient microbiome samples in our study. In line with this, *Granulosicoccus* was recruited as the most abundant metagenome-assembled bacterial genome from kelp-associated microbial communities, where their role in vitamin B12 production was highlighted (56). On the other hand, holdfast and axis were associated with a very diverse Gammaproteobacterial community, likely contributing to higher alpha diversity recorded for this algae body part (Fig. 3A and 4B). Interestingly, other reports on *Cystoseira s.l.* microbiome from our study area did not detect Acidimicrobiia and *Granulosicoccaceae* (24, 25).

Some bacterial groups, like Flavobacteriales, represented an important part of the algae microbiome (10.9%–55.9%), with no preference for thalli part, yet exhibited less host specificity, as they were present also in ambient sediment and seawater samples. Accordingly, Flavobacteriales were also reported as one of the predominant orders composing microbiomes of *Ericaria crinita*, *G. barbata,* and *Cystoseira compressa* from the Adriatic Sea (23–25). Flavobacteriales are known for their capacity to degrade a wide range of algae-derived polysaccharides, with a strong preference for algae-associated lifestyle (57). In line with our results, the same study also reported a high relative abundance of *Rhodobacteraceae* associated with all algal parts. While Blažina et al. (24) also reported Rhizobiales as a non-negligible part of the algal microbiome, this order was only detected as part of holdfast and axis-associated microbiota in our data set. In our study, we were able to find some tissue-specific bacterial taxa, which were unique to some algae thalli parts, e.g., Polyangia, which are micro predators capable of lysing cells or other microorganisms and cellulose degradations, were exclusively associated with aerocysts (58). Exclusively associated with holdfast and axis were *Nitrososphaera*, Puniceispirillales, Kiloniellales (previously isolated from macroalgae), and Parvibaculales, likely contributing to higher alpha diversities of algae thalli part communities. However, many bacterial groups were shared among holdfast and axis and sediment samples, likely representing part of recruited communities, which, however, did not survive selective pressures during the development of deciduous parts (e.g., Cytophagales, Bacteroidales, Desulfobacteria, Desulfobulbia, Planctomycetes, Desulfobulbia).

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Note the discrepancy between this study and other *Cystoseira s.l.* microbiome studies from the area likely results from major methodological differences and bioinformatic pipelines applied, which means that the comparison is not straightforward. First, different sets of primers to amplify different 16S regions were applied (V3-V4 region [59], in Blažina et al. [24], and V4-V5 region [29], this study), a different sequence approach was used (operational taxonomy unit—OTU in Blažina et al. [24] and Malfatti et al. [25] vs amplicon sequence variant—ASVs in this study), and different bioinformatic pipeline and reference database versions were employed.

Conclusions and future perspectives

We provide the first insight into the potential differences in the structure of microbiomes of two morphologically distinct but genetically identical *G. barbata* populations in the coastal anthropogenically impacted marine ecosystem. Our preliminary results suggest some environmental forcing on the structure of the algae microbiome; however, further analysis of chemical, microbiological, and other environmental factors is needed. Our baseline study allows us to formulate the first hypothesis regarding influences of environmental selective pressure and algal intrinsic factors on microbiome composition that we would like to address in the future. We show that the *G. barbata* microbiome exhibits relatively low host specificity, which was, however, somehow higher for basal algal parts than for deciduous parts. Based on these data, we suggest the second hypothesis that part of the algae microbiome is recruited from adjacent sediment communities, which becomes more specialized during algae growth, either due to the biochemical composition of different host body parts, different mechanistic basis for host–microbiome association, and/or ambient factors in the water column. Descriptive studies are important to gain insights into the diversity of host-associated microorganisms. However, these studies do not disentangle the cause–effect relationships in which the effects of the microbiota on the host (and vice versa) can be distinguished (60). Therefore, our initial findings on the microbiome of canopy-forming Fucales in the Gulf of Trieste raise interesting questions to be addressed in future research regarding the specific role of these epiphytic bacteria in macroalgal settlement, growth, and reproduction.

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DATA AVAILABILITY

Sequencing data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB60814. All other data will be made available on request.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Figures (Spectrum02204-24-S0001.docx). Figures S1-S7.
Supplemental Tables (Spectrum02204-24-S0002.xlsx). Tables S1-S7.
Table S8 (Spectrum02204-24-S0003.csv). Family-level abundance.

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Chapter 7
Development of In Situ Cultivation Methods

In addition to ex situ cultivation, we conducted preliminary in situ trials of *G. barbata* over two consecutive years (2022 and 2023). Although the results of these experiments were not published yet, they provided valuable insights into the feasibility of direct seeding methods under natural conditions. In situ approaches are attractive because, once established, they are relatively cost- and labor-efficient, requiring only periodic monitoring. However, a major challenge is protecting young germlings from intense grazing, particularly by schools of herbivorous fish and sea urchins, which can decimate recruits during their most vulnerable stages.

Two direct seeding methods (See Fig. 11) were tested on natural rock surfaces: (1) mesh bags with apical parts of *G. barbata* tied horizontally to the substrate and (2) bottomless containers used to release zygotes of *G. barbata* directly onto cleaned rocky areas. Both methods proved technically feasible in 2022, resulting in attachment and early development of recruits, which were subsequently protected with nets once they reached ~10 mm in size. However, in 2023, strong hydrodynamic conditions led to the failure of the bottomless container method, while the tied-bag method proved reliable in both years.

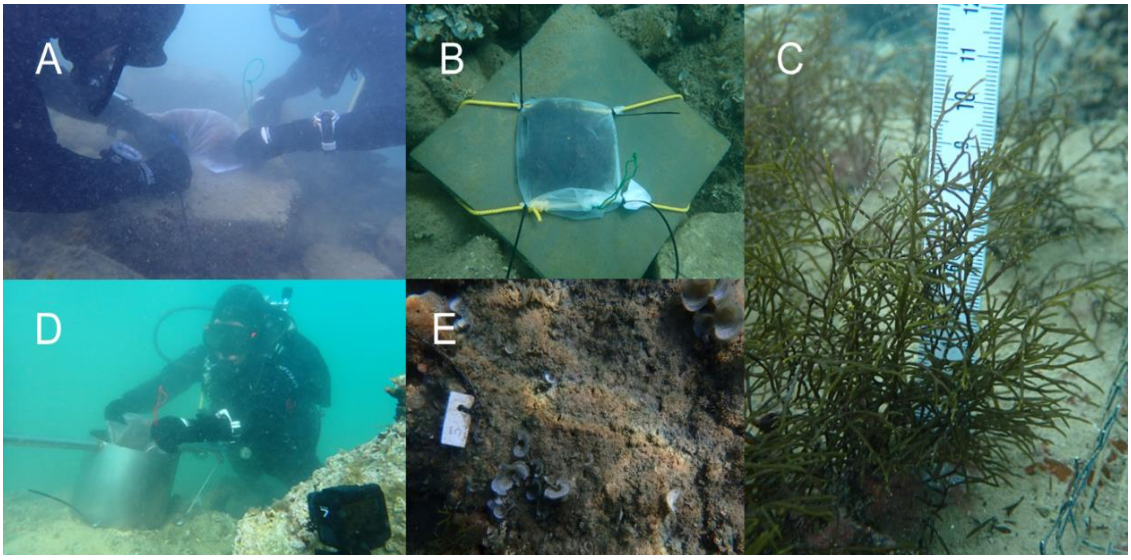


Figure 11: Two methods of in situ seeding; A, B - mesh bag with apical parts tied to the substrate; C - juvenile sporophytes growing on seeded substrate; E - seeding with bottomless container; E - seeded area marked with a tag (Photo: A, D – L.L.Zamuda, B, C, E – A. Lokovšek).

The tied-bag method is particularly noteworthy, as it had not been previously described or published. It consistently produced high recruit densities and good prospects for seedling

establishment, making it a promising option for restoration. Its main drawback, however, is that it is labor-intensive and therefore less practical for large-scale interventions. By contrast, the bottomless container technique is far more time efficient. Although it failed under high hydrodynamic conditions in 2023, it may still be suitable in more sheltered environments where wave action is lower.

A complementary experiment on sandstone slabs further tested the bottomless container method combined with different grazer protection strategies, including cages and 3D predator models (See Fig. 12). Protective cages reduced grazing pressure but quicky became overgrown with epiphytic algae, shading recruits and requiring frequent maintenance. They also proved vulnerable to extreme weather and fishing activity. Similarly, the effectiveness of 3D predator models diminished once they became fouled. These results underline the difficulty of ensuring reliable grazer protection in dynamic coastal environments.

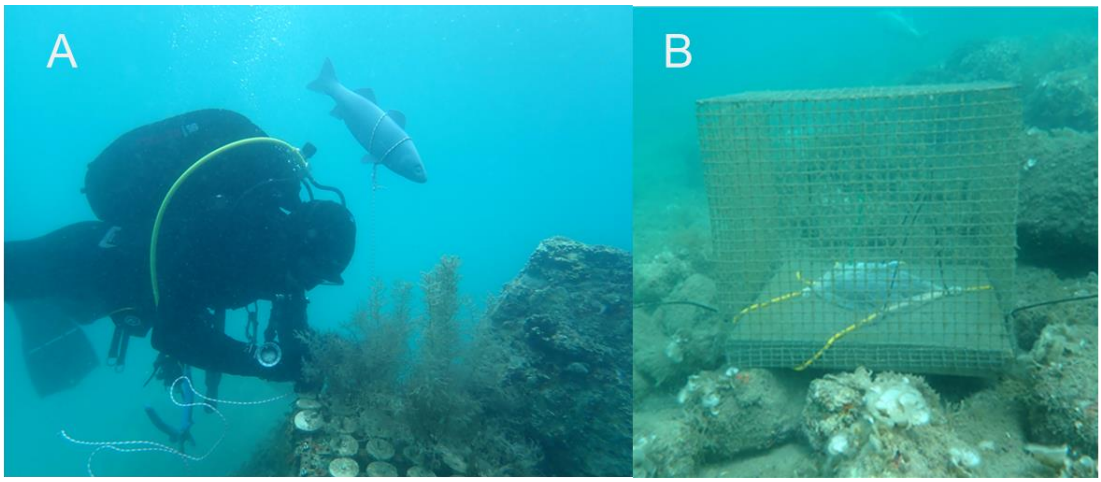


Figure 12: Complementary actions to prevent overgrazing of *G. barbata* by *S. salpa*; A - installation of fish scarecrow, 3D model of *S. salpa* predator; B - protective cage (Photo: A – L.L.Zamuda, B – A. Lokovšek).

Importantly, across both years we observed that once germlings of *G. barbata* were successfully established and protected from grazers, their growth in situ was considerably faster than in mesocosms or in open systems on land. Natural light, hydrodynamics, and nutrient conditions appear to favor development, provided that early survival is secured. This suggests that hybrid strategies that shorten the ex situ phase and transfer germlings to the field as early as possible, may provide the best balance between control and growth performance.

Overall, the two-year in situ trials confirmed that direct seeding of *G. barbata* is feasible and can lead to good establishment, though outcomes depend strongly on local conditions. In areas of high grazing pressure, protective measures are necessary but costly, labor-intensive, and only partly effective. In hydrodynamically exposed sites, the tied-bag method appears more reliable, whereas bottomless container method may be advantageous in sheltered locations where efficiency is a priority.

Building on these insights, we conducted also a preliminary test aimed at simplifying and improving *G. barbata* cultivation by integrating the most effective elements of both in situ and ex situ approaches. Fertile apical parts of *G. barbata* were seeded ex situ onto large sandstone slabs (50 × 50 × 5 cm) placed in a large plastic container (baby pool), where they were left under a few millimeters of seawater for three days, later extended to six days to enhance settlement. Upon removal of the receptacles, numerous zygotes were already visible on the sandstone surface. The container was then filled with seawater and connected to an open flow-through system, with one hose supplying seawater and another allowing outflow. Maintenance during this phase was minimal, consisting mainly of removing sediment and organic matter that accumulated on the slabs by briefly lifting and rinsing them by using gentle current from a hose. After approximately three months, the sandstone slab bearing juvenile sporophytes was transferred to the sea. To reduce sedimentation, the slab was mounted on an iron frame positioned 0.5 m above the bottom and enclosed within a protective cage to prevent grazing (See Fig. 13B). The results of this preliminary trial were very encouraging—juvenile thalli of *G. barbata* exhibited rapid growth and healthy development, reaching above 20 cm and developing fertile receptacles at 7 months (Fig. 13 – C,D,E) —suggesting that this simplified, integrated approach holds strong potential. Although further testing and replication under different environmental conditions are needed before the method can be validated, the outcomes are consistent with Lokovšek et al. (2023), demonstrating that both ex situ cultivation followed by early transfer to the sea represent time- and cost-effective strategies and that seedlings appear to thrive best under natural conditions, provided that key stressors such as sedimentation and herbivory are effectively mitigated.

Taken together, all these findings highlight the trade-offs between ex situ and in situ cultivation. Ex situ methods provide greater control, reduce early mortality, and enable hybrid strategies such as short mesocosm phases followed by transfer to lantern baskets. In situ methods, by contrast, are simpler and cheaper but more exposed to grazing and environmental variability. A promising path forward may involve combining approaches. Continued innovation in grazer deterrents and refinement of existing devices could further improve survival, but in the longer term, grazer population management may also need to be considered as part of a sustainable, ecosystem-based restoration framework.

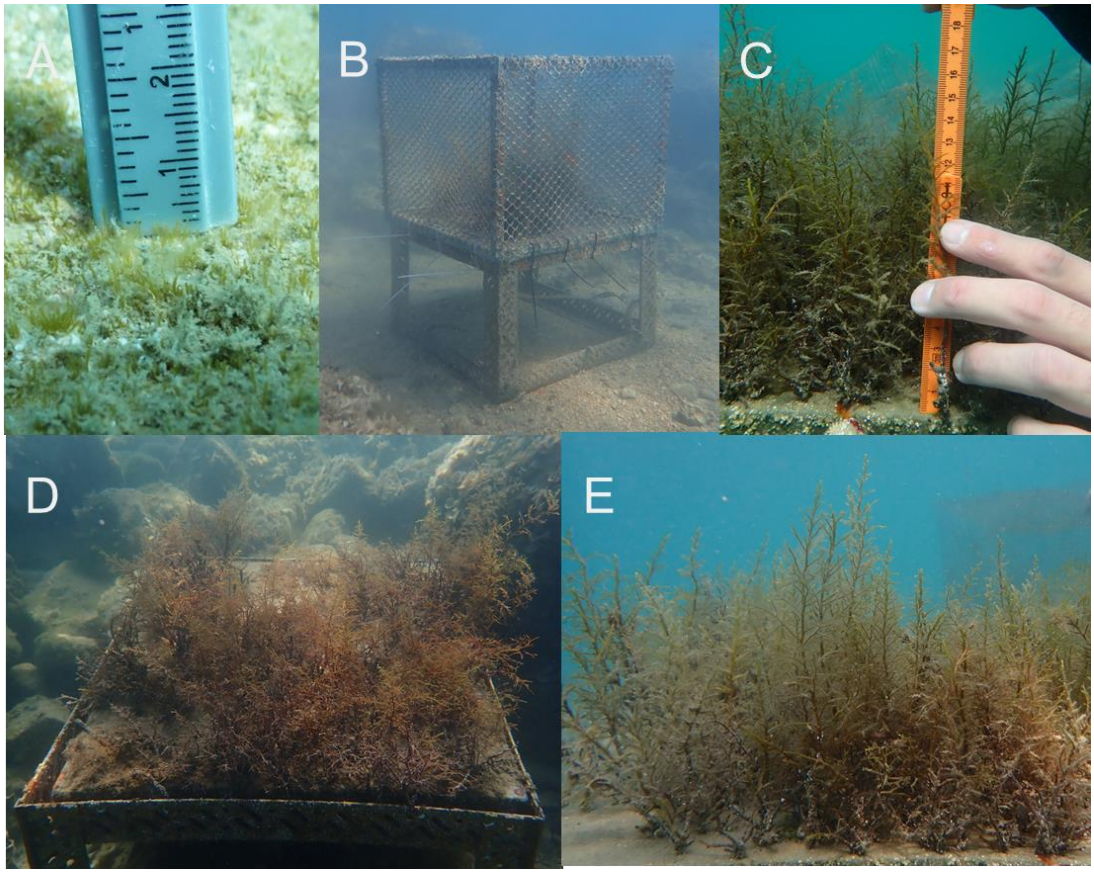


Figure 13: Testing combined approaches - ex situ seeding of *G. barbata* and early transfer to the sea; A – 4 weeks old seedlings ex situ; B - protective cage raised above the seafloor to prevent sediment depositon; C - 7 months old sporophytes; D – coverage of the seeded substrate after 7 months; E – healthy adult *G. barbata* sporophytes after 9 months (Photo: A. Lokovšek).

Chapter 8
Conclusions

This dissertation presents the ecological, physiological, and methodological foundations for the restoration of *Cystoseira* s.l. forests, with a focus on the habitat-forming brown alga *Gongolaria barbata*. Building on previous studies, it extends knowledge through five targeted studies addressing cultivation, thermal tolerance, biostimulants, genetic diversity, and the microbiome of the study species. The overall aim was to refine and test approaches that make large-scale restoration both feasible and effective.

Successful restoration must be grounded in a robust understanding of the species' biology, life cycle, limitations, and stressors. Equally important is detailed knowledge of the restoration site, which determines which interventions are possible and what outcomes are realistic. By contributing new insights in five interconnected research areas, this dissertation strengthens the scientific foundation for restoring Mediterranean brown algal forests.

8.1 Refinement of Ex Situ Cultivation Protocols

Using *G. barbata* as a study species, this work demonstrated that ex situ cultivation protocols can be improved and scaled without compromising success. The study showed that UVC sterilization provides a practical and cost-efficient alternative to manual filtration, enabling reliable water treatment at large volumes.

Comparisons between mesocosms and open systems revealed that both can support seedling development, but mesocosms offer greater control of the cultivation. Open systems, while promising, require improved safeguards against multicellular contaminants.

A hybrid approach, combining short mesocosm phases with early transfer of juveniles to floating lantern nets, proved particularly effective. This strategy reduces costs, frees laboratory space, accelerates acclimation to natural conditions, and provides protection against grazers and sedimentation. The method spreads risk across cultivation phases and shows strong potential for scaling up seedling production for restoration.

8.2 Temperature as a Critical Factor in Cultivation

The thermal tolerance study established that early life stages of *G. barbata* are highly temperature-sensitive, with optimal cultivation conditions at 15 °C. While adults tolerate a wider range, seedlings exhibited high mortality and deformities above 20 °C. At 24 °C survival was severely reduced, and at 28 °C all germlings perished within a week.

These findings clarify why earlier cultivation protocols using higher temperatures were suboptimal. They emphasize the need to align seedling cultivation with cooler seasonal windows and to anticipate the impacts of climate-driven warming and heatwaves, which could critically constrain recruitment in the wild.

8.3 Biostimulants as a Tool to Enhance Seedling Performance

This dissertation also explored the potential of algal biostimulants to strengthen early-stage resilience. Experiments with extracts from *Desmodesmus* sp., *Trichormus variabilis*, *Cylindrotheca closterium*, and a commercial kelp-based formulation (AlgatronCifo®) demonstrated that biostimulants can enhance seedling survival and growth when applied at low concentrations.

Desmodesmus extract, in particular, promoted growth comparable to commercial formulations, while high concentrations of all extracts caused cytotoxic effects. Responses were species-specific: *E. crinita* benefited more strongly than *G. barbata*.

These results highlight the promise of biostimulants as supplements to traditional nutrient media, though their application must be carefully optimized. They open new opportunities for integrating biotechnological innovation into restoration practice.

8.4 Genetic Diversity and Morphological Plasticity

The genetic study resolved long-standing questions about the infraspecific diversity of *G. barbata*. Morphologically distinct populations, such as f. *hoppei*, f. *turneri*, and f. *aurantia,* showed stable differences in form and habitat associations but no genetic divergence based on *cox1* sequences. Similarly, pleustophytic (free-floating) forms were genetically indistinguishable from attached populations.

These findings confirm that morphological diversity reflects phenotypic plasticity shaped by ecological conditions rather than underlying genetic differentiation. This has practical implications for restoration: donor selection should prioritize ecological compatibility with the target site rather than morphotype labels, broadening the potential donor pool and reducing taxonomic uncertainty.

8.5 The Role of the Microbiome

The microbiome study provided the first detailed characterization of microbial communities associated with *G. barbata*. About 75% of algal-associated Amplicon Sequence Variants overlapped with the coastal core microbiome, confirming generally low host specificity. Nevertheless, clear patterns emerged: basal parts (holdfast and axis) resembled sediment microbiota, while upper structures (branches, receptacles, aerocysts) hosted distinct assemblages shaped by the water column.

Subtle site-level differences further indicated that microbiomes are partly shaped by local conditions. These findings suggest that seedlings raised in mesocosms may develop microbial communities different from those at restoration sites, potentially influencing resilience. Restoration protocols could therefore benefit from microbiome-aware practices, such as avoiding over-sterilization and inoculating cultures with site-source microbial communities to improve acclimation.

8.6 In Situ Cultivation

Two years of in situ trials demonstrated that direct seeding of *G. barbata* is technically feasible, with outcomes strongly influenced by local hydrodynamics and grazing pressure. The novel tied-bag method proved reliable in exposed conditions but is labor-intensive, while the bottomless container method offers greater efficiency in sheltered sites. Despite the challenges of grazer protection, once recruits were established, their growth in situ outpaced that of ex situ counterparts. These results highlight the potential of hybrid strategies that minimize the ex situ phase while leveraging natural conditions for growth. Moving forward, effective and sustainable large-scale restoration will require both technical innovation and ecosystem-based management, including long-term solutions for grazer population control.

8.7 Success Criteria, Challenges, and Future Perspectives

Assessing restoration success requires more than tracking survival or growth. Long-term resilience depends on reproduction, recruitment, structural complexity, and ecosystem functioning. Standardized monitoring protocols across the Mediterranean should include these parameters, alongside genetic diversity and adaptive potential.

Scaling up remains challenging. High grazing pressure, climate stressors, incomplete distribution data, and limited funding constrain progress. Compared to seagrass or saltmarsh restoration, macroalgal restoration still lacks investment and policy integration. Embedding initiatives within marine protected area networks and coastal management frameworks will be essential for shielding restored sites from recurring impacts.

Future progress will require coordinated, multidisciplinary action. Biotechnological innovations, such as biostimulants, probiotics, and assisted selection, hold promise but must be applied cautiously. Restoration should not be isolated from conservation: active interventions must be paired with protection of existing populations and reduction of stressors.

This dissertation demonstrates that restoration of *Cystoseira* s.l. forests is achievable through a combination of ecological understanding, methodological innovation, and adaptive management. Improved cultivation methods, temperature scheduling, biostimulant use, recognition of phenotypic plasticity, and microbiome-aware practices all contribute to more resilient and scalable approaches.

Ultimately, long-term success requires that restored forests function like natural ones—supporting biodiversity, sustaining ecosystem services, and withstanding environmental variability. Only by combining science-based restoration with broader conservation and policy frameworks can these foundational habitats be rebuilt at ecologically meaningful scales.

Appendix A

Photos of the *G. barbata* forest at the Ruda site in Izola during the 2021-2025 period:

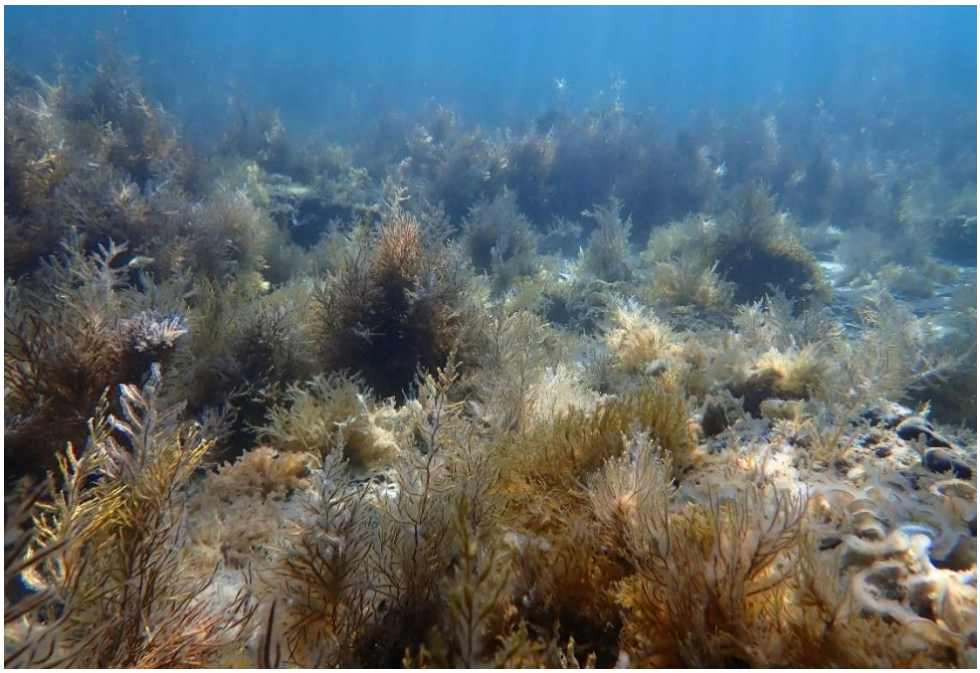


Figure 14: Healthy *G. barbata* forest - March 2022 (Photo: A. Lokovšek).



Figure 15: Well-developed adult sporophytes of *G. barbata* with receptacles - May 2022 (Photo: A. Lokovšek).

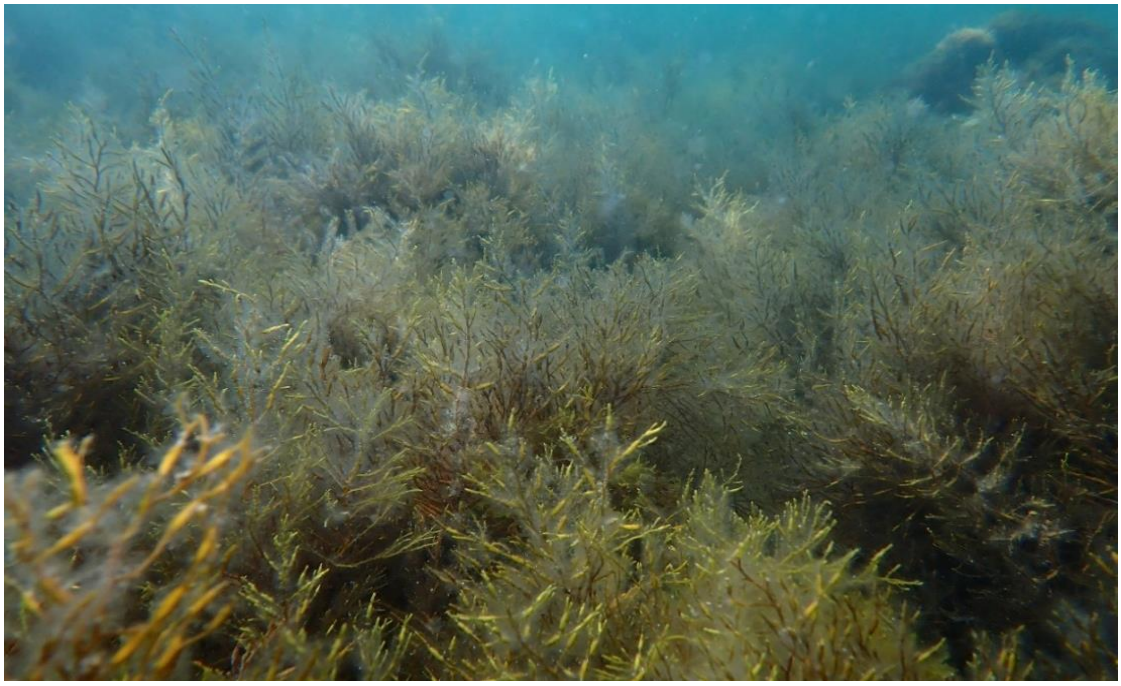


Figure 16: Still healthy *G. barbata* forest with signs of grazing - year later – March 2023 (Photo: A. Lokovšek).

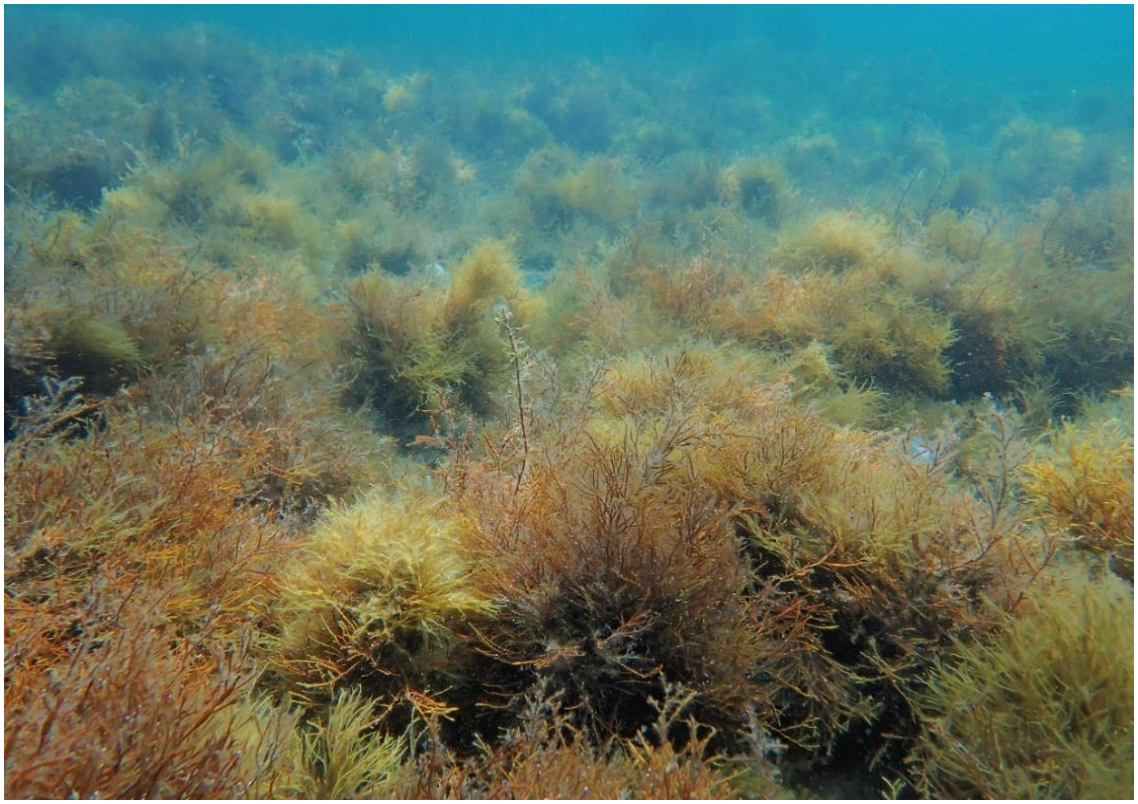


Figure 17: Due to grazing activity adult sporophytes of *G. barbata* were lower than year before - May 2023 (Photo: A. Lokovšek).

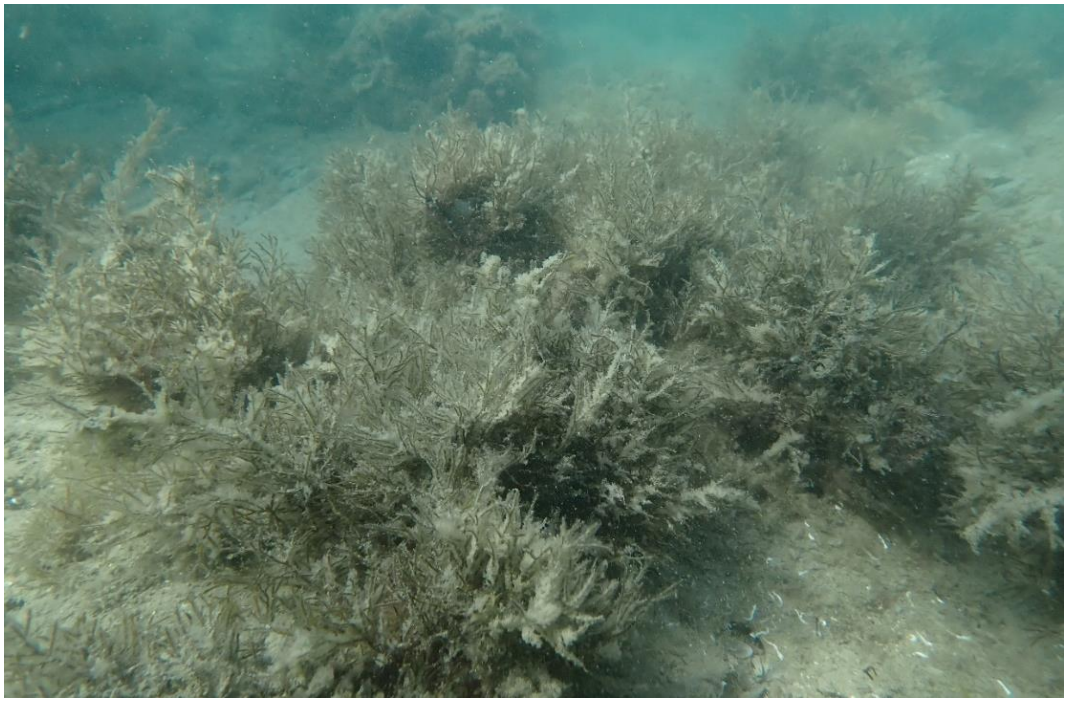


Figure 18: *G. barbata* forest covered with sediment, trapped into epiphytic overgrowth - March 2024 (Photo: A. Lokovšek).



Figure 19: *G. barbata* forest - May 2024 (Photo: A. Lokovšek).



Figure 20: *G. barbata* thalli without receptacles, probably due to grazing - bite marks visible at the end of the branches, June 2024 (Photo: A. Lokovšek).

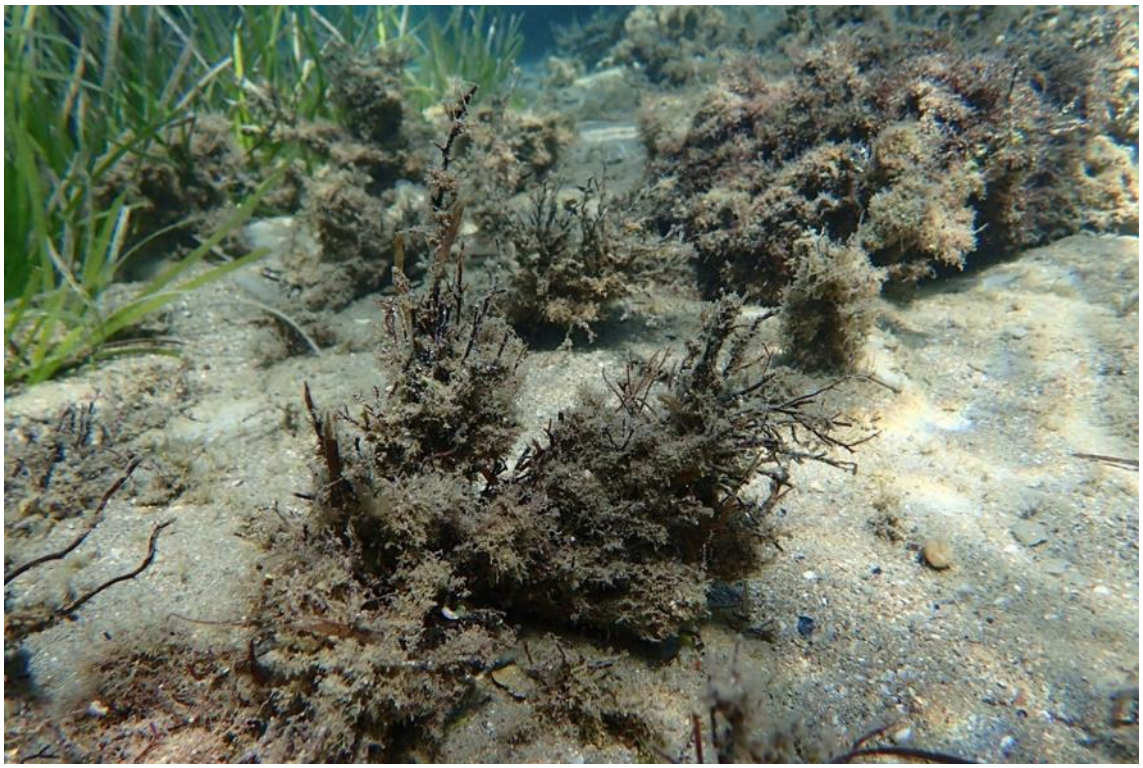


Figure 21: *G. barbata* thalli with heavy epiphytic overgrowth - July 2024 (Photo: A. Lokovšek).



Figure 22: Poor condition of *G. barbata* forest due to overgrazing, sedimentation stress and heavy epiphytic overgrowth - March 2025 (Photo: A. Lokovšek).



Figure 23: *G. barbata* forest - June 2025 (Photo: A. Lokovšek).

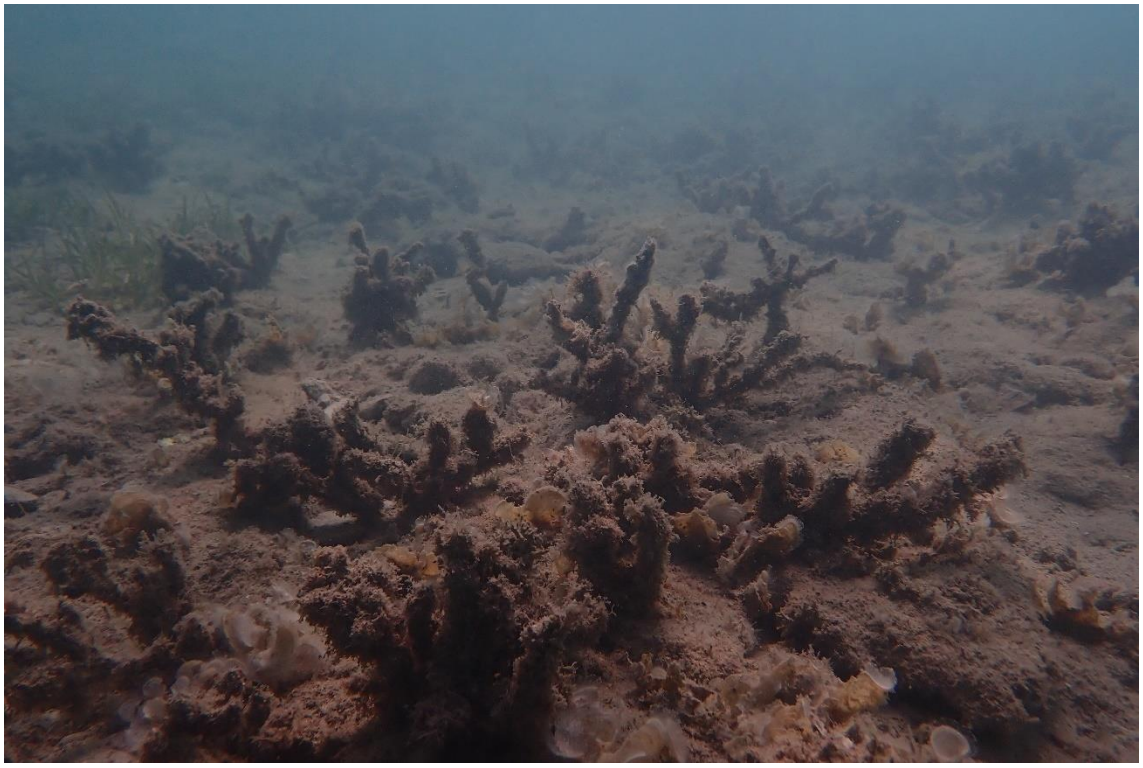


Figure 24: *G. barbata* forest - September 2025 (Photo: A. Lokovšek).



Figure 25: *G. barbata* forest with high sediment load and epiphytic overgrowth - October 2025 (Photo: A. Lokovšek).

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Publications Related to the Thesis

Journal Articles

Orlando-Bonaca, M., Savonitto, G., Asnaghi, V., Trkov, D., Pitacco, V., Šiško, M., Makovec, T., Slavinec, P., Lokovšek, A., Ciriaco, S., Chiantore, M., Kaleb, S., Descourvières, E.P., Srijemsi, M., & Falace, A. 2022. 'Where and how: New insight for brown algal forest restoration in the Adriatic', *Frontiers in Marine Science*, 9, 988584. doi:10.3389/fmars.2022.988584

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Conference Paper

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Biography

Ana Lokovšek was born on November 17, 1995, in Ljubljana, Slovenia. She attended the Biotechnical Educational Centre (BIC Ljubljana) and, after graduating, continued her studies at the Biotechnical Faculty of the University of Ljubljana. In 2018, she obtained her undergraduate degree in Biology. Fascinated by the sea, she pursued an Erasmus+ internship at the Malta National Aquarium, where she worked as an aquarist for six months, gaining valuable experience, taking care of a wide range of marine organisms from around the world. In 2019 she enrolled in the master's programme in Nature Conservation (Marine Module) at the Faculty of Mathematics, Natural Sciences and Information Technologies, University of Primorska (UP FAMNIT). During her studies, she established close collaboration with the Marine Biology Station Piran (NIB), where she completed her internship in the summer of 2020. She later participated in the international project *Plastic Pirates – Go Europe*, which addressed microplastic pollution in European rivers. In September 2021, she successfully defended her master's thesis, *"Feeding habits of painted comber (Serranus scriba) and brown comber (Serranus hepatus) in the Slovenian sea"*, under the supervision of prof. dr. Lovrenc Lipej and dr. Domen Trkov. Following the completion of her master's degree, she enrolled in the PhD programme in Ecotechnology at the Jožef Stefan International Postgraduate School in Ljubljana and began working as a young researcher at the Marine Biology Station Piran (NIB) under the supervision of Dr. Martina Orlando-Bonaca. Her doctoral research focuses on the restoration and conservation of macroalgal forests of *Cystoseira* s.l. During her PhD studies, she also actively engaged in international scientific exchange, attending several conferences and workshops. In 2023, she participated in the SeaWheat COST workshop in Kavala (Greece) and the Macroalgal Identification Course organized by Sorbonne University at the Roscoff Marine Station (France).