



# Assessment of the independent and combined effects of copper and polycyclic aromatic hydrocarbons on gametogenesis and sporophyte development of the kelp *Lessonia spicata* (Phaeophyceae, Ochrophyta)

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Received: 25 February 2021 / Revised and accepted: 21 June 2021  
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## Abstract

Coastal shores near the industrial park of Quintero Bay in central Chile exhibit increasing concentrations of copper (Cu) and polycyclic aromatic hydrocarbons (PAHs), well above international standards. This raises concern about their combined toxic effects on early development stages of kelps. Accordingly, we aimed to assess more accurately the independent and combined effects of Cu and PAHs on gametogenesis and sporophyte development in the kelp *Lessonia spicata* from central Chile by in vitro cultivation. Independent Cu and PAH trials were performed using increasing nominal concentrations of Cu and PAHs in the ranges 0.8–200  $\mu\text{g L}^{-1}$  and 0.05–100  $\mu\text{g L}^{-1}$ , respectively. Cu and PAH median effective concentrations ( $\text{EC}_{50}$ ) on gametogenesis and early sporophyte formation were calculated using DRC in the R environment. Then, combined  $\text{EC}_{50}$  Cu + PAH trials were conducted to determine their effects on gametogenesis and sporophyte formation. Cu  $\text{EC}_{50}$  values on gametogenesis and sporophyte formation were up to three orders of magnitude lower than  $\text{EC}_{50}$  reported previously on spore germination in kelps. The gametogenesis ( $\text{EC}_{50} = 1.39 \mu\text{g L}^{-1}$ ) was more sensitive to Cu than sporophyte formation ( $\text{EC}_{50} = 11 \mu\text{g L}^{-1}$ ). Inversely, sporophyte formation ( $\text{EC}_{50} = 0.04 \mu\text{g L}^{-1}$ ) was more sensitive to PAHs ( $\text{EC}_{50} = 0.11 \mu\text{g L}^{-1}$ ). Considering the entire exposure period, the combined  $\text{EC}_{50}$  Cu + PAH exposure was the most harmful and rapid for *L. spicata* microscopic stages, especially the synergistic effect on early sporophytes. This highlights the need to acquire an integrated knowledge of the seasonal variation of pollutants and their combination on highly intervened coasts.

**Keywords** *Lessonia spicata* · Copper · Polycyclic aromatic hydrocarbons · Developmental stages · Quintero Bay · Seaweeds

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## Introduction

Heavy metals (HMs) and polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants and are highly toxic and persistent in coastal environments. In the context of the co-occurrence of HMs and PAHs pollution, this raises concern about their combined effects and ecological consequences on marine environments. Environmental standards and regulations established for marine zones with a high level of anthropogenic activities are usually insufficient, because they assume strictly additive effects of pollutants. Indeed, in aquatic systems, nearly 45% of research on the co-toxic effects of metal-PAH mixtures have found more-than-additive (synergistic) mortality (Gauthier et al. 2014).



In higher plants, toxic PAHs exposure induces early developmental alterations, reduction in growth, photosynthesis inhibition, reduction in chlorophyll content, deregulation of cellular homeostasis, oxidative stress, cell death, upregulation of antioxidant systems, and mutagenic and carcinogenic effects (e.g., Jajoo et al. 2014; Ewa and Danuta 2017; Hernández-Vega et al. 2017). On the other hand, copper (Cu) is required for the normal growth and development of photosynthetic organisms, which have evolved protein Cu transporters in the plasma and organelles membranes (i.e., Cu transporter family and the group B transport family PIB-type ATPases) regulating its homeostasis in accordance with concentration levels in the environment (Blaby-Haas and Merchant, 2012; Contreras-Porcia et al. 2017). Despite its requirement as a micronutrient, above a certain threshold concentration, Cu inhibits early development and several physiological processes, such as photosynthetic electron transport, mitochondrial respiration, cell wall metabolism, oxidative stress response, and hormone signaling (Kumar et al. 2021).

To our knowledge, no studies have been conducted on the independent effects of PAHs or the interactive effects of both Cu and PAHs on the adult and early stages of kelps. In the green microalga *Desmodesmus subspicatus*, the interacting effects resulting from the equitoxic concentration treatment with cadmium and the PAH anthracene reduced the population growth rate more significantly than the substances applied independently (Baścik-Remisiewicz et al. 2011). Another study on three domesticated higher plants showed that the combined effects of chromium(VI) and phenanthrene (a PAH) on their stem lengths and above-ground fresh weights were synergistic or antagonistic, depending on the species (Hu et al. 2016).

Anthropogenic pollution from mining waste disposals and crude oil deposition can adversely affect canopy-forming seaweed populations (Aminina and Shaposhnikova 2012; Strain et al. 2014; Oyarzo-Miranda et al. 2020), which are replaced by opportunistic invertebrate species (Benedetti-Cecchi et al. 2001) and stress-tolerant opportunistic algal species that are able to resist high concentrations of pollutants (e.g., Castilla 1996; Contreras et al. 2005; Medina et al. 2005). In turn, this can directly or indirectly impact organisms at higher trophic levels and alter the entire ecosystem (Medina et al. 2005; Mineur et al. 2015; Latorre-Padilla et al. 2021). This is important because seaweeds are generally key-habitat forming species, playing important roles in aquatic ecosystems, and influencing biodiversity, the structure of communities, and the stability of ecosystems (Hurd et al. 2014; Schiel and Foster 2015). In this context, along most of the Chilean coast, two sister (intertidal) species of the kelp *Lessonia* are documented as being sensitive to anthropogenic pollution: *Lessonia berteroa*, in northern Chile, with a geographic distribution that spans at least from

17°S to 30°S, and *Lessonia spicata* in central Chile, from 29°S to 41°S (Tellier et al. 2009; González et al. 2012). In particular, one of the most recognized pollution case studies in northern Chile reports on the local extinction of *L. berteroa* populations resulting from a main single pollutant (i.e., copper, associated with mining operations) and its ecological and ecotoxicological effects on this kelp and associated communities (Correa et al. 1999, 2000; Medina et al. 2005; Contreras et al. 2007a, 2009). In central Chile, where *L. spicata* populations occur and our study is focused, the industrial park of Quintero Bay is characterized by high levels of pollution due to historical discharges of petroleum, gaseous pollutants, and atmospheric particulates; in addition, deposition occurs of HMs from diverse industrial facilities, including coal-fired power plants, a copper refinery and smelter, natural gas terminals, and cement companies (e.g., Contreras 2013; Bernalte et al. 2015; FIC-ALGAS 2016; Rueda-Holgado et al. 2016; Salmani-Ghabeshi et al. 2016). Moreover, previous studies found evidence of high concentrations of PAHs in the seawater of this zone (6.23–17.61  $\mu\text{g L}^{-1}$  (FIC-ALGAS 2016)), similar to those found in other highly industrialized areas around the world (e.g., Law et al. 1997; Maskouei et al. 2002; Nasher et al. 2013; Sinaei and Mashinchian 2014) and exceeding the maximum admissible concentrations of the EU, 2013).

In this environmental scenario of high levels of HMs and PAHs pollution, the question arises as to whether these toxicant mixtures present in the environment have additive, synergistic (more-than-additive), or antagonistic (less-than-additive) inhibition effects on development during the early life stages of kelps. Indeed, early life stages of marine algae, invertebrates, and fishes have been reported to be the most sensitive endpoints for ecotoxicological assessment of organic and metal pollutants compared to the adult stages (Connor 1972; Woltering 1984; Contreras et al. 2007a; Leal et al. 2016a; Boulais et al. 2018; Dorey et al. 2018; Morroni et al. 2018; Sekiguchi et al. 2018; Le Bihanic et al. 2020), where it is necessary to consider the effective concentration measured of the toxics along the experimental exposure (Leal et al. 2016a) and adequate ecotoxicological methodologies such as cleaning techniques (Leal et al. 2016b). In the case of the kelps *Macrocystis pyrifera* and *Undaria pinnatifida*, the established effective Cu concentrations causing 50% inhibition of meiospore germination ( $\text{Cu EC}_{50}$ ) were 157 and 231  $\mu\text{g L}^{-1}$ , respectively (Leal et al. 2016a). However, in *M. pyrifera*, sexual differentiation of gametophytes (oogonium and antheridia formation) was interrupted even at the lowest concentration tested (i.e., 54  $\mu\text{g L}^{-1}$ ) (Leal et al. 2016a). In the case of the kelp *L. spicata*, exposure to Cu concentrations higher than 7.87  $\mu\text{g L}^{-1}$  totally arrested gametophyte development after the spores settled (Contreras et al. 2007a). Therefore, our prediction is that, in kelps, the sensitivities of sexual differentiation and sporophyte development to



Cu and PAHs are even higher than the sensitivities of spore release and germination events to these toxicants. Accordingly, we performed in vitro experiments cultivating *L. spicata* from spores, to accomplish the following objectives: (i) to determine the individual effective concentrations ( $EC_{50}$ ) of Cu and PAHs (respectively) that cause 50% inhibition of gametophytes undergoing gametogenesis (sexual differentiation) and early (or embryonic) sporophyte formation and (ii) to assess whether the combined equitoxic  $EC_{50}$  Cu + PAH exposure has additive, synergistic, or antagonistic effects on gametogenesis and on sporophyte development (in early and juvenile sporophytes). To ensure a more specific evaluation of the individual and combined toxic effects of Cu and PAHs on gametogenesis and sporophyte development (objective ii), cultures were initiated from spores, but exposure treatments started only once they reached the sexually undifferentiated gametophyte stage (i.e., just before male and female gametogenesis) for gametogenesis and once they reached the formation the sporophyte development, respectively; this, in order to exclude the toxicants effects on earlier life events, such as spore release and germination.

## Materials and methods

### Sampling site and pre-treatment of reproductive sori

Reproductive fronds of 15 *Lessonia spicata* individuals were sampled randomly along 150–200 m of coastline at La Castilla Beach (33°27'17.70"S 71°40'23.50"W), in the Valparaíso Region of central Chile. This beach is 79 km away from the industrial focus of Quintero Bay and outside its influence. The segments of the fronds with sori were cut and then cleaned of epiphytes by washing them with iodine at 9%, rinsed with distilled water, cleaned superficially with ethanol at 7% using a small piece of cotton, rinsed with 0.22  $\mu$ m filtered seawater plus  $GeO_2$  at 5% m/v, and finally rinsed with 0.22  $\mu$ m filtered seawater. Fifteen randomly selected sori of 5 × 2 cm<sup>2</sup> (from the 15 *L. spicata* individuals previously sampled) were placed in 500 mL pre-treated glass bottles, containing 250 mL of 0.22  $\mu$ m filtered seawater and transported at 4 °C to the laboratory within 3 h of collection.

### In vitro experiments

#### Independent effects of Cu and PAH treatments on gametogenesis and sporophyte formation

Once in the laboratory, glass bottles with reproductive sori were kept under continuous agitation at 120 rpm on an orbital shaker (SEA STAR), under controlled conditions (16 °C and 20–30  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>) for 5 h. Then,

five glass Petri dishes per treatment (Cu, PAHs, or a control group with no toxicant addition), pre-treated according to US EPA (Environmental Protection Agency) (1999), were inoculated with approximately  $25 \times 10^3$  spores and filled with 5 mL of 0.22  $\mu$ m filtered seawater, and maintained until formation of sexually undifferentiated gametophytes. Afterwards, exposure to Cu and PAH solutions was initiated to test their effects on gametogenesis (sexual differentiation) in gametophytes and on sporophyte formation (early and juvenile sporophytes) from oogonia. More specifically, we assessed the inhibition effects of toxicants exposure on the total number of sexually differentiated gametophytes (producing oogonia or antheridia) and on the number of early sporophytes and juvenile sporophytes developed after 7, 14, and 21 days of exposure to toxicants, in comparison to control groups. For the independent Cu and PAH treatments, the development stages were exposed one time every 7 days to the nominal concentrations of Cu and PAHs in the ranges 0.8–200  $\mu$ g L<sup>-1</sup> and 0.05–100  $\mu$ g L<sup>-1</sup>, respectively, based on previous reports of Cu and PAH concentrations used in seaweeds and other organisms (e.g., Bellas et al. 2001; Contreras et al. 2007a; Barjhoux et al. 2012; Lovazzano et al. 2013; Babu et al. 2014; Sordet et al. 2014; FIC-ALGAS 2016; Leal et al. 2016a; Oyarzo-Miranda et al. 2020; Meynard et al. 2021). The PAH mixture used (QTM PAH Mix, Supelco, USA) contained 16 compounds considered PAH-priority pollutants by the US EPA, and which was diluted according to the manufacturer's instructions using dichloromethane:benzene 1:1. From this, a 200  $\mu$ g L<sup>-1</sup> stock solution was prepared. Concentrations of Cu and PAHs were renewed with an addition of stock solutions every 7 days. Cultures were maintained for 36–52 days of culture under constant aeration. For both the independent and combined Cu and PAH trials (detailed in the following sections), the number of sexually differentiated gametophytes (developed from undifferentiated gametophytes) and the number of early and juvenile sporophytes formed were counted using a Nikon Eclipse Ts2 inverted microscope, considering the cell number per stage in a total of 5 areas of 1 mm<sup>2</sup> per Petri dish. Counting of these microscopic stages was performed on days 7, 14, and 21 since the beginning of the toxicant exposures. More specifically, sexual differentiation counting considered the number of undifferentiated gametophytes that produced oogonia or antheridia (i.e., gametophytes undergoing gametogenesis) in these 5 areas of 1 mm<sup>2</sup> in each of the five Petri dishes replicates considered per treatment. When female and/or male gametophytes overlapped, they were generally counted as two different gametophytes. For early sporophyte formation, we counted the total number of early sporophytes that were produced by the oogonia present in the 5 areas of 1 mm<sup>2</sup> in each of the five Petri dishes replicates considered per treatment. For juvenile sporophyte formation, we counted the total number of juvenile sporophytes that were



produced by the oogonia present in the areas. For the counting of the number of (early and juvenile) sporophytes developed from oogonia, no distinction was made between those coming from a single or multiple female gametophytes. Development stages of this kelp species were categorized as described in Contreras et al. (2007a). The culture conditions for all experiments were photoperiod of 12:12, temperature in the range 10–15 °C, and 30–40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

#### Dose–response analysis on the effects of Cu and PAH treatments

The percentage inhibition (I%) of the independent and combined Cu and PAH treatments on the number of sexually differentiated gametophytes (with oogonia or antheridia) and sporophytes formed (for each replicate) was calculated (for days 7, 14, and 21 of culture) in relation to the control as follows:

$$I(\%) = \frac{N - N_0}{N} * 100$$

where  $N$  and  $N_0$  represent the number of gametophytes with oogonia or antheridia (undergoing gametogenesis) or sporophytes formed of the control sample and testing sample, respectively (Gao et al. 2020). Inhibitory concentrations of Cu and PAHs that caused 50% of inhibition ( $\text{EC}_{50}$ ) of gametogenesis, and sporophyte formation, were determined using the ED function of the DRC package in the statistical environment R (R Development Core Team R, 2020).

#### Combined effects of Cu + PAH trials on gametophyte and sporophyte formation

A second series of culture experiments was initiated from spores and maintained until sexually undifferentiated gametophytes formed and then the experiments took place. More specifically, the previously calculated relative  $\text{EC}_{50}$  values (for gametogenesis and early sporophytes formation) were used to perform additional independent and combined  $\text{EC}_{50}$  Cu and PAHs trials to determine the observed combined I (%) on gametogenesis, early sporophytes, and juvenile sporophytes formation for 21 days; the exception was the Cu effect on gametogenesis, for which an  $\text{EC}_{40}$  exposure was done instead of an  $\text{EC}_{50}$ , according to the data. This second series of culture experiments consisted of three subgroups of sexually undifferentiated gametophytes that were treated with the independent and combined  $\text{EC}_{50}$  Cu and PAHs solutions (five glass Petri dishes per treatment) initiating exposure at different development stages (i.e., from undifferentiated gametophytes for gametogenesis and sexually differentiated female gametophytes, for sporophyte formation). The first group of sexually undifferentiated gametophytes was devised to assess

the inhibition effects of the independent and combined  $\text{EC}_{50}$  Cu and PAHs treatments on gametogenesis (independent and combined treatments). The second and third groups were composed of two different subgroups (i.e., for early and juvenile sporophyte formation, respectively), for which exposures to toxicants were initiated once cultures reached the sexual differentiation (i.e., oogonia development) and the early sporophytes formation stages, respectively. These subgroups of experiments were designed to assess the inhibition effects of the (independent and combined)  $\text{EC}_{50}$  Cu and PAHs treatments on early sporophytes and juvenile sporophytes formation, respectively (for both,  $\text{EC}_{50}$  values of early sporophytes were used). Three control groups with no toxic addition for each subgroup (i.e., for gametogenesis and for early and juvenile sporophytes formation) were also included.

To assess the magnitude of the combined effects of the  $\text{EC}_{50}$ 's Cu and PAH treatments on gametogenesis and early sporophytes formation, observed and expected inhibition were compared as following. The observed independent I (%) of Cu and PAHs were added to predict the expected effects of the Cu + PAH mixture (response-addition model (Norwood et al. 2003)). Differences between observed (combined) and expected (adding independent) inhibitory effects of the mixture on gametogenesis, and early sporophyte and juvenile sporophyte formation, were tested against zero (i.e., additive response) using a one-tailed t-test to check the occurrence of additive, synergistic, or antagonistic effects, using the statistical software R (R Development Core Team R, 2020).

#### Statistical analyses on the independent and combined Cu and PAHs trials

To test the effects of each toxic concentration and their combination (Cu, PAHs, or Cu + PAHs) on the number of gametophytes undergoing gametogenesis, early sporophytes, and juvenile sporophytes formed, we applied an ANOVA on days 7, 14, and 21, independently, followed by post hoc Tukey multiple comparison tests. We first checked for the normality and homoscedasticity of the data by performing a Shapiro–Wilk test and a Bartlett test, respectively. All statistical analyses were performed using the statistical software R (R Development Core Team R, 2020).

## Results

### Independent effects of Cu treatments on gametophytes and sporophytes formation

After 21 days of exposure, Cu inhibition (I %) on gametogenesis were high for all treatments and varied between 70 and 98% compared to the control, within the tested concentration range of 5–200  $\mu\text{g L}^{-1}$  (Fig. 1a and Table S1 in



Supplementary information). The ANOVA and the a posteriori Tukey tests showed that the inhibitory effects of the two smallest tested Cu concentrations (5 and 10  $\mu\text{g L}^{-1}$ ) were clearly differentiated from the rest (Fig. 1a). Nonetheless, one additional and lower concentration of Cu (i.e., 0.8  $\mu\text{g L}^{-1}$ ) was newly tested for its effects on gametogenesis to calculate more precisely the independent  $\text{EC}_{50}$  of Cu on this development stage, which showed an inhibitory effect of 40% (Fig. 1a).

A wide range of Cu inhibitory effects were observed on early sporophyte formation (Fig. 1b), which varied between 22 and 100% compared to the control (Table S1 in Supplementary information). The statistical analysis showed that the inhibitory effects exerted on early sporophyte formation by the two lowest tested Cu concentrations (5 and 10  $\mu\text{g L}^{-1}$ ) were clearly differentiated from the rest, and above 40  $\mu\text{g L}^{-1}$  the effects were extremely high (96–100%) (Fig. 1b and Table S1 in Supplementary information).

Calculated  $\text{EC}_{50}$  values for Cu on gametogenesis and early sporophyte formation were  $1.39 (\pm 0.43) \mu\text{g L}^{-1}$  and  $11 (\pm 0.93) \mu\text{g L}^{-1}$ , respectively (Table 1).

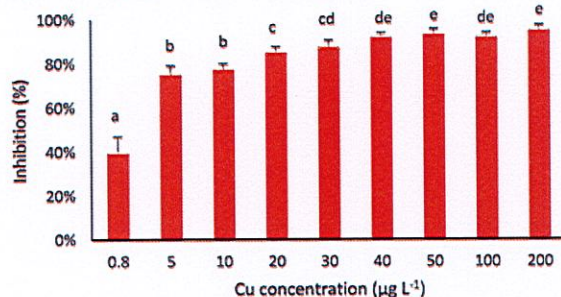
**Table 1** Effective concentrations obtained for the treatments with mixtures of Cu and PAHs ( $\mu\text{g L}^{-1}$ ), and that correspond to the concentrations of Cu and PAHs that produce a 50% inhibitory effect on the number of sexually differentiated gametophytes and on early sporophytes formed. The values indicate the mean  $\text{EC}_{50} \pm \text{SE}$

	Gametophytes $\text{EC}_{50}$ ( $\mu\text{g L}^{-1}$ )	Early sporophytes $\text{EC}_{50}$ ( $\mu\text{g L}^{-1}$ )
Cu	$1.39 \pm 0.43$	$11 \pm 0.93$
PAHs	$0.11 \pm 0.03$	$0.04 \pm 0.01$

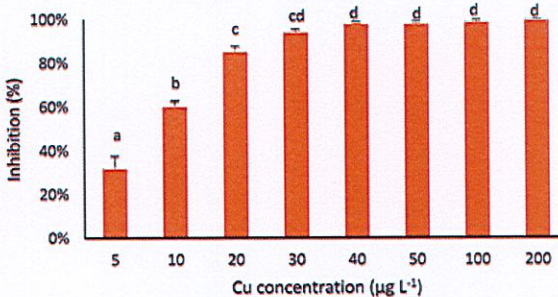
### Independent effects of PAHs treatment on gametogenesis and early sporophyte formation

Inhibitory effects of PAHs on gametogenesis increased with concentration in the range 0.05–0.5  $\mu\text{g L}^{-1}$ , with a mean I (%) varying between 8 and 55% (Fig. 1c and Table S2 in Supplementary information); nonetheless, the variation of this effect did not increase additionally with concentration in the range 1–10  $\mu\text{g L}^{-1}$  (Fig. 1c), which was corroborated by the ANOVA and the Tukey tests. By comparison, after 21 days of exposure, mean PAH inhibitory effects on early

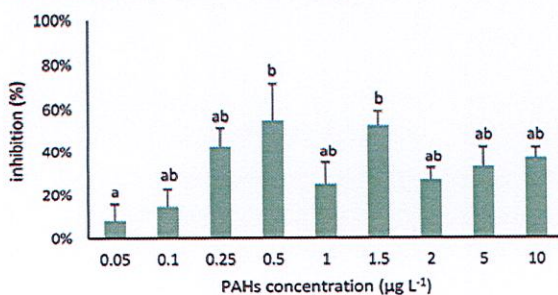
**a) Gametophytes under Cu exposure**



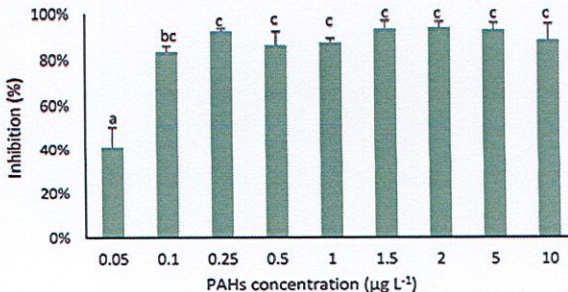
**b) Sporophytes under Cu exposure**



**c) Gametophytes under PAHs exposure**



**d) Sporophytes under PAHs exposure**



**Fig. 1** Bar plots showing inhibitory (independent) effects of tested concentrations of Cu and PAHs on the number of sexually differentiated gametophytes and early sporophytes formed after 21 days of exposure to toxicants. All life stages were exposed one time every 7 days to the following treatments: 5, 10, 20, 30, 40, 100, 200, 500, and 1000  $\mu\text{g L}^{-1}$  of Cu and 0.05, 0.1, 0.25, 0.5, 1, 1.5, 2, 5, and 10  $\mu\text{g L}^{-1}$

of the PAH mixture, respectively. One additional concentration of Cu (i.e., 0.8  $\mu\text{g L}^{-1}$ ) was newly tested for its effects on gametogenesis. Bars represent mean  $\pm \text{SE}$  ( $n=5$ ). Significant differences were determined by means of a Tukey test ( $p < 0.05$ ) and are indicated with different letters



sporophyte formation were high for all treatments, reaching their major impacts (83–100%) between 0.1 and 10  $\mu\text{g L}^{-1}$  (Fig. 1d and Table S2 in Supplementary information).

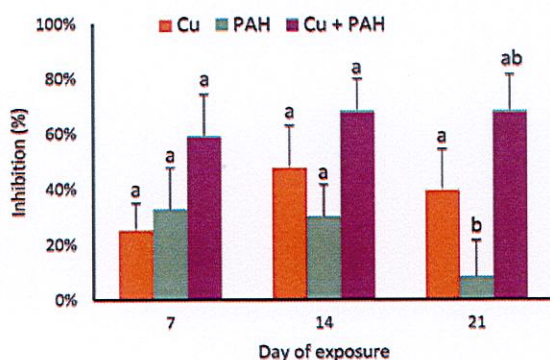
Calculated  $\text{EC}_{50}$  values for PAHs on gametogenesis and early sporophyte formation were 0.11 ( $\pm 0.03$ )  $\mu\text{g L}^{-1}$  and 0.04 ( $\pm 0.01$ )  $\mu\text{g L}^{-1}$ , respectively (Table 1).

### Combined effects of Cu + PAH trials on gametogenesis and early and juvenile sporophyte formation

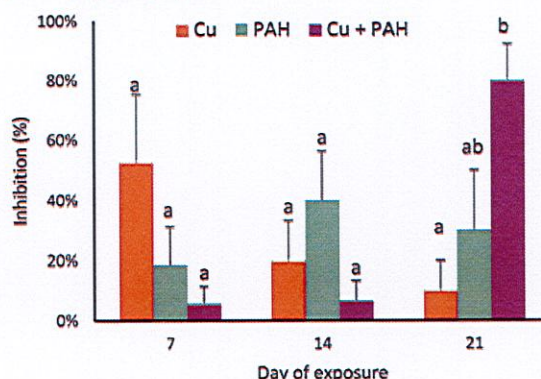
The inhibition of the  $\text{EC}_{50}$  Cu + PAH mixture on gametogenesis was higher and statistically different from the effects of the independent Cu and PAH treatments during all of the exposure time (Fig. 2a). The interaction of Cu and PAH treatment on gametogenesis was not different from an additive effect on days 7, 14, and 21 (Fig. 3).

The inhibition of the  $\text{EC}_{50}$  mixture Cu + PAHs on early sporophyte formation increased significantly over the study period (Fig. 2b), being synergistic on day 21 of culture (Fig. 3). It is noteworthy that, if we examine the raw toxicological magnitude of the independent Cu and PAH treatments on juvenile sporophyte formation, they reached extremely high and maximum individual inhibition values, particularly on day 14 of culture with more than 80% of inhibition (Fig. 2c). By comparison, the combined  $\text{EC}_{50}$  Cu + PAH treatment always exerted a less than additive (antagonistic) effect on juvenile sporophytes formation during all the exposure period (Figs. 2c and 3). Finally, a clear depigmentation or bleaching of all microscopic life stages considered here was observed under the independent  $\text{EC}_{50}$  Cu or PAH treatments but was more evident in the combined  $\text{EC}_{50}$  Cu + PAH exposure (Fig. 4). This is probably due to a low capacity of

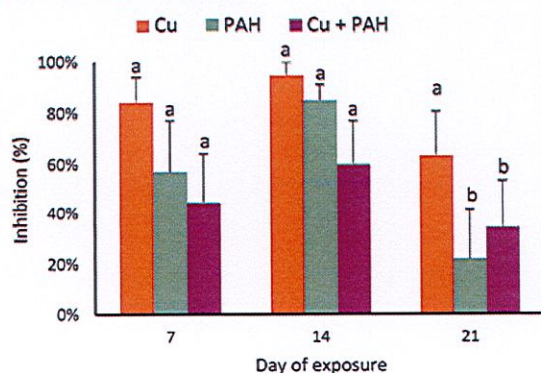
#### a) Gametophytes



#### b) Early sporophytes



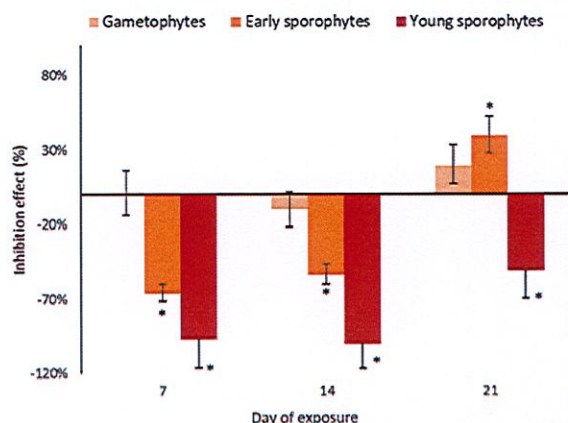
#### c) Juvenile sporophytes



**Fig. 2** Bar plots showing inhibitory (independent and combined) effects of the  $\text{EC}_{40}$  and  $\text{EC}_{50}$  copper (i.e., 0.8  $\mu\text{g L}^{-1}$  for gametophytes and 11  $\mu\text{g L}^{-1}$  for early and juvenile sporophytes),  $\text{EC}_{50}$  PAHs (i.e., 0.11  $\mu\text{g L}^{-1}$  for gametophytes and 0.04  $\mu\text{g L}^{-1}$  for early and juvenile sporophytes), and  $\text{EC}_{50}$  Cu + PAHs treatments on the number of sexu-

ally differentiated gametophytes (gametogenesis), early sporophytes, and juvenile sporophytes formed after 7, 14, and 21 days. Bars represent mean  $\pm$  SE ( $n=5$ ). Significant differences on day 21 were determined by means of a tukey test ( $p < 0.05$ ) and are indicated with different letters





**Fig. 3** Differences between the observed inhibitory effect versus the expected inhibitory effect (under the assumption of additivity) on the number of sexually differentiated gametophytes, early sporophytes, and juvenile sporophytes formed when exposed to the combined  $EC_{50}$  Cu + PAHs treatments in *L. spicata*. Bars represent mean  $\pm$  SE ( $n=5$ ). The differences were evaluated against zero (additivity) by means of a t-test; \* $p < 0.05$ . Inhibition values significantly higher or lower than zero correspond to a synergistic or antagonistic response, respectively

*L. spicata* to mitigate oxidative stress induced by HMs and PAHs, and a concomitant reduction of photosynthetic pigments and photosystem II efficiency, as discussed in the next section.

## Discussion

Our results show that in *L. spicata*, gametogenesis is the development stage most affected by Cu toxicity, whereas PAH toxicity exerted a higher impact on sporophytes

formation. Nonetheless, the most harmful effect on the early development of *L. spicata* was the toxic exposure to the combined  $EC_{50}$  Cu + PAHs, which manifested high negative effects faster and earlier (on gametogenesis) than the independent Cu or PAH treatments (on juvenile sporophyte formation).

The calculated Cu  $EC_{50}$  values on gametogenesis and early sporophyte formation in *L. spicata* were 1.39 and 11  $\mu\text{g L}^{-1}$ , respectively. These values are one to three orders of magnitudes lower than  $EC_{50}$  values reported previously for germination in *L. spicata* (i.e., 212  $\mu\text{g L}^{-1}$  (Meynard et al. 2021)) and in *M. pyrifera* (i.e., 157  $\mu\text{g L}^{-1}$  (Leal et al. 2016a)). In the present study, individual Cu inhibitory effects on gametogenesis and sporophyte formation were very high for most concentrations tested in the range 5–200  $\mu\text{g L}^{-1}$ ; gametogenesis was particularly sensitive to Cu exposure, with 70% inhibition at the lowest concentration initially tested, i.e., 5  $\mu\text{g L}^{-1}$ . In general, our findings are consistent with the results of Contreras et al. (2007a) in *L. spicata* and Leal et al. (2016a) in *M. pyrifera*, which showed that gametophyte growth and gametogenesis (but not germination of spores) are completely arrested for tested copper nominal concentrations higher than 7.87 and 100  $\mu\text{g L}^{-1}$ , respectively. The present study confirmed this pattern more accurately, by performing Cu exposure treatments on cultures only once undifferentiated gametophytes, and oogonia were formed, depending on the developmental stage assessed. The greater inhibitory effects of copper on gametogenesis and sporophyte formation compared to germination may be explained because spores depend mostly on internal lipid reserves, with triacylglycerols as the main initial energy source (Reed et al. 1999; Brzezinski et al. 1993; Steinhoff et al. 2011). On the contrary, metabolism of gametophytes and sporophytes mostly relies on photosynthesis (Steinhoff et al. 2011), which is impaired by toxic Cu exposure in

**Fig. 4** Representative images of early life stages of *Lessonia spicata* showing contrasting levels of pigmentation (i.e., pigmented versus depigmented, respectively) between the control (a–c) and the combined  $EC_{50}$  Cu + PAH treatment exposures (d–f). After the start of the experiments, we observed a, d sexually differentiated gametophytes between days 5 and 7; b, e sexually differentiated female gametophytes with egg cells fecundated and early sporophytes formed between days 15 and 30; and c, f juvenile sporophytes after 30 days. Scale bar = 50  $\mu\text{m}$





plants and algae (Aggarwal et al., 2012); additionally, high levels of copper disrupt cell division and interfere with several processes occurring in subcellular organelles, such as respiration, mitochondrial electron transport, and ATP production (Contreras-Porcia et al. 2017).

This is the first study demonstrating the direct negative impacts of PAHs exposure on early life stages of brown algae, in this case, sexual differentiation (i.e., gametogenesis) and sporophyte formation. Our findings revealed that the most important bottleneck induced by PAH toxicity was the negative impact on sporophyte formation, rather than on gametogenesis. Indeed, PAHs inhibitory effects on sporophyte formation were higher than 83% for most tested concentrations (i.e., in the range  $0.1\text{--}10\text{ }\mu\text{g L}^{-1}$ ), with the exception of the  $0.05\text{ }\mu\text{g L}^{-1}$  treatment with a mean inhibition of 40%. These effects would possibly result from the toxic action exerted directly by PAHs and/or PAH metabolites produced during the PAH xenobiotic phase I of biotransformation.

By comparison, PAH exposure had a greater negative impact on sporophyte formation than on gametogenesis, whereas Cu exposure had a greater impact on gametogenesis. This suggests that the two microscopic development processes would be affected differently by these two toxicants. Indeed, because of differences in stress sensitivity and resource/energy allocation patterns and depending on the stressor, different developmental stages of plants and invertebrates are affected at their most energy-demanding pathways (Sulmon et al. 2015). For example, we could hypothesize that, in the case of *L. spicata*, PAH stress took longer and impacted mainly growth-related traits in sporophyte development, whereas the negative impact of Cu was faster and earlier, and affected reproduction-related traits (i.e., gametogenesis). This is consistent with the fact that, in higher plants, photosynthesis, in addition to chlorophyll, carotenoid, protein biosynthesis, and gluconeogenesis, are downregulated at the gene level by toxic PAH exposure (Weisman et al. 2010), which probably affects more growth- than reproduction-related traits. This is also consistent with the fact that, in photosynthetic organisms, PAHs induce reactive oxygen species (ROS) overproduction, causing a major metabolic shift from anabolism to catabolism (e.g., Weisman et al. 2010; Sinaei and Loghmani 2019). An alternative explanation could be that higher plants and algae have evolved complex metabolic mechanisms allowing them to actively regulate copper homeostasis, such as specific Cu cellular membrane transporters, metallochaperones, and P1B-type ATPases (Blaby-Haas et al. 2012). Therefore, in contrast to the active intracellular transport of Cu, the passive uptake of PAHs and slower membrane damage due to partitioning of PAHs into lipid membranes (Gauthier et al. 2014) could be a potential explanation for the faster, greater, and earlier toxicological effect of Cu (i.e., on gametogenesis)

compared to the later effect of PAHs (on sporophyte formation). It is also noteworthy that the higher negative impacts of both the independent  $\text{EC}_{50}$  Cu or PAH trials through development were later (than the combined Cu + PAH trial effects), with juvenile sporophyte formation being highly affected (more than 80% inhibition) by both treatments on day 14 (Fig. 2c).

We were unable to discern the factors explaining the peculiarly high inhibitory effects of PAH exposure trials on *L. spicata* sporophyte development (range  $0.1\text{--}10\text{ }\mu\text{g L}^{-1}$ ) compared to the inconsistent pattern of their inhibition effects on gametogenesis, particularly for concentrations higher than  $0.5\text{ }\mu\text{g L}^{-1}$  (in the range  $1\text{--}10\text{ }\mu\text{g L}^{-1}$ ). We think that this inconsistent pattern of inhibition of gametogenesis does not depend on experimental artifacts, because the same conditions and concentrations were applied to early sporophytes formation, which showed increasing inhibition with increasing toxicant concentrations. This probably indicates that the effects of PAHs on this kelp are differentials along the life cycle stages (i.e., gametogenesis vs sporophyte formation). At the cellular level, one hypothesis could be that lipids of the cellular membranes would interact in contrasting manner with structurally different PAHs throughout the early development. This may be because the lipid content of different life stages of brown algae (i.e., zoospores, gametophytes, and adult sporophytes) would exhibit variation in their fatty acid composition with developmental stage, as suggested in *Saccharina latissima* by Steinhoff et al. (2011). Indeed, a recent study using phospholipid Langmuir monolayers as a model of bacterial membranes showed that structurally different PAH molecules are differentially incorporated into membranes (Broniatowski et al. 2017). Some of these, such as 4- and 5-ring angular and cluster PAH molecules (e.g., benzo[a]pyrene and pyrene), strongly interact with phospholipids and are incorporated into the model membranes, deeply changing their textures and fluidity, whereas large cluster PAHs (e.g., coronene and dibenzo[b,def]chrysene) cannot be incorporated and separate from the lipid matrix (Broniatowski et al. 2017). Alternatively, the inconsistent relationship between increasing PAH concentrations and inhibition impact, particularly on gametogenesis, could also arise from various loss processes that influence PAH exposures (i.e., experimental artifacts), such as volatilization, sorption, biodegradation, and aqueous photolysis of PAHs (Bragin et al. 2016). This problem may be solved by passive dosing, in which PAHs are loaded onto an inert polymer (e.g., a silicone O-ring) and then added to the test media, allowing stable exposure concentrations. Additionally, because bioavailability of PAHs is influenced by their molecular structure and size, it would be useful to determine their dissolved concentrations over time in the culture medium used for the different life stages, considering also blank treatments without addition of early life stages.



This would allow discerning the relative proportion of PAHs adsorbed by the cells, on the culture vessel walls, or that volatilize (Christensen and Rorrer 2009).

In terms of the negative effects of PAHs on photosynthetic organisms, there is also evidence in higher plants and freshwater microalgae that PAH exposure reduces the growth rate, the chlorophyll *a* and *b* levels, and other photosynthetic pigments (e.g., Jajoo et al. 2014; Kottuparambil & Park 2019); this is because PAHs inhibit the function of the photosystem II by inducing ROS overproduction (Kottuparambil & Park 2019). Decreased concentrations of photosynthetic pigments and reduction of the efficiency of the photosystem II are induced by exposure to Cu in sensitive brown and green species of algae (e.g., Han et al. 2008; Nielsen et al. 2003), which is also probably due to their low capacity to mitigate oxidative stress (e.g., Contreras et al. 2009; Sáez et al. 2015). Sensitivity of *L. spicata* to both PAH and Cu stresses, and overproduction of ROS, probably also explains the observed depigmentation of gametophytes and sporophytes under the independent EC<sub>50</sub> PAH or Cu exposures of this study, which were nonetheless less evident than the depigmentation observed under the combined EC<sub>50</sub> Cu + PAH treatment, as previously mentioned. More generally, one of the reasons why intertidal *Lessonia* species (i.e., *L. berteriana* and *L. spicata*) are not able to thrive in pollutant-enriched environments could be their insufficient physiological tolerance to HMs and other chemicals in comparison with tolerant species such as *Scytosiphon lomentaria*, *S. gracilis*, *Ulva compressa*, and *Dictyota kunthii*. In contrast, these tolerant algal species show high activities of antioxidant enzymes (i.e., catalase, glutathione peroxidase, ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase, or the thiol-dependent peroxidases peroxiredoxins), the defense enzyme lipoxygenase, and antioxidant compounds, and lower accumulation of lipoperoxides, than *Lessonia* spp. (Contreras et al. 2005, 2007b, 2009; Lovazzano et al. 2013; Sordet et al. 2014). In addition, as demonstrated recently by Oyarzo-Miranda et al. (2020), the in vitro culture of *L. spicata* using seawater from three coastal sites located at increasing distance (0–40 km) from the highly polluted industrial park area (Ventanas, Horcón, and Cachagua) led to a significant delay in gametophyte formation. Furthermore, all three seawater treatments resulted in low sporophyte production (25–50%), suggesting that pollution has sub-lethal toxic effects on the microscopic life stages of this kelp.

The maximum negative effect of the combined EC<sub>50</sub> Cu + PAH treatment manifested earlier (i.e., on gametogenesis) than the independent treatments and remained high throughout the three development stages of *L. spicata* considered here. Therefore, it was overall more harmful than the independent EC<sub>50</sub> Cu or PAH exposures. Nonetheless, the combined effect of the EC<sub>50</sub> Cu + PAH treatment on

gametogenesis was always additive during all of the exposure period (days 7, 14, and 21) (Fig. 3). On the contrary, the combined effect of the EC<sub>50</sub> Cu + PAH treatment on early sporophytes development was initially additive on days 7 to 14 but became synergistic on day 21 of exposure (Figs. 3 and 2b). In our case, the rapid manifestation of harmful impacts on gametogenesis, and the more-than-additive effect of the EC<sub>50</sub> Cu + PAH exposure on early sporophytes on day 21, could result directly from the chemical interaction of PAHs and Cu, or indirectly, from the mutual inhibition or alteration of their transport, detoxification mechanisms, and metabolic pathways. For example, in other non-algal aquatic organisms, metals inhibit the monooxygenase CYP1A, an enzyme from phase I of the detoxification of PAHs; this alteration shifts the usual metabolic pathways of PAH detoxification by inducing the formation of derivatives with greater toxicity, such as PAH-quinones (PAHQs) and, subsequently, PAHQ-derived ROS. PAHQs subsequently can inhibit the metal-binding protein metallothionein (MT), inducing a rise in the availability and toxicity of copper (Gauthier et al. 2014). Additionally, membrane damage attributed to PAHs partitioning into lipid membranes increases membrane fluidity and compromises ion homeostasis. Moreover, by deactivating P-type ATPases such as Cu-ATPase in the membrane, PAHs inhibit the efflux of Cu, which also causes a rise in intracellular copper concentration and toxicity (Gauthier et al. 2014).

In this study, the median effective concentrations of Cu and PAHs reducing *L. spicata* gametogenesis and sporophyte development by 50% (EC<sub>50</sub>) were even lower than the international standard for copper and PAHs in seawater (see FIC-ALGAS 2016; Oyarzo-Miranda et al. 2020), indicating a high sensitivity of this important kelp species to these toxicants. Indeed, the combined EC<sub>50</sub> Cu + PAH trials on gametogenesis and sporophyte formation were even more harmful than the independent Cu or PAH exposures. Nonetheless, these results should be interpreted with caution and a number of limitations should be borne in mind. First, toxic trials of this study, which used a mixture containing 16 compounds considered PAH-priority pollutants by the US EPA, are not necessarily representative of the actual PAHs along the Chilean coast. Thus, it is vital to accurately estimate the type and sources of PAHs (i.e., pyrogenic or petrogenic) and the actual ratio of low molecular weight (LMW) versus high molecular weight (HMW) PAHs, in pollutant mixtures of coastal areas affected by anthropogenic pollution. On the other hand, due to the high number of experimental units of this study, exposition to toxicants was based on nominal concentrations. It would be necessary to carry out additional experiments to specify more accurately and measure the effective or dissolved concentration of toxicants in the culture medium, because, as pointed out in the study of Leal et al. (2016a) for copper, more than 50% of this



toxicant added to the culture medium was adsorbed onto the culture vessel walls and by the cells. Nonetheless, since the nominal concentrations of Cu and PAHs that caused an inhibition of 50% of gametogenesis and sporophyte formation were very low for *L. spicata*, this consolidates the fact that this important kelp species is extremely sensitive to these toxicants. It is also necessary to establish the dynamics and fate of PAHs contingent upon mechanisms, such as photo-oxidation, evaporation, dispersion, and biodegradation, and the vertical sinking of PAH particles or their remobilization in sediments (Duran and Cravo-Laureau 2016). A better understanding of the main parameters determining the fate of copper and PAHs in particular environmental conditions (in this case along the coast of Quintero Bay in central Chile) and its analytical integration with the response of early development of kelps would aid in better characterization of the risk of copper and PAHs in this kelp and seaweeds in general. More generally, the direct effects of chemical contaminants on the performance of early development stages of kelps observed in this study could have long-term consequences for the abundance and persistence of kelp populations. Because kelps play key ecological roles (influencing biodiversity and stability of aquatic ecosystems), a decline in their population size or their local extinction could subsequently translate into indirect cascading effects at all trophic levels within a marine community.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10811-021-02552-7>.

**Acknowledgements** This work was supported by ANID FONDECYT No. 1170881, ANID PIA/BASAL FB0002, and ANID Millennium Science Initiative Program ICN 2019\_015. We also thank LEBMA ([www.lebma.cl](http://www.lebma.cl)) for technical support.

**Author contribution** L-CP, C-EG, and AM designed the study and wrote the manuscript. C-EG, AM, N-LP, AN, and FC performed the practical work and statistical analysis. C-OM and JR contributed to writing the manuscript. All authors commented on previous versions of the manuscript. Review and editing were done by L-CP and AM.

**Funding** This study was funded in Chile by ANID FONDECYT 1170881, ANID PIA/BASAL FB0002, and ANID Millennium Science Initiative Program ICN 2019\_015.

**Data availability** Derived data supporting the findings of this study are available from the corresponding author (L. Contreras-Porcía) on request.

## Declarations

**Competing interests** The authors declare no competing interests.

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