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Chronic exposure of two pesticides in jellyfish polyps

Polyps of the jellyfish *Aurelia aurita* are unaffected by chronic exposure to a combination of pesticides

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Abstract: Pesticides are a major contaminant in coastal waters and can cause adverse effects in marine invertebrates such as jellyfish. Most studies have investigated short-

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term responses of organisms to unrealistically high concentrations of pesticides; however, chronic exposure to persistent low concentrations, which are more likely to occur in the environment, are rarely analyzed. Here, we tested the response of polyps of the Moon jellyfish *Aurelia aurita*, to environmental concentrations of the herbicide atrazine and the insecticide chlorpyrifos, individually and in combination, over nine weeks. We hypothesized that exposure to individual pesticides would reduce rates of asexual reproduction and alter polyps' metabolite profiles, and that the results would be more severe when polyps were exposed to the combined pesticides. Polyps survived and reproduced (through budding) in all treatments and no differences among treatments were observed. Proton nuclear magnetic resonance (^1H NMR) spectroscopy revealed no difference in profiles of polar metabolites of polyps exposed to the individual or combined pesticides. Our results suggest that *A. aurita* polyps are unaffected by chronic exposure to atrazine and chlorpyrifos at concentrations recommended as being protective by current Australian water quality guidelines.

Graphical Abstract

Key words: Atrazine, Chlorpyrifos, Aquatic invertebrates, Aquatic toxicology, Herbicide, Insecticide, Metabolites, Stress response

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1. INTRODUCTION

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Approximately 3.5 million tons of pesticides are used globally each year (Pretty and Bharucha 2015) to maximize agricultural production by killing, controlling or incapacitating problematic plants (herbicides), insects and arachnids (insecticides) and fungi (fungicides) (FAO 2016). Although pesticides are designed and marketed to target pest species, non-target organisms are often susceptible due to the conserved nature of biochemical pathways across taxa (Damalas and Eleftherohorinos 2011; Pisa et al. 2015). As such, pesticides can pose a considerable risk to non-target organisms when they enter aquatic environments (Ormad et al. 2008; Caquet et al. 2013; Davis et al. 2013). Furthermore, since multiple pesticides are often applied to crops, chemical ‘cocktails’ are frequently detected in coastal waters where they may act synergistically or additively causing a wide range of adverse health effects in exposed aquatic species (Relyea 2009; Magnusson et al. 2010; Nunes et al. 2018). Pesticides usually persist at relatively low concentrations in coastal waters and generally exceed water quality guidelines only after periods of heavy rainfall (Davis et al. 2012; Gallen et al. 2019). Despite this, toxicological studies (Jones and Kerswell 2003; Vera Candioti et al. 2010), often investigate acute (i.e. high concentrations for short periods of time) rather than chronic exposure (i.e. persistent and low concentrations) despite chronic exposure being experienced most commonly (Negri et al. 2015; Cantin et al. 2007).

Herbicides and insecticides are the most frequently detected pesticides in river catchments, intertidal and subtidal marine environments (Shaw et al. 2010). The herbicide atrazine is one of the most commonly used and detected pesticides in the coastal waters of Queensland, Australia (APVMA 2014; Gallen et al. 2019). Atrazine is

a photosystem II (PSII) herbicide that inhibits photosynthesis by blocking electron transport in the PSII of plants (Oettmeier 1992; Jones and Kerswell 2003). Exposure to atrazine can be detrimental to animals with photosynthetic symbionts (e.g. sea anemones (Howe et al. 2017)) but can also affect non-photosynthetic animals, for example, by reducing growth rates of fish (del Carmen Alvarez and Fuiman 2005). Chlorpyrifos, is among the top five most commonly detected insecticides in Queensland's coastal waters (Shaw et al. 2010; Gallen et al. 2019). Chlorpyrifos, is an acetylcholinesterase inhibitor that disrupts nerve impulses, which can ultimately lead to paralysis and death of invertebrates (Corbett 1974; Van Emden 2004). Other effects in non-target species exposed to chlorpyrifos include reduced settlement and metamorphosis of coral larvae (Markey et al. 2007), and decreased hatching success (Mhadhbi and Beiras 2012) and muscle cholinesterase activity in fish (Botte et al. 2012). Considering the intense use of atrazine and chlorpyrifos in agriculture and that they often co-occur in aquatic environments (Shaw et al. 2010; Smith et al. 2012; O'Brien et al. 2016), research on potential interactive effects of these pesticides in marine species is warranted.

Sub-lethal effects on physiological or metabolic endpoints are essential considerations for chemical toxicity evaluation because they often manifest at low concentrations and may be linked to individual and ultimately to population health (Viant 2007; Lankadurai et al. 2013). Measurements of growth and reproduction, for example, are recognised as meaningful non-invasive parameters that provide unambiguous information about responses to pesticide exposure (Bejarano and Chandler 2003). Cellular or molecular analysis, such as metabolomics, offers insights regarding broad molecular changes that could occur in an individual as a result of chemical exposure, and, thus provides

information that may relate to the mechanistic underpinnings of observed higher-level effects (Lin et al. 2007; Bouhifd et al. 2013). Since pesticides can have physiological (Yasmin and D'Souza 2010), molecular (Van Meter et al. 2018) and cellular (Akcha et al. 2016) effects on non-target species, the integration of multiple approaches to ecotoxicology studies will provide a better understanding of the overall pesticide toxicity.

Jellyfish are often claimed to be robust to anthropogenic stressors (e.g. Purcell 2007; Richardson et al. 2009); but rigorous experiments to support such claims are scarce (Pitt et al. 2018). Indeed, recent studies indicate that some jellyfish taxa exhibit negative sub-lethal responses to climate stressors (Klein et al. 2014) and chemical pollutants (Klein et al. 2016). More robust experiments examining both lethal and sub-lethal responses to a range of different stressors are thus needed to understand how jellyfish populations may respond to changing ocean conditions.

The jellyfish genus *Aurelia* (Cnidaria: Scyphozoa) has been proposed as a model organism in ecotoxicology due its sensitivity to pollutants such as crude oil (Almeda et al. 2013; Echols et al. 2016), organic chemicals and heavy metals (Faimali et al. 2014). *Aurelia* is distributed along tropical, temperate and sub-polar coastlines of the world (Kramp 1961; Arai 1997) and like most scyphozoans, has a metagenetic life cycle that includes a pelagic medusa that reproduces sexually and a benthic polyp that reproduces asexually through budding and strobilation (Arai 1997).

Only three studies have examined the effects of pesticides on jellyfish (Costa et al. 2015; Klein et al. 2016; Rowen et al. 2017). Although these studies used a wide range of

pesticide concentrations, exposure duration never exceeded seven days, and none investigated potential interactions between pesticides. Costa et al. (2015), reported that high concentrations (5-50 mg/L⁻¹) of the pesticides eserine and chlorpyrifos inhibited pulsation and the mobility of *Aurelia aurita* ephyrae but the interactions between pesticides were not examined. Rowen et al. (2017), observed reduced growth, photosynthetic efficiency and zooxanthellae density after *Cassiopea maremetens* medusae were separately exposed to the pesticides diuron and hexazinone at environmentally relevant concentrations. Klein et al. (2016) was the only study to consider how responses to pesticides may interact with changing environmental conditions, by testing the individual and combined effects of the herbicide atrazine and salinity. In that study, medusae of the upside-down jellyfish, *Cassiopea* sp. exhibited lower survival, photosynthetic efficiency, growth and bell contraction rates when simultaneously exposed to high atrazine (20 µg/L⁻¹) and low salinity (17 ppt) conditions, highlighting the need to consider interactions between stressors (Klein et al. 2016). Longer-term studies that consider relevant chronic exposure conditions and evaluate interactive effects of common pesticides are needed to broaden our understanding of the effects of these chemicals on jellyfish, and on marine species in general.

The aim of the present study was to investigate potential interactive effects of the herbicide atrazine and insecticide chlorpyrifos on polyps of *Aurelia aurita*. We studied the effects of chronic exposure to low concentrations and measured sub-lethal response variables, including rates of asexual reproduction and profiles of polar metabolites. We hypothesised that exposure to atrazine and chlorpyrifos in combination would impart more severe effects than exposure to the individual pesticides.

2. MATERIAL AND METHODS

2.1 Experimental approach

Polyps of *Aurelia aurita* were obtained from Underwater World (Sunshine Coast, Queensland) and acclimated to laboratory conditions for two weeks. Throughout the acclimation period and experiment, polyps were maintained in a controlled temperature room at 24°C, which represents the average summer sea temperature in southern Queensland. Salinity was maintained at 35 ppt and polyps were exposed to a 12:12 hr light:dark cycle using fluorescent aquarium lights (Aqua Zonic Super Actinic Blue and Aqua Zonic Super Sun) to imitate the natural solar spectrum. Polyps were fed newly hatched *Artemia* sp. nauplii twice a week.

The experiment consisted of two orthogonal factors: atrazine (with two levels: 0 µg/L⁻¹ and 2.5 µg/L⁻¹) and chlorpyrifos (0 µg/L⁻¹ and 0.04 µg/L⁻¹). The chosen concentrations did not exceed current Australian water quality guideline values and were below the 90% trigger value for the protection of species (Authority 2010). The experiment ran for nine weeks. Replicates consisted of glass petri dishes, each containing 11 attached polyps that were immersed in 400 mL glass beakers filled with fresh filtered (10 µm) seawater. Ten replicate beakers were randomly assigned to each treatment. The water in the beakers was changed daily with fresh filtered seawater and the appropriate pesticide concentration. Every week, new stock solutions of atrazine (2-Chlor-4-Ethyl-Amino-6-Isopropylamino-1,3,5-triazine; sourced from Sigma-Aldrich, CAS number 1912-24-9) and chlorpyrifos (0, 0-diethyl 0-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate; sourced from Sigma-Aldrich, CAS 2921-88-2) were prepared by dissolving them in filtered seawater (10 µm). Stock solutions were sonicated daily prior to use, to ensure

homogenous solutions were added to the beakers. Temperature and salinity values were verified by daily measurements using a conductivity-salinity meter (TPS salinity-conductivity meter, Aqua-Cond/pH).

Response Variables

The number of polyps and the number of asexual buds were measured once a week using a dissection microscope to assess rates of survival and asexual reproduction. At the end of the experiment, 120 polyps per treatment were sacrificed for analysis of their metabolomes. Metabolomic samples were processed using the protocol of Melvin et al. (2017). Polyps were quickly transferred into plastic tubes containing 400 μ l of cold methanol and incubated at -20°C to extract metabolites. After 24 hours, 800 μ l of chloroform and 300 μ l of distilled water were added, the samples were vortexed and centrifuged (10 min, 16,000 x g, at 4°C) and the organic and polar phases were separated into glass vials. The extraction was repeated, and final samples were stored at -80°C . The polar phase was dried using a Series II centrifugal vacuum concentrator (GeneVac Technologies, England) and resuspended in 200 μ l 0.01M phosphate buffer (K_2HPO_4 and NaH_2PO_4 ; pH 7.4) made with deuterium oxide (D_2O), containing 0.05% sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal standard. Samples were transferred to 3 mm NMR tubes using glass pipettes for nuclear magnetic resonance (NMR) spectroscopy and spectra were acquired with an 800 MHz Bruker® Avance III HDX spectrometer. The NMR was equipped with a Triple (TCI) Resonance 5mm Cryoprobe with Z-gradient and automatic tuning and matching, and a SampleJet automatic sample changer controlled via the software IconNMR™ (Bruker Pty Ltd., Victoria, Australia). Spectra were acquired at 298 K, using D_2O for field locking and

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TSP (^1H δ 0.00) as an internal reference. Proton (^1H) spectra were acquired using the zg30 pulse program with 128 scans, 0.8s relaxation delay, 8.75 μs pulse width and 16 kHz spectral width (^1H δ -3.75-16.28). An edited ^1H - ^{13}C HSQC spectra was acquired for a pooled sample using the pulse sequence hsqcedetgpsisp2.2 with 32 scans and 128 experiments, 0.8 s relaxation delay, 8.75 μs pulse width and spectral widths of 12.8 kHz (^1H δ -3.23-12.79) and 33.1 kHz (^{13}C δ -9.40-155.2).

MestReNova v11.0 (Mestrelab Research S.L., Spain) was used for post processing of NMR spectra. ^1H NMR FIDs were Fourier transformed with line broadening of 0.3 Hz and HSQC spectra were processed with default Bruker post-processing parameters. All spectra were manually phase corrected, automatically baseline adjusted (ablative), and referenced and normalised to TSP (^1H δ 0.00). Once processed, discrete spectra features (i.e., peaks corresponding to metabolites) were manually integrated and exported to Excel for further processing and multivariate statistical analysis. The metabolites were tentatively assigned to ^1H NMR spectra by comparing with HSQC NMR spectra and using published reference spectra from the Human Metabolome Database (HMDB).

2.2 Measurements of pesticides concentrations

Samples from stock solution of both atrazine and chlorpyrifos were analysed through high performance liquid chromatography (HPLC) (LCMS/QTOF) to find detection limits for the pesticides and construct a standard curve. The concentrations of atrazine and chlorpyrifos in the treatments were analysed following the same methodology as Klein et al. (2016). Each week, 400 mL of seawater from three replicates per treatment were randomly selected and transferred into a 500 mL separation funnel and 100 mL of

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methylene chloride (DCM) was added, mixed and evaporated using a Roto-Vap (150 rpm, 490 mbar at 30°C). 1 mL of methanol was added to collect the precipitated material and directly injected and run using 1 x 100mm/4.6mm Agilent Infinity CA₁₈ 100Å column (Model Agilent 1290 HPLC System). Samples were analysed through HPLC (LCMS/QTOF) at a flow rate of 1mL min⁻¹ with a linear gradient starting from 0 to 50% water to acetonitrile (0-10 min) for atrazine samples and 50 to 100% water to acetonitrile (0-10 min) for chlorpyrifos samples. The analysis confirmed the mean (±SE) concentrations of atrazine and chlorpyrifos were 2.49 (±0.012) µg/L⁻¹ and 0.04 (±0.007) µg/L⁻¹ respectively and this remained stable throughout the experiment.

Data analysis

Variation in numbers of polyps among treatments was analysed using repeated measures linear mixed models (LMMs) in SPSS (IBM SPSS Statistics 25.0). Prior to analyses, the data were checked for normality and homoscedasticity using standardized residual plots and Q-Q plots and transformed if needed. The factors analysed were atrazine, chlorpyrifos and time, which was the repeated measure. A range of models (e.g. AR(1), AR(1): heterogeneous and Compound Symmetry (CS)) were investigated to assess the model of best fit by comparing several goodness-of-fit statistics (e.g. -2 restricted log likelihood, Akaike's information criteria (AIC) and Bayesian information criteria (BIC)). When significant differences were found, estimated marginal means were used to determine which means differed.

Metabolomics data were analysed for visual separation of treatments using MetaboAnalyst 4.0 (Xia and Wishart 2016). Principal Component Analysis (PCA) and

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supervised Partial Least Squares Discriminant Analysis (sPLS-DA) were performed to explore differences in the complete metabolite profiles extracted from polyps, between treatment groups.

3.RESULTS

3.1 Survival and asexual reproduction

All polyps of *Aurelia aurita* survived the experiment, no strobilation was observed, and the average number of polyps did not differ between treatments at any time point.

Numbers of polyps remained relatively constant from weeks 1-8 but increased in all treatments during week 9 ($F_{8, 221} = 10.331$, $p = <0.001$) (Fig. 1, Table 1).

3.2 Metabolomic profile

Twenty-four metabolites were identified (Fig. 2a, 2b, 2c) of which 14 were common α -amino acids: isoleucine, leucine, valine, serine, alanine, proline, glycine, d-lysine, methylhistidine, cysteine, creatine, glutamic acid, tyrosine and tryptophan; two were amines: dimethylamine and diethanolamine; three were acids: malonate, formate and acetate; three were osmoregulators: taurine, betaine and homarine; and two were metabolic energy sources: glucose and pyruvic acid. The overall metabolome profile did not differ among treatments (Fig. 3a, 3b).

4. DISCUSSION

Aurelia aurita polyps were unaffected by chronic exposure to current Australian water quality guideline values of atrazine and chlorpyrifos, either separately or in combination.

Our hypothesis that exposure to combined pesticides would be more detrimental than exposure to individual pesticides, therefore, was not supported.

Few studies are available with which to directly compare our results. To our knowledge, the only other study investigating the effects of pesticides on *A. aurita* examined the acute toxicity of eserine and chlorpyrifos on the ephyrae stage of the life-history (Costa et al. 2015). That study observed that the frequency of pulsation of the ephyrae decreased with increasing concentrations of chlorpyrifos. The exposure concentrations used in that experiment, however, greatly exceeded (by 4 orders of magnitude) those detected in the environment (Shaw et al. 2010), such that the environmental relevance of the findings are limited. Another study, by Rowen et al. (2017), used concentrations of two herbicides, diuron and hexazinone, that align with current water quality guidelines (Authority 2010) and described adverse effects on growth and photosynthetic efficiency in the zooxanthellate jellyfish *Cassiopea maremetens*. In our study, exposure to guideline concentrations for atrazine and chlorpyrifos did not cause any effect on the measured endpoints. Differences between studies may be due the exposure concentrations used, the specific pesticides studied, since diuron is more harmful than atrazine (Jones et al. 2003; Magnusson et al. 2008; Flores et al. 2013), or differences in sensitivity between species of jellyfish, since *Aurelia aurita* is not a photosynthetic species and herbicides are unlikely to affect them directly. Indeed, unrealistic high exposure concentrations often elicit an effect, and differences in species sensitivities are well established across and within taxa (Pathiratne and Kroon 2016; Giddings et al. 2019).

The concentrations of atrazine and chlorpyrifos used in our study were below the 90% value trigger for the protection of species (e.g. fish, crustaceans, molluscs, echinoderms, annelids, algae and corals) (Authority 2010). A lack of adverse sub-lethal effects following exposure to either the individual or combined pesticides is evidence that the current guidelines adequately protect the polyp life-stage of *A. aurita* jellyfish. Nevertheless, water quality guidelines are generally established based on information relevant for individual pesticides, despite monitoring data demonstrating that multiple pesticides co-occur in aquatic environments (Shaw et al. 2010; Smith et al. 2012; Gallen et al. 2019). Several studies have reported additive (Relyea 2009; Wilkinson et al. 2015) or synergistic (Cedergreen 2014) effects on aquatic organisms exposed to mixtures of different types of pesticides. LeBlanc et al. (2012) reported decreased survival of a midge (*Chironomus dilutus*) after exposure to individual and binary mixtures of three insecticides: chlorpyrifos, imidacloprid and dimethoate. Two of these insecticides have the same mode of action (chlorpyrifos and dimethoate are acetylcholinesterase inhibitors, while imidacloprid blocks acetylcholine receptors), but synergistic rather than additive effects were observed between chlorpyrifos-imidacloprid and chlorpyrifos-dimethoate, and antagonistic effects with imidacloprid-dimethoate. This highlights that even when pesticides have a common mode of action predictions about possible interactive effects are difficult and stresses the importance of testing relevant pesticide mixtures to establish reliable protection values for aquatic organisms.

The combined use of lethal and sub-lethal end-points is becoming increasingly common in ecotoxicological research due to the ability to integrate mechanistic data with higher-level biological outcomes (Ankley and Edwards 2018). As such, we evaluated both

molecular endpoints (metabolomics) and higher-level endpoints directly related to fitness (survival and reproduction) in our assessment of pesticide toxicity to *A. aurita* polyps.

The fact that no differences in the metabolite profiles were observed suggests that chronic exposure to atrazine and chlorpyrifos at current guideline concentrations presents little to no risk to *A. aurita* polyps even at the molecular level. We detected no changes in the glucose content which is consistent with Gao et al. (2019) who also found no change in the glucose content of the eelgrass (*Zostera marina* L.) after the exposure to 1 $\mu\text{g/L}^{-1}$ of atrazine, although they did observe a decrease of other types of carbohydrates (e.g. sucrose and mannose) at a higher concentration of atrazine (10 $\mu\text{g/L}^{-1}$). Glucose molecules can be used in the glycolysis process to meet energy requirements (e.g. production of ATP molecules) (Cole and Kramer 2016). As we detected sources of energy like glucose and pyruvic acid, we can consider that glycolysis is occurring which can produce alanine or follow to the Krebs cycle to ultimately produce ATP (Pörtner et al. 1984), suggesting no stress in *A. aurita* polyps.

Osmoconformers are organisms that maintain an osmotic pressure equivalent to that of the external environment using organic osmolytes, like taurine, betaine and homarine (Yancey 2005). These osmolytes have been detected in several marine invertebrates such as polychaetes (*Glycera*), snails (*Mitrella carinata*) (Yancey 2005), mussels (*Mytilus edulis*) (Tuffnail et al. 2009; May et al. 2017) and jellyfish (Stabili et al. 2019; Boco et al. 2019). These specific osmolytes were indeed present at relatively high abundances in jellyfish polyps, but no differences were observed due to pesticide exposure in the present study. A recent study by Boco et al. (2019) observed a decrease in the relative abundance of betaine in polyps of the Irukandji jellyfish, *Carukia barnesi*, when exposed

to future climate change scenarios (high temperature and low pH), demonstrating the sensitivity of osmolytes to stressors that impact their cellular homeostasis. The lack of response following pesticide exposure therefore suggests that chronic exposure to atrazine and chlorpyrifos do not present an osmotic challenge to *A. aurita* polyps. Although NMR-based metabolomics did not detect changes in *A. aurita* metabolite profiles upon exposure to pesticides, the present study has demonstrated the utility of the technique to analyse changes in the molecules of very small organisms exposed to anthropogenic stressors.

The jellyfish genus *Aurelia* appears robust to a broad range of stressors (i.e. temperature, pH and salinity) (Lucas 2001; Algueró-Muñiz et al. 2016) including chemical stressors (i.e. pollutants) (Faimali et al. 2014; Lucas and Horton 2014). Moreover, *Aurelia* is widely distributed and highly invasive (Bayha and Graham 2014), which is consistent with it being a particularly hardy species. Recent ecotoxicological studies (Faimali et al. 2014; Echols et al. 2016) have promoted the ephyrae of *Aurelia* as a model organism, but it may poorly represent the responses of other, less robust species of jellyfish. We must be cautious, therefore, not to conclude from this one study that jellyfish, as a group, are robust to pesticides. Indeed, jellyfish are a diverse group of animals that differ in ecology and physiology (Arai 1997) thereby multiple species need to be studied to understand the range of responses that jellyfish exhibit to pesticide pollution.

Ours is the first study that we are aware of investigating the potential interactive effects of two pesticides, one being a herbicide and the other an insecticide, on the polyp life-history stage of jellyfish. Studying responses of polyps is particularly important because they are benthic and sessile, and unlike the pelagic ephyrae and medusae, cannot readily

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move away from stressful environments. Animals with complex life cycles often have life-histories stages that vary in their physiological tolerance to stressors. For example, the nauplii of the copepod, *Mesocyclops leuckarti*, experienced 100% mortality when exposed to 50 µg/L⁻¹ of the fungicide triphenyltin, whereas juvenile and adult stages experienced 20% and 40% of mortality respectively (Kulkarni et al. 2013). The different life history stages of jellyfish may vary in their responses to stressors and studies that encompass the responses of different life-history stages to the same stressor are required, therefore, to confidently conclude that *Aurelia* spp. are unaffected by chronic exposure to pesticides.

5. CONCLUSIONS

The present study demonstrated that chronic exposure to current Australian water quality guideline values of atrazine and chlorpyrifos, either individually or in combination, has no effect in the survival or metabolome content of *Aurelia aurita* polyps. As pesticides generally persist in low concentrations in coastal waters, we suggest environmentally relevant concentrations or those based on water quality guidelines for future ecotoxicological studies. Moreover, given that *Aurelia* appears to be a particularly hardy species that may not represent other jellyfish taxa, we caution against concluding that jellyfish, in general, are robust to pesticide pollutants and recommend that other species and different life history stages are tested.

Supplemental Information--The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability statement--Supplemental files.

Author contributions--C. Olguin-Jacobson: conceptualization, methodology, formal analysis, data curation, writing-original draft, writing-review and editing, project administration. K.A. Pitt: conceptualization, project administration, methodology, writing review and editing. A.R. Carroll: conceptualization, methodology, formal analysis, data curation, writing review. S.D. Melvin: methodology, formal analysis, data curation, writing-review editing.

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Graphical abstract: Survival, asexual reproduction and metabolite profile of polyps of the moon jellyfish *Aurelia aurita* were unaffected after chronic exposure of two pesticides.

Fig. 1. Temporal variation in mean ($\pm$ SE) number of polyps of *A. aurita* during nine weeks chronic exposure to two pesticides; individually and in combination. Atz: atrazine, Chl: chlorpyrifos.

Fig. 2. Representative one-dimensional ^1H NMR spectrum of *Aurelia aurita* polyps. The figure has been enlarged into three sections A, B and C for better clarity. Numbers represent the identified metabolite peaks. Key to metabolites: 1. Isoleucine, 2. Leucine, 3. Valine, 4. D-Lysine, 5. Alanine, 6. Proline, 7. Glutamic acid, 8. Pyruvic acid, 9. Dymethylamine, 10. Creatine, 11. Cysteine, 12. Taurine, 13. Betaine, 14. Glycine, 15. Diethanolamine, 16. Serine, 17. Glucose, 18. Homarine, 19. Acetate, 20. γ -Methylhistidine, 21. Formate, 22. Malonate, 23. Tyrosine, 24. Tryptophan.

Fig. 3. Scores plots for A) unsupervised PCA and B) supervised Partial Least Squares Discriminant Analysis (sPLS-DA) showing no separation of experimental treatments (atrazine and chlorpyrifos). Atz: atrazine, Chl: chlorpyrifos.

Table 1. Results of repeated measures LMMs analysis comparing number of polyps among treatments and time^a

Source	Numerator df	Denominator df	F	<i>p</i>
Week	8	221.379	10.331	<0.001*
Atz	1	50.150	0.286	0.595
Chl	1	50.150	1.300	0.260
Week x Atz	8	221.379	1.136	0.340
Week x Chl	8	221.379	0.582	0.792
Atz x Chl	1	50.150	0.041	0.840
Week x Atz x Chl	8	221.379	1.065	0.389

^a The model of best fit was AR(1), BIC (Bayesian information criterion) = -391.469, AIC (Akaike's information criterion) = -406.591.

* $p < 0.05$.

Data ln transformed.

df = degrees of freedom (numerator, denominator). Atz: atrazine. Chl: chlorpyrifos.