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SHORT RESEARCH AND DISCUSSION ARTICLE

Synergistic, antagonistic, and additive effects of heavy metals (copper and cadmium) and polycyclic aromatic hydrocarbons (PAHs) under binary and tertiary combinations in key habitat-forming kelp species of Chile

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Abstract

Heavy metals (HMs) and polycyclic aromatic hydrocarbons (PAHs) are persistent toxicants in coastal environments. Notably, in comparison to individual metal toxicity, knowledge about the effects of HMs and PAHs mixtures on kelps remains scarce. Accordingly, we performed in vitro experiments to determine the individual and combined effects of Cu, Cd, and PAHs on spore release, settlement, and germination on *Macrocystis pyrifera* and *Lessonia spicata*, two key-habitat forming kelp species of the coast of the Valparaíso Region in Chile. This region concentrates highly polluting industries, mainly due to unrestrained mining and fossil-fuel energy production. Single Cu, Cd, and PAHs treatments included concentrations in the ranges 5–200, 0.125–2000, and 0.05–100 μg/L, respectively, and a toxic-free treatment. Cu, Cd, and PAHs concentrations causing 20–50% (IC20, IC50) arrested spore release, settlement, and germination were determined, and the results shown in both species that single Cu, Cd, and PAHs IC20 values were generally lower on spore release than on spore settlement and germination, probably due to the absence of a cell wall in spores compared to later stages. Binary equitoxic IC20s mixture treatments changed from an antagonistic response to another with a greater inhibitory effect on spore release, from hour 1 to 7, whereas in IC50 treatments, the response was always antagonistic. The tertiary IC20 mixture of Cu+Cd+PAHs produced generally an antagonistic effect. Remarkably, all IC20 equitoxic mixture treatments showed a synergistic response on spore settlement in both kelps, suggesting that these toxicants are extremely harmful to kelp population persistence near highly polluted sites.

Keywords Heavy metals · Polycyclic aromatic hydrocarbons (PAHs) · Combined toxicity effects · Binary and tertiary mixtures · Kelps · Early life stages

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Introduction

Heavy metals (HMs) and polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants, highly toxic, and persistent in coastal environments. Accordingly, they have been included as priority substances in the EU Water Framework Directive 2000/60/EC (European Commission 2000). HMs pollution alters the whole community structure and ecosystem function in coastal benthic areas, such as those highly impacted by mining operations. Indeed, the negative impacts of pollution on invertebrates and kelps mostly affect their microscopic early-life stages, which are more sensitive to pollutants than adults (Bellgrove et al. 1997; Contreras et al. 2007; Leal et al. 2016). On the other hand, PAHs are planar molecules

composed of three or more fused aromatic rings, are highly lipid-soluble, non-biodegradable within ecosystems, and able to cause mutagenic and carcinogenic effects (Walker et al. 2012). Despite their significance, there is a lack of research into the effects of PAHs toxicity on microscopic early life stages of kelps.

Heavy metals such as copper and cadmium were shown to independently inhibit spore settlement and gametophyte development in the kelps *Lessonia spicata* and *Macrocystis pyrifera* (Contreras et al. 2007; Leal et al. 2016, respectively). In comparison to individual toxicity tests with HMs, there are no studies on the interactive effects of HMs and/or PAHs toward early stages of kelps. In the microalga *Chlorella* sp., cell division rate was shown to be synergistically inhibited by equitoxic mixtures of Cu+Cd, whereas other binary (Cu+Zn or Cd+Zn) or tertiary mixtures (Cu+Cd+Zn) exerted an antagonistic effect (Franklin et al. 2002). In the microalga *Desmodesmus subspicatus*, the combined equitoxic concentration treatment with cadmium and anthracene (a PAH) reduced population growth rate more vigorously than the substances applied independently (Baścik-Remisiewicz et al. 2011).

Along the central Chilean coast, *Lessonia spicata* and *Macrocystis pyrifera* are the most important habitat-forming kelps species in the subtidal and intertidal ecosystems, respectively. Located in this geographical area, the neighboring districts of Puchuncaví, Quintero, and Concón (Valparaíso Region) house one of the most polluting industrial parks in Chile (Puchuncaví-Ventanas at 32° 43′ S, 71° 30′ W), concentrating fossil-fuel energy industries, mining smelters and refineries, plastic and chemical industries, cement companies, among others. The last study commissioned by the Chilean Ministry of the Environment (Ministerio del Medio Ambiente (MMA) 2013) determined high median concentrations of HMs in the seawater and coastal sediments, and high bioaccumulation in the biota, exceeding international quality standards such as those of the US Environmental Protection Agency (e.g., in seawater it was 151 and 2 times higher for copper and cadmium, respectively) (Araya 2019; Oyarzo-Miranda et al. 2020) as well as high concentrations of PAHs in seawater (6.23-17.61 μg/L, FIC-ALGAS 2016). Thus, in this environmental scenario of high levels of HMs and PAHs pollution, our aims were (i) to determine in vitro the individual concentrations of these toxics that cause 20% and 50% inhibition (IC20 and IC50) as well as (ii) the combined effects of binary and tertiary mixtures of these toxics, on spore release, settlement. and germination of *L. spicata* and *M. pyrifera* from central Chile. The latter is particularly important because anthropogenic enrichment of HMs and PAHs usually occurs together in the environment (Gauthier et al. 2014). We chose *L. spicata* and *M. pyrifera* because these provide habitat and refuge for associated organisms, regulate the biodiversity of intertidal and subtidal communities, and influence the stability of ecosystems (Teagle et al. 2017). Thus, this information will be crucial to understand the combined effect of these pollutants in seaweeds, which in turn would be helpful for environmental normative (still missing in Chile) in marine zones with a high level of anthropogenic activities.

Sampling and toxicological trials

Glass vials and Petri dishes for HMs and PAHs trials were pretreated according to US E.P.A. (Environmental Protection Agency) (1999). Reproductive fronds of *L. spicata* and sporophylls from *M. pyrifera* were sampled at two sites 60 km apart and outside the influence of the industrial focus. Three small discs of 5 × 2 cm^2 excised from fertile tissue were placed in three 5-mL glass vials per treatment. Based on the reports of concentrations used in seaweeds and other organisms (i.e., Bellas et al. 2001; Collén et al. 2003; Contreras et al. 2007; Kumar et al. 2010, 2012; Barjhoux et al. 2012; Lovazzano et al. 2013; Babu et al. 2014; Leal et al. 2016), the glass vials were filled with test solutions that contained increasing nominal concentrations (in μg/L) of copper (5, 10, 20, 30, 40, 50, 100, 200, 500, and 1000), cadmium (0.125, 0.25, 0.5,1, 10, 50, 100, 250, 350, 500, and 1000), and PAHs (0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10, and 100), and a treatment with no toxic addition as control (0.22-μm filtered seawater). The PAHs mixture used (QTM PAH Mix, Supelco, USA) contained 16 compounds considered PAH-priority pollutants by the US EPA. We registered the number of spores released after 1, 2, 3, 5, and 7 h using a Neubauer chamber on an upright Leica ICC50 HD microscope (Wetzlar, Germany). For spore settlement and germination, five glass Petri dishes per treatment (50 × 10 mm) were inoculated with approximately 25×10^3 spores and filled with 5 mL of the aforementioned test solutions. The number of settled and germinated spores was monitored during 72 h in an inverted microscope Nikon Eclipse Ts2 (Melville, NY, USA). The obtained concentration response curves were fitted to the four-parameter log-logistic model (Van der Vliet and Ritz 2013) using the ED function of the DRC package in the statistical environment R (R Core Development Team 2017); this allowed determining IC20 and IC50 values of copper, cadmium, and PAHs that cause 20–50 % inhibition of spore release, settlement (48 h), and germination (72 h) in both kelps. Then, IC20 and IC50 values were used to assess the combined effects of equitoxic mixtures of HMs and PAHs on each life stage, in which each toxic of the mixture was present at the concentration exerting the same toxic effect. To test the individual effects of each toxic concentration (Cu, Cd, or PAHs) on spore release, settlement, and germination, an ANOVA was applied, followed by a post hoc Tukey multiple comparison test. Individual inhibitory effects of each metal or PAHs were added to predict the effects of each mixture (response-addition model, Norwood et al. 2003). Differences between observed and expected inhibitory effects of mixtures

Table 1 Inhibitory concentrations (μg/L) used in HMs and PAHs mixture experiments, which correspond to 20% and 50% inhibitory concentrations effects (IC20s and IC50s) of Cu, Cd, and PAHs on the number of released, settled, and germinated spores for (a) *L. spicata* and (b) *M. pyrifera*

(a)	*Lessonia spicata*			
	Spore release		Settlement	Germination
Toxic	IC20	IC50	IC20	IC20
Cu	12	36	12.6	212
Cd	0.02	1	537	17
PAH	76*	100*	0.43	0.42
(b)	*Macrocystis pyrifera*			
	Spore release		Settlement	Germination
Toxic	IC20	IC50	IC20	IC20
Cu	21	33	41	205
Cd	0.08	0.71	4.08	785
PAH	0.043	0.25	0.07	10

The asterisk indicates that for the PAHs toxicity experiment on spore release in *L. spicata*, we were only able to determine a low inhibitory concentration (LOIC), which was used in mixture experiments; while 100 μg/L, the maximum solubility achieved for the 16 compounds PAHs mixture, was used as a high inhibitory concentration

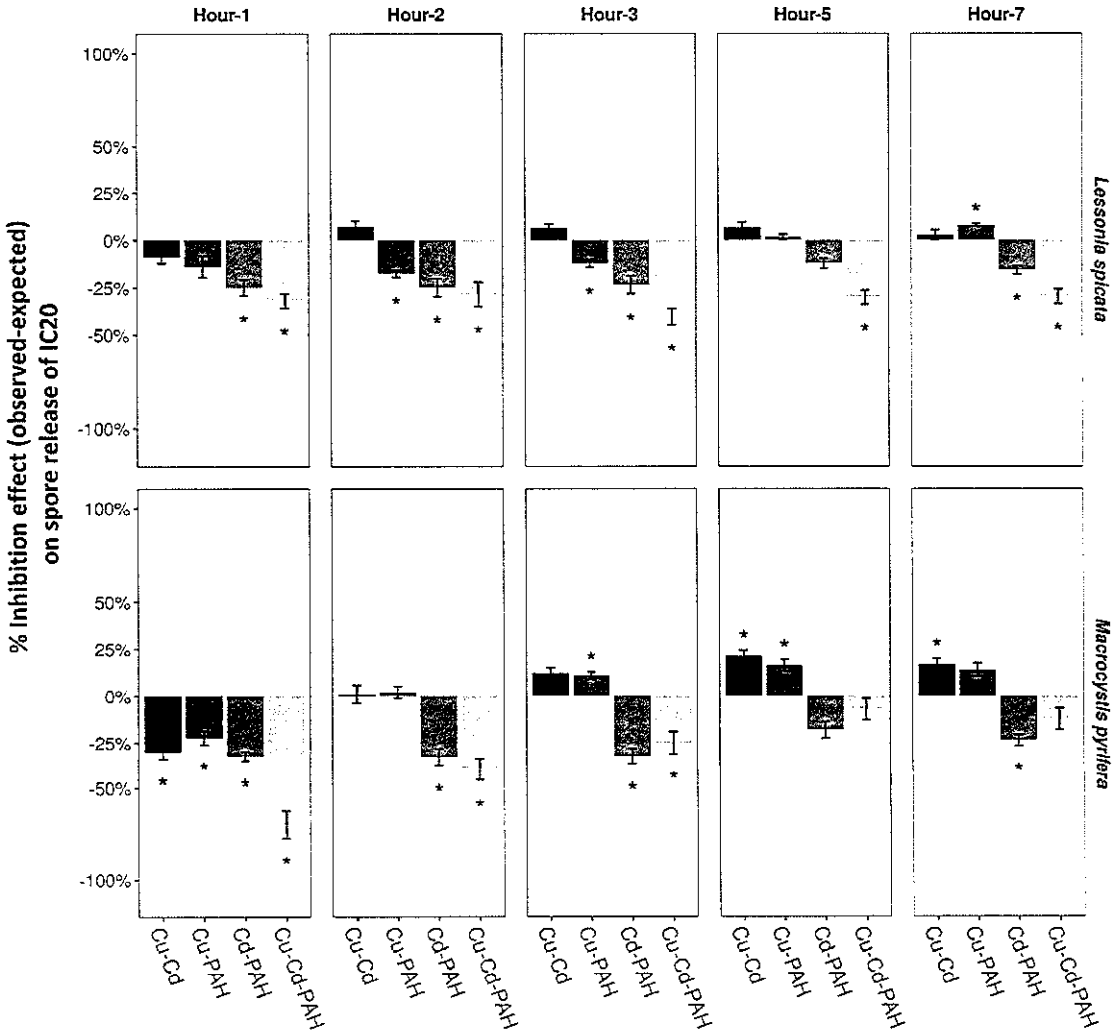

Fig. 1 Effects of IC20 mixtures on kelp spore release. Differences between observed versus expected inhibition effects on the number of spores released of the IC20 mixture treatments with Cu, Cd, and PAHs, after 1, 2, 3, 5, and 7 h of exposure. The IC20 values used correspond to those indicated in Table I. n=3, errors bars indicate ± 1 SEM. Differences were tested against zero by a t-test; *p<0.05. Observed inhibition values significantly greater or less than zero correspond to synergistic or antagonistic responses, respectively

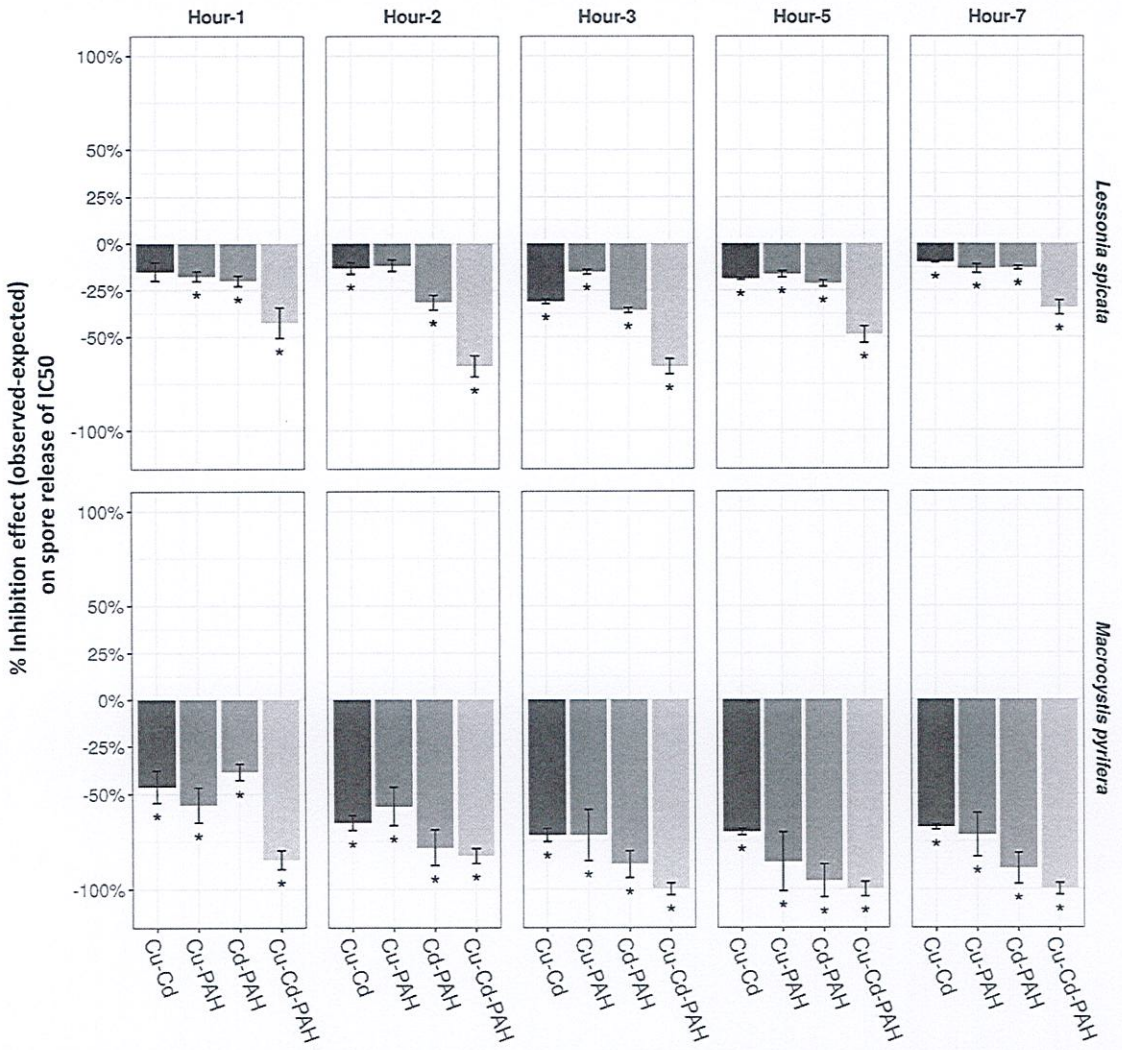

Fig. 2 Effects of IC50 mixtures on kelp spore release. Differences between observed versus expected inhibition effects on the number of spores released of the IC50 mixture treatments with Cu, Cd, and PAHs, after 1, 2, 3, 5, and 7 h of exposure. The IC50 values used correspond to those indicated in Table 1. n=3, errors bars indicate ± 1 SEM. Differences were tested against zero by a t-test; *p<0.05. Observed inhibition values significantly greater or less than zero correspond to synergistic or antagonistic responses, respectively

were tested against zero by a t-test to check the occurrence of additive, synergistic, or antagonistic effects. All statistical analyses were performed using the statistical software R (R Core Development Team 2017).

Results

After 7, 48, and 72 h of exposure, a general trend of negative effects was observed on the number of released, settled, and germinated spores, as a function of increasing Cu (Cd or PAHs) concentration (ANOVA tests on toxic concentration effects are found in Tables 1S to 6S). Table 1 a and b show IC20 and IC50 values (for metals and PAHs) on the number of released, settled, and germinated spores in *L. spicata* and *M. pyrifera*. Independent tolerance to Cu, Cd, or PAHs was highly variable, depending on the life cycle stage and species; nonetheless, generally, both kelps were more sensitive to pollutants during the stage of spore release, than during settlement or germination. The exception was the high tolerance of *L. spicata* to PAHs concerning spore release. In this case, we were unable to obtain the full inhibitory concentration range, so we used instead 76 μg/L (the lowest inhibitory concentration tested, or LOIC) and 100 μg/L (maximum solubility achieved for the PAHs mixture) as the lowest and highest inhibitory concentrations, respectively, in the mixture experiments.

Regarding the combined effects of toxics on spore release, the binary and tertiary mixtures (Cd+PAHs and Cu+Cd+PAHs) were always antagonistic from hour 1 to 7 in both kelps (Fig. 1). However, binary combinations with Cu evolved, from antagonism at hour 1 to additivity in *L. spicata* and to synergism in *M. pyrifera* at hour 7 (Fig. 1).

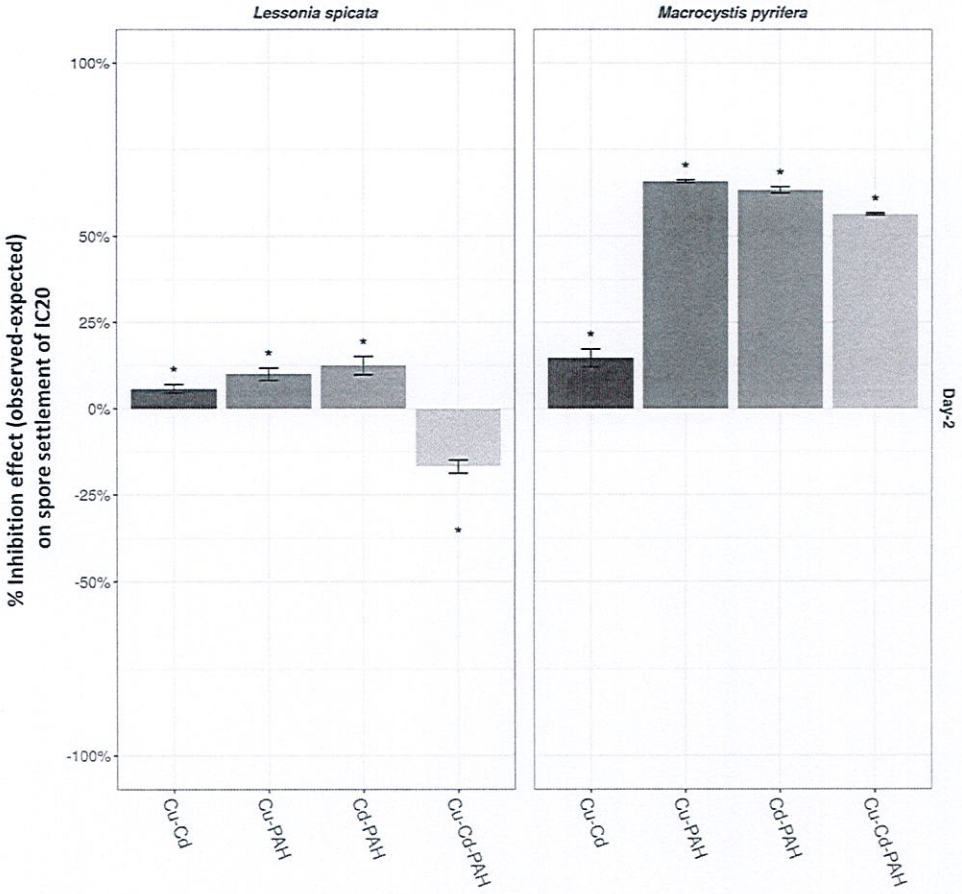

Fig. 3 Effects of IC20 mixtures on kelp spore settlement. Differences between observed versus expected inhibition effects on the number of settled spores of the IC20 mixture treatments with Cu, Cd, and PAHs, after 48 h of exposure. The IC20 values used correspond to those indicated in Table 1. n=5; errors bars indicate ± 1 SEM. Differences were tested against zero by a t-test; *p<0.05. Observed inhibition values significantly greater or less than zero correspond to synergistic or antagonistic responses, respectively

Exposure to all binary and tertiary mixtures using IC50 values resulted in antagonistic effects on spore release from hour 1 to 7 in both kelps (Fig. 2). A similar contrasting response pattern was observed at low versus high inhibitory concentrations exposure on population growth of the green alga *Desmodesmus subspicatus* (Baścik-Remisiewicz et al. 2011).

Regarding the combined effects of IC20 treatments on settlement of both kelps, all binary mixtures showed a synergistic negative effect on the number of settled spores, and the impacts were stronger in *M. pyrifera* (Fig. 3). The effect of the tertiary mixture Cu+Cd+PAHs on settlement was also synergistic in *M. pyrifera*, whereas in *L. spicata* was antagonistic (Fig. 3). Regarding the combined effects of IC20 mixtures on germination (Fig. 4), the Cu+Cd exposure was synergistic in *L. spicata* and additive in *M. pyrifera*; the binary mixtures Cu+PAHs and Cd+PAHs were antagonistic in *L. spicata* and additive in *M. pyrifera*. The tertiary mixture Cu+Cd+PAHs was antagonistic in both species (Fig. 4).

Discussion and perspectives

The magnitude of inhibitory effects under individual toxic action of Cu, Cd, and PAHs were greater on spore release than on spore settlement and germination. The higher sensitivity of spores in comparison to later life stages of algae could be in part explained by the absence of a cell wall, which is only formed during settlement and cell adhesion (Maggs and Callow 2002; Bouzon et al. 2006; Petrone et al. 2011). In fact, a thickening of the cell wall and overproduction of polysaccharides were induced in adults of the brown alga *Padina gymnospora* by high levels of heavy metals, in order to prevent their absorption, as well as protect the photosystems in two green algae species (e.g., Andrade et al. 2010; Zeroual et al. 2020). In addition, the biosorption of PAHs such as phenanthrene would be positively correlated with the lipid and nonhydrolyzable carbon fractions of algae (Zhang et al. 2013), which are more abundant in the plasmatic membrane than in the cellular wall. On the other hand, the binary mixtures with Cu became more harmful (additive or synergistic) on spore release from hour 1 to 7 than the binary mixture Cd+PAHs; on the contrary, the tertiary mixture Cu+Cd+PAHs was antagonistic in most cases (spore release, settlement, and germination). Increasing effects of mixtures with time (especially binary mixtures with Cu) could be caused by the slower intracellular accumulation of metals, in comparison to the faster metal accumulation of the adsorption mechanism upon the cell surface (Geddie and Hall 2019).

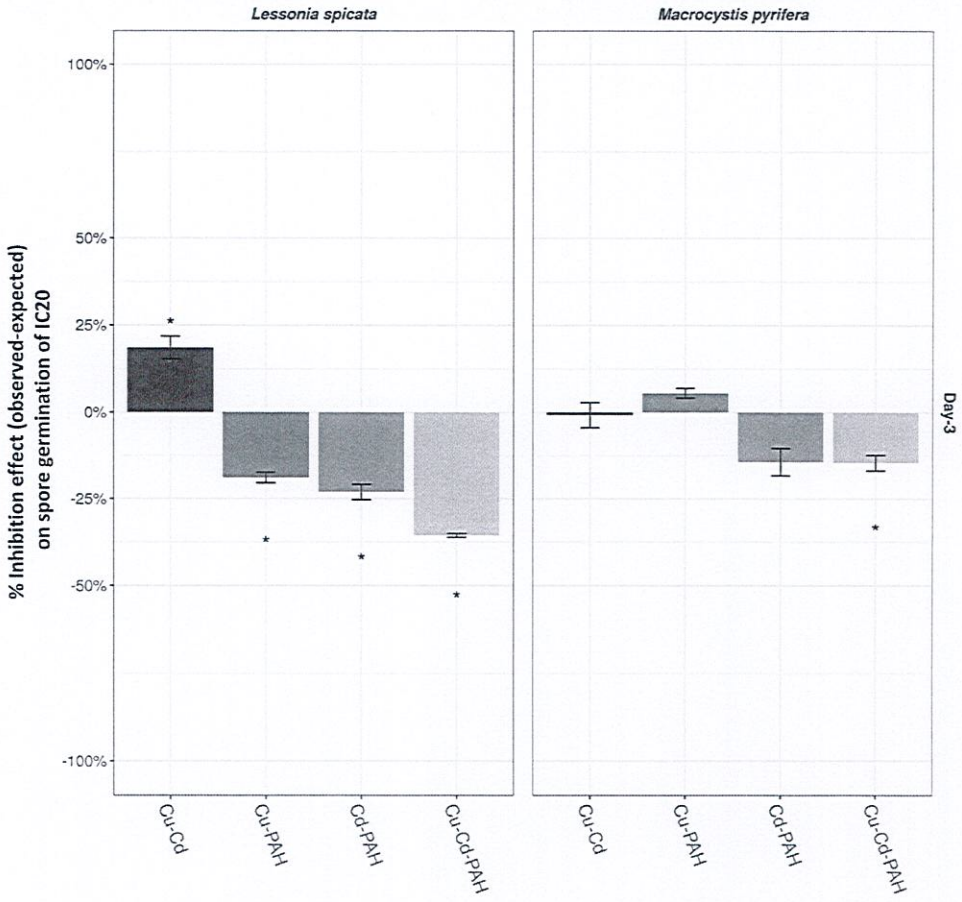

Fig. 4 Effects of IC20 mixtures on kelp spore germination. Differences between observed versus expected inhibition effects on the number of germinated spores of the IC20 mixture treatments with Cu, Cd, and PAHs, after 72 h of exposure. The IC20 values used correspond to those indicated in Table 1. n=5; errors bars indicate ± 1 SEM. Differences were tested against zero by a t-test; *p<0.05. Observed inhibition values significantly greater or less than zero correspond to synergistic or antagonistic response, respectively

Synergism versus antagonism, especially the response herein observed under binary compared to tertiary mixtures, could be explained by the competitive (extracellular) binding as well as (intracellular) uptake of metals (and probably PAHs). For example, the binary mixture Cu+Cd affected synergistically the growth of the microalga *Chlorella* sp. (Franklin et al. 2002). This could be because cadmium and copper co-exposure increases copper (extracellularly and intracellularly) but inhibits cadmium uptake, compared to each metal present by itself (e.g., Franklin et al. 2002; Andrade et al. 2006). On the contrary, Cu+Cd+Zn exposure had an antagonistic effect on *Chlorella* sp. growth, as was also observed in our study for spore release and settlement of the two kelps under the Cu+Cd+PAHs (IC20) exposure. This is probably caused by a competitive binding of metals (and probably PAHs) on the cell surface (Franklin et al. 2002). Antagonism could also be associated with the metal-induced release of exudates by kelps, which have compounds able to chelate in a non-toxic form or act as carriers of metals (e.g., Sordet et al. 2014). The antagonistic effects on early stages of kelps observed in this study under the combined HMs and PAHs exposures could also be explained by the existence of Cation-π interactions, which allow strong bonding between HMs and PAHs, such as between Cu and phenanthroline (Rodgers and Armentrout 2016). On the other hand, the adhesive secreted by kelp spores to settle on substrates contains sulfide, phosphorus, calcium, and magnesium among other important ions; the last two are probably involved in the gelation of the adhesive (Petrone et al. 2011). Since sulfide precipitates in the presence of copper and cadmium (as CuS and CdS) (Choi et al. 2006; Gharabaghi et al. 2012; Pohl 2020), possible explanations for the synergistic negative effects on spore settlement in *L. spicata* and *M. pyrifera* under the combined treatments with Cu and Cd could be sulfide precipitation or the interruption of gelation by metals. Nonetheless, additional hypotheses should be explored. In conclusion, our study demonstrated that combined treatments with Cu, Cd, and PAHs would produce harmful effects on early life stages of *L. spicata* and *M. pyrifera*. These effects were not always predicted by the simple addition of the individual effects of each contaminant. Moreover, inhibitory effects under individual and binary mixtures exposures with HMs and PAHs differed greatly between the two species, and were stage specific; one reason could be that seaweeds from different environments (e.g., intertidal versus subtidal kelps) probably have different types of cellular membrane transporters and chaperones to

regulate metals (Blaby-Haas and Merchant 2012) and PAHs homeostasis. Finally, it is of utmost importance the field evaluation of impacts of toxics on both kelps (and other species) under scenarios of increasing levels of contamination.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-13261-6.

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Availability of data and materials Raw data were generated at Universidad Andrés Bello (Santiago, Chile). Derived data supporting the findings of this study are available from the corresponding author L. Contreras-Porcia on request.

Author contribution L. Contreras-Porcia conceived the project and designed the experiments. C. Espinoza-González, A. Núñez, and F. Castañeda carried out the experiments. Material preparation, data collection, and analysis were performed by C. Espinoza-González, A. Núñez, A. Meynard, and L. Contreras-Porcia. The first draft of the manuscript was written by A. Meynard and L. Contreras-Porcia, and all authors commented on previous versions of the manuscript. Writing-review and editing by A. Meynard and L. Contreras-Porcia. All authors read, edited, and approved the final manuscript.

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Declaration

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Consent to participate "Not applicable."

Consent to publish "Not applicable."

Competing interests The authors declare no competing interests.

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