1. DATOS DEL PROYECTO

IP 1: Jesús R. Aboal Viñas
IP 2: M. Teresa Boquete Seoane

TÍTULO DEL PROYECTO (ACRÓNIMO): Desvelando el impacto de la exposición crónica a metales pesados en especies de macroalgas fundacionales: un enfoque multidisciplinario e integrador (CoastProtect)

TITLE OF THE PROJECT (ACRONYM): *Unveiling the impacts of chronic heavy metal exposure on key macroalgal foundation species: a multidisciplinary and integrative approach (CoastProtect)*

2. JUSTIFICACIÓN Y OBJETIVO

Pollution constitutes one of the most powerful drivers of biodiversity and ecosystem transformation (IPBES, 2019). Marine ecosystems, especially those in coastal areas, are particularly vulnerable since they are subject to both land- and ocean-based pollution sources (Halpern *et al.*, 2008). Due to their potential toxicity, persistence, and capacity to biomagnify in the food chain, pollutant exposure poses an important threat to all living organisms through lethal and sub-lethal effects at the individual level that might have far-reaching consequences at higher hierarchical levels: populations, communities, and even entire ecosystems (Díez *et al.*, 1999; Kim *et al.*, 2003; Johnston *et al.*, 2015). The cascading effects of pollution can thus jeopardize ecosystems' integrity and undermine their resilience, especially when they impact species with critical ecosystem roles like foundation species (Mayer-Pinto *et al.*, 2020).

Marine macroalgae are considered key coastal foundation species because they create, maintain, and modify habitats that support a range of flora and fauna (Olafsson, 2016). In addition to their important role as primary producers, they provide food, shelter, and breeding grounds for multiple species (Taylor and Cole, 1994; Tait and Schiel, 2011). Besides, macroalgae provide important ecosystem services like nursery grounds for coastal fisheries species, or their own exploitation for human consumption, agricultural fertilization, biofuel production, and for different industrial and pharmaceutical applications (Macreadie *et al.*, 2017). Previous research has shown that macroalgae are particularly sensitive to changes in environmental parameters related to climate change which can lead to the retreat or complete loss of macroalgae in coastal areas and cause a significant deterioration of their associated community assemblages (Mangialajo *et al.*, 2008; Smale and Wernberg, 2013; Macreadie *et al.*, 2017); our knowledge of how and to what extent pollutant exposure impact macroalgae, however, is much more limited (Mayer-Pinto *et al.*, 2020) hindering our capacity to develop effective management and protection policies for coastal ecosystems.

Macroalgae, particularly brown macroalgae (class Phaeophyceae), are known to accumulate high concentrations of pollutants in their thalli, especially heavy metals (HMs), reason why they have been routinely used in marine biomonitoring studies since the 1950s (Rainbow, 1995; Salgado *et al.*, 2005; García-Seoane *et al.*, 2018). Based on the literature published in this field, our research group just released the most extensive, complete, and up-to-date meta-analysis of the worldwide temporal patterns of the concentrations of HMs in these organisms for the past 68 years. We showed that the concentrations of HMs in brown macroalgae decreased significantly, between 60-84% worldwide, especially since the early 1970s (Aboal *et al.*, 2023) (Fig. 1), and proposed three potentially, non-mutually exclusive mechanisms for this global trend: (i) a decline in marine pollution as a result of the global environmental protection actions implemented since the 1970s (at least in developed countries); (ii) a general decrease in pollutant bioavailability as a consequence of the changes in marine physicochemical conditions

derived from climate change; and (iii) a reduction in pollutant uptake by macroalgae as an adaptive response to pollutant exposure. The extent to which each of these mechanisms contributes to the observed results is virtually unknown mostly because the vast majority of research involving macroalgae and environmental pollution has focused on the field of marine biomonitoring. Comparatively, research assessing how macroalgae respond to pollution from a biological/ecological perspective is extremely scarce. Only recently it has been demonstrated that macroalgae growing natively in clean areas and transplanted into polluted ones can reach higher pollutant concentrations than macroalgae native from polluted sites (Hédouin *et al.*, 2008; García-Seoane *et al.*, 2020) (Fig. 2). This evidence points towards a potentially adaptive response whose mechanistic basis remains unexplored.

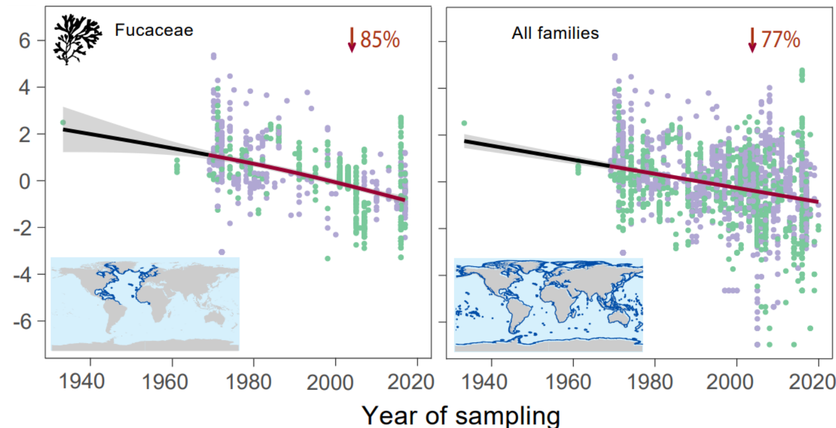

Figure 1: Temporal changes in the log-transformed concentrations of Cd (µg g^{-1}) in samples of the Fucaceae family (left graph) and all families analyzed (right graph). The geographic distribution of the corresponding families is shown within each graph. Solid line: generalized additive model fit; grey shaded area: 95% confidence interval; green dots: samples from areas without any anthropogenic pressure; purple dots: areas subjected to anthropogenic pressure. Source: (Aboal *et al.*, 2023).

All in all, there exists a significant gap of knowledge about how macroalgae are responding to pollution, and whether and to what extent these responses have consequences on the overall fitness of the algae (e.g. growth, reproductive output) and ultimately, on the ecosystems they create. With CoastProtect, for the first time, we aim to fill part of this gap by investigating the response of brown macroalgae to chronic pollutant exposure. We will use a multidisciplinary approach to study the different dimensions of this response encompassing its metabolic, morphological, physiological, ecological, and molecular components. In addition, we will evaluate whether and to what extent this response compromises macroalgal fitness. This non-oriented project will thus generate new knowledge that will enlighten our mechanistic understanding of the ecological interaction between pollutants and macroalgae. This basic, but more profound knowledge at the key species level, will lay the foundation for a better understanding and predictive capacity of the ecological and evolutionary consequences of marine environmental pollution at higher organizational levels.

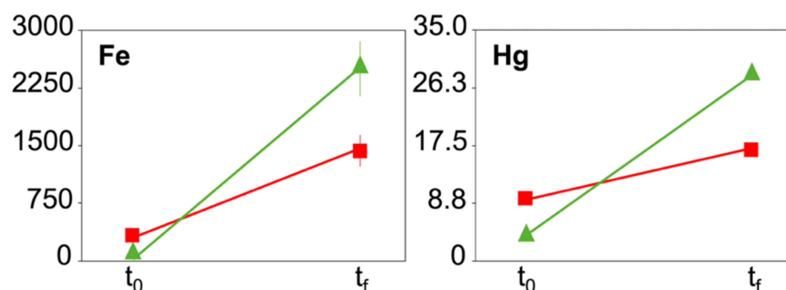

Figure 3: Changes in the mean concentrations (µg g^{-1} d.w. ± SE, n = 5 replicates) of Fe and Hg in transplants of *Fucus vesiculosus* exposed in a highly polluted site for 90 days. Green triangles:

macroalgae transplanted from an unpolluted to a polluted site; red squares: macroalgae native from a polluted site and autotransplanted within it; t_0: beginning of the experiment; t_f: end of the experiment. Adapted from: (García-Seoane *et al.*, 2020).

3. METODOLOGÍA Y PLAN DE TRABAJO

We will use the brown macroalga *Fucus vesiculosus* L. as a model, and we designed three main lines of work to simultaneously study and integrate all the components of its response to chronic HM exposure.

First of all, we will define the phenotypic traits involved in the response of *F. vesiculosus* to chronic HM exposure. Not only will we study the physiological traits involved, but also its potential metabolic, morphologic, and ecological, components. For this, we considered the different mechanisms that allow macroalgae to take up HMs, identified the phenotypic traits involved, and will assess whether there is a shift in their phenotypic value as a result of pollution. The investigated traits include:

i) Metabolism: by turning off macroalgal metabolism, we will assess to what extent metabolic regulatory processes contribute to macroalgal HM uptake, that is, we will be able to quantify the relative contribution of active vs. passive HM uptake processes.
ii) Morphology: we will assess whether the specific leaf area (responsible for the amount of HMs retained as particles in the surface of the thallus) and the ratio dry to wet weight (potentially responsible for some of the variation in the final concentrations of HMs among samples) differ between individuals of *F. vesiculosus* growing under different levels of pollution.
iii) Physiology: we will assess whether the concentrations of polysaccharides – alginates and fucoidans (responsible for the amount of HMs in the extracellular space) differ between individuals of *F. vesiculosus* growing under different levels of pollution.
iv) Physiology: we will assess whether the amount and activity of cationic transmembrane transporters (responsible for the transfer of HMs between the intra and extracellular compartments of the cells) differ between individuals of *F. vesiculosus* growing under different levels of pollution.
v) Ecology: we will assess whether the functional composition of the microbiota associated to macroalgae (potentially involved in HM homeostasis) differs between individuals of *F. vesiculosus* growing naturally under different levels of pollution. Additionally, we will experimentally examine the role of the microbiome on the capacity of macroalgae to take up HMs.

Second, we will determine the molecular basis of the response of *F. vesiculosus* to chronic HM exposure. For this, we will use a next generation sequencing that will allow us to:

i) Look for a signature of genetic differentiation among populations of *F. vesiculosus* subjected to different levels of HMs in the field for multiple generations. We will compare DNA sequences between individuals from populations growing in clean vs. highly polluted environments to test whether this species has undergone genetic adaptations.
ii) Test whether epigenetic mechanisms are involved in the response of *F. vesiculosus* to HM pollution. It is now well established that epigenetic mechanisms including DNA methylation, histone modifications and histone variants, and non-coding RNAs (collectively known as the epigenome), can modify the way genes are expressed within the cells by controlling the chromatin structure (Li *et al.*, 2007; Bartee *et al.*, 2017). It has also been demonstrated that the epigenome can be rapidly modified in response to external environmental stimuli leading to important phenotypic changes that could contribute to organisms phenotypic adaptation to environmental stress (Mirouze and Paszkowski, 2011; Dowen *et al.*, 2012; Foust *et al.*, 2016; Furlong *et*

al., 2020; Boquete *et al.*, 2021). Epigenetic modifications in response to environmental stress can disappear when the stress ceases, i.e. can be reversible (Wang *et al.*, 2011; Boquete *et al.*, 2021), leading to the environmental acclimation of species. On the other hand, some epigenetic modifications can be transmitted across generations (Verhoeven *et al.*, 2010; Rubenstein *et al.*, 2016; Weyrich *et al.*, 2016) providing an opportunity for epigenetic modifications to be adaptive. Research on how epigenetic mechanisms might contribute to macroalgal adaptation/acclimation lags well behind that of other taxa. In the case of Phaephyceae, some studies have looked at DNA methylation changes in response to developmental changes (Yang *et al.*, 2021), or have described overall methylation patterns (Cock *et al.*, 2010). Only one recent study has investigated the role of DNA methylation on temperature acclimation in the kelp species *Saccharina latissim a*(Scheschonk *et al.*, 2022). So far, experimental studies about the potential contribution of epigenetic mechanisms to HM tolerance has mostly been obtained for angiosperms (Greco *et al.*, 2012; Taspinar *et al.*, 2018; Galati *et al.*, 2021), a couple of bryophytes (Ghosh *et al.*, 2019; Boquete *et al.*, 2022), and one algal species (Kumar *et al.*, 2012). In CoastProtect we will test, for the first time in the Phaeophyceae, whether epigenetic mechanisms, specifically changes in DNA methylation in response to HM stress, underlie the acclimation and/or adaptive response of *F. vesiculosus* to these pollutants.

Third, we will determine the potential trade-offs between the response of *F. vesiculosus* to chronic HM exposure and its fitness. It is plausible that phenotypic changes aimed at reducing the stress generated by HMs compromise macroalgal productivity and/or reproduction. For example, a reduction in the specific leaf area to limit the number of particles that can be attached to the surface of the thalli could lead to a decreased photosynthetic rate and, consequently, decreased growth. Also, an increased production of extracellular polysaccharides to enhance the capacity of macroalgae from polluted sites to maintain HMs outside the cells could limit the amount energy and resources that can be allocated to growth and/or reproduction. Thus, we will measure:

i) Chlorophyll fluorescence and spectral reflectance curves as proxies for photosynthetic performance which, together with the specific leaf area, will inform us about the growth capacity of *F. vesiculosus* under different levels of pollution
ii) The number and biomass of reproductive structures as proxies for reproductive investment of *F. vesiculosus* growing under different levels of pollution.

The project will develop around three main experiments (experimental approaches) involving transplants and native samples of *F. vesiculosus* that will allow us to fulfil these objectives:

Experimental approach 1; "*Preparation and exposure of dead and living macroalgal transplants*": we will collect samples consisting of entire thalli of *F. vesiculosus* in a previously characterized unpolluted area in the NW Spain (X=506689; Y= 4737483; UTM 29 N ETRS89). These will be taken to the laboratory in polyethylene bags (at 5ºC) where the three most apical healthy dichotomies will be selected and cut with glass equipment. This selection results in similarly-aged, homogeneous material (García-Seoane *et al.*, 2021). An aliquot of this material will be dried, stored, and later used as a reference for the initial concentrations of HM in the samples (t_0 = unexposed samples). Half of the remaining material will be killed by oven-drying it using a temperature ramp in an oven, and the other half will be left untouched (alive and submerged in seawater at 5ºC). Both dead and living shoots will be used to prepare the transplants. For this, we will put 11 g of fresh or 1.8 g of dried material (difference in weight due to the estimated loss of ~5/6 during drying) in 9 x 9 cm fiberglass mesh flat bags (2 mm mesh size). At each of 25 selected sampling sites based on the expected HM contamination level, we will expose five replicates of each type of transplant for 15 days (Vázquez-Arias *et al.*, 2023) (n = 250 samples). Additionally, four replicate transplants of each type will be transported to

each of the sampling sites but not exposed, to control for any changes that might have occurred due to transportation (n = 100 samples). Whenever native *F. vesiculosus* is spotted close to the site, we will also collect three samples of ten thalli each at the end of the exposure period for its comparison with the transplanted materials (n = 75 samples). At each site, we will also collect samples of seawater every hour for the entire duration of the exposure time using an Automatic Portable Sampler (ISCO 3700). Given the need for power supply and surveillance of the equipment, our 25 sampling sites will be located in private marinas located along the coast of Galicia. We already have granted permission to install the equipment in all the marinas. After the exposure period, we will bring the transplants to the laboratory (in polyethylene bags at 5ºC) and process them to determine the Total concentrations of Ag, Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn by ICP–MS (Agilent 7700x) at the Research Support Services Unit from Universidade de Santiago de Compostela (USC). Seawater samples collected by the automatic sampling device will be combined to form a composite sample representative of the transplants' exposure period. We will determine the pH, Eh, conductivity, and turbidity in each of these samples (n = 25 in total) as well as the concentrations of Ag, Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn by ICP–MS (Agilent 7700x).

Experimental approach 2; "*Collection of native samples*": we will collect samples of *F. vesiculosus* growing naturally in 15 polluted and 15 unpolluted sites selected based on our knowledge of the pollution status of the Galician coast (Viana *et al.*, 2010). The selected polluted sites are characterized by their significant enrichment in As, Cd, Cr, Cu, Hg, Ni, Pb and Zn, due to their proximity to densely populated areas and industries. The unpolluted sites are located in open sea areas away from point pollution sources and their marine biota (including macroalgae) and surface sediments show low concentrations of these metals. During a field campaign in 2021 we confirmed the presence of *F. vesiculosus* all these sites. Five replicate samples consisting of at least ten thalli of *F. vesiculosus* will be handpicked at each site (n = 150 samples in total), brought to the laboratory in polyethylene bags (at 5ºC), and processed to determine specific leaf area (SLA), dry to wet weight ratio, chlorophyll fluorescence and spectral reflectance, reproductive effort, the concentrations of polysaccharides, the subcellular location of HMs, and the functional composition of the microbiome.

Experimental approach 3; "*Cross-transplants experiment*": we will reciprocally transplant living thalli of *F. vesiculosus* between three polluted and three unpolluted sites located in the Galician coast (all six sites are included in the collection of sites selected for experimental approach 2). The three polluted sites are located within rías nearby industrialized environments including an iron and steel plant (X = 566905 Y = 4816449; UTM 29 N ETRS89), a paper pulp industry/chlor-alkali plant (X = 525918 Y = 4694978), and an old ceramics factory (X = 531815 Y =4688350). First, we will handpick ca. 60 samples consisting of entire thalli of *F. vesiculosus* at each of the six sites. To minimize the variability among individual samples, we will only collect thalli of similar sizes. These will be taken to the laboratory in polyethylene bags (at 5ºC). In order to establish the baseline concentrations at the beginning of the experiment (t_0), we will separate and store five thalli from each site (n = 30 samples), which will be later analyzed in the same way as transplanted thalli. The remaining thalli will be attached to rocks (previously collected on the coast) by applying waterproof silicone sealant to their holdfast (basal disc that serves as substrate attachment point) in order to imitate natural conditions. In total, 30 individual thalli of *F. vesiculosus* will be exposed at each site, including 5 replicate thalli from each of the six origin sites. These will be distributed into 5 rocks, each containing one thallus per site (i.e. 5 rocks per site, with 6 thalli per rock; n = 180 samples in total). In addition, we will separate 15 thalli from each of the sites and eliminate the microorganisms associated to their surface (see section 3.2.7 for more details). Five replicates of each of these microbiome-depleted transplants will be exposed together with the regular transplants only in the three polluted locations to test

for the effect of the microbiome in the capacity of *F. vesiculosus* to take up HMs (n = 90 samples). At the end of the experiment we will have 300 samples in total.

All transplants will be labelled with plastic tags indicating both the origin and the exposure sites and will be left on the coast for 90 days. To avoid the loss of material without preventing water flow, the stones with the attached thalli will be covered with polyethylene nets of 2 cm mesh size. Transplants exposed within the same site in which the thalli were collected (i.e., in their origin environment) will hereafter be referred to as autotransplants. Those exposed in sites other than the original site, will be referred to as transplants. The rocks will be exposed in the intertidal zone amongst native populations of *F. vesiculosus*. This methodology has been successfully employed before by our group in (García-Seoane *et al.*, 2020) (Fig. 3).

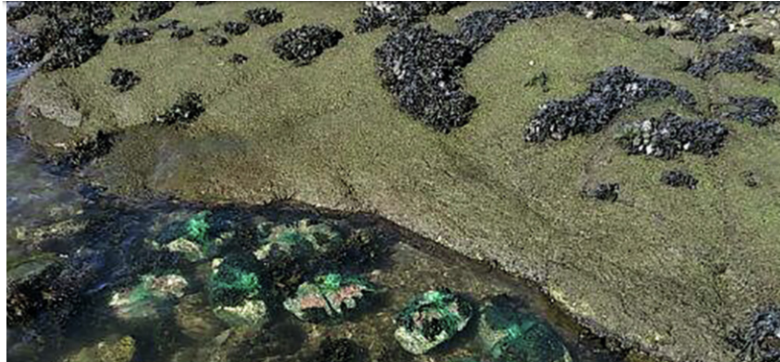

Figure 3. Transplantation systems as exposed in the field. Each system consists of one rock containing thalli of *Fucus vesiculosus* from each of the experimental sites covered with a polyethylene mesh net (picture from the experiment carried out by (García-Seoane *et al.*, 2020).

At the end of the experiment, thalli will be detached from the rocks, cleaned in the surrounding seawater, placed inside clean polyethylene bags, and transported to the laboratory (at 5°C). We will separate the apical segments (1 cm long) from each thallus with a glass spatula to analyze newly grown, standardized material containing the most recent and physiologically active parts of the thallus.

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5. RECURSOS HUMANOS Y MATERIALES DISPONIBLES PARA LA EJECUCIÓN DEL PROYECTO

With regards to the human resources, the research team of the project will be composed by the two PIs (Dr. Jesús R. Aboal Viñas and Dr. M. Teresa Boquete) and Dr. Carlos Real and Dr. Rubén Villares, Associate Professors at the USC. Additionally, early career researchers Carme Pacín and Antón Vázquez Arias will constitute the working team.

Our laboratory and university facilities have most of the equipment necessary to carry out the project:

<u>GENERAL EQUIPMENT</u>:

- Laboratory oven: 2 P-Selecta Digitronic heaters; P-Selecta Dry-big heater.

- Precision balance: Mettler Toledo ML203/01, AJ100, AB135-S, XP26, B502-S, AB104.
- Mills: Ultracentrifugal Retsch ZM 200 (titanium-niobium box and titanium rings); tangential Retsch MM 400 (2 sets of zirconium oxide cups).
- pH meters, conductivity meters, oximeters: conductivity meters (Hanna HI9811-5; Crison 524); pH meters/REDOX/ORP (Orion 4 Star; Crison pH meter 507); oximeters (Crison Oxi 320; Hanna); turbidimeter or nephelometer (Lutron TU-2016)
- Automatic Portable Sampler: 2 ISCO Model 3700FS.

ELEMENTAL ANALYSIS:

- Elemental analysis: Milestone DMA80 mercury autoanalyzer (Direct Mercury Analyzer) at our laboratory and Ethos-1, Milestone and ICP–MS (Agilent 7700x) at the Research Support Services Unit from Universidade de Santiago de Compostela.

CHLOROPHYLL FLUORESCENCE, FOLIAR REFLECTANCE, LEAF SCANNER:

- Spectrometer: UniSpec Spectral Analysis System, PP Systems; Fluorometers: Photosynthesis Yield Analyzer Fluorometer, PAM-2000 and MINI-PAM, Heiz Walz GMBH, Leaf Area Meter: LICOR LI-3100C.

ATTENUATED TOTAL REFLECTANCE-FOURIER TRANSFORM INFRARED SPECTROSCOPY:

- Cary 630 FTIR-ATR, Agilent Technologies

VEHICLES:

- Peugeot Partner Tepee 4x4 Dangel Extreme Plus.