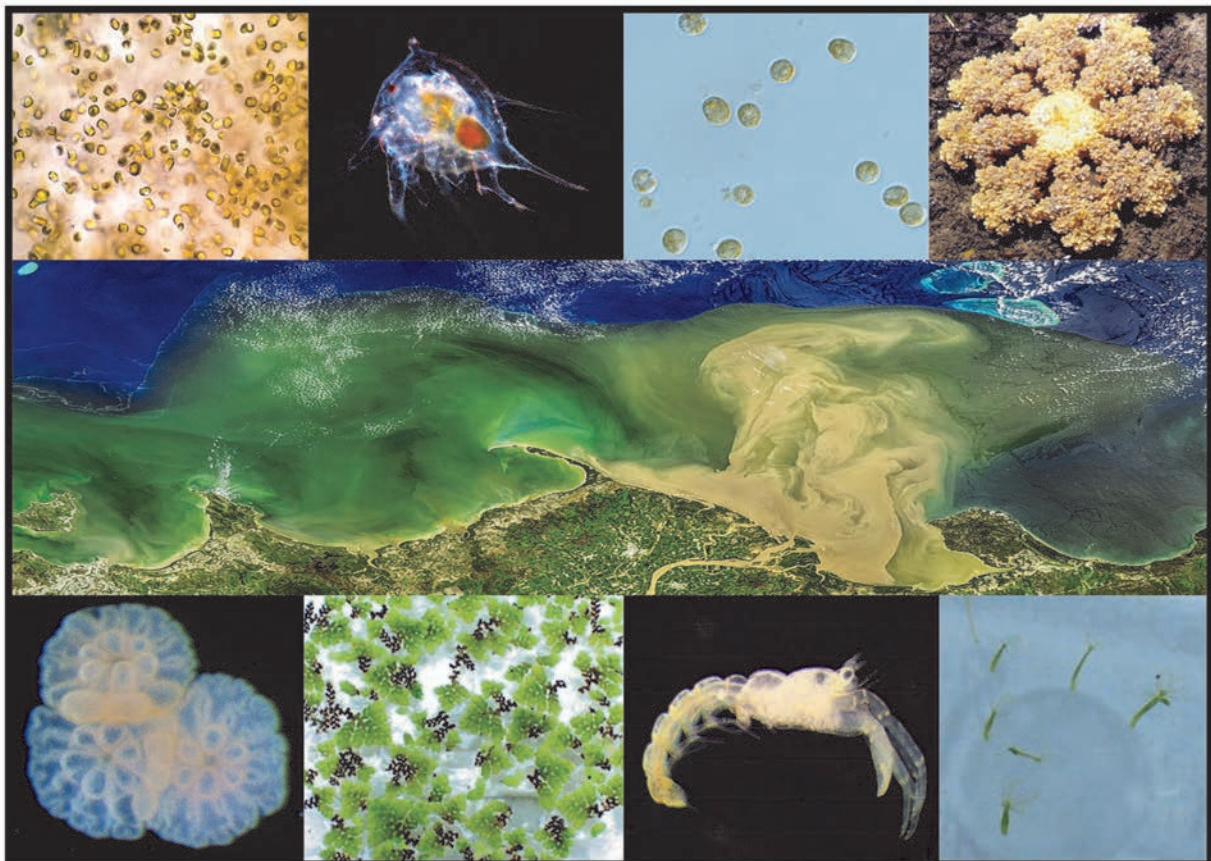# Ecotoxicology of pesticides on the Great Barrier Reef for guideline development and risk assessments

Andrew P. Negri, Shelley Templeman, Florita Flores, Joost W. van Dam, Marie Thomas, Madeline McKenzie, Laura S. Stapp, Sarit Kaserzon, Reinier M. Mann, Rachael Smith, Michael St. J. Warne and Jochen Mueller

Ecotoxicology of pesticides on the Great Barrier Reef for guideline development and risk assessments

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Cover photographs: (Front cover) Images of some of the test species used in the ecotoxicology tests (Image credit: Australian Institute of Marine Science and James Cook University) and the Burdekin River Plume 11 February 2019 (Image credit: NASA-OBPG's Landsat 8 image). (Back cover) Australian Institute of Marine Science jetty (Image credit: AIMS)

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ACRONYMS

DAWE………Department of Agriculture, Water and Environment
DoEE ....Department of the Environment and Energy
eAtlas……….Environmental research, maps and data for tropical Australia
........................https://eatlas.org.au/
GBR..............Great Barrier Reef
NESP ....National Environmental Science Program
OGBR………..Office of the Great Barrier Reef
DES...............Department of Environment and Science [Queensland]
RRRC............Reef and Rainforest Research Centre Limited
TWQ..............Tropical Water Quality
WQIP…………Water Quality Improvement Plan

ABBREVIATIONS

ANZG............Australian and New Zealand Governments
ΔF/F$_m$′...........Effective quantum yield – proportional to photosynthetic efficiency at a given
........................light intensity
EC$_{10}$ ....Effect concentration (10%): concentration of a pesticide that affects 10% of
........................test organisms or causes a 10% effect on organisms
EC$_{50}$ ....Effect concentration (50%): concentration of a pesticide that affects 50% of
........................test organisms or causes a 50% effect on organisms
ms-PAF ....Multi-substance - potentially affected fraction
NEC ....No effect concentration: concentration below which there is no effect on test
........................organisms
NOEC............No observed effect concentration: the highest test concentration that does not
........................cause an effect that significantly differs from the control
PCx ....Protective concentration: concentration that should protect x% of species.
........................Typically x equals 99,95, 90 or 80.
PAM…………Pulse amplitude modulation (fluorometry) for measuring ΔF/F$_m$′
PSII................Photosystem II
REP…………..Relative equivalent potencies (RePs) of the PSII herbicides (EC$_{50,diuron}$ /
........................EC$_{50,herbicide}$)
SGR…………..Specific growth rate
SSD…………..Species sensitivity distribution
SSW………….Synthetic soft water
WQGV……….Water quality guideline value

ACKNOWLEDGEMENTS

EXECUTIVE SUMMARY

Over 50 land-sourced pesticides have been detected in waters of the Great Barrier Reef (GBR) and its catchments. Previous studies on the risks posed by pesticides have mainly focused on five priority PSII herbicides. However, other pesticides are increasingly being used and detected, for which there are few fate, persistence and toxicity data.

In order to contribute to improved water quality guideline values (WQGVs) and assessments of the potential risks posed by these "alternate" pesticides to the GBR and its catchments, this study conducted a series of ecotoxicity tests for 21 pesticides on 16 tropical aquatic species. The pesticide and taxa combinations were chosen based on data-gaps identified by the Water Quality and Investigation team of Queensland Department of Environment and Science (DES), which was developing species sensitivity distributions (SSDs) for a broader list of priority pesticides used in the Great Barrier Reef catchments. The herbicides tested were: 2,4-D, bromacil, diuron, fluroxypyr, fluometuron, haloxyfop, hexazinone, imazapic, isoxaflutole, MCPA, metribuzin, prometryn, propazine, simazine, tebuthiuron and triclopyr. The insecticides tested were imidacloprid, fipronil and diazinon and the fungicides chlorothalonil and propiconazole.

In total 52 marine and 39 freshwater chronic growth and reproduction estimates of toxicity were reported. Fourteen of these values were greater than the maximum concentrations tested, indicating low risks to those species. An additional 63 toxicity estimates (including effects on photosynthetic efficiency or less sensitive biological effects) were reported. In order to facilitate uptake in SSDs for updating national WQGVs, the tests and their results were described in species-specific Appendices A-P in a format that corresponds with the quality assessment criteria outlined in Warne et al. (2018a). All data used to derive effect concentrations (EC_{10} and EC_{50}) and no effect concentrations (NEC) have been uploaded to eAtlas with direct links in each Appendix.

For a great number of tests, several toxicity metrics were reported and the most sensitive ecologically relevant endpoints for each freshwater and marine test species were presented in separate summary tables. The EC_{10} and NEC values in these tables are the appropriate values for inclusion in SSDs for WQGV derivation. For most marine species we reported toxicity thresholds values as both EC_{10} and NECs, and the end-user can select which is most appropriate for the application. NECs are preferred (Warne et al., 2018a) but selecting the lowest value will be more conservative (protective).

Several general observations on the toxicity tests could be made:-

- the toxicities of each of the pesticides tested were dependent on species and mode of action;
- most herbicides tested were less toxic than the reference Photosystem II herbicide diuron (growth in both marine and freshwater plants tested);
- most non-PSII herbicides had far less effect on the growth of both marine and freshwater marine microalgae (than PSII herbicides);
- non-PSII herbicides (e.g. isoxaflutole) sometimes had similar growth inhibition potencies as diuron towards freshwater macrophytes;
- concentration-dependent inhibition of photosynthesis ($\Delta F/Fm'$) was observed for all PSII herbicides to all marine and freshwater microalgae and macrophytes tested. The only non-PSII herbicide that caused appreciable inhibition of $\Delta F/Fm'$ was isoxaflutole to the

freshwater macrophytes *Azolla pinnata* and *Lemna aequinoctialis*. There were strong linear correlations between inhibition of ΔF/Fm′ with inhibition of growth for both marine and freshwater phototrophic species, highlighting the effectiveness and sensitivity of measuring ΔF/Fm′ using the non-invasive pulse amplitude modulation fluorometer;

- the neonicotinoid insecticide imidacloprid was moderately toxic to hermit crab larvae (*Coenobita variabilis*) and coral larvae (*Acropora tenuis*), while larvae of the second arthropod (the barnacle *Amphibalanus amphitrite*) were insensitive. Two other insecticides, fipronil and diazinon, were more toxic to coral larvae than imidacloprid but not tested on other species.
- the fungicide propiconazole was moderately toxic to *A. tenuis* larvae, *A. amphitrite* larvae and less toxic to the marine microalgae *Tisochrysis lutea.* Chlorothalonil, another fungicide, was only tested on coral larvae and was found to be far more toxic, with an NEC of 2.4 µg L^{-1}.

The intention of this project was to fill data gaps and to increase the number of toxicity estimates to 5 species from 4 marine phyla and 8 species from 4 freshwater phyla. The number of toxicity tests conducted ranged from one to five for different species and pesticide combinations. Consequently, it was difficult to identify patterns of toxicity for each of the pesticides; however, these patterns will become apparent when the toxicity data presented here are combined with available toxicity data to generate new SSDs and WQGVs.

The success of this project in deriving toxicity data that can be used to improve national WQGVs rests with the collaboration with end user groups that guided the selection of pesticide-taxa combinations, the choice of test criteria and the format of data presentation. The data should directly feed into: the development of national guidelines for ecosystem protection; improving relevance of pesticide guidelines for tropical marine and freshwater aquatic ecosystems; developing toxic equivalency values and therefore toxicity-based pollutant loads and expanding the number of pesticides included in the multisubstance-potentially affected fraction (ms-PAF) values and their relevance to tropical species; as well as chemical risk assessments for pesticide registration and review. The toxicity data will contribute to improving estimates for meeting the Reef 2050 Water Quality Improvement Plan's 2025 pesticide target as reported in the Reef Water Quality report cards; as well as measuring current condition of pesticide risk in Great Barrier Reef Regional report cards and regional Water Quality Improvement Plans (WQIP); relative risk assessments for alternate pesticides (for on-ground decision making by industries); expanding pesticide related ecological risk assessments to be reported in future Scientific Consensus Statements; and expansion and improvement of the information used in the Pesticide Decision Support Tool (Warne & Neale, 2019).

1.0 INTRODUCTION

1.1 Pesticides in the Great Barrier Reef and its catchments

Declining water quality, including pesticide contamination from coastal agriculture, is considered one of several serious pressures faced by tropical marine and freshwater ecosystems globally (Castillo et al., 1997; Haynes et al., 2000b; Fu et al., 2003; Ali et al., 2014). Over 50 contemporary pesticides (including herbicides, insecticides and fungicides) have been detected in the nearshore marine and freshwater systems of the Great Barrier Reef (GBR) and its catchments (Devlin et al., 2015; Warne et al., 2020). Coastal waters of the GBR are adjacent to vast areas of agriculture, and pesticide contamination is strongly associated with wet season runoff (Kennedy et al., 2012a; Kennedy et al., 2012b; Smith et al., 2012; Turner et al., 2012). However, water quality monitoring programs have identified pesticides in these waters year-round (Smith et al., 2012; Gallen et al., 2019), which is at least partially due to the persistence of many of the most commonly detected herbicides (Mercurio et al., 2014; Mercurio et al., 2015; Mercurio et al., 2016).

The risks posed by pesticides to marine and freshwater species depend on the exposure concentrations, duration of the exposures and the toxicity (Devlin et al., 2015). Monitoring of pesticides in the GBR lagoon initially focussed on five of the most commonly detected "priority" Photosystem II (PSII) herbicides: ametryn, atrazine, diuron, hexazinone and tebuthiuron, but the scope of monitoring has since broadened to over 40 herbicides, insecticides and fungicides (Gallen et al., 2019). The ecological risks posed by pesticides have been assessed by comparing concentrations from monitoring programs against national (ANZG, 2018) or GBR-relevant (GBRMPA, 2010) default water quality guideline values (WQGVs). Recently, cumulative risks posed by multiple co-occurring pesticides detected in the environment have been assessed by predicting the total toxicity using the multi-substance - potentially affected fraction (ms-PAF) (see Gallen et al., (2019)) that was further developed by the Australian and Queensland Governments (2019a, 2019b). The ms-PAF method depends on the availability of reliable toxicity data for all pesticides detected (Traas et al., 2002), and ideally the toxicity metrics should be consistent with national WQGVs (Warne et al., 2018b). However, currently reliable WQGVs are not available for most of the pesticides detected in the GBR and its catchments due to a lack of relevant toxicity data. More targeted toxicity testing is therefore required to improve current WQGVs for some pesticides and to develop WQGVs where they do not exist (Davis et al., 2014; Warne et al., 2018b).

1.2 Ecotoxicity tests for development of water quality guidelines for emerging pesticides

National WQGVs (referred to by ANZG (2018) as default GVs) are derived using species sensitivity distributions (SSDs) where data availability allows (Warne et al., 2018a). This process involves several steps that include: (i) assessing the quality of available ecotoxicity data for a given pesticide against formal criteria; (ii) selecting the most appropriate and/or sensitive toxicity thresholds for each species and pesticide, and (iii) modelling a cumulative frequency distribution (termed SSD) of the toxicity thresholds against pesticide concentrations for at least five, but preferably eight or more, species (from at least four phyla) (Warne et al., 2018a). The resulting SSD is assumed to represent the relationship between the concentration

of a pesticide and its predicted effect on an aquatic community. WQGVs are derived from SSDs as protective concentrations (PCx) for a proportion of a community. For example, measured pesticide concentrations below PC99, PC95, PC90 and PC80 should protect at least 99%, 95%, 90% and 80% of species in aquatic communities, respectively. The highest level of reliability in the WQGVs are obtained when the data that are included in the SSDs represent a large number and high diversity of species that are characteristic of the receiving environment; when toxicity data are used from chronic exposures and biological effects that are ecologically relevant (effects on survival, reproduction or growth); and when the SSD model provides a good fit to the dataset. The many experimental considerations and conditions that contribute to the derivation of high quality WQGVs can be found in Warne et al. (2018a).

Ideally, risk and monitoring assessments of pesticides in the GBR and its catchments will be conducted using the most current and comprehensive WQGVs available. Revision of the limited and dated national WQGVs for pesticides (ANZG, 2018) are overdue and, therefore, Warne et al. (2018b) and King et al. (2017a; 2017b) recently proposed updates for 27 GBR-relevant pesticides based on all available contemporary data. Nevertheless, there remain many data gaps, especially for marine species. Existing and proposed WQGVs for marine species will continue to include data from freshwater species until more appropriate marine data are available.

1.3 Objective

In order to improve WQGVs for pesticides, the objective of this project was to derive new toxicity threshold data for tropical marine and freshwater species. The project specifically targeted current data gaps based on consultation with the Qld Department of Environment and Science (DES, Project RP129) which is developing SSDs to for priority pesticides used in the Great Barrier Reef catchments and to update national WQGVs. All toxicity tests were conducted in accordance with current criteria for deriving WQGVs (Warne et al., 2018a), allowing them to directly feed into development of: (i) national and GBR ecosystem protection guidelines; (ii) toxic equivalency values; and (iii) toxic loads and multi-substance potentially affected fraction values. The toxicity data will also be available to directly feed into: improving relevance of pesticide guidelines for tropical marine and freshwater aquatic ecosystems; developing toxic equivalency values and therefore toxicity-based pollutant loads and expanding the number of pesticides included in the multisubstance-potentially affected fraction (ms-PAF) values and their relevance to tropical species; as well as chemical risk assessments for pesticide registration and review. The toxicity data will also go towards improving estimates for meeting the Reef 2050 Water Quality Improvement Plan's 2025 pesticide target as reported in the Reef Water Quality report cards; as well as measuring current condition of pesticide risk in Great Barrier Reef Regional report cards and regional Water Quality Improvement Plans (WQIP); relative risk assessments for alternate pesticides (for on-ground decision making by industries); expanding pesticide related ecological risk assessments to be reported in future Scientific Consensus Statements; and expansion and improvement of the information used in the Pesticide Decision Support Tool (Warne & Neale, 2019).

2.0 APPROACH AND METHODOLOGY

2.1 Pesticide and taxa selection

Pesticides and taxa to be tested in the current project were chosen based on data gaps identified by the Water Quality and Investigation team of DES (Project RP129). The pesticides (Table 1) were prioritised based on: (i) their detection in the GBR (e.g. Gallen et al., (2019)) and its catchments (e.g., Huggins et al. (2017)) in monitoring programs and (ii) those that needed extra toxicity data to fulfil the minimum requirements to develop WQGVs from marine and freshwater SSDs (Warne et al., 2018a). The project aimed to increase the current toxicity datasets for SSD development to at least five marine species and at least eight freshwater species (from at least four phyla in each case) for each of the identified pesticides. The current national (ANZG, 2018) and recently proposed (King et al., 2017a; King et al., 2017b; Warne et al., 2018b) WQGVs for pesticides are dominated by toxicity data from tests on temperate aquatic species. To partially address this bias, the current project selected test species that are found in the tropics. The common and widely studies photosystem II herbicide diuron was used as a reference toxicant for many of the toxicity tests.

Table 1. Required toxicity data to increase current toxicity datasets to reach at least five marine species and eight freshwater species (from at least four phyla in each case) for each of the prioritised pesticides (in order of priority for each pesticide type).

Pesticide type	Priority Pesticides	Number of tests required	
		Marine	Freshwater
Herbicides	Imazapic	4	7
	Metribuzin	4	0
	Hexazinone	2	3
	Tebuthiuron	4	0
	Haloxyfop	5	7
	Bromacil	3	2
	Propazine	4	3
	2,4-D	3	0
	Simazine	2	0
	Fluroxypyr	4	2
	MCPA	1	0
	Isoxaflutole	0	4
	Fluometuron	0	1
	Triclopyr	0	3
	Prometryn	0	1
Insecticides	Imidacloprid	1	0
Fungicides	Propiconazole	3	0

Pesticide SSDs that include both phototrophs and heterotrophs are often bimodal since herbicides selectively target phototrophs and insecticides selectively target heterotrophs. When multimodal or bimodal SSDs are observed or expected, only the most sensitive taxonomic subgroup is used to derive WQGVs (Warne et al., 2018a). Therefore, in the current project only phototrophs were used to test herbicides and heterotrophs to test insecticides. No toxicity tests using fungi were available for the fungicides propiconazole and chlorothalonil, so a combination of phototrophs and heterotrophs were tested.

2.2 Toxicity tests

The toxicity tests conducted in this project are listed in Tables 2 to 5. The tests were conducted in three laboratories: marine tests were performed at the Australian Institute of Marine Science in Townsville and Darwin, while the freshwater tests (and marine test for *Cassiopea maremetens* tests) were conducted at TropWATER, James Cook University, Townsville.

The toxicity test methods are described in the individual species' toxicity reports in [Appendices A-P](). All toxicity tests met the minimum criteria for inclusion in SSDs to derive national WQGVs (Warne et al., 2018a). The methods of the toxicity tests are presented in tabular format in the appendices to facilitate quality assessment of the generated data, based on the criteria presented in Warne et al. (2018a). All tests were chronic and evaluated ecologically relevant biological endpoints, reporting results as effect concentrations (e.g. EC_{10} where 10% of individuals or a population are affected) and sometimes also no effect concentrations (NECs). Both EC_{10}s and NECs from chronic tests can be directly included in SSDs (Warne et al., 2018a). The ecologically relevant effects measured included inhibition of growth (including specific growth rate, frond number, surface area, biomass, stem length) and larval development and settlement larval development and settlement. Other endpoints measured included effects of herbicides on photosynthetic efficiency and jellyfish statolith number and symbiont density. Brief descriptions of the endpoints that were assessed are provided below.

2.2.1 Effects of pesticides on growth

The chronic effects of contaminants on growth are considered ecologically relevant (Warne et al., 2018a) and measurements of growth are particularly well suited to quantifying the effects of herbicides on aquatic microalgae and macrophytes.

Microalgae
The rapid growth rates of microalgae allow for chronic exposure testing in a short period (Warne et al., 2018a). Additionally, microalgae play important ecological roles in primary productivity and food for zooplankton and changes in their abundance, composition and nutritional value may initiate an indirect bottom-up effect on higher trophic levels. In this project the inhibition of specific growth rate (SGR) of marine and freshwater microalgae by herbicides was quantified from standard ecotoxicology protocols guided by methods outlined in OECD test 201 (OECD, 2011).

Macrophytes
The inhibition of growth rate (biomass increase, increase in frond number, surface area increase and stem length increase) in freshwater macrophytes was quantified from standard ecotoxicology protocols similar to methods outlined in OECD test 221 (OECD, 2006b), OECD TG 238 (OECD, 2014) and tropical methods (Brown et al., 1994; Riethmuller et al., 2003; Pease et al., 2016). High growth rates for *L. aequinoctialis* under tropical conditions required test duration to be limited to 4 days due to potential issues associated with overcrowding and associated growth limitations confounding effects. Although not strictly adhering to the minimum timeframe (7 days) as required for chronic assessment for temperate species, (as outlined in Warne et al. (2018a)), the combination of relatively high growth rates and the use of multiple ecologically relevant endpoints (surface area and frond number) should provide sufficient certainty of evidence of ecological impairment to be considered a chronic response.

The Supervising Scientist (DAWE) also consider the 4 day *L. aequinoctialis* protocol to be a chronic assessment for these reasons (van Dam pers. comm.)

Jellyfish
Cassiopea spp. are scyphozoan endosymbiotic jellyfish that possess an atypical behaviour of resting upside-down on shallow coastal waters. Their endosymbiotic zooxanthellae are located within amoebocytes (Arai, 1997) throughout the oral epidermal tissue and oral arms. The only rigid structure found in these jellyfish are small hexagonally shaped crystalline structures (statoliths) that accumulate with age in the jellyfish (Hopf & Kingsford, 2013).

Change in surface area relative to the control animals was used as a proxy for growth in the jellyfish over 14 days. A number of studies (Klein et al., 2016; Rowen et al., 2017) have shown that stressors and toxicants (including herbicides) can significantly affect growth (as reflected in changes in bell diameter or bell surface area). Changes in bell size can indicate reduced energy resourcing, potentially through inhibition of the photosynthetic efficiency of the endosymbiont, even with heterotrophic food resource availability. Statoliths are considered to have utility as an age proxy, particularly as they are more resistant to environmental stresses (e.g. food, salinity) than bell size (Hopf & Kingsford, 2013). Changes in statolith number can reflect shifts in resource allocation under stress (Hopf & Kingsford, 2013). The symbiotic zooxanthellae are contained in amboecytes within oral epidermal tissues. Zooxanthellae density (using cell number per unit area of bell tissue mm^2) was assessed to determine if zooxanthellae numbers changed through expulsion or other removal processes (e.g. ingestion by host) during herbicide exposure. Zooxanthellae density was standardised to the bell area to account for differences in jellyfish size. Inhibition in growth (as bell surface area), statolith number, symbiont density in the upside-down jellyfish was assessed using previously published methods (Hopf & Kingsford, 2013; Rowen et al., 2017).

2.2.2 Effects of pesticides on invertebrate larvae

Coral larval metamorphosis
Coral reproduce by generating larvae, either sexually or asexually, and the process of larval settlement, attachment and metamorphosis into a sessile primary polyp is a critical step in recruitment necessary to maintain coral reef populations (Harrison & Wallace, 1990). Metamorphosis success is one of the most sensitive early life history stages to contaminant stress (Reichelt-Brushett & Harrison, 2000; Negri et al., 2016). The current project applied a larval metamorphosis assay that follows the methods applied in multiple similar studies (Negri & Heyward, 2000; Negri et al., 2005; Markey et al., 2007; Negri et al., 2016; Negri et al., 2018; Nordborg et al., 2018). In this case the larvae were chronically exposed to pesticides for 48-h in static exposures as per Nordborg et al., (2018). Metamorphosis was assessed after a further 24-h and larvae were considered normal and functional if larvae had changed from free swimming or casually attached pear-shaped forms to squat, firmly attached, disc-shaped structures with pronounced flattening of the oral–aboral axis and with septal mesenteries radiating from the central mouth region (Heyward & Negri, 1999).

Barnacle larval development
Adult barnacles were induced to spawn and freshly hatched nauplii larvae exposed in a static system for four days to increasing pesticide concentrations following previously described methods (van Dam et al., 2016). Test results were derived from the ability of the larvae to

successfully complete the four consecutive moults to nauplii stage VI and subsequent metamorphosis into cyprid larvae, within the four-day test duration.

Hermit crab larval development
Adult hermit crabs were allowed to naturally spawn and freshly hatched larvae exposed in a static system for six days to increasing pesticide concentrations following previously described methods (van Dam et al., 2018). Test results were derived from the ability of the larvae to successfully complete two consecutive moults and transition from zoeae stage I to megalopae larvae, within the six-day test duration.

2.2.3 Effects of herbicides on photosynthetic efficiency

While inhibition of growth is the most common ecologically relevant effect applied in SSDs for the toxicity of herbicides to aquatic phototrophs, many herbicides have also been shown to affect photosynthetic efficiency in tropical species, including corals (Cantin et al., 2007), crustose coralline algae (Negri et al., 2011a), foraminifera (van Dam et al., 2012), jellyfish (Rowen et al., 2017) and seagrass (Haynes et al., 2000a). Effects of herbicides on photosynthesis can be measured using the sensitive and non-invasive technique of Pulse Amplitude Modulation (PAM) fluorometry (Ralph et al., 2007). Using PAM fluorometry, the inhibition of effective quantum yield ($\Delta F/Fm'$) by PSII herbicides is proportional to the inhibition of photosynthetic efficiency at a given irradiance (Schreiber et al., 2007), and has been demonstrated as a rapid, sensitive and non-invasive alternative for growth measurements in microalgal toxicity tests involving PSII herbicides (Magnusson et al., 2008; Muller et al., 2008). However, this sensitive photophysiological response may not be suitable as an ecologically relevant measure of whole organism stress for microalgae to non-PSII herbicides where the mode of action does not involve PSII. Further comparisons between the inhibition of growth and $\Delta F/Fm'$ as endpoints for herbicide toxicity in aquatic species are needed and, therefore, the current project compared these biological and photophysiological effects for a broader range of marine and freshwater species.

2.3 Pesticide analysis

All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) according to the methods in Mercurio et al. (2015). Samples taken at the start and end of pesticide exposures for all tests were analysed for the test pesticide (See Appendices A-P for details).

2.4 Data analysis

The national WQGVs are derived from SSDs using statistical endpoints such as NEC, EC_x (x ≤ 10) and no observed effect concentration (NOEC), in that order of preference (Warne et al., 2018a). This project derived chronic EC_{10} and NEC values where possible for each test. In most cases, nonlinear sigmoidal regressions were used to estimate most EC_{10} values, while Bayesian non-linear models were applied to derive NEC and sometimes EC_{10} values. Models were chosen based on the quality of the fit to experimental data and, therefore, differed between pesticide and test. All statistical analyses and resulting estimates of toxicity were based on measured pesticide concentrations (average of start and end concentrations).

Effect concentrations (EC_x) that inhibit growth or reproduction were estimated from nonlinear regression using GraphPad Prism V 8.0. or the DRC package in R (Ritz & Streibig, 2005; Ritz et al., 2015). In DRC, regression models evaluated included log-logistic, Weibull and Brain-cousins hormesis models of different levels of parametrisation. Model comparisons were conducted using the Akaike Information Criterion (AIC) and models that best described the data were applied to derive appropriate estimates of toxicity (EC_x). The associated 95% confidence intervals (CI) were estimated using the delta method. In some cases, EC_x values were estimated from Bayesian non-linear gaussian model using the R package jagsNEC (See below).

The estimations of NEC were calculated in R (Version 3.6.1). Proportional decline in response (1-inhibition) was modelled as a function of log concentration of each pesticide using a Bayesian non-linear gaussian, beta or binomial model using the R package jagsNEC (Fisher et al., 2019). This model has been specifically developed to derive NECs but also allows the estimation of EC_{10} and EC_{50} values and is adapted from Fox (2010). Models were run with 10,000 Markov chain Monte Carlo (MCMC) iterations after an initial 'burn-in' period of 20,000 iterations and for five separate chains. Trace plots were used to evaluate model fits and were found to have relatively good mixing in all cases.

3.0 RESULTS AND DISCUSSION

3.1 Results summary

Tests on 21 pesticides were conducted with 16 tropical aquatic species. In total 52 marine and 39 freshwater chronic growth and reproduction toxicity estimates were derived (91 total). Fourteen of these values were greater than the maximum concentrations that could be tested. An additional 63 toxicity estimates (including effects on photosynthetic efficiency or less sensitive biological effects) were reported. Summaries of the results of all tests are provided in the following Tables:-

Table 2	Marine	Most sensitive ecologically relevant endpoint	e.g. inhibition of growth or reproduction
Table 3		Other biological effects	e.g. inhibition of photosynthesis
Table 4	Freshwater	Most sensitive ecologically relevant endpoint	e.g. inhibition of growth or reproduction
Table 5		Other biological effects	e.g. inhibition of photosynthesis and less sensitive measures of growth

Toxicity data in Tables 2 to 5 were obtained from the individual species' toxicity test reports provided in Appendices A-P, in order to facilitate direct application in SSDs. All concentration-response data used to derive effect concentrations (EC_{10} and EC_{50}) and NECs have been uploaded to eAtlas with direct links in each Appendix. The concentration-response curves used to predict the effect concentrations are provided in Appendices A-P.

As per the guidance material for derivation of water quality guidelines (Warne et al., 2018a), where more than one ecologically relevant biological effect was identified for an individual species-pesticide combination, data from the most sensitive endpoint should be used in SSDs. The most sensitive endpoints for each freshwater and marine test species are presented in Table 2 (marine) and Table 4 (freshwater). The EC_{10} and NEC values in Table 2 and Table 4 are, therefore, generally the appropriate values for inclusion in SSDs for WQGV derivation. Other biological effect thresholds, including EC_{10}s and NECs, for inhibition of photosynthetic efficiency as well as less sensitive growth effects, are listed in Table 3 and Table 5.

We reported toxicity thresholds values as both EC_{10} and NECs for many of the tests in Table 2, and the end-user can select that which is most appropriate for the application. Both measures of toxicity are acceptable for deriving national WQGVs (Warne et al., 2018a). NECs are preferred but selecting the lowest value will be more conservative (protective). Professional judgment (i.e. on the rigour or reliability of data) can be applied when selecting the most appropriate toxicity estimate. Some predicted EC_{10} values were lower than associated NECs, yet in these cases confidence intervals often overlapped, indicating the values were not substantially different. Non-linear regression was usually applied to estimate EC_x, while NEC values were derived from the NEC model described in Fox (2010), which assumes that there is no effect across an initial concentration range up to the estimated NEC threshold value, after which the effect increases exponentially with increasing concentration. A smooth non-linear model represents a fundamentally different shape to an NEC model, with the differences in fit generally most apparent in the lower concentration range. Consequently, it is not surprising that EC10s were sometimes lower than NECs.

Table 2. Summary of pesticide toxicity threshold values for marine taxa (most sensitive ecologically relevant endpoint). Modelled no effect concentration (NEC) and effect concentrations (EC$_{10}$ and EC$_{50}$). All concentrations are in µg L^{-1} (95% confidence intervals).

Pesticide	Phylum	Pesticide type[2]	Species common name	Species scientific name	Most sensitive biological effect	NEC (95% CI)[3]	EC$_{10}$ (95% CI)[3]	EC$_{50}$ (95% CI)[3]	Summary Appendix each test
Diuron	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	1.68 (1.53 – 1.90)	1.94 (1.75 – 2.14)	6.27 (6.02 – 6.54)	Appendix D
	Bacillariophyta		Diatom	*Chaetoceros muelleri*		1.47 (1.15 – 1.83)	1.79 (1.60 – 1.98)	12.4 (11.8 – 13.0)	Appendix B
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		2.75 (2.56 – 2.93)	2.54 (2.34 – 2.75)	4.45 (4.31 – 4.59)	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		2.27 (1.99 – 2.49)	1.64 (1.41 – 1.86)	5.24 (4.91 – 5.57)	Appendix E
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		0.781 (0.438 – 1.30)	0.600 (0.402 – 0.800)	3.96 (3.40 – 4.52)	Appendix F
Bromacil	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	5.53 (4.33 – 6.44)	4.89 (4.01 – 5.91)	19.3 (17.7 – 21.0)	Appendix D
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		16.6 (15.4 – 20.6)	18.3 (16.9 – 19.9)	27.7 (26.7 – 28.7)	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		1.75 (1.29 – 2.40)	0.985 (0.788 – 1.18)	6.68 (6.22 – 7.14)	Appendix E
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		1.96 (1.57 – 2.37)	1.94 (1.55 – 2.34)	6.80 (6.31 – 7.28)	Appendix F
Fluroxypyr	Haptophyta	H	Golden–brown algae	*Tisochrysis lutea*	Growth (SGR[1])	Unreliable NEC	> 6300	> 6300	Appendix F
Haloxyfop	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	> 3700	> 3700	> 3700	Appendix D
	Bacillariophyta		Diatom	*Chaetoceros muelleri*		> 4,570	> 4,570	> 4,570	Appendix B
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		> 3000	> 3000	> 3000	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		Unreliable NEC	3740 (3560 – 3930)	5930 (5740 – 6110)	Appendix E
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		4180 (3800 – 4710)	4000 (3650 – 4350)	4380 (4160 – 4600)	Appendix F
Hexazinone	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	4.58 (4.34 – 4.78)	3.96 (3.40 – 4.57)	8.50 (7.99 – 9.06)	Appendix D
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		71.7 (63.4 – 91.0)	78.7 (57.8 – 92.0)	100 (96.1 – 141)	Appendix C
	Cnidaria		Jellyfish	*Cassiopea maremetens*	Bell surface area		31.3 (8.96 – 75.1)	176 (92.0 – 364)	Appendix A

Imazapic	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	363,000 (341,000 – 386,000)	410,000 (362,000 – 462,000)	790,000 (760,000 – 825,000)	Appendix D
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		> 165,000	> 165,000	> 165,000	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		Unreliable NEC	> 20800	> 20800	Appendix E
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		471 (283 – 861)	783 (399 – 1170)	4320 (3180 – 5460)	Appendix F
MCPA	Haptophyta	H	Golden–brown algae	*Tisochrysis lutea*	Growth (SGR[1])	Unreliable NEC	21800 (7670 – 35900)[4]	> 20,000,000	Appendix F
Metribuzin	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	2.21 (1.97 – 2.82)	2.66 (2.21 – 3.18)	13.4 (12.3 – 14.5)	Appendix D
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		23.6 (21.3 – 27.5)	22.3 (16.2 – 25.9)	33.5 (30.2 – 50.4)	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		6.66 (4.67 – 7.80)	4.14 (3.50 – 4.77)	18.5 (17.4 – 19.5)	Appendix E
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		0.499 (0.287 – 1.24)	0.721 (0.355 – 1.09)	3.11 (2.46 – 3.75)	Appendix F
Propazine	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	27.8 (24.2 – 31.1)	42.0 (37.1 – 47.3)	188 (177 – 201)	Appendix D
	Bacillariophyta		Diatom	*Chaetoceros muelleri*		12.9 (9.29 – 32.0)	21.5 (18.4 – 25.0)	98.2 (91.7 – 105)	Appendix B
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		45.1 (37.0 – 51.1)	50.8 (44.8 – 57.4)	86.5 (83.0 – 90.1)	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		29.3 (22.2 – 34.5)	27.2 (22.4 – 32.0)	121 (111 – 130)	Appendix E
	Haptophyta		Golden brown algae	*Tisochrysis lutea*		14.4 (10.8 – 20.9)	18.5 (15.2 – 21.9)	56.5 (51.0 – 62.0)	Appendix F
Simazine	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	48.0 (44.0 – 51.0)	38.4 (33.0 – 44.2)	184 (173 – 195)	Appendix D
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		320 (234 – 452)	257 (226 – 294)	387 (361 – 416)	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		37.5 (27.9 – 46.3)	37.6 (33.0 – 42.2)	154 (145 – 162)	Appendix E
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		70.0 (55.3 – 80.3)	60.2 (51.9 – 68.4)	206 (194 – 218)	Appendix F
Tebuthiuron	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	22.7 (20.3 – 25.2)	27.5 (24.2 – 31.2)	112 (106 – 119)	Appendix D
	Bacillariophyta		Diatom	*Chaetoceros muelleri*		16.0 (13.0 – 19.1)	26.8 (23.9 – 29.9)	187 (179 – 195)	Appendix B
	Dinoflagellata		Zooxanthellae	*Cladocopium goreaui*		107 (84.6 – 136)	138 (108 – 173)	331 (300 – NA)	Appendix C
	Chlorophyta		Green algae	*Tetraselmis sp.*		20.6 (15.7 – 24.6)	18.4 (15.4 – 21.4)	69.9 (65.5 – 74.4)	Appendix E

	Haptophyta		Microalgae	*Tisochrysis lutea*		63.1 (42.5 – 71.5)	35.9 (30.6 – 41.1)	112 (106 – 118)	Appendix F
2,4–D	Cryptophyta	H	Microalgae	*Rhodomonas salina*	Growth (SGR[1])	> 279,000	> 279,000	> 279,000	Appendix D
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*		15300 (6980 – 28400)	40700 (28800 – 52500)	172000 (61500 – 283000)	Appendix F
Imidacloprid	Cnidaria	I	Coral larvae	*Acropora tenuis*	Larval settlement	263 (195 – 295)	273 (208 – 305)	348 (307 – 396)	Appendix G
	Arthropoda		Barnacle	*Amphibalanus amphitrite*	Larval development	> 1660	> 1660	> 1660	Appendix H
	Arthropoda		Hermit crab		Larval development	102 (38.7 – 175)	43.3 (2.92 – 83.6)	390 (262 – 517)	Appendix I
Fipronil	Cnidaria	I	Coral larvae	*Acropora tenuis*	Larval settlement	12.3 (7.13 – 19.1)	13.9 (8.46 – 21.1)	29.1 (20.2 – 41.6)	Appendix G
Diazinon	Cnidaria	I	Coral larvae	*Acropora tenuis*	Larval settlement	38.0 (20.4 – 51.3)	40.8 (22.4 – 53.8)	54.7 (52.3 – 57.0)	Appendix G
Chlorothalonil	Cnidaria	F	Coral larvae	*Acropora tenuis*	Larval settlement	2.42 (1.63 – 3.89)	2.76 (1.90 – 4.42)	5.95 (4.40 – 8.82)	Appendix G
Propiconazole	Cnidaria	F	Coral larvae	*Acropora tenuis*	Larval settlement	269 (123 –468)	330 (171 – 537)	1008 (704 – 1689)	Appendix G
	Arthropoda		Barnacle	*Amphibalanus amphitrite*	Larval development	878 (829 – 907)	568 (425 – 710)	1020 (936 – 1100)	Appendix H
	Haptophyta		Golden–brown algae	*Tisochrysis lutea*	Growth (SGR[1])	2980 (2660 – 3230)	2710 (2300 – 3110)	4840 (4640 – 5040)	Appendix F

[1]SGR = specific growth rate
[2]Pesticide type: H = herbicide, I = insecticide, F = fungicide
[3]All concentrations are in µg L^{-1} (95% confidence intervals)
[4]Extrapolated

Table 3. Summary of pesticide toxicity threshold values for marine taxa effective quantum yield (ΔF/Fm'). Modelled effect concentrations (EC_{10} and EC_{50}). All concentrations are in µg L^{-1} (95% confidence intervals).

Pesticide	Phylum	Species common name	Species scientific name	EC_{10} (95% CI)[1]	EC_{50} (95% CI)[1]	Summary Appendix each test
Diuron	Cryptophyta	Microalgae	*Rhodomonas salina*	0.43 (0.38 – 0.48)	1.71 (1.63 – 1.80)	Appendix D
	Bacillariophyta	Diatom	*Chaetoceros muelleri*	0.97 (0.81 – 1.15)	4.25 (3.96 – 4.55)	Appendix B
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	0.29 (0.26 – 0.33)	1.20 (1.15 – 1.26)	Appendix C
Bromacil	Cryptophyta	Microalgae	*Rhodomonas salina*	0.59 (0.45 – 0.75)	3.56 (3.19 – 3.98)	Appendix D
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	2.54 (2.29 – 2.82)	8.36 (8.01 – 8.69)	Appendix C
Haloxyfop	Cryptophyta	Microalgae	*Rhodomonas salina*	> 3,700	> 3,700	Appendix D
	Bacillariophyta	Diatom	*Chaetoceros muelleri*	> 4,570	> 4,570	Appendix B
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	> 3,000	> 3,000	Appendix C
Hexazinone	Cryptophyta	Microalgae	*Rhodomonas salina*	1.81 (1.63 – 1.99)	5.85 (5.61 – 6.09)	Appendix D
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	8.36 (7.14 – 9.80)	33.8 (30. –37.6)	Appendix C
	Cnidaria	Jellyfish	*Cassiopea maremetens*	3.40 (1.39 – 6.71)	82.0 (59.1 – 119)	Appendix A
Imazapic	Cryptophyta	Microalgae	*Rhodomonas salina*	> 790,000	> 790,000	Appendix D
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	> 165,000	> 165,000	Appendix C
Metribuzin	Cryptophyta	Microalgae	*Rhodomonas salina*	0.60 (0.50 – 0.71)	2.95 (2.72 – 3.18)	Appendix D
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	2.31 (2.08 – 2.56)	8.75 (8.39 –9.12)	Appendix C
Propazine	Cryptophyta	Microalgae	*Rhodomonas salina*	5.85 (4.90 – 6.91)	39.5 (37.1 – 42.1)	Appendix D
	Bacillariophyta	Diatom	*Chaetoceros muelleri*	8.12 (7.04 – 9.33)	48.6 (45.6 – 51.7)	Appendix B
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	5.42 (4.94 – 5.95)	18.7 (18.0 – 19.5)	Appendix C
Simazine	Cryptophyta	Microalgae	*Rhodomonas salina*	9.28 (8.41 – 10.2)	59.2 (56.7 – 61.8)	Appendix D
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	28.8 (23.9 – 35.3)	93.3 (84.6 – 102)	Appendix C
Tebuthiuron	Cryptophyta	Microalgae	*Rhodomonas salina*	2.66 (2.31 – 3.06)	16.0 (15.1 – 17.0)	Appendix D
	Bacillariophyta	Diatom	*Chaetoceros muelleri*	6.95 (5.79 – 8.27)	47.7 (44.1 – 51.5)	Appendix B
	Dinoflagellata	Zooxanthellae	*Cladocopium goreaui*	6.37 (4.79 – 8.50)	41.0 (36.3 – 46.3)	Appendix C
2,4–D	Cryptophyta	Microalgae	*Rhodomonas salina*	> 279,000	> 279,000	Appendix D

[1]All concentrations are in µg L^{-1} (95% confidence intervals)

Table 4. Summary of pesticide toxicity threshold values for freshwater taxa (most sensitive ecologically relevant endpoint). Modelled effect concentrations (EC$_{10}$ and EC$_{50}$). All concentrations are in µg L^{-1} (95% confidence intervals).

Pesticide	Phylum	Pesticide type[6]	Species common name	Species scientific name	Most sensitive biological effect	EC$_{10}$ (95% CI)[7]	EC$_{50}$ (95% CI)[7]	Summary Appendix each test
Diuron	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	11.2 (9.87 – 12.8)	24.7 (23.1 – 26.4)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	6.13 (3.86 – 9.20)	28.4 (23.3 – 34.7)	Appendix M
	Chlorophyta		Green algae	*Raphidocelis subcapitata*	Growth (SGR[1])	5.32 (4.31 – 6.47)	20.6 (18.5 – 22.8)	Appendix P
	Pteridophyta		Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	3.28 (1.96 – 5.02)	13.6 (11.1 – 16.8)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	3.73 (2.94 – 4.65)	24.1 (21.8 – 26.8)	Appendix N
Bromacil	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	14.6 (12.8 – 16.7)	26.3 (24.9 – 27.8)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	12.9 (10.1 – 16.6)	36.8 (33.1 – 40.6)	Appendix M
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	14.2 (11.5 – 17.3)	51.8 (47.1 – 57.0)	Appendix N
Fluometuron	Pteridophyta	H	Fern	*Azolla pinnata*	Biomass (SGR-B[3])	3.96 (0.145 – 22.1)	119 (50.6 – 403)	Appendix J
Fluroxypyr	Pteridophyta		Fern	*Azolla pinnata*	Biomass (SGR-B[3])	2,620 (1,590 – 4,400)	6,190 (5,150 – 7,170)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	4,730 (4,080 – 5,440)	18,100 (16,900 – 19,300)	Appendix N
Haloxyfop	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	2,180 (1,630 – 2,930)	7,810 (6,960 – 9,160)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	311 (190 – 486)	921 (771 – 1120)	Appendix M
	Chlorophyta		Green algae	*Raphidocelis subcapitata*	Growth (SGR[1])	>10,200	>10,200	Appendix P
	Pteridophyta		Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	78.4 (47.0 – 122)	808 (662 – 979)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	223 (158 – 311)	1,450 (1,200 – 1,770)	Appendix N
	Tracheophyta – Magnoliopsida		Stonewort	*Ceratophyllum demersum*	Biomass (SGR-B[3])	207 (8.40 – 1,390)	1,190 (576 – 2,390)	Appendix K
Hexazinone	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	22.8 (20.1 – 25.5)	51.3 (48.7 – 54.0)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	12.6 (7.45 – 19.4)	52.0 (42.8 – 62.6)	Appendix M
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	33.9 (27.1 – 41.4)	110 (101 – 120)	Appendix N
Imazapic	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	38,100 (21,800 – 57,900)	>190,000	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	>198,000	>198,000	Appendix M
	Chlorophyta		Green algae	*Raphidocelis subcapitata*	Growth (SGR[1])	27,500 (16,800 – 41,700)	432,000 (282,000 – 855,000)	Appendix P
	Pteridophyta		Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	31.6 (15.4 – 55.7)	372 (268 – 546)	Appendix J
	Cyanophyta		Cyanobacteria	*Microcystis aeruginosa*	Growth (SGR[1])	9,370 (5,090–15,600)	102,000 (84,500–127,000)	Appendix O
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	29.2 (11.0 – 65)	298 (206 – 581)	Appendix N

	Tracheophyta – Magnoliopsida		Stonewort	*Ceratophyllum demersum*	Length (SGR-L[3])	7.25 (0 – 35.4)	67.8 (25.6 – 148)	Appendix K
Isoxaflutole	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	>2,570	>2,570	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	>798	>798	Appendix M
	Pteridophyta		Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	1.69 (0.711 – 3.46)	84.2 (58.5 – 129)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	0.721 (0.241 – 1.55)	4.87 (3.21 – 7.64)	Appendix N
Prometryn	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	5.29 (2.20 – 10.9)	22.0 (16.1 – 29.4)	Appendix L
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	7.75 (6.00 – 9.85)	30.9 (27.5 – 34.7)	Appendix N
Propazine	Chlorophyta	H	Green algae	*Chlorella* sp.	Growth (SGR[1])	72.4 (61.7 – 83.3)	178 (168 – 189)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	Growth (SGR[1])	54.4 (43.8 – 66.4)	153 (140 – 167)	Appendix M
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	27.0 (23.2 – 31.2)	171 (161 – 182)	Appendix N
Triclopyr	Pteridophyta	H	Fern	*Azolla pinnata*	Biomass (SGR-B[3])	2,540 (1,660 – 4,330)	7,250 (6,040 – 8,580)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Biomass (SGR-B[3])	8,540 (5,940 – 11,300)	33,900 (29,500 – 40,800)	Appendix N
	Tracheophyta – Magnoliopsida		Stonewort	*Ceratophyllum demersum*	Biomass (SGR-B[3])	68.4 (18.1 – 145)	356 (252 – 467)	Appendix K

[1]SGR = specific growth rate
[2]SGR-SA = specific growth rate-surface area
[3]SGR-B = specific growth rate-biomass
[4]SGR-FC = specific growth rate-frond number
[5]SGR-L = specific growth rate-length
[6]Pesticide type: H = herbicide
[7]All concentrations are in µg L^{-1} (95% confidence intervals)

Table 5. Summary of pesticide toxicity threshold values for freshwater taxa (for alternative biological effects and endpoints). Modelled effect concentrations (EC$_{10}$ and EC$_{50}$). All concentrations are in µg L^{-1} (95% confidence intervals).

Pesticide	Phylum	Pesticide type[6]	Species common name	Species scientific name	Biological effect	EC$_{10}$ (95% CI)[7]	EC$_{50}$ (95% CI)[7]	Summary Appendix each test
Diuron	Chlorophyta	H	Green algae	*Chlorella* sp.	ΔF/Fm'	2.32 (1.99 – 2.68)	8.73 (8.16 – 9.33)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	ΔF/Fm'	1.94 (0.938 – 1.28)	14.5 (12.4 – 17.0)	Appendix M
	Chlorophyta		Green algae	*Raphidocelis subcapitata*	ΔF/Fm'	2.66 (1.71 – 4.10)	9.21 (7.96 – 10.6)	Appendix P
	Pteridophyta		Fern	*Azolla pinnata*	ΔF/Fm'	2.01 (1.09 – 3.32)	10.4 (8.23 – 13.0)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	6.00 (4.83 – 7.36)	23.7 (21.4 – 26.1)	Appendix N
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	1.24 (0.995 – 1.40)	7.03 (6.53 – 7.58)	Appendix N
Bromacil	Chlorophyta	H	Green algae	*Chlorella* sp.	ΔF/Fm'	11.0 (8.80 – 13.1)	21.4 (19.6 – 23.5)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	ΔF/Fm'	37.8 (31.6 –45.2)	43.8 (42.0 – 45.8)	Appendix M
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	17.3 (14.0 – 21.0)	63.9 (58.6 – 69.7)	Appendix N
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	4.34 (3.68 – 5.07)	19.4 (18.2 – 20.6)	Appendix N
Fluometuron	Pteridophyta	H	Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	32.0 (21.1 – 45.9)	360 (298 – 444)	Appendix J
	Pteridophyta		Fern	*Azolla pinnata*	ΔF/Fm'	29.6 (20.2 – 41.6)	505 (433 – 591)	Appendix J
Fluroxypyr	Pteridophyta		Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	6,450 (4,450 – 8,930)	17,760 (14,680 – 21,780)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	5,380 (4,020 – 7,020)	19,500 (17,500 – 21,700)	Appendix N
Haloxyfop	Pteridophyta		Fern	*Azolla pinnata*	Biomass (SGR-B[3])	208 (132 – 320)	876 (723 – 1,052)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	282 (179 – 440)	2,380 (1,950 – 3,020)	Appendix N
Hexazinone	Chlorophyta	H	Green algae	*Chlorella* sp.	ΔF/Fm'	29.5 (N.D.)	34.0 (N.D.)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	ΔF/Fm'	5.85 (4.07 – 7.97)	22.6 (19.7 – 25.7)	Appendix M
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	4.27 (3.27 – 5.50)	31.0 (27.8 – 34.4)	Appendix N
Imazapic	Pteridophyta	H	Fern	*Azolla pinnata*	Biomass (SGR-B[3])	47.0 (22.8 – 76.8)	127 (102 – 162)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	60.7 (39.7 – 86.1)	254 (220 – 292)	Appendix N
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	> 915	> 915	Appendix N
Isoxaflutole	Chlorophyta	H	Green algae	*Chlorella* sp.	ΔF/Fm'	>2,570	>2,570	Appendix L
	Pteridophyta		Fern	*Azolla pinnata*	Biomass (SGR-B[3])	1.80 (0.383 – 5.61)	212 (107 – 630)	Appendix J
	Pteridophyta		Fern	*Azolla pinnata*	ΔF/Fm'	1.92 (0.873 – 3.72)	197 (136 – 318)	Appendix J

	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	0.766 (0.443 – 1.13)	2.57 (2.07 – 3.26)	Appendix N
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	10.6 (5.44 – 20.7)	129 (93.3 – 204)	Appendix N
Prometryn	Chlorophyta	H	Green algae	*Chlorella* sp.	ΔF/Fm'	1.19 (0.182 – 3.11)	15.6 (9.98 – 24.1)	Appendix L
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	10.7 (8.86 – 12.7)	38.8 (35.5 – 42.4)	Appendix N
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	2.01 (1.79 – 2.44)	12.1 (11.3 – 13.0)	Appendix N
Propazine	Chlorophyta	H	Green algae	*Chlorella* sp.	ΔF/Fm'	29.7 (20.9 – 39.9)	138 (122 – 155)	Appendix L
	Chlorophyta		Green algae	*Desmodesmus asymmetricus*	ΔF/Fm'	11.7 (5.91 – 20.3)	69.3 (53.5 – 90.2)	Appendix M
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Frond number (SGR-FC[4])	32.5 (25.9 – 39.9)	171 (158 – 186)	Appendix N
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	ΔF/Fm'	11.0 (8.04 – 14.4)	77.1 (68.7 – 86.6)	Appendix N
Triclopyr	Pteridophyta	H	Fern	*Azolla pinnata*	Surface Area (SGR-SA[2])	6,563 (N.D.)	9,800 (N.D.)	Appendix J
	Tracheophyta – Liliopsida		Duckweed	*Lemna aequinoctialis*	Surface Area (SGR-SA[2])	12,200 (10,100 – 14,600)	31,400 (28,700 – 34,600)	Appendix N
	Tracheophyta – Magnoliopsida		Stonewort	*Ceratophyllum demersum*	Length (SGR-L[4])	3,030 (246 – 5,810)	8,540 (2,640 – 14,400)	Appendix K

[1]SGR = specific growth rate
[2]SGR-BSA = specific growth rate-surface area
[3]SGR-B = specific growth rate-biomass
[4]SGR-FC = specific growth rate-frond number
[5]SGR-L = specific growth rate-length
[6]Pesticide type: H = herbicide
[7]All concentrations are in µg L^{-1} (95% confidence intervals)

3.2 Marine taxa

3.2.1 PSII herbicides and marine species

Toxicity tests using marine autotrophs were performed on seven PSII herbicides, including the reference herbicide diuron (Table 2 and Table 4). Diuron was the most toxic of all PSII herbicides with respect to growth (primarily SGR), with EC_{50} values ranging between 4.0 and 12.4 µg L^{-1}. The relative equivalent potencies (RePs) of the PSII herbicides ($EC_{50,diuron}$/$EC_{50,herbicide}$) are presented in Table 6 and (based on the average RePs) indicate the order of toxicity (i.e., highest to lowest ReP): diuron > metribuzin > bromacil > hexazinone > propazine > tebuthiuron > simazine. However, this was not always consistent between species. For example, metribuzin was more toxic than diuron to *T. lutea* but far less toxic to *C. goreaui* and *Tetraselmis* sp. and hexazinone was over an order of magnitude more toxic to *R. salina* than to *C. goreaui*.

Table 6. Summary of relative potencies (ReP) relative to the reference herbicide diuron for marine taxa based on SGR ($EC_{50,diuron}$/$EC_{50,herbicide}$). ND denotes values could not be determined. Empty spaces mean we did not run that test.

Herbicide	*Rhodomonas salina*	*Chaetoceros muelleri*	*Cladocopium goreaui*	*Tetraselmis sp.*	*Tisochrysis lutea*	Average ReP
Diuron[PSII]	1	1	1	1	1	1
Bromacil[PSII]	0.32		0.16	0.78	0.58	0.46
Hexazinone[PSII]	0.74		0.045			0.39
Metribuzin[PSII]	0.47		0.13	0.28	1.27	0.54
Propazine[PSII]	0.033	0.13	0.051	0.043	0.070	0.065
Simazine[PSII]	0.034		0.011	0.034	0.019	0.025
Tebuthiuron[PSII]	0.056	0.066	0.013	0.075	0.035	0.049
Imazapic[1]	0.0000079		ND	ND	0.00092	0.00046
Fluroxypyr[2]					ND	ND
Haloxyfop[3]	ND	ND	ND	0.00088	0.00090	0.00089
MCPA[2]					ND	ND
2,4-D[2]	ND				0.000023	0.000023

[PSII]Photosystem II inhibitor
[1]Acetohydroxyacid synthase (AHAS) inhibitor
[2]Auxin mimic
[3]Acetyl-CoA carboxylase (ACCase) inhibitor

The PSII herbicides all inhibited photosynthetic efficiency ($\Delta F/F_m'$) and showed a similar order of toxicity to growth (Table 4 and Table 7). The orders of toxicity were similar and the relationship between inhibition of growth and inhibition of $\Delta F/F_m'$ was linear for the four species (Figure 1). The correlation plot of EC_{50} values for both endpoints had a slope of 3.4, showing that inhibition of $\Delta F/F_m'$ is more sensitive than inhibition of SGR to PSII herbicides. Previous work by Magnusson *et al*. (2008) had demonstrated a relationship between SGR and $\Delta F/F_m'$ inhibition by PSII herbicides that was closer to 1:1 for two tropical benthic microalgae; *Navicula* sp. and *Nephroselmis pyriformis*. There is a clear link between inhibition in $\Delta F/F_m'$ and decreasing growth rates; however, the direct link between the binding of PSII herbicides to the D1 protein (reducing electron transport and causing damage to PSII) with growth is not necessarily expected to be 1:1 for all taxa and experimental conditions. Light intensity and the light acclimation history have large influences on the relationships between photophysiology,

primary production and growth (Ralph et al., 2007). However, the results from this project reinforce the notion that inhibition of $\Delta F/F_m'$ in marine microalgae by individual PSII herbicides is a very good indicator of effects on growth.

Table 7. Summary of relative potencies (ReP) relative to the reference herbicide diuron for marine taxa based on ΔF/Fm'. ND denotes that values could not be determined. Empty spaces mean we did not run that test.

	Rhodomonas salina	*Chaetoceros muelleri*	*Cladocopium goreaui*	Average ReP
Diuron[PSII]	1	1	1	1
Bromacil[PSII]	0.48		0.14	0.31
Hexazinone[PSII]	0.29		0.036	0.16
Metribuzin[PSII]	0.58		0.14	0.36
Propazine[PSII]	0.043	0.087	0.064	0.065
Simazine[PSII]	0.029		0.013	0.021
Tebuthiuron[PSII]	0.11	0.089	0.029	0.075
Imazapic[1]	ND		ND	ND
Fluroxypyr[2]				
Haloxyfop[3]	ND	ND	ND	ND
MCPA[2]				
2,4-D[2]	ND			ND

[PSII]Photosystem II inhibitor

[1]Acetohydroxyacid synthase (AHAS) inhibitor

[2]Auxin mimic

[3]Acetyl-CoA carboxylase (ACCase) inhibitor
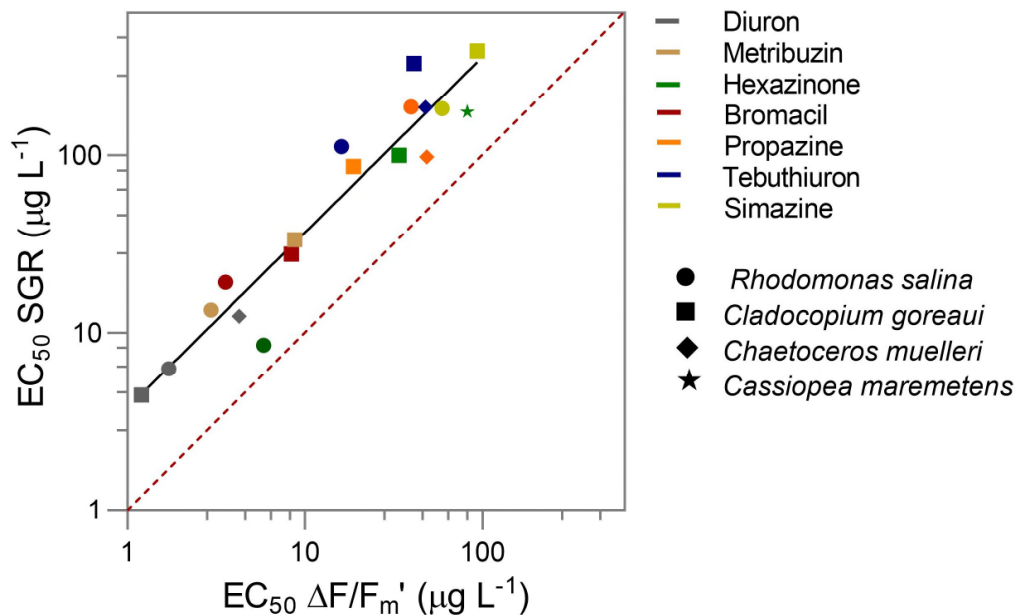
Figure 1. Linear relationship between inhibition of growth and inhibition of effective quantum yield (ΔF/Fm′) for marine taxa. Comparison of EC50 values [EC50(SGR) = 3.36 * EC50(ΔF/Fm′) + 13.2; R² = 0.72] of seven PSII herbicides to four species. Dashed line indicates 1:1 relationship.

3.2.2 Non-PSII herbicides and marine species

The non-PSII herbicides were far less toxic than PSII herbicides to all marine microalgae species tested (Table 2). The acetohydroxyacid synthase inhibitor imazapic was tested on 4 species and was most toxic to *T. lutea* (NEC = 471 µg L^{-1}) and was an order of magnitude less toxic to *R. salina,* while no toxicity was observed for the other species at the highest concentrations tested. Other marine microalgae are similarly insensitive to imazapic, for example, no effect on SGR of the marine microalgae *Navicula sp.* and *Nephroselmis pyriformis* were observed after 10 d exposure at concentrations of up to 1,500 µg L^{-1} (Magnusson, 2009).

The marine microalgae were also insensitive to the auxin mimic (growth regulator) herbicides 2,4-D, fluroxypyr and MCPA (Table 2). 2,4-D had SGR NEC and EC$_{10}$ values in excess of 15,000 µg L^{-1} for *T. lutea* and were not reached for *R. salina* at 279,000 µg L^{-1}. Fluroxypyr and MCPA were only tested on *T. lutea* and had EC$_{10}$ values in excess of 6,000 µg L^{-1} (Table 2). NECs reported for the effects of fluroxypyr and MCPA were unreliable ([Appendix F](#)). Auxin regulators are primarily used as selective herbicides for controlling broadleaves (dicots) (King et al., 2017a). This pathway is unlikely to be present in microalgae, explaining the observed lack of toxicity.

Of the five autotrophs tested, only the SGRs of *Tetraselmis* sp. and *T. lutea* were affected by the acetyl-CoA carboxylase inhibitor haloxyfop in the concentration range tested (Table 2). Both species had NEC and/or EC$_{10}$ values of ~ 4000 µg L^{-1}, while the other three microalgae species were not affected at similar concentrations. ACCase inhibitors, such as haloxyfop, target the eukaryotic form of the enzyme rather than the prokaryotic form (King et al., 2017b) and the microalgae tested here are unlikely to contain the eukaryotic ACCase enzyme in their plastids (Huerlimann & Heimann, 2013), likely explaining the resistance observed.

Another factor to consider with respect to the sensitivity of marine species is whether the structures of 2,4-D, MCPA, imazapic and fluroxypyr may affect their bioavailability in seawater. All contain a carboxylic acid group (COOH), which may complex with Mg^{2+} and Ca^{2+} ions in seawater, or stabilise the herbicides at the seawater:air interface (Tang et al., 2011). Both mechanisms could reduce the bioavailability of each of these herbicides to marine species accounting for the low toxicities reported.

Photosynthetic efficiency ($\Delta F/F_m'$) was not affected by non-PSII herbicides in any of the microalgae tested (Table 3).

3.2.3 Insecticides and marine species

The neonicotinoid insecticide imidacloprid was moderately toxic to hermit crab larvae (*C. variabilis* (NEC 102 µg L^{-1}) and coral larvae (*A. tenuis* NEC 263 µg L^{-1}), while larvae of the second arthropod (the barnacle *A. amphitrite*) were insensitive (Table 2). The insect nicotinic acetylcholine receptor (nAChR) is the main target for imidacloprid (Zhang et al., 2000) and this is largely conserved across Arthropoda so differences in sensitivity between the crab and barnacle larvae were not expected. Two other emerging insecticides fipronil and diazinon were more toxic to coral larvae, exhibiting NEC values of 12.3 and 38 µg L^{-1}, respectively. Fipronil is a gamma-aminobutyric acid blocker, while diazinon is an acetylcholinesterase inhibitor. Both target pathways that may be present in coral larvae, but more additional work is needed to confirm the sensitivity of coral larvae to these insecticides. However, these results and the

moderate response of coral larvae to imidacloprid are consistent with the earlier work by Markey et al (2007), which showed coral larvae can be sensitive to a broad range of insecticides.

3.2.4 Fungicides and marine species

The fungicide propiconazole was moderately toxic to *A. tenuis* larvae, *A. amphitrite* larvae and less toxic to *T. luetea* (NECs of 269, 878 and 2980 µg L^{-1}, respectively; (Table 2)). The mode of action of propiconazole is inhibition of ergosterol synthesis (critical to cell wall formation in fungi) and its toxicity is assumed to be relatively specific to fungi (King et al., 2017b). This likely explains the moderate to low sensitivity of the species tested here and future tests should be performed on non-target marine fungi. Chlorothalonil was only tested on coral larvae (*A. tenuis*) and was found to be far more toxic than propiconazole, with an NEC of 2.4 µg L^{-1} (Table 2). This fungicide inactivates sulfhydryl enzymes resulting in glutathione depletion and is broadly toxic to a wide variety of other aquatic species at similar concentrations (Van Scoy & Tjeerdema, 2014), but is not as toxic to coral larvae as the mercury-containing fungicide MEMC (Markey et al., 2007).

3.3 Freshwater taxa

3.3.1 Effect of herbicides on growth in freshwater taxa

The sensitivity of freshwater species to herbicides was strongly dependent on both species and mode of action. Algae were more sensitive to PSII herbicides than to non-PSII herbicides. Of the seven species tested, *Azolla pinnata* (freshwater fern) was considered the most sensitive species overall, with four of the eleven herbicides tested exhibiting the greatest effect on growth rate as either biomass or surface area of this species (Table 4). Two of the six PSII herbicides exhibited the greatest effect on growth rate in *A. pinnata*, indicating that this species is very sensitive to PSII herbicide exposure. Of the remaining four PSII herbicides, three had a larger effect on algal growth than on macrophyte growth. The non-PSII herbicides tested were considerably less toxic to algae than aquatic macrophytes and the aquatic fern (Table 4).

Ceratophyllum demersum was very sensitive to both imazapic and triclopyr. Triclopyr as a synthetic auxin exhibited a strong hormetic effect on stem growth in *C. demersum* with mean stem length increasing by up to 60% in comparison to the controls in the lower triclopyr concentrations. *L. aequinoctialis* responses to the two growth rate variables (frond number and surface area) varied by herbicide. Surface area was a more sensitive response variable for seven of the ten herbicides tested, and also for all PSII herbicides except hexazinone. No valid data could be obtained for the effects of hexazinone on surface area due to poor growth rates. In contrast, two of the four non-PSII herbicides (isoxaflutole and triclopyr) inhibited frond number to a greater extent than surface area in *L. aequinoctialis*. Isoxaflutole was also the most toxic of all herbicides tested on L. *aequinoctialis*. The sensitivity between the two growth rate metrics (surface area and biomass) for *A. pinnata* was also herbicide dependent. However, there was no obvious link between mode of action and response sensitivity. Surface area was a more sensitive response for haloxyfop, imazapic and isoxaflutole while biomass was more sensitive to fluometuron, fluroxypyr and triclopyr. The RePs for the effects of herbicides on growth vs the potency of diuron of freshwater taxa are presented in Table 8.

Table 8. Summary of relative potencies (ReP) relative to the reference herbicide diuron for freshwater taxa based on growth rates. ND denotes values could not be determined. Empty spaces mean we did not run that test.

Herbicide	*Chlorella* sp.	*D. asymmetricus*	*R. subcapitata*	*A. pinnata*	*L. aequinoctialis*	Average ReP
Diuron[PSII]	1	1	1	1	1	1
Bromacil[PSII]	0.94	0.77			0.47	0.73
Fluometuron						
Hexazinone[PSII]	0.48	0.55			0.22	0.42
Prometryn[PSII]	1.12				0.78	0.95
Propazine[PSII]	0.14	0.19			0.14	0.16
Imazapic[1]	ND	ND	0.000048	0.037	0.081	0.039
Isoxaflutole[4]	ND	ND		0.16	4.87	2.51
Fluroxypyr[2]					0.0013	0.0013
Haloxyfop[3]	0.0032	0.031	ND	0.017	0.017	0.017
Triclopyr[2]						

[PSII]Photosystem II inhibitor
[1]Acetohydroxyacid synthase (AHAS) inhibitor
[2]Auxin mimic
[3]Acetyl-CoA carboxylase (ACCase) inhibitor
[4]4-hydroxyphenyl-pyruvate-dioxygenase inhibitor

3.3.2 Effect of herbicides on photosynthetic efficiency in freshwater taxa

Inhibition of photosynthetic efficiency (ΔF/Fm′) by PSII herbicides was greatest in *L. aequinoctialis,* with four of the six herbicides exhibiting the strongest response on this species (Table 5). Isoxaflutole was the only non-PSII herbicide tested to have a measurable effect on ΔF/Fm′. This response was only seen in aquatic macrophytes, with neither *D. asymmetricus* nor *Chlorella* sp. exhibiting inhibition at the highest concentrations of isoxaflutole tested. Imazapic was not sensitive to *L. aequinoctialis* at the highest concentration tested, while fluroxypyr, haloxyfop and triclopyr were not assessed. The relative equivalent potencies ReP for the effects of herbicides on ΔF/Fm′ vs the potency of diuron on freshwater taxa is presented in Table 9.

Table 9. Summary of relative potencies (ReP) relative to the reference herbicide diuron for freshwater taxa based on ΔF/Fm′. ND denotes values could not be determined. Empty spaces mean we did not run that test.

Herbicide	*Chlorella* sp.	*D. asymmetricus*	*R. subcapitata*	*A. pinnata*	*L. aequinoctialis*	Average ReP
Diuron[PSII]	1	1	1	1	1	1
Bromacil[PSII]	0.41	0.33			0.36	0.37
Fluometuron				0.021		0.021
Hexazinone[PSII]	0.26	0.64			0.23	0.38
Prometryn[PSII]	0.56				0.58	0.57
Propazine[PSII]	0.063	0.21			0.091	0.12
Imazapic[1]					ND	
Isoxaflutole[4]	ND			0.053	0.054	0.054
Fluroxypyr[2]						
Haloxyfop[3]						
Triclopyr[2]						

[PSII]Photosystem II inhibitor
[1]Acetohydroxyacid synthase (AHAS) inhibitor
[2]Auxin mimic
[3]Acetyl-CoA carboxylase (ACCase) inhibitor
[4]4-hydroxyphenyl-pyruvate-dioxygenase inhibitor

As for marine taxa, there was a strong linear relationship between inhibition of growth and inhibition of $\Delta F/F_m'$, in this case for five freshwater species and four PSII herbicides (Figure 2). The correlation plot of EC_{50} values for both endpoints had a slope of 1.43, showing a greater sensitivity of $\Delta F/F_m'$ than growth to PSII herbicides. This relationship was closer to unity than for marine taxa, highlighting the direct link between inhibition in $\Delta F/F_m'$ and decreasing growth rates in response to PSII herbicides.
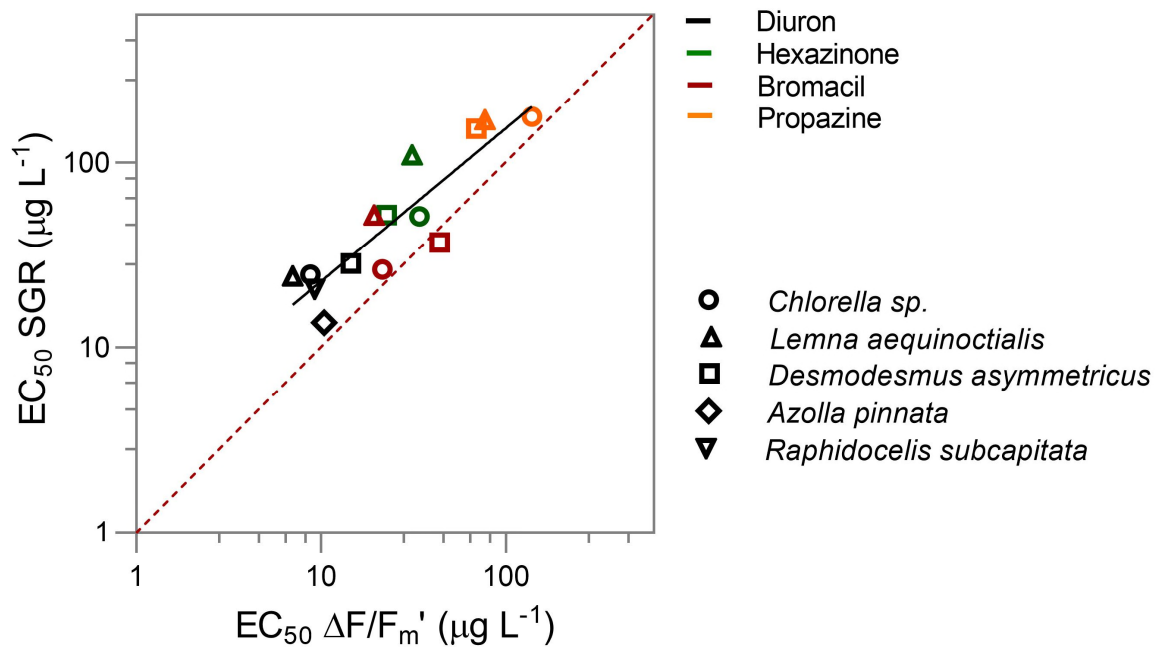
Figure 2. Linear relationship between inhibition of growth and inhibition of effective quantum yield ($\Delta F/F_m'$) for freshwater taxa. Comparison of EC_{50} values [$EC_{50}(SGR) = 1.43 * EC_{50}(\Delta F/Fm') + 15.4$; $R^2 = 0.78$] of four PSII herbicides to five species. Dashed line indicates 1:1 relationship.

4.0 CONCLUSION

This project was conceived and planned to fill specific gaps in aquatic toxicity data for emerging pesticides detected in the GBR and its catchments, increasing the number of toxicity values to five marine species belonging to four phyla and eight freshwater species belonging to four phyla. The collaboration with end-user groups (including Water Quality and Investigation Team of Qld DES) guided the selection of pesticide-taxa combinations, the choice of test criteria and the format of data presentation to best contribute to the derivation of new national WQGVs.

There were more data gaps in pesticide WQGVs for marine taxa than freshwater taxa (ANZG, 2018) and very few toxicity data for tropical species relevant to the GBR and its catchments. The large number of data gaps and the preference for chronic toxicity data (Warne et al., 2018a) meant that many of the test species chosen were microalgae. However, tropical freshwater macrophytes along with a tropical barnacle, a hermit crab and coral were also applied in the suite of tests. The project also developed and successfully applied growth toxicity tests for cultures of the coral symbiont *Cladocopium goreaui* (zooxanthellae) and *Cassiopea maremetens* (upside-down jellyfish).

The project collaborators further developed scripts to derive no effect concentrations (NECs) (Fisher et al., 2019), which are the preferred toxicity estimates for inclusion in SSDs to derive WQGVs (Warne et al., 2018a). NECs were derived for most marine tests and, in the most part, were consistent with EC_{10} values, offering end-users a selection most appropriate for the application. NECs are preferred for WQGV derivation but selecting the lower of the NEC or EC10 values will be more conservative (protective).

In total, the study conducted a series of ecotoxicity tests for 21 pesticides on 16 tropical aquatic species. 52 marine and 39 freshwater chronic growth and reproduction toxicity values were reported. Fourteen of these values were greater than the maximum concentrations that could be tested, indicating low risks to those species. An additional 63 toxicity values (including effects on photosynthetic efficiency or less sensitive biological effects) were reported. Since the data gaps for freshwater and marine species varied between pesticides, the number of tests conducted ranged from one to five for different species and pesticide combinations. It was difficult to identify patterns of toxicity for each of the emerging pesticides; however, this will become apparent when the toxicity data presented here are combined with currently available toxicity data to generate new SSDs. Regardless, several general observations on the toxicity tests could be made:-

- The toxicities of each of the pesticides tested here were dependent on species and mode of action.
- Most herbicides tested were less toxic than the reference photosystem II herbicide diuron (growth in both marine and freshwater).
- Most non-PSII herbicides were far less toxic than PSII herbicides to growth in both marine and freshwater marine microalgae.
- Non-PSII herbicides (e.g. isoxaflutole) sometimes had similar growth inhibition potencies as diuron towards freshwater macrophytes.
- Dose-dependent inhibition of photosynthesis ($\Delta F/Fm'$) was observed for all PSII herbicides to all marine and freshwater microalgae and macrophytes tested. The only non-PSII herbicide which caused appreciable inhibition of $\Delta F/Fm'$ was isoxaflutole to

the freshwater macrophytes *Lemna aequinoctialis* and *Azolla pinnata*. There were strong linear correlations between inhibition of ΔF/Fm′ with inhibition of growth for both marine and freshwater species, highlighting the relationship between these physiological and biological endpoints and the sensitivity of measuring ΔF/Fm′ using the non-invasive pulse amplitude modulation fluorometer.

- The neonicotinoid insecticide imidacloprid was moderately toxic to hermit crab larvae (*C. variabilis*) and coral larvae (*A. tenuis*), while larvae of the second arthropod (the barnacle *A. amphitrite*) were insensitive. Two other insecticides, fipronil and diazinon, were more toxic than imidacloprid to coral larvae but were not tested on other species.
- The fungicide propiconazole was moderately toxic to *A. tenuis* larvae, *A. amphitrite* larvae and less toxic to *T. lutea.* Chlorothalonil was only tested on coral larvae and was found to be far more toxic than propiconazole, with an NEC of 2.4 µg L^{-1}.

It is important to note that the toxicity data generated from the present study are not intended to be applied in isolation or be directly compared against concentrations measured in the GBR or its catchments. The risk to coastal marine and freshwater biota posed by these pesticides is best quantified by comparisons of measured values in the field against high quality WQGVs that are derived from multiple diverse taxa. The present data contributes to generate quality SSDs and associated WQGVs. Furthermore, pesticides are generally not present in isolation, but are instead detected in complex mixtures (Kennedy et al., 2012a; Kennedy et al., 2012b; Smith et al., 2012; Turner et al., 2012; Warne et al., 2020). Therefore, it is critical that the cumulative risks posed by co-occurring pesticides are assessed by predicting the total toxicity using a method that determines the risk of mixtures, such as the ms-PAF method (Traas et al., 2002). This approach has been further developed by the Australian and Queensland Governments (2019a, 2019b) and applied in pesticide monitoring by Gallen et al. (2019) and the Queensland DES (https://arcg.is/0Cj8SP; http://arcg.is/1fOGWz). Likewise, our recent study shows how ms-PAF can be used to adjust WQGVs for climate warming and heatwave events (Negri et al., 2020), which are becoming more frequent and intense (Lough et al., 2018). However, to appreciate the risks posed by emerging pesticides in combination with other pressures, further targeted multiple stressor toxicity testing (similar to the current project) is required (Davis et al., 2014; Warne et al., 2018b).

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APPENDICES: TOXICITY REPORTS BY SPECIES

Each of the following Appendices represent a stand-alone description of the pesticide toxicity tests performed on an individual species. Most of the experimental information is in table form so that the tests can be assessed against criteria required to meet standards for contribution towards the national WQGVs (Warne et al., 2018a). Table 10 summarises the Appendices.

Table 10. Summary of Appendices

Appendix	Marine/ freshwater	Autotroph/ heterotroph	Species scientific name	Species common name
Appendix A	Marine	Autotroph	*Cassiopea maremetens*	Jellyfish
Appendix B			*Chaetoceros muelleri*	Diatom
Appendix C			*Cladocopium goreaui*	Coral symbiont (dinoflagellate)
Appendix D			*Rhodomonas salina*	Microalgae
Appendix E			*Tetraselmis* sp.	Green microalgae
Appendix F			*Tisochrysis lutea*	Golden-brown microalgae
Appendix G		Heterotroph	*Acropora tenuis*	Coral larvae
Appendix H			*Amphibalanus amphitrite*	Barnacle larvae
Appendix I			*Coenobita variabilis*	Hermit crab larvae
Appendix J	Freshwater	Autotroph	*Azolla pinnata*	Mosquitofern
Appendix K			*Ceratophyllum demersum*	Hornwort
Appendix L			*Chlorella* sp.	Green microalgae
Appendix M			*Desmodesmus asymmetricus*	Green algae
Appendix N			*Lemna aequinoctialis*	Lesser duckweed
Appendix O			*Microcystis aeruginosa*	Cyanobacteria
Appendix P			*Raphidocelis subcapitata*	Green microalgae

Appendix A: Marine: *Cassiopea maremetens*

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The herbicide and the mode of action that was used in toxicity test for this species was:

- Hexazinone – PSII inhibitor

Test species: *Cassiopea maremetens*

Test phylum: Cnidaria

Biological effect: Inhibition of effective quantum yield, growth as bell surface area, statolith number and symbiont (zooxanthellae) density.

Summary

The effects of hexazinone exposure were assessed on growth of the Upside-down jellyfish *Cassiopea maremetens* over a 14-day exposure period. The concentrations of hexazinone that inhibited 10% and 50% of effective quantum yield ($\Delta F/Fm'$), bell surface area (mm^2), statolith number and zooxanthellae density (cells mm^{-2}) of *C. maremetens* relative to controls (EC_{10} and EC_{50}, respectively) were calculated from 4-parameter sigmoidal model concentration-response curves. The toxicity thresholds (EC_{10}; EC_{50} in $\mu g\ L^{-1}$) for hexazinone were $\Delta F/Fm'$ (3.40; 81.96), bell surface area (31.32; 176) and statolith number (36.05; 304) respectively. No effects on symbiont density (cells mm^{-2}) were observed at the highest hexazinone concentration (302 $\mu g\ L^{-1}$).

Methods

The inhibition of growth in *C. maremetens* by hexazinone was tested in static –renewal conditions for a 14 day exposure period (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$), statolith number, and zooxanthellae density (cells mm^{-2}) was also tested in a 14 day exposure period. Details of the experimental methods are provided in Tables A1 to A3. Original data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/3c716ba9-42b3-4736-8521-479d17e9b99e.

Table A1. Source of *Cassiopea maremetens*, its culturing and test conditions.

Source of tests species	Test individuals sourced from Reef HQ Townsville with parental stock originally from Lake Magellan, Sunshine Coast, Queensland.			
Maintenance conditions of test species	Test individuals were held in aquaria in the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University, Townsville, Queensland. Individuals were held in 10-20 L plastic tanks partially filled with natural 0.5 μm filtered seawater under a 12:12 hr light:dark cycle (146 ± 15 μmol photons $m^{-2}s^{-1}$) at 25 ± 2°C and were fed *Artemia* nauplii every other day.			
Test endpoints	Inhibition of growth as bell surface area	Inhibition of statolith number	Inhibition of zooxanthellae density	Inhibition of effective quantum yield ($\Delta F/Fm'$, proportional to photosynthetic efficiency)
Test duration	14 days			
Test chambers	250 mL plastic tanks			
Test volume	150 mL			

Assessment of inhibition	• Growth assessed as bell surface area from measured bell diameter; statoliths manually counted from two statocysts per jellyfish; zooxanthellae cells extracted and counted as per Zamoum & Furla (2012) and standardized to the bell surface area of digested tissue volume. • Effective quantum yield was assessed via mini pulse amplitude modulated fluorometer (mini-PAM; WALZ, Germany).

Table A2. Measured physico-chemical parameters of test media for *Cassiopea maremetens*.

Light intensity (mean ± SD, n=6)	146 ± 15 µmol photons $m^{-2}s^{-1}$ over a 12:12 hr L:D cycle
Test media temperature (mean ± SD, averaged day 2 – 14, n= 70)	$25 \pm 1°C$
pH (mean ± SD, averaged day 2 – 14, n= 70)	8.0 ± 0.17
Salinity (mean ± SD, averaged day 2 – 14, n= 70)	36 ± 0.5

Table A3. Test criteria for inhibition of size, statolith number, effective quantum yield and zooxanthellae density of *Cassiopea maremetens*.

Exposure duration	14 days			
Biological effect metric	Inhibition of growth as a measure of bell surface area	Inhibition of statolith number per statocyst	Inhibition of zooxanthellae as a measure of cell density per mm^2 of jellyfish bell surface area	Inhibition of the effective quantum yield ($\Delta F/Fm'$) which is proportional to photosynthetic efficiency for a given light intensity
Biological endpoint definition	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce growth, statolith number, zooxanthellae number or $\Delta F/Fm'$ by 10% and 50%, respectively, in comparison to control / solvent control treatments.			
Controls used	Hexazinone was dissolved in the carrier solvent acetone (final concentration 0.01% v/v). A separate seawater control (natural 0.5 µm filtered seawater) with no solvent was included in the experiment			
Test, treatment and replicate numbers	Concentration-response curves from one definitive test with 7 treatment concentrations. 5 replicates for all treatment concentrations except $\Delta F/Fm'$ (15 replicates).			
Test acceptability criteria	pH range <0.5, salinity range < 1.0, temperature range < 2.5 °C, <10% mortality in Controls			
Characteristics of the test organism	Actively feeding animals free of overt disease and deformity			
Type of test media	Natural, 0.5 µm filtered seawater control			
Toxicant (common name; IUPAC Name; CAS no.; purity)	Hexazinone: 3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione; 51235-04-2; ≥ 98%. Batch: BCBT6090			
Preparation of toxicant stock	100 mg L^{-1} hexazinone in Milli-Q® water.			
Exposure type	Static renewal (every 48 hours post-feeding)			
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015).			

Reference toxicant	None
Concentration-response relationship	• ECx: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OEC 2006). The concentrations of herbicide that inhibited 10% and 50% of the biological effect metrics relative to controls (EC_{10} and EC_{50}, respectively) were calculated from concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Table A4)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade hexazinone (> 98%) was used for preparation of stock and purchased from Sigma-Aldrich.
Randomisation	48 hours (following feeding and water changes)

Summary of results

The toxicity of hexazinone to *Cassiopea maremetens* is presented in Table A4 and Figure A1. Toxicity was assessed relative to the combined control and solvent control responses. Hexazinone did not inhibit zooxanthellae density at the maximum concentration of 302 µg L^{-1}.

Table A4. Modelled toxicity estimates for the inhibitory effects of hexazinone on bell size as surface area (mm^2), statolith number, effective quantum yield (ΔF/Fm′) and zooxanthellae density (cells mm^{-2}) of *Cassiopea maremetens* (Figure A1). All concentrations in µg L^{-1} (95% confidence intervals).

	EC_{10} (95% CI)	EC_{50} (95% CI)
Effective quantum yield	3.40 (1.39 – 6.71)	82.0 (59.1 – 119)
Bell surface area	31.3 (8.96 – 75.1)	176 (92.0 – 364)
Statolith count	36.0 (8.87 - 102)	304 (160 – 1210)
Zooxanthellae density	>302	>302
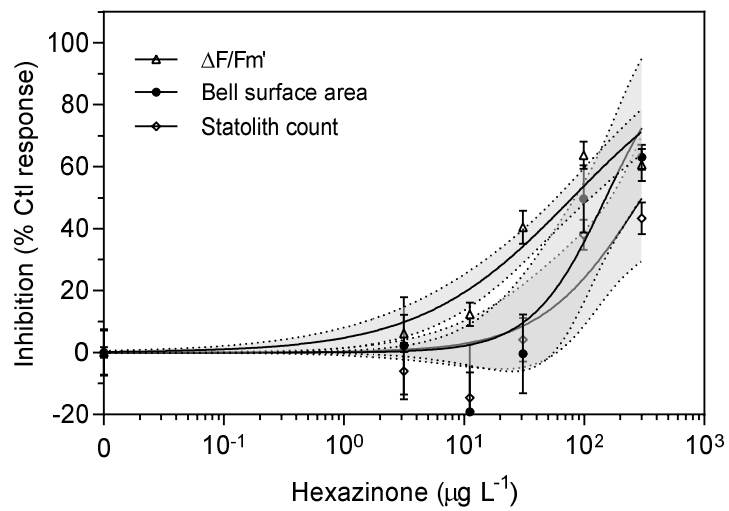
Figure A1. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 14-day effective quantum yield (ΔF/Fm′), bell surface area, and statolith count of *C. maremetens* (mean ± SEM) following herbicide exposure to hexazinone at increasing concentrations. All concentrations are reported in µg L^{-1} (n = 15 for each treatment for ΔF/Fm′, and n = 5 for each treatment for both bell surface area and statolith count, bars not visible are smaller than symbol).

Appendix B: Marine: *Chaetoceros muelleri*

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Contributing authors: Thomas, M.C., Flores, F., Kaserzon, S. Thompson, J., Fisher, R. and Negri A.P.

The herbicides used in toxicity tests for this species and their mode of action were:
- Diuron - PSII inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Propazine - PSII inhibitor
- Tebuthiuron - PSII inhibitor

Test species: *Chaetoceros muelleri* (marine)

Test phylum: Bacillariophyta

Biological effect: Inhibition of specific growth rate and effective quantum yield

Summary

The effects of four herbicides were tested on growth and photosynthetic efficiency of the marine ochrophyte diatom *Chaetoceros muelleri* in culture over 72 h exposures. The concentrations of each herbicide that inhibited 10% and 50% of the culture's specific growth rate (SGR) and effective quantum yield ($\Delta F/Fm'$) relative to controls (EC_{10} and EC_{50}, respectively) were calculated from concentration-response curves (4-parameter sigmoidal models). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of square root concentration of each measured herbicide using a Bayesian non-linear gaussian model. The toxicity thresholds for SGR (NEC, EC_{10}, EC_{50} in µg L^{-1}) were as follows: diuron (1.47, 1.79, 12.4), tebuthiuron (12.9, 21.5, 98.2) and propazine (16.0, 26.8, 187). No effects on SGR or $\Delta F/Fm'$ were observed for haloxyfop at the highest concentration tested. The inhibition of $\Delta F/Fm'$ over 24 h occurred at lower concentrations than observed for SGR, but the order of herbicide potencies towards both biological effects were similar.

Methods

The inhibition of the specific growth rate in *Chaetoceros muelleri* by each herbicide was tested in static 72 h exposures (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$) was tested in static 24 h exposures (acute). Details of the experimental methods used in the *C. muelleri* toxicity tests are provided in Tables A1 to A3. Original data including SGR, $\Delta F/Fm'$ and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/b250174f-54ce-4c29-ba0d-6ece10359fd3.

Table A1. Source of *Chaetoceros muelleri*, its culturing and test conditions.

Source of tests species	In-house culture (strain CS-176), purchased from Australian National Algae Supply Service, Hobart.	
Maintenance conditions of test species (culture conditions, light, temp etc)	Cultures were maintained in 500 mL Erlenmeyer flasks using Guillard's f/2 medium, aerated and maintained at 26 ± 1 °C, 35 psu and under a 12:12 h light:dark cycle (100-110 µmol photons m^{-2} s^{-1}.	
Test endpoint	1. Specific growth rate (SGR) of culture in log growth phase	2. Inhibition of effective quantum yield (proportional to photosynthetic efficiency)
Test duration	72 h (inhibition of SGR)	24 h (inhibition of $\Delta F/Fm'$)
Test chambers	20 mL glass scintillation vials	48-well plates
Test volume	10 mL	1mL
Starting density	$3x10^3$ cells mL^{-1}	$1x10^6$ cells mL^{-1}

| Counting of cells, calculation of SGR | Cells counted on flow cytometer as per Trenfield et al. (2015). SGR calculated as per OECD test 201 (OECD, 2011). | |

Table A2. Measured physico-chemical parameters of test media for *Chaetoceros muelleri*.

Light intensity (mean ± SD, n = 1 measurement at start of test)	100 -110 µmol photons $m^{-2}s^{-2}$ over a 12:12 h L:D cycle
Temperature (mean ± SD, logged 10 min intervals)	27.5 ± 0.4 °C
Dissolved oxygen (mean ± SD, averaged 0 and 72 h, n = 64)	8.3 ± 0.2 mg L^{-1}
pH (mean ± SD, averaged 0 and 72 h, n = 64)	8.24 ± 0.2
Salinity (mean ± SD, averaged 0 and 72 h, n = 64)	34.6 ± 0.8 psu

Table A3. Test criteria for specific growth rate and effective quantum yield of *Chaetoceros muelleri*.

Exposure duration	SGR 72 h	$\Delta F/Fm'$ 24 h
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase of biomass over 72 h (OECD, 2011).	Inhibition of the effective quantum yield ($\Delta F/Fm'$) which is proportional to photosynthetic efficiency for a given light intensity (Schreiber et al., 2002; Schreiber et al., 2007).
Biological endpoint definition	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce SGR by 10% and 50%, respectively, in comparison to control treatments. No effect concentration (NEC) is the concentration below which the herbicides are not expected to cause a reduction in SGR.	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce $\Delta F/Fm'$ by 10% and 50%, respectively, in comparison to control treatments.
Controls used	Diuron was dissolved using the carrier solvent ethanol (final concentration < 0.001 % v/v in all exposure treatments). Haloxyfop was dissolved in dimethyl sulfoxide (final concentration < 0.006 % v/v in all exposure treatments). No solvent carrier was used for the preparation of the tebuthiuron and propazine stock solutions.	

Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve SGR ($\Delta F/Fm'$)	Replicates per concentration
Diuron	1	8 (9)	5
Propazine	1	7 (9)	5
Tebuthiuron	1	8 (8)	5
Haloxyfop	1	6 (6)	5

| Test acceptability criteria | • Control SGR ≥ 0.92 day^{-1} as per OECD (OECD, 2011). Observed average control SGR of all tests: 1.54 ± 0.11 day^{-1} (mean ± SD, n = 20)
• The coefficient of variation (CV) of mean SGR in controls ≤ 10% as per OECD (OECD, 2011). Observed control CV: < 5% in all tests | $\Delta F/Fm'$ control measurements > 0.45 (Schreiber et al., 2007). |

Characteristics of the test organism	4-day old culture in exponential growth phase, starting density 3×10^3 cells mL^{-1}	4-day old culture in exponential growth phase, starting density 1×10^6 cells mL^{-1}
Type of test media	Natural, 0.5 µm polypropylene-filtered coastal seawater (19°16'19.60"S; 147° 3'40.93"E) spiked with test solution.	
Toxicant (common name; IUPAC Name; CAS no.; purity)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Diuron (DCMU); 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98% • Haloxyfop-p-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98% • Propazine; 6-chloro-2-N,4-N-di(propan-2-yl)-1,3,5-triazine-2,4-diamine; 139-40-2; > 98% • Tebuthiuron; 1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea; 34014-18-1; ≥ 98%	
Preparation of toxicant stock	Stock solutions (8.5-50 mg L^{-1}) of all herbicides were prepared in Milli-Q® water or filtered seawater. Diuron was dissolved using the carrier solvent ethanol (final concentration < 0.001 % v/v in exposures). Haloxyfop was dissolved in dimethyl sulfoxide (final concentration < 0.006 % v/v in exposure). No solvent carrier was used for the preparation of the tebuthiuron and propazine stock solutions.	
Exposure type	Static	
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015).	
Reference toxicant	Diuron at 4 µg l^{-1}	
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal models, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.0.0, San Diego, CA, USA), see Figure A1. • NEC: Binomial exponential decay regression using the R package jagsNEC (Fisher et al., 2019), see Figure A2.	
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR (or ΔF/Fm') relative to controls (EC$_{10}$ and EC$_{50}$, respectively) were calculated from concentration-response curves (4-parameter sigmoidal models) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.0.0, San Diego, USA). • No effect concentration (NEC) values were calculated in R statistical package (v 3.5.1) and the proportional decline in SGR (1-inhibition) was modelled as a function of square root measured concentration of each herbicide using a Bayesian non-linear gaussian model using the R package jagsNEC (Fisher et al., 2019).	
Data variance	95% Confidence Limits (CL) (see Tables A4 and a5)	
Test solutions, blanks and/or controls tested for	Controls were tested for contamination. Analytical grade herbicides (> 98% purity) were used for preparation of all stock solutions.	

contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	

Summary of results

The toxicity of four herbicides to *C. muelleri* is presented in Tables A4 and A5 and Figures A1 and A2. The non-PSII herbicide haloxyfop did not inhibit SGR and ΔF/Fm′ in *C. muelleri* at the maximum concentration of 4570 µg L^{-1}. Higher concentrations of haloxyfop could not be tested due to the stock solution being at its practical solubility limit in seawater.

Table A4. Modelled toxicity estimates for the inhibitory effects of four herbicides on the specific growth rate (SGR) of *Chaetoceros muelleri* (Figs. A1 and A2). All concentrations in µg L^{-1} (95% confidence intervals).

	NEC (95% CI)	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	1.47 (1.15 – 1.83)	1.79 (1.60 – 1.98)	12.4 (11.8 – 13.0)
Propazine	12.9 (9.29 – 32.0)	21.5 (18.4 – 25.0)	98.2 (91.7 – 105)
Tebuthiuron	16.0 (13.0 – 19.1)	26.8 (23.9 – 29.9)	187 (179 – 195)
Haloxyfop	> 4,570	> 4,570	> 4,570

Table A5. Modelled toxicity estimates for the inhibitory effects of four herbicides on the photosynthetic efficiency (ΔF/Fm′) of *Chaetoceros muelleri* (Fig. A1). All concentrations in µg L^{-1} (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	0.97 (0.81 – 1.15)	4.25 (3.96 – 4.55)
Propazine	8.12 (7.04 – 9.33)	48.6 (45.6 – 51.7)
Tebuthiuron	6.95 (5.79 – 8.27)	47.7 (44.1 – 51.5)
Haloxyfop	> 4,570	> 4,570
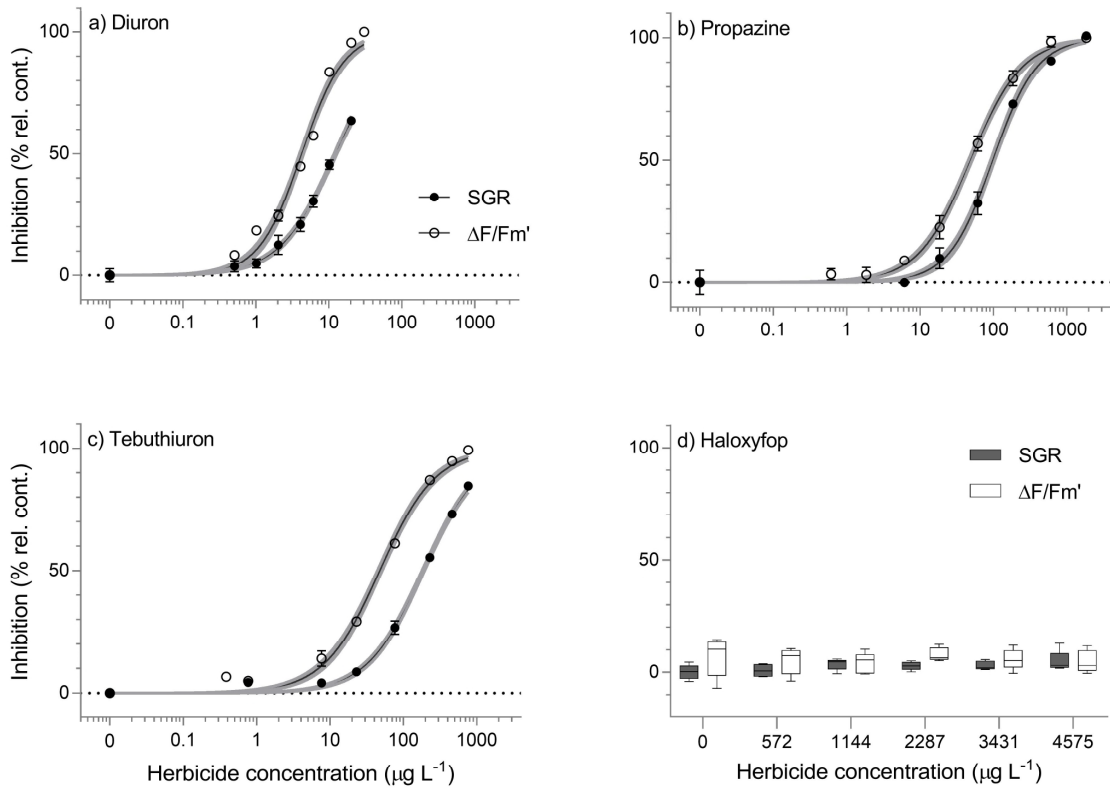
Figure A1. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 3-day specific growth rate (SGR) and effective quantum yield (ΔF/Fm′) of *Chaetoceros muelleri* (mean ± SD) following herbicide exposure to a) diuron; b) tebuthiuron; and c) propazine and boxplot showing inhibition of 3-day specific growth rate (SGR) and effective quantum yield (ΔF/Fm′) in response to d) haloxyfop. All concentrations in µg L^{-1} (n = 5 for each treatment, bars not visible are smaller than symbol).
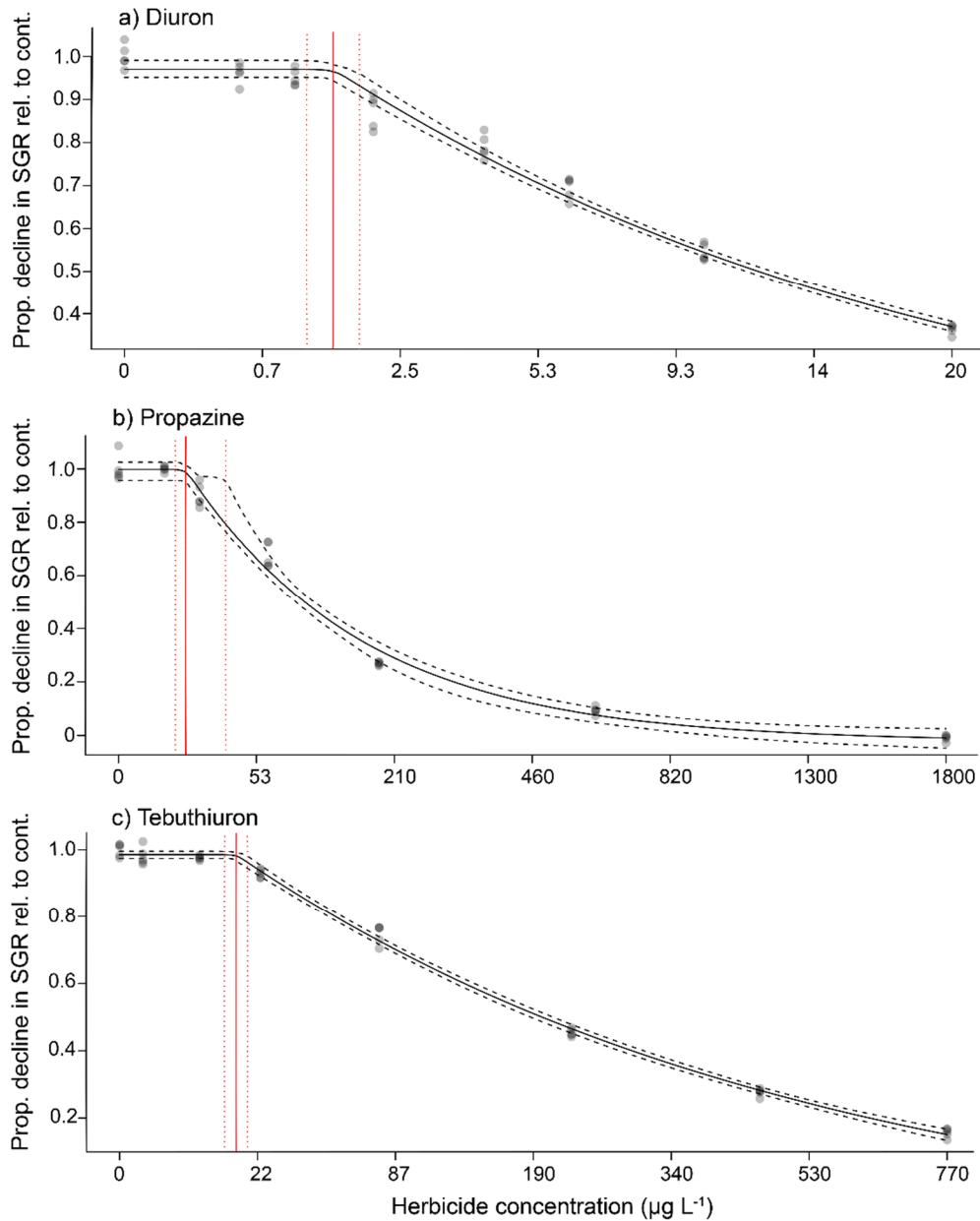
Figure. A2. Bayesian non-linear gaussian model fit on the proportional decline in **3-day specific growth rate (SGR)** of *Chaetoceros muelleri* relative to the control treatment (solid black line) and 95% confidence intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of a) diuron; b) tebuthiuron; and c) propazine. All concentrations in µg L⁻¹.

Appendix C: Marine: *Cladocopium goreaui*

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Contributing authors: Marzonie M., Flores F., Sadoun, N.,Valada-Mennuni, A., Thomas, M., Elisei, G., Negri A.P.

The herbicides used in toxicity tests for this species and their mode of action were:

- Diuron - Photosystem II (PSII) inhibitor
- Bromacil - PSII inhibitor
- Haloxyfop - acetyl CoA carboxylase (ACCase) inhibitor
- Hexazinone - PSII inhibitor
- Imazapic – acetohydroxyacid synthase (AHAS) inhibitor
- Metribuzin - PSII inhibitor
- Propazine - PSII inhibitor
- Simazine - PSII inhibitor
- Tebuthiuron - PSII inhibitor

Test species: *Cladocopium goreaui* (marine)

Test phylum: Dinoflagellata

Biological effect: Inhibition of specific growth rate and effective quantum yield

Summary

The effects on growth and photosynthetic efficiency of nine herbicides were tested on *Cladocopium goreaui* (formerly *Symbiodinium* clade C, (LaJeunesse et al., 2018)) over 14 d exposures. The concentrations of each herbicide that inhibited 10% and 50% of the culture's specific growth rate (SGR) and effective quantum yield ($\Delta F/Fm'$) relative to controls (EC_{10} and EC_{50}, respectively) were calculated from concentration-response curves (4-parameter sigmoidal models). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of log measured concentration of each herbicide using a Bayesian non-linear gaussian model. The toxicity thresholds for SGR (NEC, EC_{10}, EC_{50} in $\mu g\ L^{-1}$) were as follows: diuron (2.8, 2.5, 4.5), bromacil (17, 18, 28), metribuzin (24, 22, 34), propazine (45, 51, 87), hexazinone (72, 79, 100), tebuthiuron (107, 138, 331) and simazine (320, 257, 387). The inhibition of $\Delta F/Fm'$ occurred at lower concentrations than observed for SGR. The toxicity thresholds for $\Delta F/Fm'$ (EC_{10}, EC_{50} in $\mu g\ L^{-1}$) were as follows: diuron (0.3, 1.2), metribuzin (2.3, 8.8), bromacil (2.5, 8.4), propazine (5.4, 19), hexazinone (8.4, 34), tebuthiuron (6.4, 41) and simazine (29, 93). No effects on SGR and $\Delta F/Fm'$ were observed for haloxyfop and imazapic at the highest concentrations tested.

Methods

The inhibition of specific growth rate and effective quantum yield ($\Delta F/Fm'$) of *Cladocopium goreaui* was tested in static 14-d exposures (chronic). All test tubes were rearranged every 1-2 days to ensure that all cultures experienced similar temperature and light conditions. Details of the experimental methods used in the *Cladocopium goreaui* toxicity tests are provided in Tables A1 to A3. Original data including SGR, $\Delta F/Fm'$ and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/9f681349-004f-4407-b5f3-a6db6a3aa611.

Table A1. Source of *Cladocopium goreaui,* its culturing and test conditions.

Source of tests species	Australian Institute of Marine Science culture (ID: SCF 055-01.10). Monoclonal strain isolated from *Acropora tenuis* from Magnetic Island, QLD, Australia.
Maintenance conditions of test species (culture conditions, temp, light)	Cultures were maintained in 75 cm^2 culture flasks in IMK nutrient media under 27 ± 1°C and light intensity 60 - 75 µmol photons m^{-2}s^{-1} under 14:10 h light:dark light cycle
Test duration	14 days
Test chambers	50 mL polypropylene conical centrifuge tube
Test volume	30 mL IMK media tube^{-1}
Starting density	1.7-2.7 x 10^4 cells mL^{-1}
Test endpoint	1. Inhibition of specific growth rate (SGR) of culture in logarithmic growth phase	2. Inhibition of effective quantum yield (ΔF/Fm', proportional to photosynthetic efficiency)
Counting of cells, calculation of SGR	Samples fixed in glutaraldehyde (0.5% v/v final concentration) and surfactant (Pluronic F68; 0.1% v/v final concentration) as per Marie et al. (2014). Cell counts were performed as per Trenfield et al. (2015). SGR calculated as per OECD test no. 201 (OECD, 2011).	

Table A2. Measured physico-chemical parameters of test media for *Cladocopium goreaui*.

Light intensity (mean ± SD, averaged across all treatments)	71 ± 8 µmol photons m^{-2} s^{-1} over a 12:12 h L:D cycle
Temperature (mean ± SD, logged 5-10 min intervals)	27 ± 0.6 °C
Dissolved oxygen, (mean ± SD, averaged 0 and 14 d, n = 152)	7.8 ± 0.3 mg L^{-1}
pH (mean ± SD, averaged 0 and 14 d, n = 152)	7.8 ± 0.5
Salinity (mean ± SD, averaged 0 and 14 d, n = 152)	32.5 ± 0.7 psu

Table A3. Test criteria for specific growth rate and effective quantum yield of *Cladocopium goreaui*.

Test duration	14 d	
Biological effect metric	Inhibition of the mean specific growth rate – the logarithmic increase of biomass over 14 d (OECD, 2011).	Inhibition of the effective quantum yield (ΔF/Fm') which is proportional to photosynthetic efficiency for a given light intensity using microscopy pulse amplitude modulated (PAM) fluorometry (microscopy-PAM, Walz GmbH, Germany; PAM settings were MF =10-12; SI = 2; SW = 0.8; OG = 3) (Schreiber et al., 2007).
Biological endpoint definition	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce SGR by 10% and 50%, respectively, in comparison to control treatments. No effect concentration (NEC) is the	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce ΔF/Fm' by 10% and 50%, respectively, in comparison to control treatments.

	concentration below which the herbicides are not expected to cause a reduction in SGR.		
Controls used	Diuron and metribuzin were dissolved using the carrier solvent ethanol (≤ 0.002% v/v in all exposure treatments). Haloxyfop and simazine were dissolved in the carrier solvent dimethyl sulfoxide (DMSO; ≤ 0.006% v/v in all exposure treatments). No solvent carrier was used for the preparation of the remaining herbicide stock solutions.		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Diuron	1[1]	7	6
Bromacil	1[3]	8	4
Hexazinone	1[1]	7	6
Metribuzin	1	8	6
Propazine	1[1]	8	4
Simazine	1	8	5
Tebuthiuron	1[1]	7	5
Haloxyfop	1	7	5
Imazapic	1	10	3
	[1] and [3] were rangefinders were conducted prior to the definitive experiments		
Test acceptability criteria	• Control SGR ≥ 0.1 day^{-1} (Rogers & Davis, 2006; Sakami, 2008; Hennige et al., 2009; Klueter et al., 2017). Observed mean control SGR of all tests: 0.13 ± 0.02 day^{-1} (mean ± SD, n = 54). • The coefficient of variation (CV) of mean SGR in controls ≤ 10% as per (OECD, 2011). Observed control CV: ≤ 10% in all tests.	ΔF/Fm′ control measurements > 0.30 (Hennige et al., 2009; Karim et al., 2015). Observed control ΔF/Fm' = 0.35 ± 0.04.	
Characteristics of the test organism (e.g. length, mass, age)	14-d old culture in exponential growth phase, starting density 1.7 – 2.7 x 10^4 cells		
Type of test media	Natural, 0.2 µm polypropylene-filtered coastal seawater (19°16'19.60"S; 147° 3'40.93"E) spiked with test solution.		
Toxicant (common name; IUPAC Name; CAS no., purity)	All chemicals were analytical grade purchased from Sigma-Aldrich. • Diuron (DCMU); 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98% • Bromacil; 5-bromo-3-butan-2-yl-6-methyl-1H-pyrimidine-2,4-dione; 314-40-9; 98.5% • Haloxyfop-P-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98% • Hexazinone; 3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-dione; 51235-04-2; 99.5% • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98% • Metribuzin; 4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one; 21087-64-9; 99.5% • Propazine; 6-chloro-2-N,4-N-di(propan-2-yl)-1,3,5-triazine-2,4-diamine; 139-40-2; > 98%		

	• Simazine; 6-chloro-2-N,4-N-diethyl-1,3,5-triazine-2,4-diamine; 122-34-9; 99% • Tebuthiuron; 1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea; 34014-18-1; ≥ 98%
Preparation of toxicant stock	Stock solutions (5 - 600 mg L^{-1}) were prepared in Milli-Q® water or filtered seawater. Diuron and metribuzin were dissolved using the carrier solvent ethanol (≤ 0.002% v/v in all exposure treatments). Haloxyfop and simazine were dissolved in the carrier solvent dimethyl sulfoxide (DMSO; ≤ 0.006% v/v in all exposure treatments). No solvent carrier was used for the preparation of the remaining herbicide stock solutions.
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted averaged of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Diuron at 6 µg l^{-1}
Concentration-response relationship.	• ECx: 4-parameter sigmoidal models, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism using the software GraphPad Prism (v 7.05, San Diego, CA, USA), see Figure A1. • NEC: Binomial exponential decay regression using the R package jagsNEC (Fisher et al., 2019), see Figure A2.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR (or ΔF/Fm') relative to controls (EC$_{10}$ and EC$_{50,}$ respectively) were calculated from concentration-response curves (4-parameter sigmoidal models) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 7.05, San Diego, CA, USA). • No effect concentration (NEC) values were calculated in R statistical package (v 3.5.3) and the proportional decline in SGR (1-inhibition) was modelled as a function of log measured concentration of each herbicide using a Bayesian non-linear gaussian model using the R package jagsNEC (Fisher et al., 2019).
Data variance	95% Confidence Limits (CL) (see Tables A4 and A5)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade herbicides (98-99.5% purity) were used for preparation of all herbicide stock solutions.

Summary of results

The toxicity of nine herbicides to *C. goreaui* is presented in Tables A4 (SGR) and A5 (ΔF/Fm') and Figures A1 to A3. The non-PSII herbicides haloxyfop and imazapic did not inhibit SGR or ΔF/Fm' at the maximum concentration of 3,000 µg L^{-1} and 165,000 µg L^{-1}, respectively. Higher concentrations could not be tested because at higher concentrations imazapic affected the pH of the test media.

Table A4. Modelled toxicity estimates for the inhibition of specific growth rate (SGR) of nine herbicides on *Cladocopium goreaui* (Figs. A1-A3). NA indicates values could not be calculated. All concentrations in µg L^{-1} (95% confidence intervals).

	NEC (95% CI)	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	2.75 (2.56 – 2.93)	2.54 (2.34 – 2.75)	4.45 (4.31 – 4.59)
Bromacil	16.6 (15.4 – 20.6)	18.3 (16.9 – 19.9)	27.7 (26.7 – 28.7)
Haloxyfop	> 3,000	> 3,000	> 3,000
Hexazinone	71.7 (63.4 – 91.0)	78.7 (57.8 – 92.0)	100 (96.1 – 141)
Imazapic	> 165,000	> 165,000	> 165,000
Metribuzin	23.6 (21.3 – 27.5)	22.3 (16.2 – 25.9)	33.5 (30.2 – 50.4)
Propazine	45.1 (37.0 – 51.1)	50.8 (44.8 – 57.4)	86.5 (83.0 – 90.1)
Simazine	320 (234 – 452)	257 (226 – 294)	387 (361 – 416)
Tebuthiuron	107 (84.6 – 136)	138 (108 – 173)	331 (300 – NA)

Table A5. Modelled toxicity estimates for inhibition of photosynthetic efficiency (ΔF/Fm') of nine herbicides on *Cladocopium goreaui* (Fig. A1 and A3). All concentrations in µg L^{-1} (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	0.29 (0.26 – 0.33)	1.20 (1.15 – 1.26)
Bromacil	2.54 (2.29 – 2.82)	8.36 (8.01 – 8.69)
Haloxyfop	> 3,000	> 3,000
Hexazinone	8.36 (7.14 – 9.80)	33.8 (30.6 – 37.6)
Imazapic	> 165,000	> 165,000
Metribuzin	2.31 (2.08 – 2.56)	8.75 (8.39 – 9.12)
Propazine	5.42 (4.94 – 5.95)	18.7 (18.0 – 19.5)
Simazine	28.8 (23.9 – 35.3)	93.3 (84.6 – 102)
Tebuthiuron	6.37 (4.79 – 8.50)	41.0 (36.3 – 46.3)
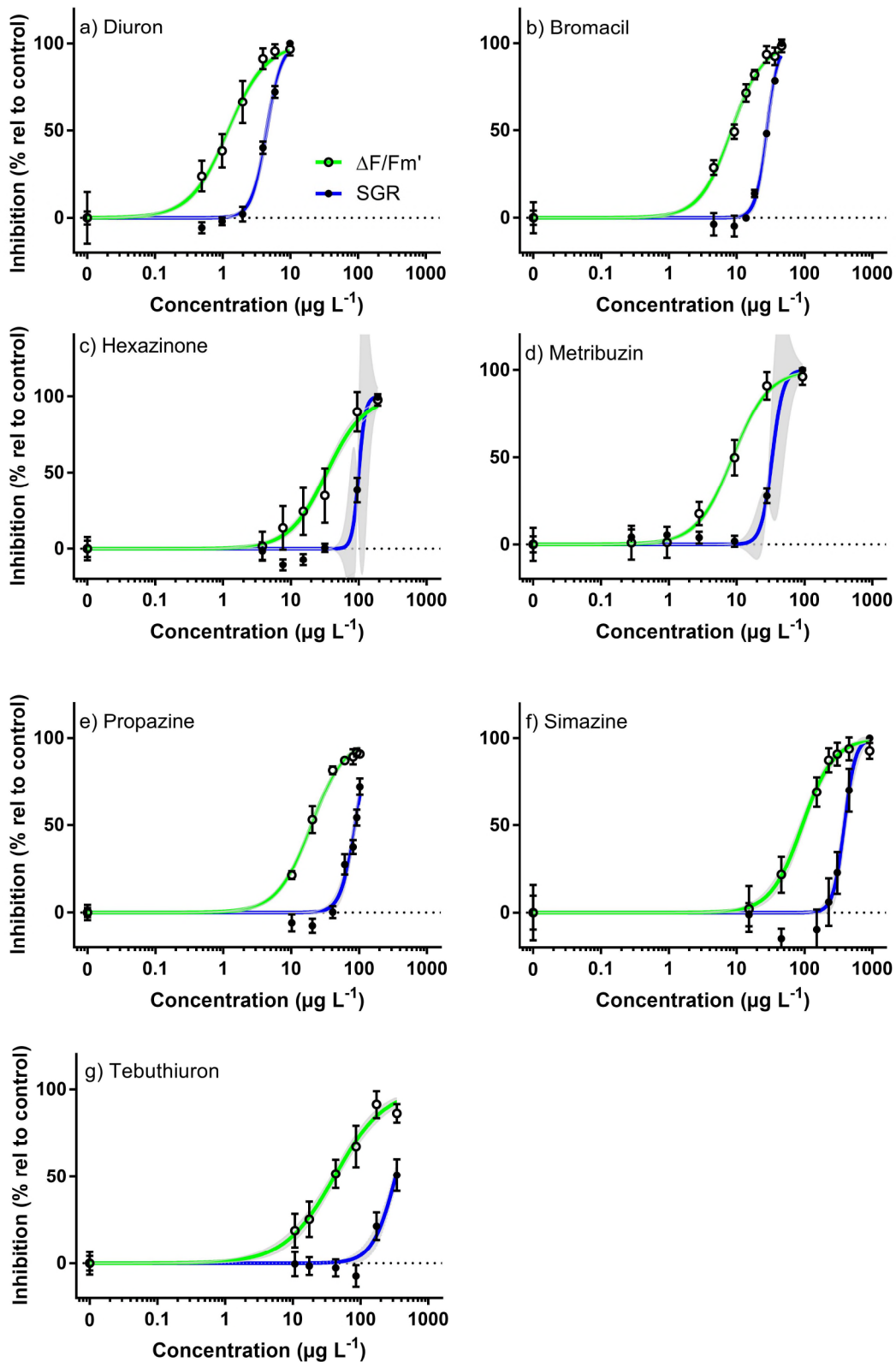
Figure A1. Concentration-response curves for EC$_x$ derivation. Sigmoidal, 4-parameter curve fit (solid line) and 95% confidence intervals (shaded area) on the relative percent inhibition of 14-day specific growth rate (SGR; closed circle, mean ± SD) and effective quantum yield (ΔF/F$_m$′; open circle, mean ± SD) of *Cladocopium goreaui* following herbicide exposure to a) diuron; b) bromacil; c) hexazinone; d) metribuzin; e) propazine; f) simazine; and g) tebuthiuron at increasing concentrations. All concentrations in µg L^{-1} (error bars not visible are smaller than symbol).
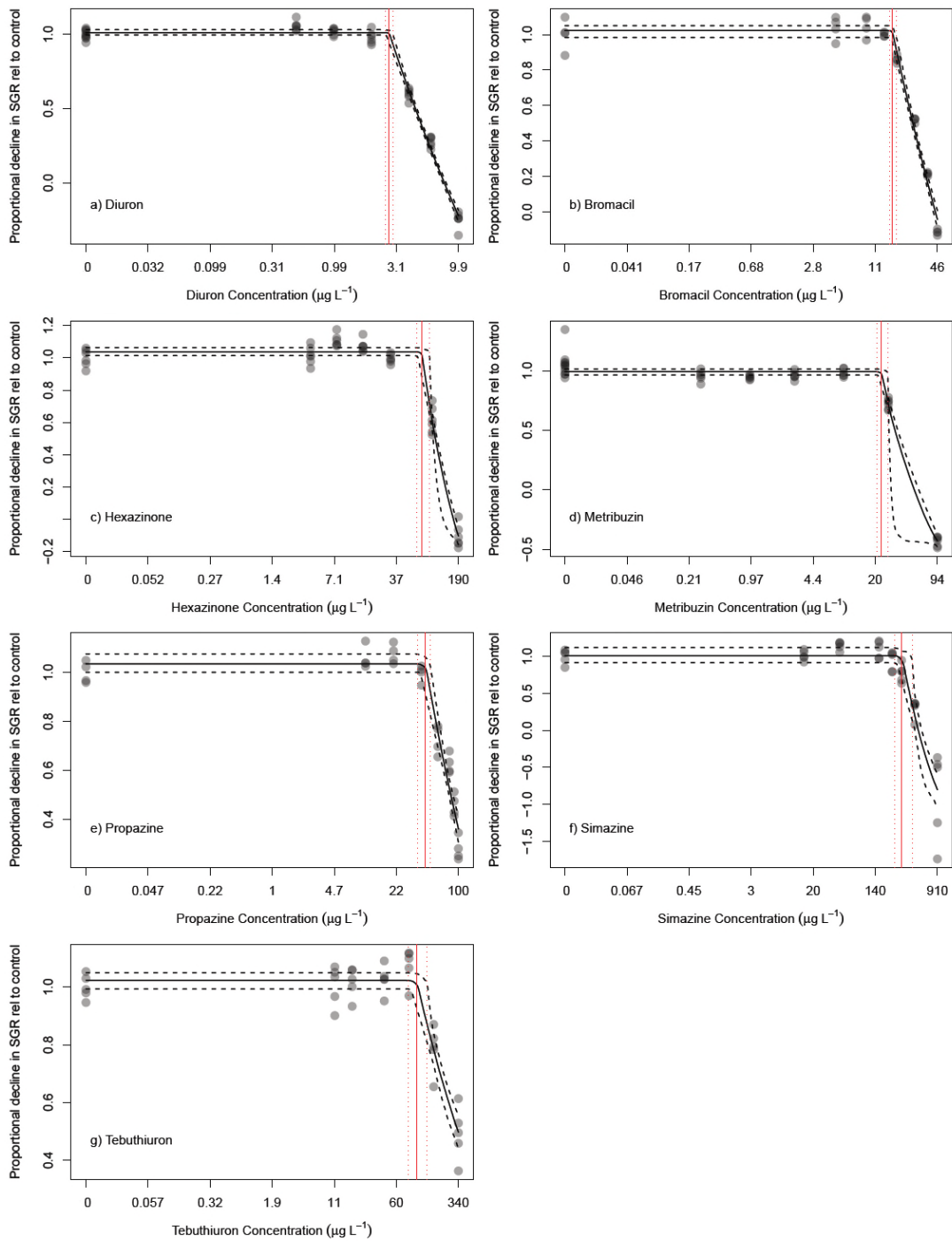
Figure A2. Bayesian non-linear gaussian model fits (except for propazine in which a gamma model was a better fit) on the proportional decline of specific growth rate (SGR) of *Cladocopium goreaui* relative to the control treatment (solid black line) and 95 % confidence intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of a) diuron; b) bromacil; c) hexazinone; d) metribuzin; e) propazine; f) simazine; and g) tebuthiuron. All concentrations in µg L⁻¹.
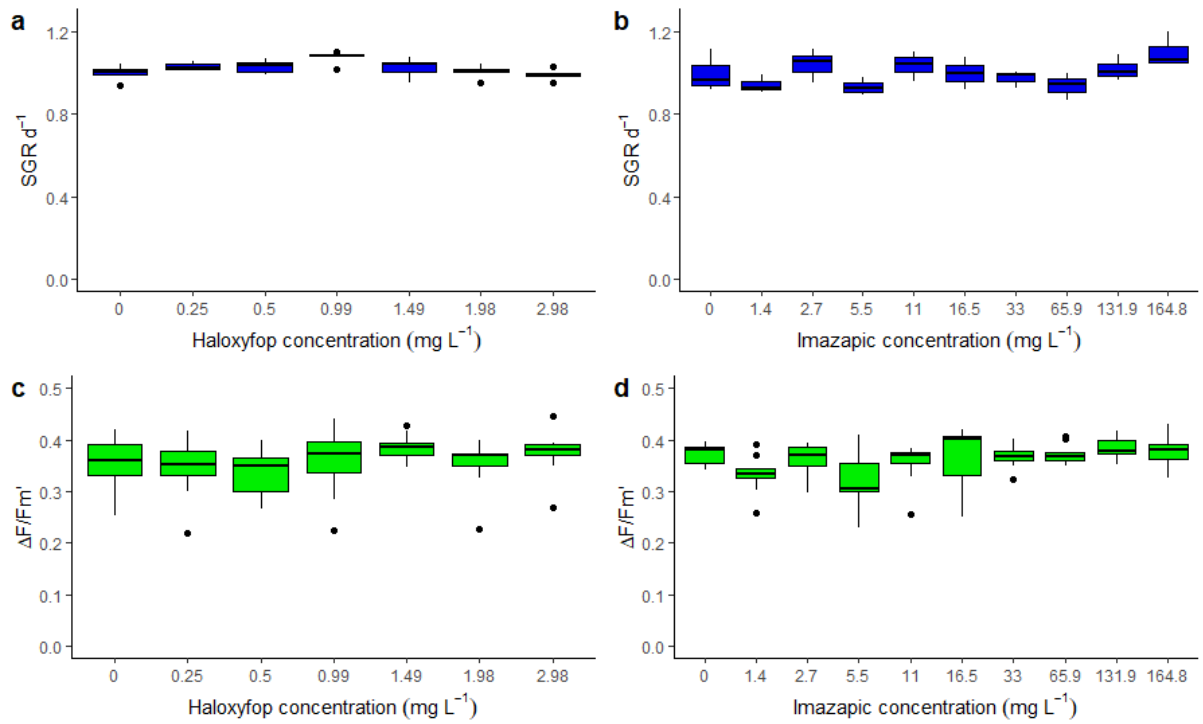
Figure A3. Boxplots of the specific growth rate (SGR d^{-1}) and effective quantum yields (ΔF/Fm') of *Cladocopium goreaui* in response to haloxyfop (a, c) and imazapic (b, d). All concentrations in mg L^{-1}.

Appendix D: Marine: *Rhodomonas salina*

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Contributing authors: Thomas, M.C., Flores, F., Kaserzon, S., Fisher, R. and Negri A.P.

The herbicides used in toxicity tests for this species and their mode of action were:
- Diuron - PSII inhibitor
- Metribuzin - PSII inhibitor
- Hexazinone - PSII inhibitor
- Tebuthiuron - PSII inhibitor
- Bromacil, - PSII inhibitor
- Simazine - PSII inhibitor
- Propazine - PSII inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- 2,4-D - auxin mimic

Test species: *Rhodomonas salina* (marine)

Test phylum: Cryptophyta

Biological effect: Inhibition of specific growth rate and effective quantum yield

Summary

The effects of ten herbicides were tested on growth of the cryptophyte *Rhodomonas salina* in culture over 72 h exposures. The concentrations of each herbicide that inhibited 10% and 50% of the culture's specific growth rate (SGR) and effective quantum yield ($\Delta F/Fm'$) relative to controls (EC_{10} and EC_{50}, respectively) were calculated from concentration-response curves (4-parameter sigmoidal models). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of log concentration of each herbicide using a Bayesian non-linear gaussian model. The toxicity thresholds for SGR (NEC, EC_{10}, EC_{50} in µg L^{-1}) were as follows: diuron (1.7, 1.9, 6.3), metribuzin (2.2, 2.7, 13), hexazinone (4.6, 4.0, 8.5), bromacil (5.5, 4.9, 19), tebuthiuron (23, 28, 112), simazine (48, 38, 184), propazine (28, 42, 188), imazapic (363,000, 410,000; 790,000). No effects on SGR were observed for haloxyfop and 2,4-D at the highest concentrations tested. The inhibition of $\Delta F/Fm'$ over 24 h occurred at lower concentrations than observed for SGR, but the order of herbicide potencies towards both biological effects were similar. No effects on $\Delta F/Fm'$ were observed for imazapic, haloxyfop and 2,4-D at the highest concentrations tested.

Methods

The inhibition of the specific growth rate in *Rhodomonas salina* by each herbicide was tested in static 72 h exposures (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$) was tested in static 24 h exposures (acute). Details of the experimental methods are provided in Tables A1 to A3. Original data including SGR, $\Delta F/Fm'$ and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/abe661f3-558d-4c4e-b655-12e1fbdcd5a1.

Table A1. Source of *Rhodomonas salina*, its culturing and test conditions.

Source of tests species	In-house culture (strain CS-24/01), purchased from Australian National Algae Supply Service, Hobart.	
Maintenance conditions of test species (culture conditions, light, temp etc)	Cultures were maintained in 500 mL Erlenmeyer flasks using Guillard's f/2 medium, aerated and maintained at 26 ± 1 °C, 35 psu and under a 12:12 h light:dark cycle (90-100 µmol photons m^{-2} s^{-1}).	
Test endpoint	1. Inhibition of specific growth rate (SGR) of culture in log growth phase	2. Inhibition of effective quantum yield (ΔF/Fm′, proportional to photosynthetic efficiency)
Test duration	72 h (inhibition of SGR)	24 h (inhibition of ΔF/Fm′)
Test chambers	20 mL glass scintillation vials	48-well-plates
Test Volume	10 mL	1 mL
Starting density	3×10^3 cells mL^{-1}	3.5×10^5 cells mL^{-1}
Counting of cells, calculation of SGR	Cells counted on flow cytometer as per Trenfield et al. (2015). SGR calculated as per OECD test 201 (OECD, 2011)	

Table A2. Measured physico-chemical parameters of test media for *Rhodomonas salina*.

Light intensity (mean ± SD, n = 1 measurements at start of test)	90 - 100 µmol photons m^{-2}s^{-2} over a 12:12 h L:D cycle
Temperature (mean ± SD, logged 10 min intervals)	26.0 ± 0.6 °C
Dissolved oxygen (mean ± SD, averaged 0 and 72 h, n = 168)	8.0 ± 0.4 mg L^{-1}
pH (mean ± SD, averaged 0 and 72 h, n = 168)	8.5 ± 0.4
Salinity (mean ± SD, averaged 0 and 72 h, n = 168)	34.2 ± 0.6 psu

Table A3. Test criteria for specific growth rate and effective quantum yield of *Rhodomonas salina*.

Exposure duration	SGR 72 h	ΔF/Fm′ 24 h
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase of biomass over 72 h (OECD, 2011)	Inhibition of the effective quantum yield (ΔF/Fm′) which is proportional to photosynthetic efficiency for a given light intensity (Schreiber et al., 2002; Schreiber et al., 2007).
Biological endpoint definition	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce SGR by 10% and 50%, respectively, in comparison to control treatments. No effect concentration (NEC) is the concentration below which the herbicides are not expected to cause a reduction in SGR.	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce ΔF/Fm′ by 10% and 50%, respectively, in comparison to control treatments.
Controls used	Diuron and simazine were dissolved using the carrier solvent ethanol (final concentration < 0.001 % v/v in all exposure treatments). Haloxyfop was dissolved in the carrier dimethyl sulfoxide (final concentration < 0.006 % v/v in all exposure treatments). No solvent carrier was used for the preparation of the remaining herbicide stock solutions.	
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve: SGR (ΔF/Fm′)	Replicates per concentration
Diuron	1*	8 (8)	5
Metribuzin	1	7 (7)	5
Hexazinone	1	7 (7)	5

Bromacil	1	8 (8)	5
Tebuthiuron	1	7 (7)	5
Simazine	1*	8 (8)	5
Propazine	1	9 (9)	5
Imazapic	1	7 (6)	4
Haloxyfop	1	7 (6)	5
2,4-D	1	7 (6)	5

*rangefinders were conducted prior to the definitive experiment

Test acceptability criteria	• Control SGR ≥ 0.92 day^{-1} as per (OECD, 2011). Observed mean control SGR of all tests: 1.20 ± 0.07 day^{-1} (mean ± SD, n = 50) • The coefficient of variation (CV) of mean SGR in controls ≤ 10% as per (OECD, 2011). Observed control CV: < 5% in all tests	ΔF/Fm′ control measurements > 0.45 (Schreiber et al., 2007).
Characteristics of the test organism	4-day old culture in exponential growth phase, starting density 3x10^3 cells mL^{-1}	4-day old culture in exponential growth phase, starting density 3.5x10^5 cells mL^{-1}
Type of test media	Natural, 0.5 μm polypropylene-filtered coastal seawater (19°16'19.60"S; 147° 3'40.93"E) spiked with test solution.	
Toxicant (common name; IUPAC Name; CAS no.; purity)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Diuron (DCMU); 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98% • Metribuzin; 4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one; 21087-64-9; 99.5% • Hexazinone; 3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-dione; 51235-04-2; 99.5% • Tebuthiuron; 1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea; 34014-18-1; ≥ 98% • Bromacil; 5-bromo-3-butan-2-yl-6-methyl-1H-pyrimidine-2,4-dione; 314-40-9; 98.5% • Propazine; 6-chloro-2-N,4-N-di(propan-2-yl)-1,3,5-triazine-2,4-diamine; 139-40-2; > 98% • Simazine; 6-chloro-2-N,4-N-diethyl-1,3,5-triazine-2,4-diamine; 122-34-9; 99% • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98% • Haloxyfop-p-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98% • 2,4-D; 2-(2,4-dichlorophenoxy)acetic acid; 94-75-7; ≥ 98%	
Preparation of toxicant stock	Stock solutions (5-2,000 mg L^{-1}) of all herbicides were prepared in Milli-Q® water or filtered seawater. Diuron and simazine were dissolved using the carrier solvent ethanol (< 0.001 % (v/v) in exposures). Haloxyfop was dissolved in dimethyl sulfoxide (DMSO) (≤ 0.006 % (v/v) in exposure). No solvent carrier was used for the preparation of the remaining herbicide stock solutions.	
Exposure type	Static	
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the	

	Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Diuron at 4 µg L^{-1}
Concentration-response relationship	• EC_x: 4-parameter sigmoidal models, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.0.0, San Diego, CA, USA). see Figure A1. • NEC: Binomial exponential decay regression using the R package jagsNEC (Fisher et al., 2019), See Figure A2.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR (or ΔF/Fm′) relative to controls (EC_{10} and $EC_{50,}$ respectively) were calculated from concentration-response curves (4-parameter sigmoidal models) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.0.0, San Diego, CA, USA). • No effect concentration (NEC) values were calculated in R statistical package (v 3.5.1) and the proportional decline in SGR (1-inhibition) was modelled as a function of square root measured concentration of each herbicide using a Bayesian non-linear gaussian model using the R package jagsNEC (Fisher et al., 2019).
Data variance	95% Confidence Limits (CL) (see Tables A4 and A5)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade herbicides (98-99.5% purity) were used for preparation of all stock solutions.

Summary of results

The toxicity of ten herbicides to *R. salina* is presented in Table A4, Table A5 and Figures A1 and A2. The non-PSII herbicides 2,4-D and haloxyfop did not inhibit SGR in *R. salina* at the maximum concentration of 279,000 µg L^{-1} and 3,700 µg L^{-1}, respectively. Imazapic, haloxyfop and 2,4-D had no effect on ΔF/Fm′ in *R. salina* at the maximum concentrations of 790,000 µg L^{-1}, 279,000 µg L^{-1} and 3,700 µg L^{-1}, respectively. Higher concentrations could not be tested because at higher concentrations both imazapic and 2,4-D affected the pH and because haloxyfop had reached its practical solubility limit in seawater (at 3,700 µg L^{-1}).

Table A4. Modelled toxicity estimates for the inhibition of ten herbicides on the specific growth rate (SGR) of *Rhodomonas salina* (Figs. A1 and A2). All concentrations in µg L-1 (95% confidence intervals).

	NEC (95% CI)	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	1.68 (1.53-1.90)	1.94 (1.75 - 2.14)	6.27 (6.02 - 6.54)
Metribuzin	2.21 (1.97 - 2.82)	2.66 (2.21 - 3.18)	13.4 (12.3 -14.5)
Hexazinone	4.58 (4.34 – 4.78)	3.96 (3.40 – 4.57)	8.50 (7.99 – 9.06)
Bromacil	5.53 (4.33 – 6.44)	4.89 (4.01 - 5.91)	19.3 (17.7 - 21.0)
Tebuthiuron	22.7 (20.3 - 25.2)	27.5 (24.2 - 31.2)	112 (106 - 119)
Simazine	48.0 (44.0 – 51.0)	38.4 (33.0 – 44.2)	184 (173 - 195)
Propazine	27.8 (24.2 – 31.1)	42.0 (37.1 - 47.3)	188 (177 – 201)
Imazapic	363,000 (341,000 - 386,000)	410,000 (362,000 - 462,000)	790,000 (760,000 - 825,000)
Haloxyfop	> 3,700	> 3,700	> 3,700
2,4-D	> 279,000	> 279,000	> 279,000

Table A5. Modelled toxicity estimates for inhibition of ten herbicides on the photosynthetic efficiency (ΔF/Fm′) of *Rhodomonas salina* (Fig. A1). All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	0.43 (0.38 - 0.48)	1.71 (1.63 - 1.80)
Metribuzin	0.60 (0.50 - 0.71)	2.95 (2.72 - 3.18)
Hexazinone	1.81 (1.63 – 1.99)	5.85 (5.61 - 6.09)
Bromacil	0.59 (0.45 - 0.75)	3.56 (3.19 – 3.98)
Tebuthiuron	2.66 (2.31 - 3.06)	16.0 (15.1 – 17.0)
Simazine	9.28 (8.41 - 10.2)	59.2 (56.7 – 61.8)
Propazine	5.85 (4.90 - 6.91)	39.5 (37.1 – 42.1)
Imazapic	> 790,000	> 790,000
Haloxyfop	> 3,700	> 3,700
2,4-D	> 279,000	> 279,000
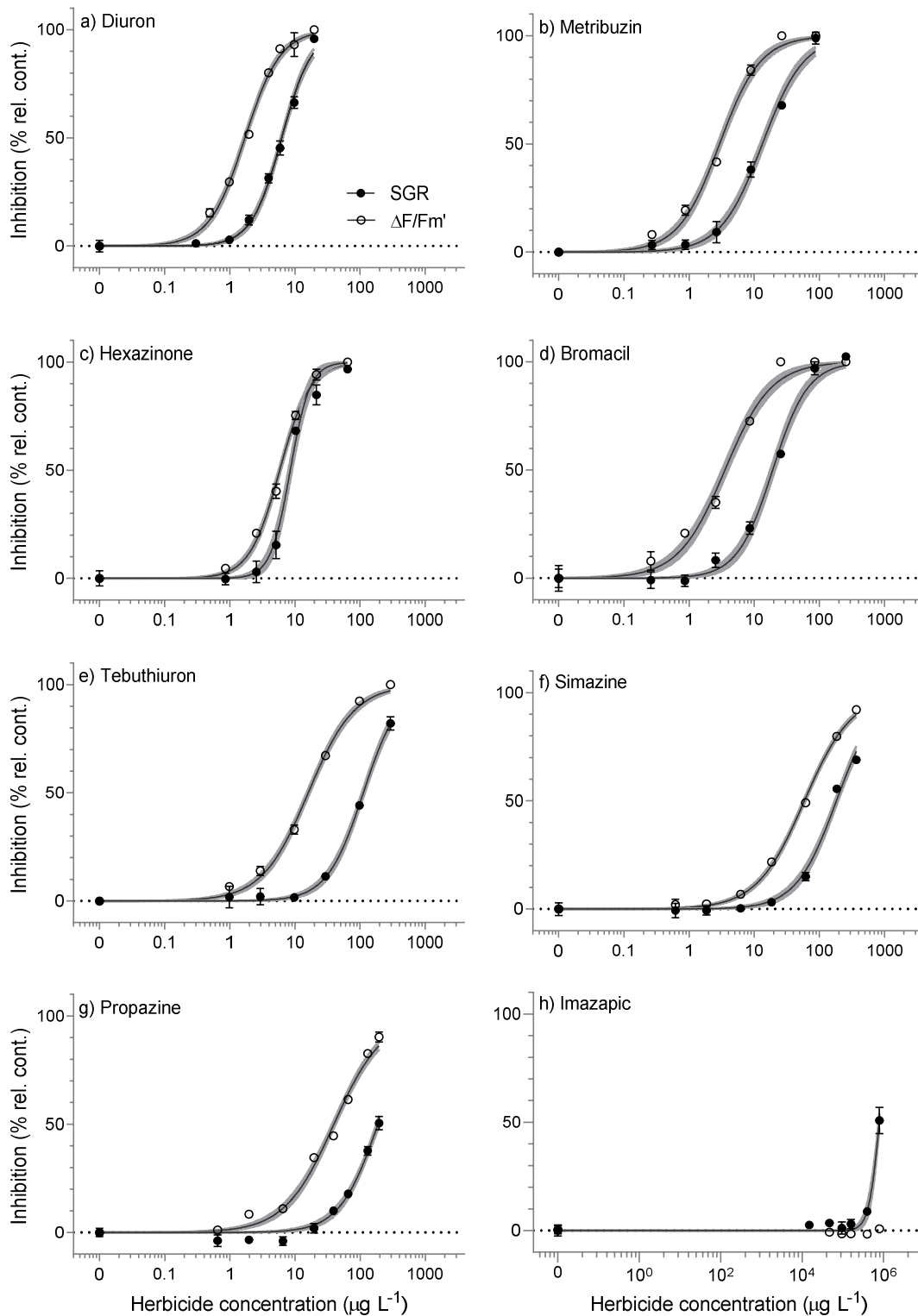
Figure A1. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 3-day specific growth rate (SGR) and effective quantum yield (ΔF/Fm′) of *Rhodomonas salina* (mean ± SE) following herbicide exposure to a) diuron; b) metribuzin; c) hexazinone; d) bromacil; e) tebuthiuron; f) simazine; g) propazine; and h) imazapic at increasing concentrations. All concentrations are reported in μg L-1 (n = 5 for each treatment, bars not visible are smaller than symbol).
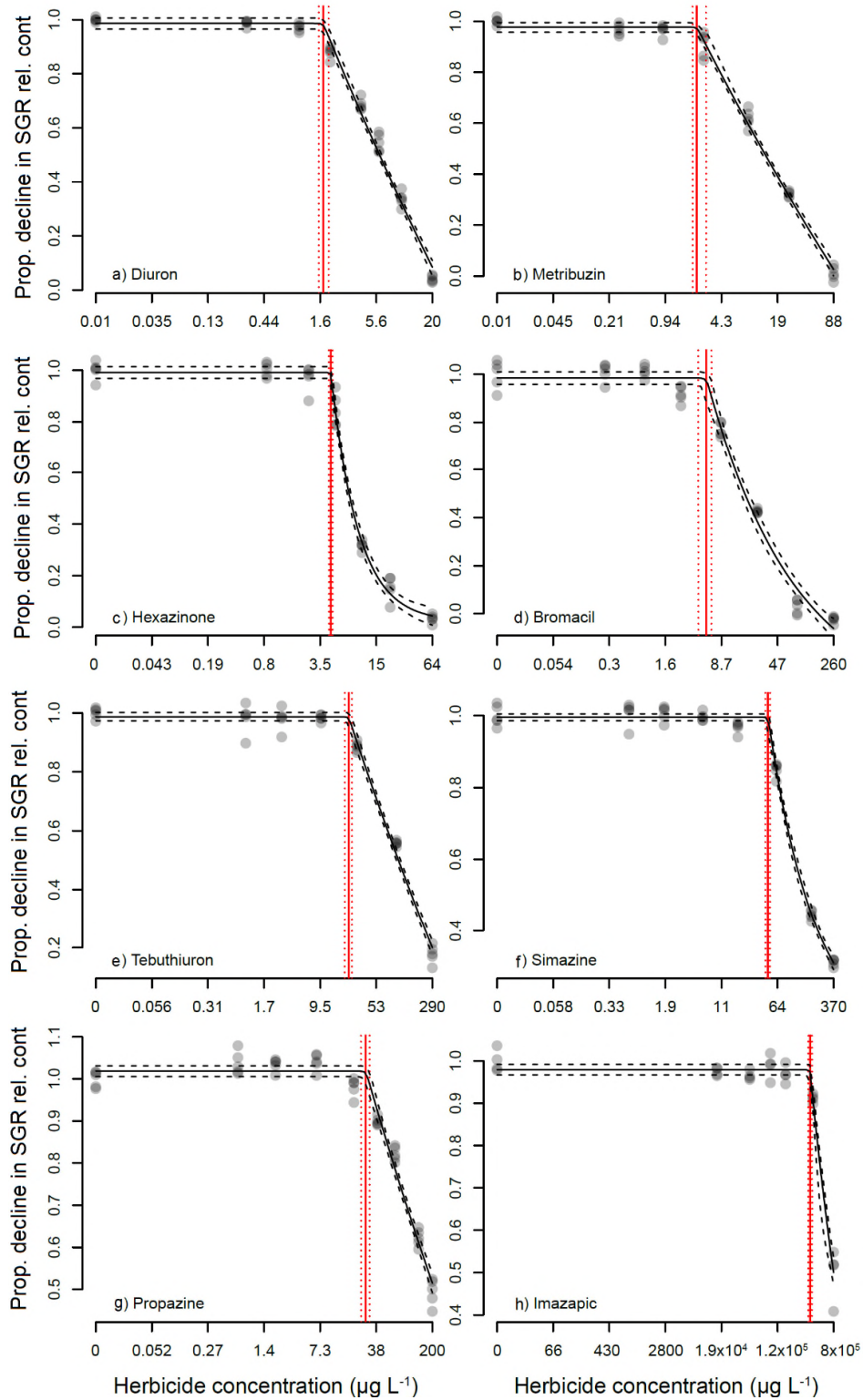
Figure. A2. Bayesian non-linear gaussian model fit on the proportional (prop.) decline in 3-day specific growth rate (SGR) of *Rhodomonas salina* relative to the control (rel. cont.) treatment (solid black line) and 95% confidence intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of a) diuron; b) metribuzin; c) hexazinone; d) bromacil; e) tebuthiuron; f) simazine; g) propazine; and h) imazapic. All concentrations in µg L^{-1}.

Appendix E: Marine: *Tetraselmis* sp.

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Contributing authors: van Dam, J.W., Stapp, L.S., Kaserzon, S., Fisher, R. and Negri A.P.

The herbicides used in toxicity tests for this species and their mode of action were:
- Diuron - PSII inhibitor
- Metribuzin - PSII inhibitor
- Tebuthiuron - PSII inhibitor
- Bromacil - PSII inhibitor
- Simazine - PSII inhibitor
- Propazine - PSII inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor

Test species: *Tetraselmis* sp*.* (marine)

Test phylum: Chlorophyta

Biological effect: Inhibition of specific growth rate

Summary

The inhibitory effects of eight herbicides on the specific growth rates (SGR) of the chlorophyte *Tetraselmis* sp*.* were determined by exposing cultures of *Tetraselmis* sp*.* to different pesticide concentrations over 72 h. Regression models were used to calculate the concentrations of each herbicide that inhibited 10% and 50% of the culture's SGR relative to controls (EC_{10} and EC_{50}, respectively). In order to determine the model which best described the data for each pesticide, various regression models of different levels of parametrization were evaluated and compared using the Akaike Information Criterion (AIC). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of log concentration of each herbicide using a Bayesian non-linear gaussian model. The toxicity thresholds for SGR (NEC; EC_{10}; EC_{50} in µg L^{-1}) were as follows: diuron (2.3; 1.6; 5.2), metribuzin (6.7; 4.1; 18.5), tebuthiuron (21; 18; 70), bromacil (1.8; 1; 6.7), simazine (38; 38; 154), propazine (29; 27; 121) and haloxyfop (13 [unreliable]; 3,740; 5,930). No effects on SGR were observed for imazapic at the highest concentrations tested (> 20,800 µg L^{-1}).

Methods

The inhibition of the specific growth rate of *Tetraselmis* sp*.* by each herbicide was tested in static 72 h exposures (chronic). Details of the experimental method are provided in Tables A1 to A3. Original data including SGR and physico-chemical data (all start and end of test measurements) can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/4a8d5927-0619-4f7e-8894-2e3aaf8d3aed.

Table A1. Source of *Tetraselmis sp.*, its culturing and test conditions.

Source of tests species	Australian Institute of Marine Science in-house culture (strain CS-317), purchased from Australian National Algae Supply Service, Hobart.
Maintenance conditions of test species (culture conditions, light, temp etc)	Cultures were maintained in-house in 500 mL Erlenmeyer flasks using EDTA-free Guillard's f/2 medium. Cultures were transferred weekly under aseptic conditions and maintained at 28 ± 1 °C, 33 ± 1.5 psu and under a 12:12 h light:dark cycle (80 – 100 µmol photons m^{-2} s^{-1}).
Test endpoint	Inhibition of specific growth rate (SGR) of culture in log growth phase
Test duration	72 h
Test chambers	125 mL Erlenmeyer flasks
Test Volume	50 mL
Starting density	2.5×10^3 cells mL^{-1}
Counting of cells, calculation of SGR	Start of test cell counts conducted using haemocytometer; end of test cells count conducted using flow cytometry as per Trenfield (2015). SGR calculated as per OECD (2011).

Table A2. Range of measured physico-chemical parameters of test media for all test solutions at the start of test for total number of tests performed with *Tetraselmis sp.* (n = 26).

Light intensity (µmol photons $m^{-2}s^{-2}$ over a 12:12 L:D cycle)	80 - 100
Temperature (°C)	27 - 29
Dissolved oxygen (mg L^{-1}). Exposure solutions were always within 0.3 mg L^{-1} of corresponding control solutions	8.3 - 8.7
pH (units). Exposure solutions were always within 0.1 pH unit of corresponding control solutions	8.1 - 8.2
Salinity (psu). Exposure solutions were always within 0.6 psu of corresponding control solutions.	32 - 33

Table A3. Test criteria for specific growth rate of *Tetraselmis sp.*

Exposure duration	72 h		
Biological effect metric	Estimated effect concentrations, EC_{10} and EC_{50}, for the concentrations that reduce SGR by 10% and 50%, respectively, relative to control treatments. No effect concentration (NEC) is the threshold below which the toxicants are not expected to cause a reduction in SGR.		
Biological endpoint definition	Inhibition of the average specific growth rate - the logarithmic increase of biomass over 72 h.		
Controls used	Seawater controls, no carrier or toxicant		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Diuron	2	16	2
Metribuzin	2	16	2
Tebuthiuron	2	16	2
Bromacil	2	16	2
Simazine	2	16	2
Propazine	2	16	2
Imazapic	2	16	2
Haloxyfop	2	16	2
Test acceptability criteria	i. Control SGR ≥ 0.92 day^{-1} as per (OECD, 2011). Observed average control SGR: 1.02 ± 0.06 day^{-1} (mean ± SD, *n* = 16 tests). ii. The coefficient of variation (CV) of mean SGR in controls ≤ 10% as per OECD (2011). Observed control CV: < 6% in all tests		

Characteristics of the test organism	5-day old culture in exponential growth phase, starting density 2.5×10^3 cells mL^{-1}
Type of test media	Natural, 0.5 µm filtered seawater spiked with pesticide stock (acetone or DMSO carrier < 0.02% v/v) in ultrapure water. Nutrient source added: quarter strength EDTA-free f/2 media.
Toxicant (common name; IUPAC Name; CAS no.; supplier; purity)	• Diuron; 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; Merck; ≥ 98%) • Metribuzin; 4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one; 21087-64-9; Merck; ≥ 99.5%) • Tebuthiuron; 1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea; 34014-18-1; Merck; ≥ 98%) • Bromacil; 5-Bromo-3-sec.-butyl-6-methyluracil; 314-40-9; Merck; ≥ 98%) • Propazine; 6-chloro-2-N,4-N-di(propan-2-yl)-1,3,5-triazine-2,4-diamine; 139-40-2; Merck; ≥ 99%) • Simazine; 6-Chloro-*N,N'*-diethyl-1,3,5-triazine-2,4-diamine; 122-34-9; Merck; ≥ 98%) • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; Merck; ≥ 98.5%) • Haloxyfop-p-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; Merck; ≥ 98%)
Preparation of toxicant stock	Stock solutions (100 – 1,000 mg L^{-1}) of pesticides were prepared in ultrapure water. Simazine, tebuthiuron and haloxyfop-p-methyl were dissolved using the carrier dimethyl sulfoxide (DMSO) (≤ 0.02 % (v/v) in exposure). Diuron, imazapic, metribuzin, bromacil and propazine were dissolved in acetone (≤ 0.01 % (v/v) in exposure). Stock solutions stored refrigerated and in the dark. Stock used to spike filtered seawater to obtain test solutions of desired concentration.
Exposure type	Static
Measured contaminant concentrations	Pesticide concentrations (2-3 per test) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All pesticide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS) at the University of Queensland, using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system). (Mercurio et al., 2015).
Reference toxicant	Diuron
Concentration-response relationship	• EC$_x$: regression models, fitted to the percent inhibition and measured pesticide concentrations using the DRC package in R (Ritz & Streibig, 2005; R Development Core Team, 2015). Concentration-response relationships presented in Figure A1. • NECs were estimated using jagsNEC package in R (R Development Core Team, 2015; Fisher et al., 2019). Proportional decline in SGR was modelled using a Bayesian non-linear gaussian model. NEC models presented Figure A2.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis were conducted following prescribed procedures (OECD, 2006a). The package DRC in R (Ritz & Streibig, 2005; R Development Core Team, 2015) was used to model the test data and to determine pesticide concentrations that inhibited 10% and 50% of the SGR relative to controls (EC$_{10}$ and EC$_{50}$, respectively). Regression models evaluated included log-logistic, Weibull and hormesis models of different levels of parametrization. Model

	comparisons were conducted using the Akaike Information Criterion. The model that best described the data was applied to derive estimates of toxicity. The associated 95% confidence limits were estimated using the delta method. • No effect concentration (NEC) values were calculated in R (v 3.5.3) (R Development Core Team, 2015). The proportional decline in SGR was modelled as a function of the log measured concentration of each pesticide using a Bayesian non-linear gaussian model using the package jagsNEC in R (R Development Core Team, 2015; Fisher et al., 2019). Trace plots were used to evaluate model fits and were found to have relatively good mixing in all cases. Bayesian 95% credible intervals (confidence limits) based on the upper 97.5th and lower 2.5th percentile of the posterior sample for the NEC parameter estimate.
Data variance	95% confidence limits (for EC_x) or Bayesian 95% credible intervals (for NECs) (Table A4).
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade pesticides (all ≥ 98% as described above) were used for preparation of all stock solutions. No pesticides were measured in any of the control solutions.

Summary of results

The toxicity of eight herbicides to *Tetraselmis* sp. is presented in Table A4 and Figures A1 and A2. The acetohydroxyacid synthase inhibitor imazapic did not inhibit SGR in *Tetraselmis* sp. at the maximum concentration of 20,800 μg L^{-1}.

Table A4. Modelled toxicity estimates for the inhibition of eight herbicides on the specific growth rate (SGR) of *Tetraselmis sp.* (Figs. A1 and A2). All concentrations in μg L-1 (95% confidence intervals).

	NEC (95% CI)	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	2.27 (1.99 – 2.49)	1.64 (1.41 – 1.86)	5.24 (4.91 – 5.57)
Metribuzin	6.66 (4.67 – 7.80)	4.14 (3.50 – 4.77)	18.5 (17.4 – 19.5)
Tebuthiuron	20.6 (15.7 – 24.6)	18.4 (15.4 – 21.4)	69.9 (65.5 – 74.4)
Bromacil	1.75 (1.29 – 2.40)	0.99 (0.79 – 1.18)	6.68 (6.22 – 7.14)
Simazine	37.5 (27.9 – 46.3)	37.6 (33.0 – 42.2)	154 (145 – 162)
Propazine	29.3 (22.2 – 34.5)	27.2 (22.4 – 32.0)	121 (111 – 130)
Imazapic	Unreliable NEC*	> 20800	> 20800
Haloxyfop	Unreliable NEC*	3740 (3560 – 3930)	5930 (5740 – 6110)

* Although a NEC was provided by the model (Figure A2), no concentration-response relationship was observed and confidence around the supplied NEC was extremely low. Therefore, the NEC was deemed unreliable and should not be used in a regulatory context.
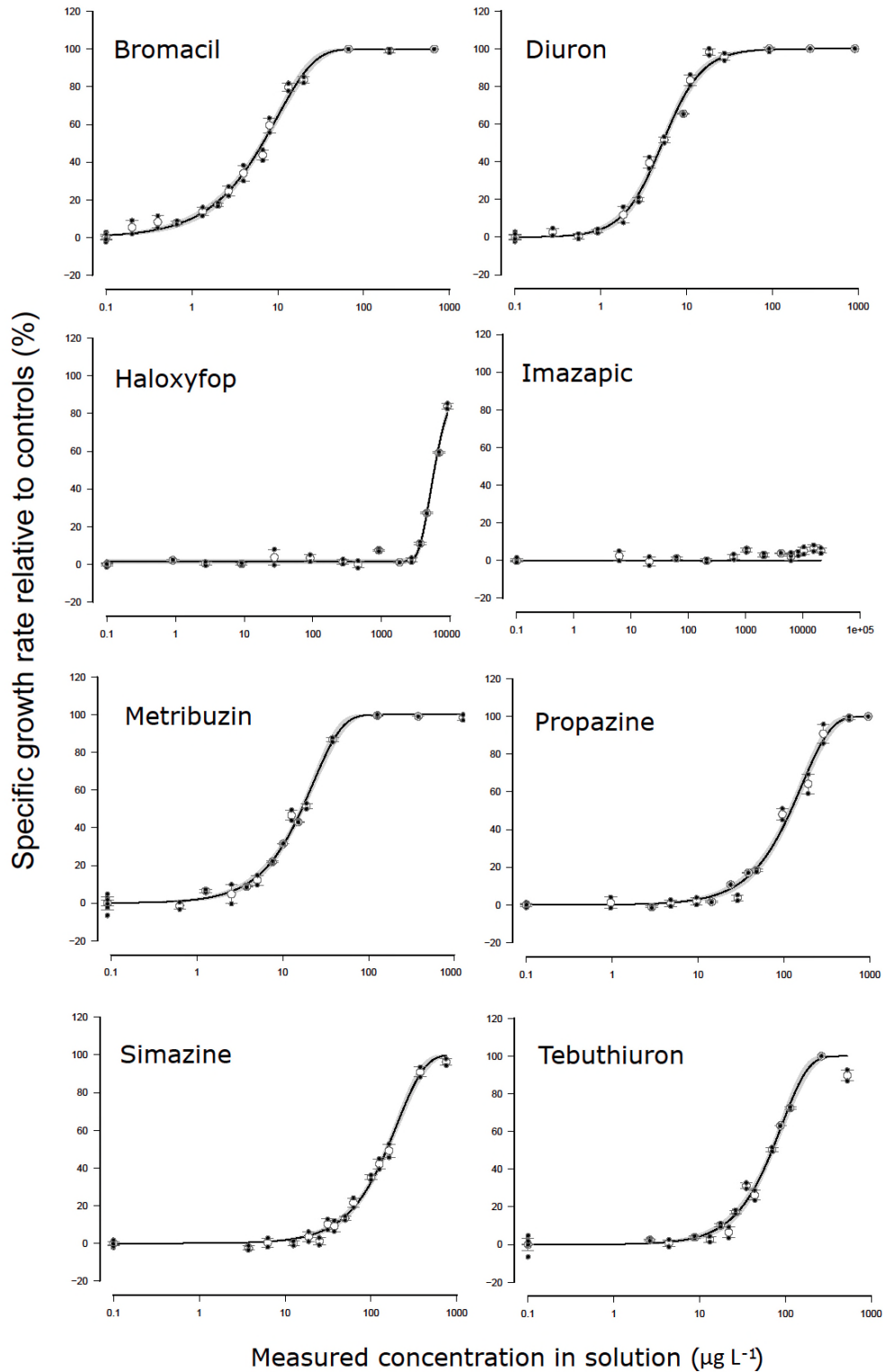
Figure A1. Relative inhibition of specific growth rate of *Tetraselmis sp.* in response to 72-h exposures to increasing concentrations of the respective pesticide. Open circles represent the treatment mean ± SE and closed circles represent individual treatment replicates. All data are expressed relative to control values and the upper limit of the concentration response curve was fixed at 100%. The solid black line is the fitted regression model, the shaded areas represent the model's 95% confidence limits. Best-fitting models (based on Akaike Information Criterion) were 3-parameter log-logistic (diuron), Weibull type I 4-parameter (haloxyfop) and Weibull type II 3-parameter (bromacil, metribuzin, propazine, simazine and tebuthiuron). All concentrations are reported in µg L-1. Note the dissimilar scaling on the horizontal axis.
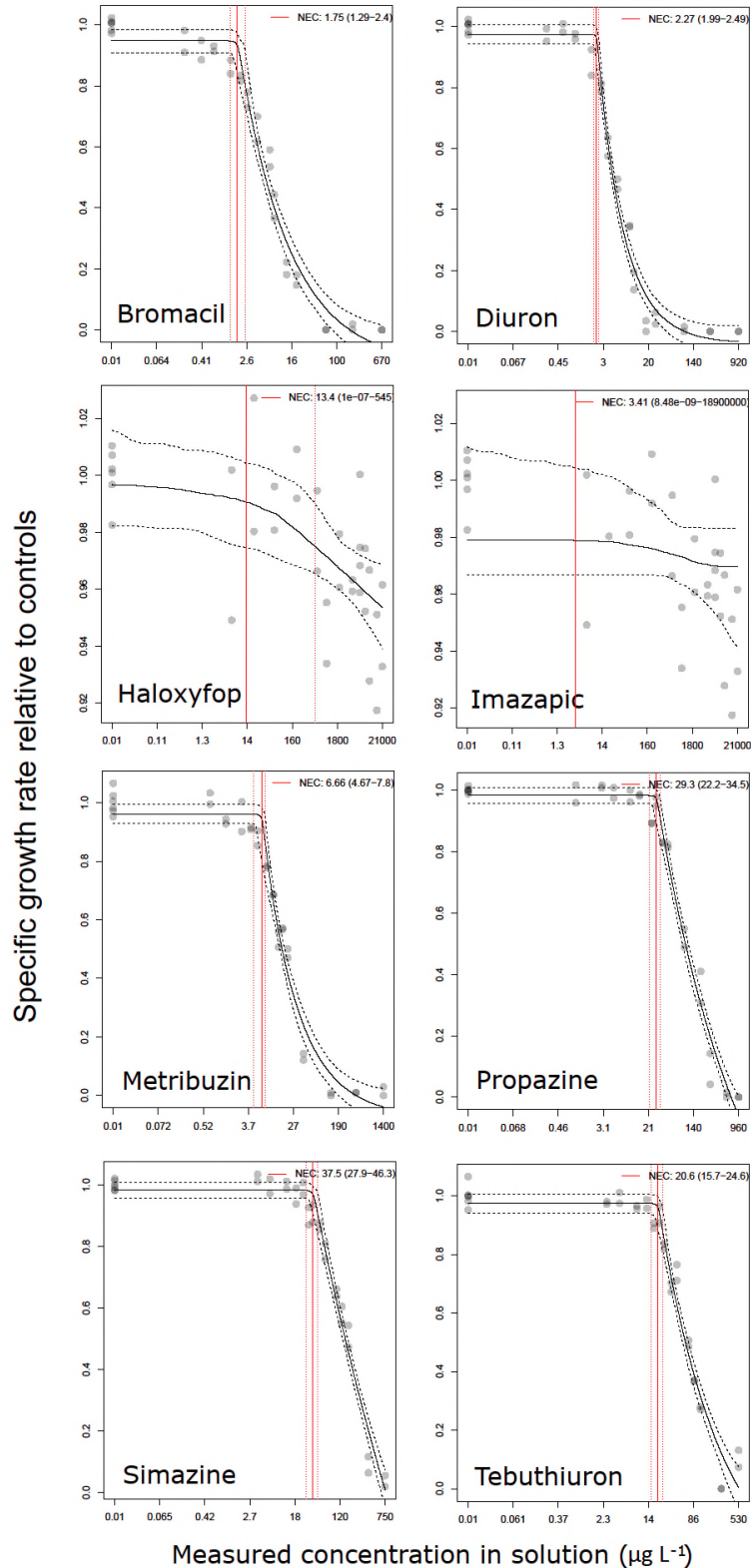
Figure A2. Bayesian non-linear gaussian model fit on the proportional decline in 3-day specific growth rate (SGR) of *Tetraselmis sp.* relative to the control treatment (solid black line) and Bayesian 95% credible (confidence) intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of the respective pesticide. All concentrations in µg L-1. Note the dissimilar scaling on the axes.Appendix F: Marine: Tisochrysis lutea

Appendix F: Marine: *Tisochrysis lutea*

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Contributing authors: van Dam, J.W., Stapp, L.S., Kaserzon, S., Fisher, R. and Negri A.P.

The herbicides used in toxicity tests for this species and their mode of action were:
- Diuron - PSII inhibitor
- Metribuzin - PSII inhibitor
- Tebuthiuron - PSII inhibitor
- Bromacil - PSII inhibitor
- Simazine - PSII inhibitor
- Propazine - PSII inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- MCPA – auxin mimic
- 2,4-D - auxin mimic
- Fluroxypyr - auxin mimic

The fungicide used in toxicity tests for this species and its mode of action was:
- Propiconazole - sterol biosynthesis inhibitor

Test species: *Tisochryisis lutea* (marine)

Test phylum: Haptophyta

Biological effect: Inhibition of specific growth rate

Summary

The inhibitory effects of eleven herbicides and one fungicide on the specific growth rate (SGR) of the haptophyte *Tisochrysis lutea* (formerly known as *Isochrysis galbana*) were determined by exposing cultures of *T. lutea* to different pesticide concentrations over 72 h. Regression models were used to calculate the concentrations of each herbicide that inhibited 10% and 50% of the culture's SGR relative to controls (EC_{10} and EC_{50}, respectively). In order to determine the model which best described the data for each pesticide, various regression models of different levels of parametrization were evaluated and compared using the Akaike Information Criterion (AIC). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of log concentration of each herbicide using a Bayesian non-linear gaussian model. The toxicity thresholds for SGR (NEC; EC_{10}; EC_{50} in µg L^{-1}, respectively) were as follows: diuron (0.8; 0.6; 4.0), metribuzin (0.5; 0.7; 3.1), tebuthiuron (63; 36; 112), bromacil (2.0; 1.9; 6.8), simazine (70; 60; 206), propazine (14; 19; 57), haloxyfop (4,180; 4,000; 4,380), imazapic (471; 783; 4,320), 2,4-D (15,300; 40,700; 172,000), MCPA (43 [unreliable]; 21,800; > 1 kg L^{-1}) and propiconazole (2,980; 2,710; 4,840). No effects on SGR were observed for fluroxypyr at the highest concentrations tested (6,300 µg L^{-1}).

Methods

The inhibition of the specific growth rate of *Tisochrysis lutea* by each pesticide was tested in static 72 h exposures (chronic). Details of the experimental method are provided in Tables A1 to A3. Original data including SGR and physico-chemical data (all start and end of test measurements) can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/91967f34-b24d-4352-b6b0-526e54ec052f.

Table A1. Source of *Tisochrysis lutea*, its culturing and test conditions.

Source of tests species	Australian Institute of Marine Science in-house culture (strain CS-177), purchased from Australian National Algae Supply Service, Hobart.
Maintenance conditions of test species (culture conditions, light, temp etc)	Cultures were maintained in-house in 500 mL Erlenmeyer flasks using EDTA-free Guillard's f/2 medium. Cultures were transferred weekly under aseptic conditions and maintained at 28 ± 1 °C, 33 ± 1.5 psu and under a 12:12 h light:dark cycle (80 – 100 µmol photons m^{-2} s^{-1}).
Test endpoint	Inhibition of specific growth rate (SGR) of culture in log growth phase
Test duration	72 h
Test chambers	125 mL Erlenmeyer flasks
Test Volume	50 mL
Starting density	$3x10^3$ or $1x10^4$ cells mL^{-1}
Counting of cells, calculation of SGR	Start of test cell counts conducted using haemocytometer; end of test cell counts conducted using flow cytometry as per Trenfield (2015). SGR calculated as per OECD (2011).

Table A2. Range of measured physico-chemical parameters of test media for all test solutions at the start of test for total number of tests performed with *Tisochrysis lutea* (n = 32).

Light intensity (µmol photons $m^{-2}s^{-2}$ over a 12:12 L:D cycle)	80 - 100
Temperature (°C)	27 - 29
Dissolved oxygen (mg L^{-1}). Exposure solutions were always within 0.4 mg L^{-1} of corresponding control solutions	7.7 - 8.7
pH (units). Exposure solutions were always within 0.2 pH unit of corresponding control solutions	7.9 - 8.3
Salinity (psu). Exposure solutions were always within 0.9 psu of corresponding control solutions, except for a single value in an imazapic test where a difference of 1.6 psu was measured between control and the highest exposure solution.	28 - 33

Table A3. Test criteria for specific growth rate of *Tisochrysis lutea*.

Exposure duration	72 h		
Biological effect metric	Estimated effect concentrations, EC_{10} and EC_{50}, for the concentrations that reduce SGR by 10% and 50%, respectively, relative to control treatments. No effect concentration (NEC) is the threshold below which the toxicants are not expected to cause a reduction in SGR.		
Biological endpoint definition	Inhibition of the average specific growth rate - the logarithmic increase of biomass over 72 h (OECD, 2011).		
Controls used	Seawater controls, no carrier or toxicant		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Diuron	3	18	3
Metribuzin	3	18	3
Tebuthiuron	2	16	2
Bromacil	3	18	3
Simazine	2	16	2
Propazine	3	18	3
Imazapic	3	18	3
Haloxyfop	2	15	2
2,4-D	4	21	3
MCPA	2	16	3
Fluroxypyr	3	18	3
Propiconazole	2	16	2

Test acceptability criteria	iii. Control SGR ≥ 0.92 day^{-1} as OECD (2011). Observed average control SGR: 1.41 ± 0.23 day^{-1} (mean ± SD, *n* = 32 tests). iv. The coefficient of variation (CV) of mean SGR in controls ≤ 10% as OECD (2011). Observed control CV: < 7% in all tests
Characteristics of the test organism	4-day old culture in exponential growth phase, starting density 3x10^3 or 1x10^4 cells mL^{-1}
Type of test media	Natural, 0.5 µm filtered seawater spiked with pesticide stock (acetone or DMSO carrier < 0.02% v/v) in ultrapure water. Nutrient source added: quarter strength EDTA-free f/2 media.
Toxicant (common name; IUPAC Name; CAS no.; supplier; purity)	• Diuron; 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; Merck; ≥ 98%) • Metribuzin; 4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one; 21087-64-9; Merck; ≥ 99.5%) • Tebuthiuron; 1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea; 34014-18-1; Merck; ≥ 98%) • Bromacil; 5-Bromo-3-sec.-butyl-6-methyluracil; 314-40-9; Merck; ≥ 98%) • Propazine; 6-chloro-2-N,4-N-di(propan-2-yl)-1,3,5-triazine-2,4-diamine; 139-40-2; Merck; ≥ 99%) • Simazine; 6-Chloro-*N,N'*-diethyl-1,3,5-triazine-2,4-diamine; 122-34-9; Merck; ≥ 98%) • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; Merck; ≥ 98.5%) • Haloxyfop-p-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; Merck; ≥ 98%) • 2,4-D; 2,4-D, 2,4-Dichlorophenoxyacetic acid; 94-75-7; Merck; ≥ 98%) • MCPA; 4-Chloro-2-methylphenoxyacetic acid; 94-74-6; Merck; ≥ 98%) • Fluroxypyr; [(4-Amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid ;69377-81-7; Merck; ≥ 98%) • Propiconazole; 1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole; 60207-90-1; Merck; ≥ 98%)
Preparation of toxicant stock	Stock solutions (100 – 1,000 mg L^{-1}) of pesticides were prepared in ultrapure water. Simazine, tebuthiuron and haloxyfop-p-methyl were dissolved using the carrier dimethyl sulfoxide (DMSO) (≤ 0.02 % (v/v) in exposure). Diuron, imazapic, metribuzin, bromacil, 2,4-D, propazine, MCPA, fluroxypyr and propiconazole were dissolved in acetone (≤ 0.01 % (v/v) in exposure). Stock solutions stored refrigerated and in the dark. Stock used to spike filtered seawater to obtain test solutions of desired concentration.
Exposure type	Static
Measured toxicant concentrations	Pesticide concentrations (2-3 per test) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All pesticide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS) at the University of Queensland, using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015).
Reference toxicant	Diuron
Concentration-response relationship	• EC$_x$: regression models, fitted to the percent inhibition and measured pesticide concentrations using the DRC package (Ritz & Streibig, 2005; R Development Core Team, 2015)

	• Concentration-response relationships presented in Figure A1. • NECs were estimated using jagsNEC package in R (R Development Core Team, 2015; Fisher et al., 2019). Proportional decline in SGR was modelled using a Bayesian non-linear gaussian model. NEC models presented Figure A2.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis were conducted following prescribed procedures (OECD, 2006a). The package DRC in R (Ritz & Streibig, 2005; R Development Core Team, 2015) was used to model the test data and to determine pesticide concentrations that inhibited 10% and 50% of the SGR relative to controls (EC_{10} and EC_{50}, respectively). Regression models evaluated included log-logistic, Weibull and hormesis models of different levels of parametrization. Model comparisons were conducted using the Akaike Information Criterion. The model that best described the data was applied to derive estimates of toxicity. The associated 95% confidence limits were estimated using the delta method. • No effect concentration (NEC) values were calculated in R (v 3.5.3) (R Development Core Team, 2015; Trenfield et al., 2015). The proportional decline in SGR was modelled as a function of the log measured concentration of each pesticide using a Bayesian non-linear gaussian model using the package jagsNEC in R (R Development Core Team, 2015; Fisher et al., 2019). Trace plots were used to evaluate model fits and were found to have relatively good mixing in all cases. Bayesian 95% credible intervals (confidence limits) based on the upper 97.5th and lower 2.5th percentile of the posterior sample for the NEC parameter estimate.
Data variance	95% confidence limits (for EC_x) or Bayesian 95% credible intervals (for NECs) (Table A4).
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade pesticides (all ≥ 98% as described above) were used for preparation of all stock solutions. No pesticides were measured in any of the control solutions.

Summary of results

The toxicity of twelve pesticides to *T. lutea* is presented in Table A4 and Figures A1 and A2. The auxin mimic herbicide fluroxypyr did not inhibit SGR in *T. lutea* at the maximum concentration of 6,300 µg L^{-1}.

Table A4. Modelled toxicity estimates for the inhibition of eleven herbicides and one fungicide on the specific growth rate (SGR) of *Tisochrysis lutea* (Figs. A1 and A2). All concentrations in µg L-1 (95% confidence intervals).

	NEC (95% CI)	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	0.78 (0.44 – 1.30)	0.60 (0.40 - 0.80)	3.96 (3.40 - 4.52)
Metribuzin	0.50 (0.29 – 1.24)	0.72 (0.36 - 1.09)	3.11 (2.46 - 3.75)
Tebuthiuron	63.1 (42.5 – 71.5)	35.9 (30.6 - 41.1)	112 (106 - 118)
Bromacil	1.96 (1.57 - 2.37)	1.94 (1.55 - 2.34)	6.80 (6.31 - 7.28)
Simazine	70.0 (55.3 – 80.3)	60.2 (51.9 - 68.4)	206 (194 - 218)
Propazine	14.4 (10.8 – 20.9)	18.5 (15.2 - 21.9)	56.5 (51.0 - 62.0)
Imazapic	471 (283 – 861)	783 (399 – 1170)	4,320 (3180 – 5460)
Haloxyfop	4,180 (3,800 – 4,710)	4,000 (3650 – 4350)	4,384 (4170 – 4600)
2,4-D	15,300 (6980 – 28,400)	40,700 (28,800 – 52,500)	172,000 (61,500 – 283,000)
MCPA	Unreliable NEC*	21,800 (7680 – 35,900)	> 20,000,000
Fluroxypyr	Unreliable NEC*	> 6,300	> 6,300
Propiconazole	2980 (2660 – 3230)	2,710 (2300 – 3110)	4,840 (4640 – 5040)

* Although a NEC was provided by the model (Figure A2), no concentration-response relationship was observed and confidence around the supplied NEC was extremely low. Therefore, the NEC was deemed unreliable and should not be used in a regulatory context.
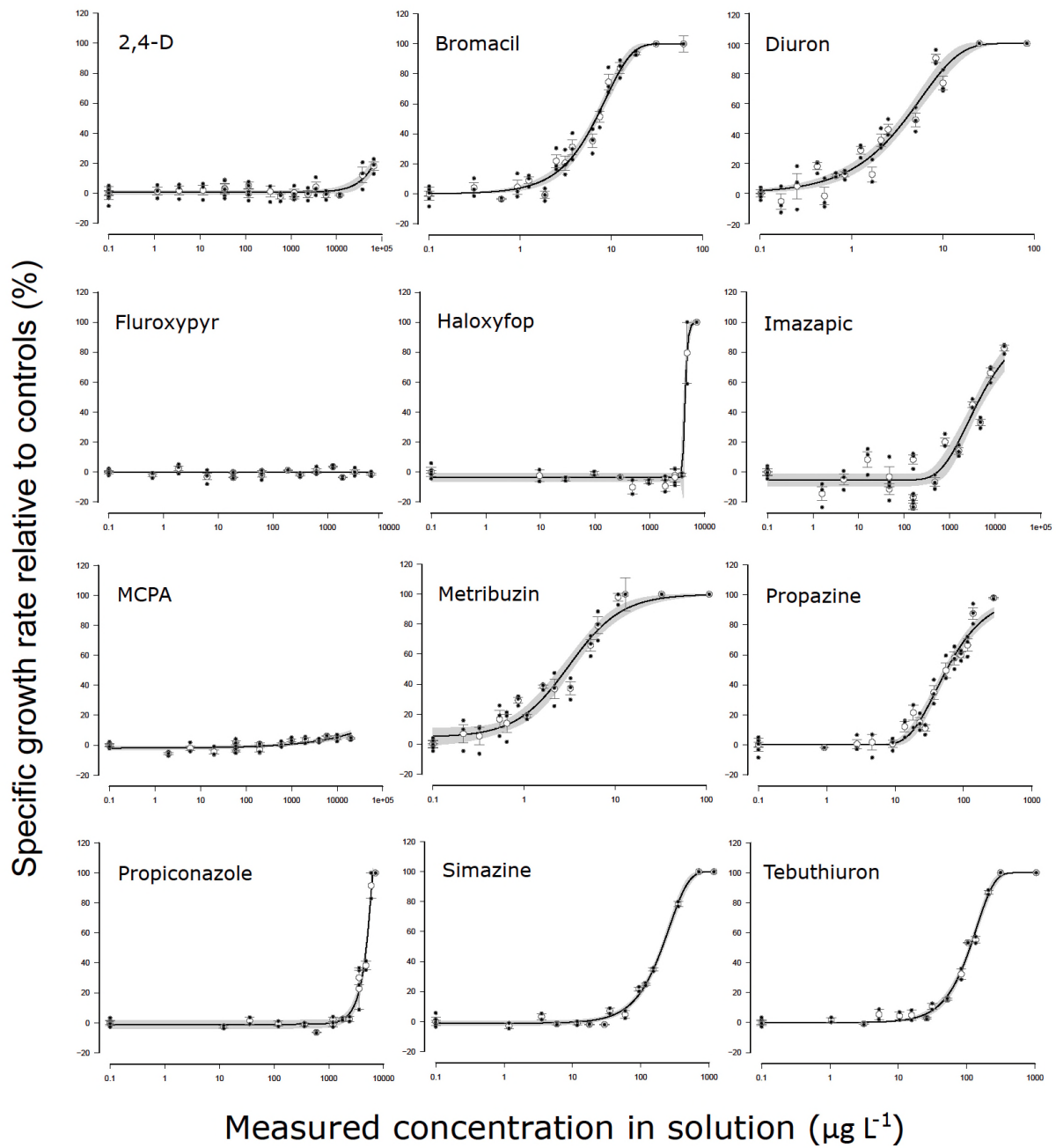
Figure A1. Relative inhibition of specific growth rate of *Tisochrysis lutea* in response to 72-h exposures to increasing concentrations of the respective pesticides. Open circles represent the treatment mean ± SE and closed circles represent individual treatment replicates. All data are expressed relative to control values and the upper limit of the concentration response curve was fixed at 100%. The solid black line is the fitted regression model, the shaded areas represent the model's 95% confidence limits. Best-fitting models (based on Akaike Information Criterion) were 4-parameter log-logistic (2,4-D, metribuzin), 5-parameter log-logistic (propiconazole), Weibull type I 3-parameter (propazine), Weibull type I 4-parameter (haloxyfop, imazapic), Weibull type II 3-parameter (bromacil, diuron, tebuthiuron) and Weibull type II 4-parameter (MCPA, simazine). All concentrations are reported in µg L-1. Note the dissimilar scaling on the horizontal axis.
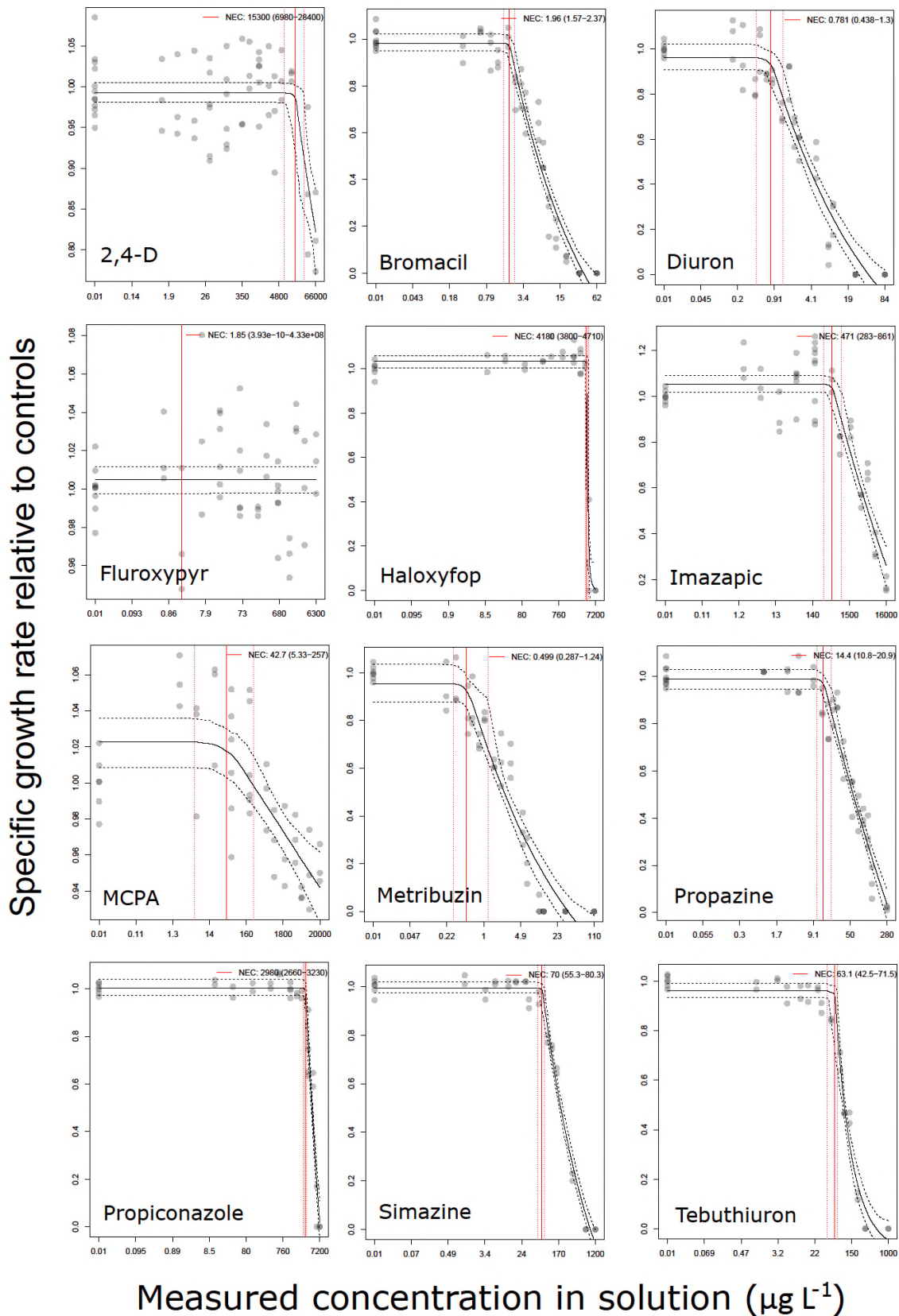
Figure A2. Bayesian non-linear gaussian model fit on the proportional decline in 3-day specific growth rate (SGR) of *Tisochrysis lutea* relative to the control treatment (solid black line) and Bayesian 95% credible (confidence) intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of the respective pesticide. All concentrations in µg L-1. Note the dissimilar scaling on the axes.

Appendix G: Marine: *Acropora tenuis*

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Contributing authors: Flores, F., Ricardo, G.F., Kaserzon, S., Negri, A.P.

The pesticides that were used in toxicity tests for this species and their mode of action were:

- Diazinon – acetylcholinesterase (AChE) inhibitor (insecticide)
- Fipronil – GABA disruptor (insecticide)
- Imidacloprid – blocks nicotinic acetylcholine receptors (insecticide)
- Propiconazole – sterol biosynthesis inhibitor (fungicide)
- Chlorothalonil – reduces glutathione molecules to alternate chemicals (fungicide)

Test species: *Acropora tenuis* (marine)

Test phylum: Cnidaria

Biological effect: Reproductive, failure of larvae to metamorphose

Summary

The effects of three insecticides (diazinon, fipronil, imidacloprid) and two fungicides (chlorothalonil, propiconazole) on the metamorphosis of *Acropora tenuis* larvae were tested over 48 h exposures. No effect concentration (NEC) values and concentrations of each pesticide that inhibited 10% and 50% of larval settlement relative to controls (EC_{10} and EC_{50}, respectively) were calculated from the proportion of metamorphosed larvae as a function of log concentration of each pesticide using a Bayesian non-linear beta model (except for imidacloprid and propiconazole in which a binomial model was a better fit) (Fisher et al., 2019). The toxicity thresholds for larval settlement (NEC, EC_{10}, EC_{50} in $\mu g\ L^{-1}$) were as follows: chlorothalonil (2.4, 2.8, 6.0); fipronil (12.3, 13.9, 29.1); diazinon (38.0, 40.8, 54.7); imidacloprid (263, 273, 347); and propiconazole (269, 330, 1008).

Methods

The metamorphosis of planktonic larvae into sessile juvenile polyps is a critical step in the recruitment of corals (Heyward & Negri, 1999). The inhibition of coral larval metamorphosis by pesticides was tested in static 48 h exposures (chronic), with metamorphosis initiated by the addition of crustose coralline algae (CCA) extract (Negri et al., 2011b; Negri et al., 2016). Details of the current experimental methods are provided in Tables A1 to A3. Original data including percent larval settlement and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/da9fc37d-e74b-477d-8cd5-79178cda968c.

Table A1. Source of *Acropora tenuis* and test conditions.

Source of tests species	Trunk Reef (18°23' S, 146°48' E) and Falcon Island (18°46' S, 146°32' E), Great Barrier Reef
Maintenance conditions of test species	Larval cultures were maintained in 500 L flow-through tanks with aeration and filtered seawater (0.5 µm) under 26-27°C (range), which was equivalent to the water temperature at the collection site.
Test duration	48 h
Test chambers	20 mL glass vials
Test volume	10 mL
Starting density	12-14 larvae/10 mL
Test endpoint	Metamorphosis of planula larvae

Table A2. Measured physico-chemical composition of test media for *Acropora tenuis*.

Light intensity (mean, averaged across treatments)	60 µmol photons m^{-2} s^{-1} over a 12:12 h L:D cycle
Temperature (mean ± SD, logged 5 min intervals)	26.7 ± 0.7 °C
Dissolved oxygen (averaged 0 and 48 h, n = 60)	8.1 ± 0.2 mg L^{-1}
pH (mean ± SD, averaged 0 and 48 h, n = 60)	8.2 ± 0.1
Salinity (mean ± SD, averaged 0 and 48 h, n = 60)	36 ± 1 psu

Table A3. Test criteria for inhibition of larval metamorphosis of *Acropora tenuis*.

|			
Exposure duration	48 h		
Biological effect metric	Inhibition of metamorphosis of planula larvae		
Biological endpoint definition	No effect concentration (NEC) is the concentration below which the pesticides are not expected to cause a reduction in metamorphosis of larvae. Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce larval metamorphosis by 10% and 50%, respectively, in comparison to control treatments.		
Controls used	All chemicals were dissolved using the carrier solvent dimethyl sulfoxide (DMSO; final concentration < 0.01% (v/v) in exposures; n=12-18 replicates)		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Diazinon	1	9	6
Fipronil	1	10	6
Imidacloprid	1	9	6
Chlorothalonil	1	10	6
Propiconazole	1*	8	6
	* rangefinder conducted prior to the definitive experiment		
Test acceptability criteria	Metamorphosis was scored as normal if larvae had changed from free swimming or casually attached pear-shaped forms to squat, firmly attached, disc-shaped structures with pronounced flattening of the oral–aboral axis and with septal mesenteries radiating from the central mouth region (Heyward & Negri, 1999). Larval metamorphosis ≥70% in the controls was considered acceptable as an endpoint based upon multiple similar studies using this species (Negri et al., 2011b; Negri et al., 2016).		

Characteristics of the test organism	Larvae were all competent to metamorphose and were 7 – 10 d old, measuring approximately 1 mm in length.
Type of test media	Natural, 0.5 µm polypropylene-filtered coastal seawater (19°16'19.60"S; 147° 3'40.93"E) spiked with test solution.
Toxicant (common name; IUPAC Name; CAS no.; purity)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Diazinon; *O,O*-diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate; 333-41-5; 98.5% • Fipronil; (*RS*)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)pyrazole-3-carbonitrile; 120068-37-3; ≥ 95% • Imidacloprid; 1-((6-chloro-3-pyridinyl)methyl)-4,5-dihydro-N-nitro-imidazol-2-amine; 138261-41-3; ≥ 98% • Propiconazole; 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole; 60207-90-1; 99.1% • Chlorothalonil; (2,4,5,6-tetrachlorobenzene-1,3-dicarbonitrile); 1897-45-6; 99.3%
Preparation of toxicant stock	Stock solutions (5 mg L^{-1}) of all pesticides were prepared in Milli-Q® water using the carrier solvent dimethyl sulfoxide (DMSO, final concentration < 0.01% (v/v) in exposures).
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016). Copper samples were analysed by inductively coupled plasma-mass spectrometry following nitric acid hot block digestion at the Townsville Laboratory Services, Queensland (NATA Accreditation No. 14698).
Reference toxicant.	Copper as CuCl$_2$ was used as a reference toxicant.
Concentration-response relationship.	Binomial exponential decay regression using the jagsNEC package in R (Fisher et al., 2019) see Figure A1.
Statistical method or model used to determine effect of toxicant on test species	No effect concentration (NEC) values and concentrations of each pesticide that inhibited 10% and 50% of larval settlement relative to controls (EC$_{10}$ and EC$_{50}$, respectively) were calculated from the proportion of metamorphosed larvae as a function of log concentration of each pesticide using a Bayesian non-linear beta model (except for imidacloprid and propiconazole in which a binomial model was a better fit) using the package jagsNEC (Fisher et al., 2019) in R statistical package (v 3.5.3).
Data variance	95% Confidence Limits (CL) (see Table 4)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade pesticides (95 - 99.3% purity) were used for preparation of all stock solutions.

Summary of results

The modelled toxicity estimates (NEC, EC_{10} and EC_{50}) of the pesticides are presented in Table A4 and Figure A1.

Table A4. Modelled toxicity estimates for the inhibition of coral larval metamorphosis by diazinon, fipronil, imidacloprid, chlorothalonil, propiconazole and copper to *A. tenuis* (from Fig. A1). All concentrations in µg L-1 (95% confidence limits).

	NEC (95% CI)	EC_{10} (95% CI)	EC_{50} (95% CI)
Diazinon	38.0 (20.4 – 51.3)	40.8 (22.4 – 53.8)	54.7 (52.3 – 57.0)
Fipronil	12.3 (7.13 – 19.1)	13.9 (8.46 – 21.1)	29.1 (20.2 – 41.6)
Imidacloprid	263 (200 – 295)	273 (211 – 306)	347 (306 – 417)
Chlorothalonil	2.42 (1.63 – 3.89)	2.76 (1.90 – 4.42)	5.95 (4.40 – 8.82)
Propiconazole	269 (123 - 468)	330 (171 – 537)	1008 (704 – 1689)
Copper	7.41 (5.75 – 8.45)	7.79 (6.13 – 8.82)	10.2 (8.58 – 11.5)
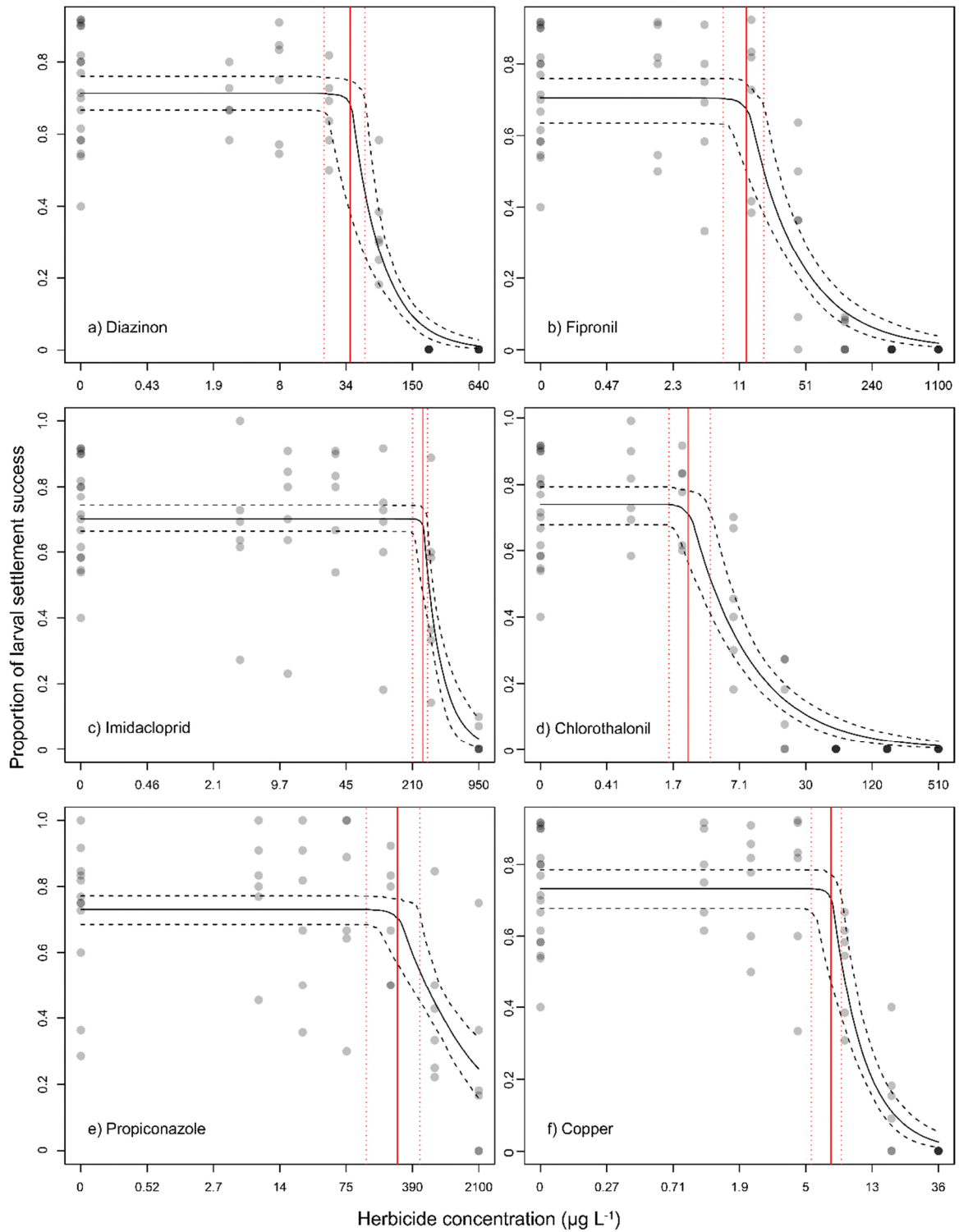
Figure A1. Concentration-response relationships for the toxicity of five pesticides and the reference toxicant copper to coral larval metamorphosis. Bayesian non-linear beta model fit (binomial model fit for imidacloprid and propiconazole) on the proportional decline of coral larval metamorphosis of *Acropora tenuis* relative to the solvent control treatment (solid black line) and 95% confidence intervals (dashed black line) and the derived no effect concentrations (red line) with 95% confidence intervals (red dashed line) of a) Diazinon; b) Fipronil; c) Imidacloprid; d) Chlorothalonil; e) Propiconazole; and f) Copper. All concentrations are in µg L-1.

Appendix H: Marine: *Amphibalanus amphitrite*

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Contributing authors: van Dam, J.W., Stapp, L.S., Kaserzon, S., Fisher, R. and Negri A.P.

The pesticides used in toxicity tests for this species and their mode of action were:
- Imidacloprid – blocks nicotinic acetylcholine receptors (insecticide)
- Propiconazole - sterol biosynthesis inhibitor (fungicide)

Test species: *Amphibalanus amphitrite* (marine)

Test phylum: Arthropoda/Crustacea (Cirripedia)

Biological effect: Inhibition of larval development

Summary

The inhibitory effects of the insecticide imidacloprid and the fungicide propiconazole on larval development of the acorn barnacle *Amphibalanus amphitrite* were determined by exposing newly hatched staged II nauplii to increasing concentrations of the pesticides over 96 h. Regression models were used to calculate the concentrations of each herbicide that inhibited 10% and 50% of the culture's SGR relative to controls (EC_{10} and EC_{50}, respectively). In order to determine the model which best described the data for each pesticide, various regression models of different levels of parametrization were evaluated and compared using the Akaike Information Criterion (AIC). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of log concentration of each herbicide using a Bayesian non-linear gaussian model. No effects on larval development were observed for the insecticide imidacloprid at the highest concentration tested (> 1660 µg L^{-1}). Toxicity estimates (NEC, EC_{10} and EC_{50}) for inhibition of larval development for the fungicide propiconazole were 878, 568, and 1020 µg L^{-1}, respectively.

Methods

The inhibition of the larval development of newly hatched staged II nauplii of *Amphibalanus amphitrite* by both pesticides was tested in static 96 h exposures (chronic). Details of the experimental methods are provided in Tables A1 to A3. Original data including inhibition of the larval development and physico-chemical data (all start and end of test measurements) can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/f0f68320-ad62-43fc-b73d-ab96b3321fe4.

Table A1. Source of *Amphibalanus amphitrite*, its culturing and test conditions.

Source of tests species	Australian Institute of Marine Science in-house culture for several generations, originally sourced from Darwin Harbour, NT
Maintenance conditions of test species (culture conditions, light, temp etc)	Broodstock barnacles were grown on bricks, fed, maintained and spawned under conditions as described by van Dam (2016).
Test endpoint	% successful transition to cyprid relative to controls
Test duration	96 h
Test chambers	250 mL customised glass funnels (van Dam et al., 2016).
Test volume	100 mL chamber^{-1}
Starting density	0.5 larva mL^{-1}
Counting of larvae	Test vessels drained over nitrile mesh, larval developmental stages scored using stereomicroscope (van Dam et al., 2016).

Table A2. Range of physico-chemical parameters measured in test media for all test solutions at the start of test for total number of tests performed with *Amphibalanus amphitrite* (n = 3).

Light intensity (µmol photons m^{-2}s^{-2} over a 12:12 L:D cycle)	80 - 100
Temperature (°C)	27 - 29
Dissolved oxygen (mg L^{-1}). Exposure solutions were always within 0.4 mg L^{-1} of corresponding control solutions	8.2 – 9.0
pH (units). Exposure solutions were always within 0.3 pH unit of corresponding control solutions	7.9 - 8.3
Salinity (psu). Exposure solutions were always within 0.5 psu of corresponding control solutions.	30 - 33

Table A3. Test criteria for larval development rate of *Amphibalanus amphitrite*.

Exposure duration	96 h		
Biological effect metric	Estimated effect concentrations, EC_{10} and EC_{50}, for the concentrations that reduce larval development rate by 10% and 50%, respectively, relative to control treatments. No effect concentration (NEC) is the threshold below which the toxicants are not expected to cause a reduction in larval development rate.		
Biological endpoint definition	Successful transition of newly hatched nauplii to cyprid within 96 h (van Dam et al., 2016).		
Controls used	Seawater controls, no carrier or toxicant		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Imidacloprid	2	16	3
Propiconazole	1	8	3
Test acceptability criteria	≥ 80% successful transition in controls (van Dam et al., 2016). Observed average % transition ± SD in these tests: 87.3 ± 2.4% (*n* = 3)		
Characteristics of the test organism	Newly hatched (< 4 h) stage II nauplii of the acorn barnacle *Amphibalanus amphitrite*		
Type of test media	Natural, 0.5 µm filtered seawater spiked with pesticide stock (acetone carrier < 0.06% v/v) in ultrapure water. Daily addition of 1 x 10^5 cells mL^{-1} of the diatom *Chaetoceros muelleri*. Gentle, continuous aeration (~1 bubble s^{-1}) from bottom of funnel (van Dam et al., 2016).		
Toxicant (common name; IUPAC Name; CAS no.; supplier; purity)	• Imidacloprid; N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide; 138261-41-3; Merck; ≥ 99.9%) • Propiconazole; 1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole; 60207-90-1; Merck; ≥ 98%)		
Preparation of toxicant stock	Stock solutions (100 – 1,000 mg L^{-1}) of pesticides were prepared in ultrapure water. Imidacloprid and propiconazole were dissolved in acetone (≤ 0.06 % (v/v) in exposure). Stock solutions stored refrigerated and in the dark. Stock used to spike filtered seawater to obtain test solutions of desired concentration.		
Exposure type	Static		
Measured toxicant concentrations	Pesticide concentrations (n=3 per test) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All pesticide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS) at the University of Queensland, using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015).		
Reference toxicant	Copper		

Concentration-response relationship	- EC_x: regression models, fitted to the percent inhibition and measured pesticide concentrations using the DRC package in R (Ritz & Streibig, 2005; R Development Core Team, 2015). Concentration-response relationships presented in Figure A1. - NECs were estimated using jagsNEC package in R (R Development Core Team, 2015; Fisher et al., 2019). Proportional decline in larval development was modelled using a Bayesian non-linear gaussian model. NEC models presented Figure A2.
Statistical method or model used to determine effect of toxicant on test species	- Regression analysis were conducted following prescribed procedures (OECD, 2006a). The package DRC in R (Ritz & Streibig, 2005; R Development Core Team, 2015) was used to model the test data and to determine pesticide concentrations that inhibited 10% and 50% of larval development relative to controls (EC_{10} and EC_{50}, respectively). Regression models evaluated included log-logistic, Weibull and hormesis models of different levels of parametrization. Model comparisons were conducted using the Akaike Information Criterion. The model that best described the data was applied to derive estimates of toxicity. The associated 95% confidence limits were estimated using the delta method. - No effect concentration (NEC) values were calculated in R (v 3.5.3) (R Development Core Team, 2015). The proportional decline in larval development was modelled as a function of the log measured concentration of each pesticide using a Bayesian non-linear gaussian model using the package jagsNEC in R (R Development Core Team, 2015; Fisher et al., 2019). Trace plots were used to evaluate model fits and were found to have relatively good mixing in all cases. Bayesian 95% credible intervals (confidence limits) based on the upper 97.5th and lower 2.5th percentile of the posterior sample for the NEC parameter estimate.
Data variance	95% confidence limits (for EC_x) or Bayesian 95% credible intervals (for NECs) (Table A4).
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade pesticides (all ≥ 98% as described above) were used for preparation of all stock solutions. No pesticides were measured in any of the control solutions.

Summary of results

The toxicity of the insecticide imidacloprid and the fungicide propiconazole to the larval development of *A. amphitrite* is presented in Table A4 and Figures A1 and A2. The neonicotinoid insecticide imidacloprid did not inhibit larval development in *A. amphitrite* at the maximum concentration of 1,660 µg L^{-1}.

Table A4. Modelled toxicity estimates for the inhibition of the insecticide imidacloprid and the fungicide propiconazole on larval development of *A. amphitrite* (Figs. A1 and A2). All concentrations in µg L-1 (95% confidence intervals).

	NEC (95% CI)	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Imidacloprid	> 1660*	> 1660	> 1660
Propiconazole	878 (829 – 907)	568 (425 – 710)	1020 (936 – 1100)

* Although a NEC was provided by the model (Figure A2), no concentration-response relationship was observed and confidence around the supplied NEC was extremely low. Therefore, the NEC was deemed unreliable and should not be used in a regulatory context.
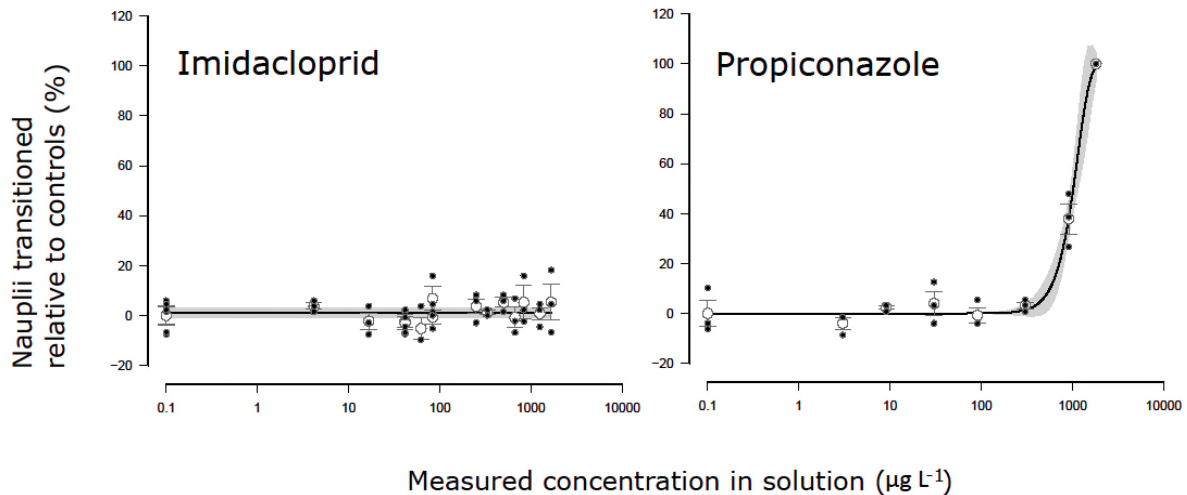
Figure A1. Relative inhibition of larval development of *Amphibalanus amphitrite* in response to 96-h exposures to increasing concentrations of imidacloprid and propiconazole. Open circles represent the treatment mean ± SE and closed circles represent individual treatment replicates. All data are expressed relative to control values and the upper limit of the concentration response curve was fixed at 100%. The solid black line is the fitted regression model, the shaded areas represents the model's 95% confidence limits. The best-fitting model (based on Akaike Information Criterion) for the propiconazole response was a Weibull type II 3-parameter. All concentrations are reported in µg L-1.
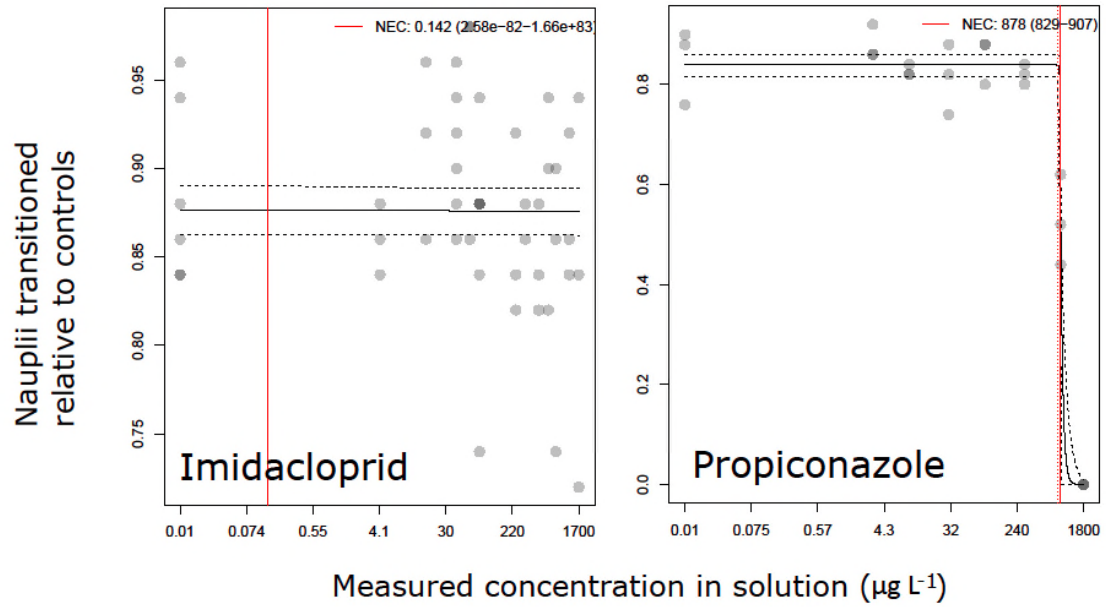
Figure A2. Bayesian non-linear gaussian model fit on the proportional decline in 4-day larval development of *Amphibalanus amphitrite* relative to the control treatment (solid black line and Bayesian 95% credible (confidence) intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of the respective pesticide. All concentrations in µg L-1.

Appendix I: Marine: *Coenobita variabilis*

Contact: j.vandam@aims.gov.au

Contributing authors: van Dam, J.W., Stapp, L.S., Kaserzon, S., Fisher, R. and Negri A.P.

The insecticide used in toxicity tests for this species and the mode of action were:
- Imidacloprid – blocks nicotinic acetylcholine receptors

Test species: *Coenobita variabilis* (terrestrial adult with marine larvae)

Test phylum: Arthropoda/Crustacea (Decapoda)

Biological effect: Inhibition of larval development

Summary

The inhibitory effects of the insecticide imidacloprid on larval development of the acorn barnacle *Coenobita variabilis* were determined by exposing newly hatched stage I zoea larvae to different imidacloprid concentrations over 144 h. Regression models were used to calculate the concentrations of each herbicide that inhibited 10% and 50% of the culture's SGR relative to controls (EC_{10} and EC_{50}, respectively). In order to determine the model which best described the data for each pesticide, various regression models of different levels of parametrization were evaluated and compared using the Akaike Information Criterion (AIC). No effect concentration (NEC) values were calculated from the proportional decline in SGR as a function of log concentration of each herbicide using a Bayesian non-linear gaussian model. Toxicity estimates for inhibition of *C. variabilis* larval development for the insecticide imidacloprid were 102, 43.3, and 390 µg L^{-1} for the NEC, EC_{10} and EC_{50}, respectively.

Methods

The inhibition of the larval development of free-swimming stage I zoea of *Coenobita variabilis* by imidacloprid was tested in static 144 h exposures (chronic) following (van Dam et al., 2018). Details of the experimental methods are provided in Tables A1 to A3. Original data including inhibition of the larval development and physico-chemical data (all start and end of test measurements) can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/769b9efa-9fb0-40c5-98e4-d3ac354371e0.

Table A1. Source of *Coenobita variabilis*, its culturing and test conditions.

Source of tests species	Broodstock was locally collected off the shore (Darwin, Australia – 12°23'8.70"S, 130°50'34.59"E)
Maintenance conditions of test species (culture conditions, light, temp etc)	Broodstock was maintained in custom-built, flat-bottomed enclosures as described by (van Dam et al., 2018).
Test endpoint	% successful transition to megalopa relative to controls
Test duration	144 h
Test chambers	Transparent polystyrene cell culture 6-well plates (Nunc; Thermo Scientific).
Test Volume	10 mL chamber^{-1}
Starting density	0.1 larva mL^{-1}
Counting of cells, calculation of SGR	Larval developmental stages scored using stereomicroscope as per van Dam et al (2018).

Table A2. Range of physico-chemical parameters measured in test media for all test solutions at the start of test for total number of tests performed with *Coenobita variabilis* (n = 2).

Light intensity (µmol photons m^{-2}s^{-2} over a 12:12 L:D cycle)	80 - 100
Temperature (°C)	27 - 28
Dissolved oxygen (mg L^{-1}). Exposure solutions were always within 0.4 mg L^{-1} of corresponding control solutions	8.0 – 8.1
pH (units). Exposure solutions were always within 0.3 pH unit of corresponding control solutions	8.0 – 8.4
Salinity (psu). Exposure solutions were always within 0.5 psu of corresponding control solutions.	31 - 32

Table A3. Test criteria for larval development rate of *Coenobita variabilis*.

Exposure duration	144 h
Biological effect metric	Estimated effect concentrations, EC$_{10}$ and EC$_{50}$, for the concentrations that reduce larval development rate by 10% and 50%, respectively, relative to control treatments. No effect concentration (NEC) is the threshold below which the toxicants are not expected to cause a reduction in larval development rate.
Biological endpoint definition	Successful transition of newly hatched stage I zoea to megalopa within 144 h (van Dam et al., 2018).
Controls used	Seawater controls, no carrier or toxicant
Replication	2 consecutive tests contributed to the definitive concentration-response curve. There were 3 replicates for each of the 12 concentrations
Test acceptability criteria	≥ 80% successful transition in controls (van Dam et al., 2016). Observed average % transition ± SD in these tests: 100% ± 0% (*n* = 2)
Characteristics of the test organism	Newly hatched free-swimming stage I zoea of the hermit crab *Coenobita variabilis*
Type of test media	Natural, 0.5 µm filtered seawater spiked with insecticide stock (acetone carrier < 0.05% v/v) in ultrapure water.
Toxicant (common name; IUPAC Name; CAS no.; supplier; purity)	Imidacloprid; N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide; 138261-41-3; Merck; ≥ 99.9%)
Preparation of toxicant stock	Stock solutions (2 – 200 mg L^{-1}) of imidacloprid were prepared in ultrapure water. Imidacloprid was dissolved in acetone (≤ 0.05 % (v/v) in exposure). Stock solutions stored refrigerated and in the dark. Stock used to spike filtered seawater to obtain test solutions of desired concentration.
Exposure type	Static
Measured toxicant concentrations	Imidacloprid concentrations (3 per test) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All pesticide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS) at the University of Queensland, using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015).
Reference toxicant	Copper
Concentration-response relationship	• EC$_x$: regression models, fitted to the percent inhibition and measured pesticide concentrations using the DRC package in R (Ritz & Streibig, 2005; R Development Core Team, 2015). Concentration-response relationships presented in Figure A1. • NECs were estimated using jagsNEC package in R (R Development Core Team, 2015; Fisher et al., 2019). Proportional decline in larval development was modelled using a Bayesian non-linear gaussian model. NEC models presented Figure A2.

Statistical method or model used to determine effect of toxicant on test species	• Regression analysis were conducted following prescribed procedures (OECD, 2006a). The package DRC in R (Ritz & Streibig, 2005; R Development Core Team, 2015) was used to model the test data and to determine pesticide concentrations that inhibited 10% and 50% of larval development relative to controls (EC_{10} and EC_{50}, respectively). Regression models evaluated included log-logistic, Weibull and hormesis models of different levels of parametrization. Model comparisons were conducted using the Akaike Information Criterion. The model that best described the data was applied to derive estimates of toxicity. The associated 95% confidence limits were estimated using the delta method. • No effect concentration (NEC) values were calculated in R (v 3.5.3) (R Development Core Team, 2015). The proportional decline in larval development was modelled as a function of the log measured concentration of each pesticide using a Bayesian non-linear gaussian model using the package jagsNEC in R(R Development Core Team, 2015; Fisher et al., 2019). Trace plots were used to evaluate model fits and were found to have relatively good mixing in all cases. Bayesian 95% credible intervals (confidence limits) based on the upper 97.5th and lower 2.5th percentile of the posterior sample for the NEC parameter estimate.
Data variance	95% confidence limits (for EC*x*) or Bayesian 95% credible intervals (for NECs) (Table A4).
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade imidacloprid was used for preparation of stock solution. No imidacloprid were measured in any of the control solutions.

Summary of results

The toxicity of the insecticide imidacloprid to the larval development of *C. variabilis* is presented in Table A4 and Figures A1 and A2.

Table A4. Modelled toxicity estimates for the inhibition of the insecticide imidacloprid on larval development of *C. variabilis* (Figs. A1 and A2). All concentrations in µg L-1 (95% confidence intervals).

	NEC (95% CI)	EC_{10} (95% CI)	EC_{50} (95% CI)
Imidacloprid	102 (38.7 – 175)	43.3 (2.92 – 83.6)	390 (262 – 517)
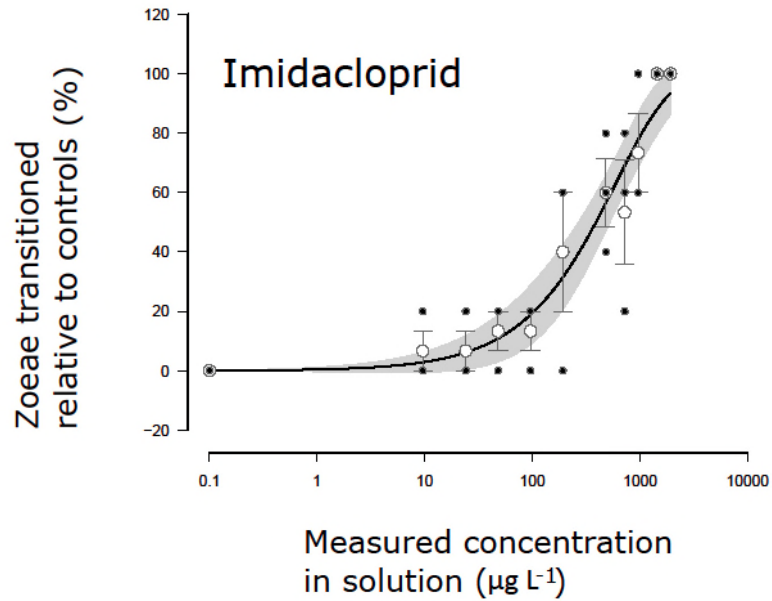
Figure A1. Relative inhibition of larval development of *Coenobita variabilis* in response to 144-h exposures to increasing concentrations of imidacloprid. Open circles represent the treatment mean ± SE and closed circles represent individual treatment replicates. All data are expressed relative to control values and the upper limit of the concentration response curve was fixed at 100%. The solid black line is the fitted regression model, the shaded areas represents the model's 95% confidence limits. The best-fitting model (based on Akaike Information Criterion) for the imidacloprid response was a Weibull type II 3-parameter. All concentrations are reported in µg L-1.
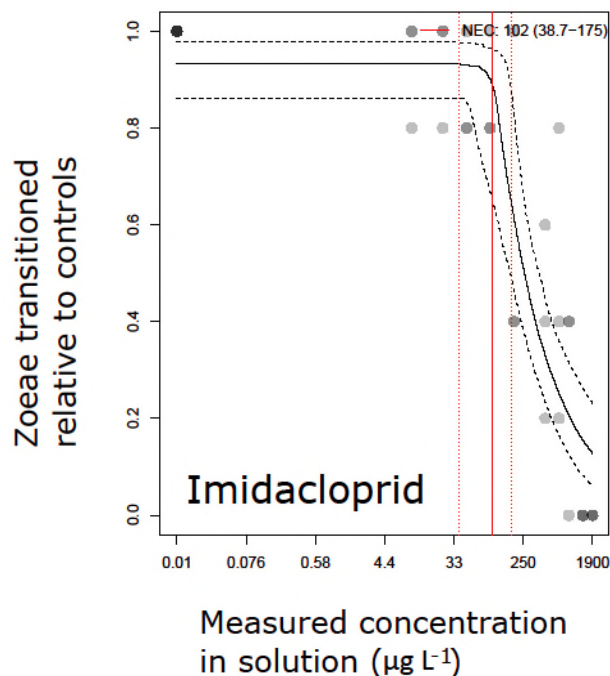
Figure A2. Bayesian non-linear gaussian model fit on the proportional decline in 6-day larval development rate of *Coenobita variabilis* relative to the control treatment (solid black line and Bayesian 95% credible (confidence) intervals (black dashed line) and the derived no effect concentration (NEC) (red line) and 95% confidence interval (red dashed line) of the respective pesticide. All concentrations in µg L-1.

Appendix J: Freshwater: *Azolla pinnata*

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Contributing authors: Ballantyne S., Le Gal, A-S., Templeman, M.A., McKenzie, M.R., Williams, C.D.

The herbicides used in toxicity tests for this species and their mode of action were:
- Diuron - PSII inhibitor
- Fluometuron – PSII Inhibitor
- Fluroxypyr – auxin mimic
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Isoxaflutole - 4-hydroxyphenyl-pyruvate-dioxygenase inhibitor
- Triclopyr – auxin mimic

Test species: *Azolla pinnata* (freshwater)

Test phylum: Pteridophyta – Filicopsida

Biological effect: Inhibition of specific growth rate – surface area, inhibition of specific growth rate – biomass and effective quantum yield

Summary of test results

The effect of seven herbicides (diuron, fluometuron, fluroxypyr, haloxyfop, imazapic, isoxaflutole and triclopyr) were assessed on growth of the freshwater macrophyte *Azolla pinnata* over 14 day exposures. The concentrations that inhibited 10% and 50% of specific growth rate (SGR) as surface area (SGR-SA) or biomass (SGR-B) of *A. pinnata* relative to control response (EC_{10} and EC_{50}, respectively) were calculated from 4-parameter sigmoidal model concentration-response curves. The toxicity thresholds for SGR-SA (EC_{10}; EC_{50} in $\mu g\ L^{-1}$) were as follows: diuron (3.28; 13.6), fluometuron (32.0; 360), fluroxypyr (6,450; 17,760), haloxyfop (78.4; 808), imazapic (31.6; 372), isoxaflutole (1.69; 84.2) and triclopyr (N.D.; 9,370). The toxicity thresholds for SGR-B (EC_{10}; EC_{50} in $\mu g\ L^{-1}$) were as follows: fluometuron (3.96; 119), fluroxypyr (2,620; 6,190), haloxyfop (208; 870), imazapic (47.0; 127), isoxaflutole (1.80; 212) and triclopyr (2,540; 7,250). The inhibition of $\Delta F/Fm'$ (EC_{10}; EC_{50} in $\mu g\ L^{-1}$) were as follows: diuron (2.01; 10.4), fluometuron (29.6; 505), and isoxaflutole (1.92; 197). Fluroxypyr, haloxyfop, imazapic and triclopyr were not assessed for $\Delta F/Fm'$.

Methods

The inhibition of the surface area specific growth rate (SGR-SA) and biomass specific growth rate (SGR-B) in *Azolla pinnata* by diuron, fluometuron, fluroxypyr, haloxyfop, imazapic, isoxaflutole and triclopyr were tested in static-renewal 14 day exposure periods (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$) was also assessed in static-renewal 14 day exposure periods. Details of the experimental methods are provided in Tables A1 to A3. Original data including SGR and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/e0eebe28-d26b-4644-ad20-16403bbce3f4.

Table A1. Source of *Azolla pinnata*, its culturing and test conditions.

Source of tests species	James Cook University in-house culture, parental stock supplied by Watergarden Paradise Nursery, NSW.
Maintenance conditions of test species (culture conditions, light, temp, etc)	Cultures were maintained in 10 L tubs containing 3 – 5 L IRRI2 medium (Pereira & Carrapiço, 2009) at 26 ± 1 °C, under a 12:12 hr light:dark cycle (65-77µmol photons m^{-2} s^{-1}).
Test endpoints	Inhibition of surface area specific growth rate (SGR - SA)	Inhibition of biomass specific growth rate (SGR-B)	Inhibition of effective quantum yield (ΔF/Fm′, proportional to photosynthetic efficiency)
Test duration	14 days (solution renewal at 7 days)
Test chambers	250 mL glass
Test volume	100 mL
Starting density	Four fronds each comprising eight ramets per replicate (Brown, 1994)
Calculation of SGR and ΔF/Fm′	• Frond surface area automatically assessed from photographs using ImageJ (Rueden & Eliceiri, 2019) and SGR calculated as per OECD TG238 (OECD, 2011). • Effective quantum yield was assessed via mini pulse amplitude modulated fluorometer (mini-PAM; WALZ, Germany).

Table A2. Measured physico-chemical parameters of test media for *Azolla pinnata*.

Light intensity (mean ± SD, n = 14 measurements across chamber)	90 ± 6 µmol photons m^{-2}s^{-1} over a 12:12 hr L:D cycle
Chamber temperature (mean ± SD, logged at 15 min intervals)	26.6 ± 0.5 °C
pH (mean ± SD, averaged 0, 7 and 14 days, n = 292)	5.93 ± 0.7
Electrical conductivity (mean ± SD, averaged 0, 7 and 14 days, n = 283)	34.4 ± 13 µS cm^{-1}
Test media temperature (mean ± SD, averaged 0, 7 and 14 days, n = 292)	25.7 ± 0.7 °C

Table A3. Test criteria for specific growth rate (surface area and biomass) and effective quantum yield of *Azolla pinnata*.

Exposure duration	14 days		
Biological effect metric	Inhibition of the mean specific growth rate (SGR-SA) - the logarithmic increase of surface area over 14 days (OECD, 2011).	Inhibition of the mean specific growth rate (SGR-B) - the logarithmic increase in biomass over 14 days (OECD, 2011).	Inhibition of the effective quantum yield (ΔF/Fm') which is proportional to photosynthetic efficiency for a given light intensity
Biological endpoint definition	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce SGR-SA, SGR-B or ΔF/Fm' by 10% and 50%, respectively, in comparison to control and / or solvent control treatments.		
Controls used	Imazapic and fluroxypyr were dissolved in the carrier solvent methanol (final concentration 0.01 % v/v). All other herbicides except triclopyr were dissolved in the carrier solvent acetone (final concentration 0.01 % v/v). No carrier solvent was used for triclopyr. A separate control treatment with no solvent was included for each experiment.		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Diuron	1	7	3
Fluometuron	2	14	3
Fluroxypyr	2	15	3

Haloxyfop	1	7	3
Imazapic	2	14	3
Isoxaflutole	1	8	3
Triclopyr	1	8	3

Test acceptability criteria	• Control SGR - SA ≥ 0.0495 day^{-1} as per (OECD, 2011). Observed mean control SGR: 0.119 ± 0.02 day^{-1} (mean ± SD, n = 54) • The coefficient of variation (CV) of mean SGR in controls ≤ 35% of each experiment as per (OECD, 2011). Observed control CV for any one test: <26%	• Control SGR - B ≥ 0.0495 day^{-1} as per (OECD, 2011). Observed mean control SGR: 0.148 ± 0.03 day^{-1} (mean ± SD, n = 36) • The coefficient of variation (CV) of mean SGR in controls ≤ 35% of each experiment as per (OECD, 2011). Observed control CV for any one test: <18%
Characteristics of the test organism	Actively growing culture free of overt disease and deformity. Starting density four fronds each comprising eight ramets.	
Type of test media	IRRI2 – synthetic media (Pereira & Carrapiço, 2009)	
Toxicant (common name; IUPAC Name; CAS no.; purity; batch)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Diuron (DCMU): 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98%; Batch: BCBS1743 • Fluometuron: 1,1-dimethyl-3-(α,α,α-trifluoro-m-tolyl)urea; 2164-17-2; > 98%; Batch: BCBW2049 • Fluroxypyr: 4-amino-3,5-dichloro-6-fluoro-2-pyridyloxyacetic acid; 69377-81-7; ≥ 98%. Batch: SZBF100XV • Haloxyfop-p-methyl: methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98%; Batch: BCBT1738 • Imazapic: 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%. Batch: BCBZ6821 • Isoxaflutole: (5-cyclopropyl-1,2-oxazol-4-yl)(α,α,α-trifluoro-2-mesyl-p-tolyl)methanone; 141112-29-0; ≥ 98%. Batch: BCBT2782 • Triclopyr: [(3,5,6-trichlor-2-pyridinyl)oxy]acetic acid; 5535-06-3; ≥ 98%; Batch: BCBW3270	
Preparation of toxicant stock	Diuron, fluometuron, haloxyfop and isoxaflutole (20 – 1,000 mg L^{-1}) were dissolved using the carrier solvent acetone (final concentration < 0.01% v/v in all exposure treatments). Fluroxypyr and imazapic (100 – 1,000 mg L^{-1}) were dissolved in the carrier solvent methanol (final concentration < 0.01% v/v in all exposure treatments). Triclopyr (0.25 - 70 mg L^{-1}) was dissolved directly into IRRI2 with no solvent carrier.	
Exposure type	Static-renewal. Test solution replacement at 7 days	
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).	
Reference toxicant	Diuron experiments conducted as a reference tests for this species	
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.	

Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR relative to controls (EC_{10} and EC_{50}, respectively) were calculated from concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Tables A4, A5 and A6)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade herbicides (≥ 98% purity) was used for preparation of stock solution. Analytical grade chemicals used for preparation of test and culture media.
Randomisation	Daily randomisation

Summary of results

The toxicity of seven herbicides to *Azolla pinnata* is presented in Table A4 (SGR-SA), Table A5 (SGR-B), Table A6 (ΔF/Fm') and Figure A1. Toxicity was assessed relative to control and/or solvent control responses. The 95% confidence intervals could not be determined for SGR-SA for triclopyr. SGR-B was not assessed for diuron and ΔF/Fm' was not assessed for fluroxypyr, haloxyfop, imazapic or triclopyr.

Table A4. Modelled toxicity estimates for the inhibition of seven herbicides on the surface area specific growth rate (SGR - SA) of *Azolla pinnata* (Figure A1, Figure A2). N.D. – Not able to be determined. All concentrations in µg L-1 (95% confidence intervals).

	EC_{10} (95% CI)	EC_{50} (95% CI)
Diuron	3.28 (1.96 – 5.02)	13.6 (11.1 – 16.8)
Fluometuron	32.0 (21.1 – 45.9)	360 (298 – 444)
Fluroxypyr	6,450 (4,450 – 8,930)	17,760 (14,680 – 21,780)
Haloxyfop	78.4 (47.0 - 122)	808 (662 - 979)
Imazapic	31.6 (15.4 – 55.7)	372 (268 – 546)
Isoxaflutole	1.69 (0.711 – 3.46)	84.2 (58.5 – 129)
Triclopyr	6,563 (N.D.)	9,800 (N.D.)

Table A5. Modelled toxicity estimates for the inhibition of seven herbicides on the biomass specific growth rate (SGR - B) of *Azolla pinnata* (Figure A1). N/A - Not assessed. All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	N/A	N/A
Fluometuron	3.96 (0.145 – 22.1)	119 (50.6 – 403)
Fluroxypyr	2,620 (1,590 – 4,400)	6,190 (5,150 – 7,170)
Haloxyfop	208 (132 - 320)	876 (723 – 1,052)
Imazapic	47.0 (22.8 – 76.8)	127 (102 – 162)
Isoxaflutole	1.80 (0.383 – 5.61)	212 (107 - 630)
Triclopyr	2,540 (1,660 – 4,330)	7,250 (6,040 – 8,580)

Table A6. Modelled toxicity estimates for inhibition of seven herbicides on the photosynthetic efficiency (ΔF/Fm′) of *Azolla pinnata* (Figure A1). N/A - Not Assessed. All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	2.01 (1.09 – 3.32)	10.4 (8.23 – 13.0)
Fluometuron	29.6 (20.2 – 41.6)	505 (433 – 591)
Fluroxypyr	N/A	N/A
Haloxyfop	N/A	N/A
Imazapic	N/A	N/A
Isoxaflutole	1.92 (0.873 – 3.72)	197 (136 – 318)
Triclopyr	N/A	N/A
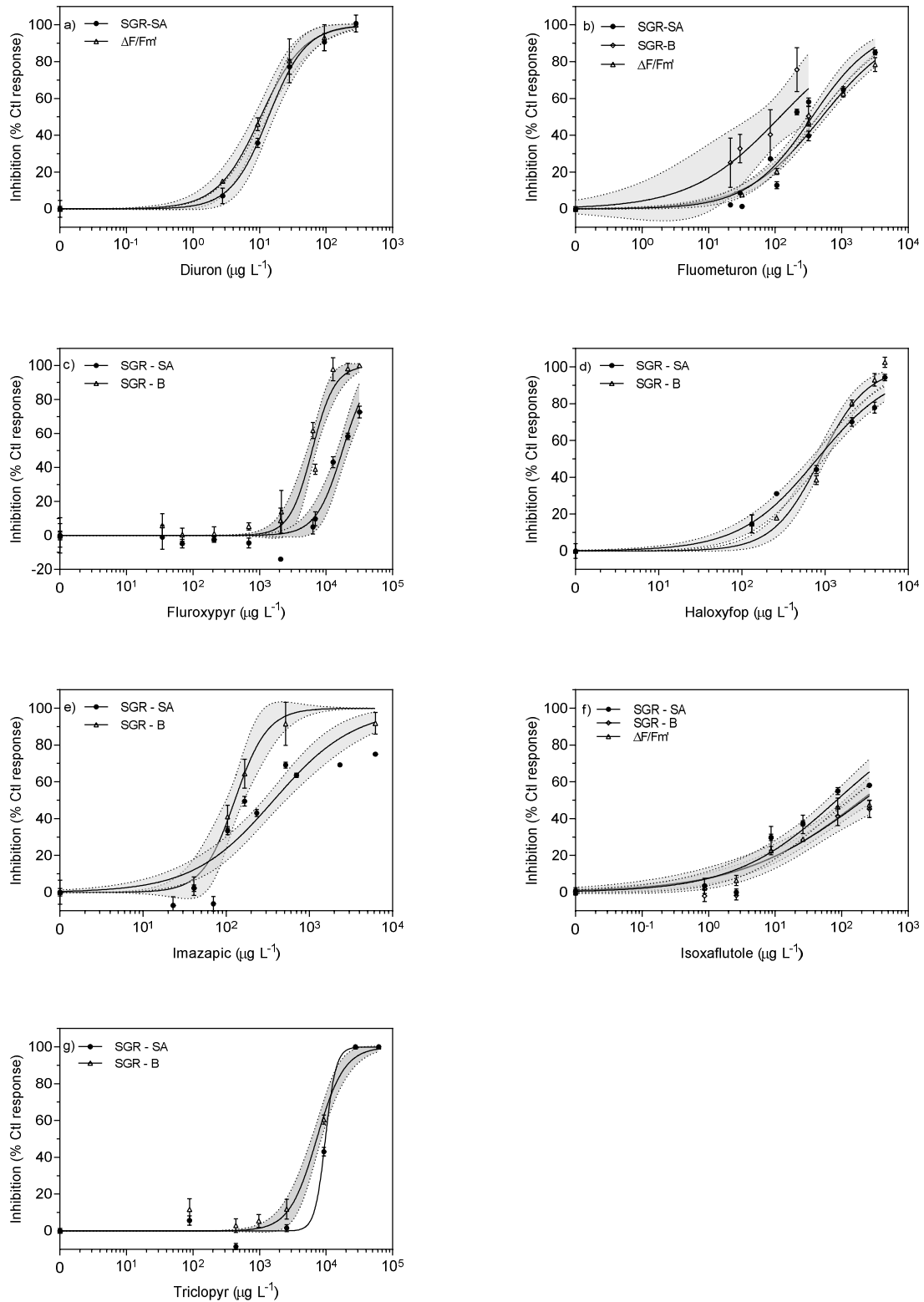
Figure A1 a-g. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 14-day surface area specific growth rate (SGR-SA), biomass specific growth rate (SGR-B) and effective quantum yield (ΔF/Fm′) of *Azolla pinnata* (mean ± SEM) following herbicide exposure to a) diuron; b) fluometuron; c) fluroxypyr; d) haloxyfop; e) imazapic; f) isoxaflutole and g) triclopyr at increasing concentrations. All concentrations are reported in μg L-1 (n = 3 for each treatment, bars not visible are smaller than symbol).

Appendix K: Freshwater: *Ceratophyllum demersum*

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The herbicides and their mode of action that were used in toxicity tests for this species were:
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Triclopyr - auxin mimic

Test species: *Ceratophyllum demersum*

Test phylum: Tracheophyta – Magnoliopsida (eudicotyledon) (IUCN, 2020)

Biological effect: Inhibition of specific growth rate (biomass and stem length)

Summary

The effects of three herbicides were tested on growth of the aquatic macrophyte *Ceratophyllum demersum* in culture over 7 day exposures. The concentrations of each herbicide that inhibited 10% and 50% of the macrophyte's biomass specific growth rate (SGR) and stem length SGR relative to controls (EC$_{10}$ and EC$_{50}$, respectively) were calculated from concentration-response curves (4-parameter sigmoidal models or Weibull model). The toxicity thresholds for biomass SGR (EC$_{10}$; EC$_{50}$ in µg L^{-1}) were as follows: triclopyr (88.3; 458) and haloxyfop (207; 1,190). The toxicity thresholds for stem length SGR (EC$_{10}$; EC$_{50}$ in µg L^{-1}) were as follows: imazapic (7.25; 67.8) and triclopyr (3,030; 8,540). The sensitivity of *C. demersum* responses for the two metrics varied depending on the herbicide.

Methods

The inhibition of the specific growth rate in *Ceratophyllum demersum* by each herbicide was tested in static 7 day exposures (chronic). Details of the experimental methods used in the *Ceratophyllum demersum* toxicity tests are provided in Tables A1 to A3. Original data including SGR (biomass), SGR (stem length) and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/b88b2d44-1f18-4657-a89f-0bdcced8302d.

Table A1. Source of *Ceratophyllum demersum*, its culturing and test conditions.

Source of test species	James Cook University in house culture, parental stock purchased from Watergarden Paradise Aquatic Nursery, Bass Hill, NSW.
Maintenance conditions of test species (culture conditions, light, temp etc)	Cultures were maintained in 500 L outdoor plastic tanks in recirculating dechlorinated tap water, aerated and maintained at ambient outdoor temperature and lighting. Test replicates selected 48 h in advance and acclimated in dechlorinated tap water, 26 ± 2 °C, under a 12:12 hr light:dark cycle (102 ± 9 µmol photons m^{-2} s^{-1}).
Test endpoint	1. Inhibition of biomass specific growth rate (SGR - B)	2. Inhibition of stem length specific growth rate (SGR-L)
Test duration	7 days (inhibition of SGR - B)	7 days (inhibition of SGR - L)
Test chambers	250 mL glass jars
Test volume	150 mL
Starting size	Approx. 35 mm plant with 5 whorls and apical tip and free from overt deformity and buds
Calculation of SGR	Individual plants were blotted to remove excess moisture and weighed to 3 decimal places at beginning and end of experiment as per OECD TG 238 (OECD, 2014) and Riethmuller et al (2003). SGR calculated as per OECD TG 238 (OECD, 2011).	Individual plants were photographed and measured using ImageJ (Rueden & Eliceiri, 2019) at beginning and end of experiment as per OECD TG 238 (OECD, 2014) and Riethmuller et al ((2003)). SGR calculated as per OECD TG 238 (OECD, 2011).

Table A2. Measured physico-chemical parameters of test media for *Ceratophyllum demersum*.

Light intensity (mean ± SD, n = 7 measurements across shelf)	90 ± 6 µmol photons $m^{-2}s^{-1}$ over a 12:12 hr L:D cycle
Chamber temperature (mean ± SD, logged at 15 min intervals)	26.6 ± 0.5 °C
Temperature (mean ± SD, averaged 0 and 7 days, n = 56)	25.8 ± 0.5 °C
pH (mean ± SD, averaged 0 and 7 days, n = 56)	7.57 ± 0.27
Electrical conductivity (mean ± SD, averaged 0 and 7 days, n = 56)	184 ± 8 $µS.cm^{-1}$

Table A3. Test criteria for specific growth rate (biomass and length) of *Ceratophyllum demersum*.

Exposure duration	7 days - Biomass	7 days – Stem Length
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase in biomass over 7 days (OECD, 2011)	Inhibition of the mean specific growth rate - the logarithmic increase in length over 7 days. (Riethmuller et al., 2003; OECD, 2014).
Biological endpoint definition	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce SGR by 10% and 50%, respectively, in comparison to control treatments.	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce SGR by 10% and 50%, respectively, in comparison to control treatments.
Controls used	Imazapic was dissolved using the carrier solvent methanol (final concentration < 0.01 % v/v in all exposure treatments). Haloxyfop was dissolved in the carrier acetone (final concentration < 0.01% v/v in all exposure treatments). No solvent carrier was used for the preparation of Triclopyr stock solution.	

Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Haloxyfop	1	8	5
Imazapic	1	8	5

Triclopyr	2	12	5
Test acceptability criteria	• Control SGR - B ≥ 0.0495 day^{-1} as per (OECD, 2011). Observed mean control SGR - B of all tests: 0.074 ± 0.023 day^{-1} (mean ± SD, n = 20) • The coefficient of variation (CV) of mean SGR - B in controls ≤ 35% as per (OECD, 2011). Observed control CV: < 35% in all tests		• Control SGR - L ≥ 0.0495 day^{-1} as per (OECD, 2011). Observed mean control SGR - L of all tests: 0.064 ± 0.020 day^{-1} (mean ± SD, n = 20) • The coefficient of variation (CV) of mean SGR in controls ≤ 35% as per . Observed control CV: < 35% in all tests (Riethmuller et al., 2003; OECD, 2014).
Characteristics of the test organism	Actively growing culture with no buds, no lateral branches and free of overt disease and deformity.		Actively growing culture with no buds, no lateral branches and free of overt disease and deformity.
Type of test media	Autoclaved, recirculating dechlorinated tap water		
Toxicant (common name; IUPAC Name; CAS no.; purity)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%; Batch: BCBZ6821 • Haloxyfop-p-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98%; Batch: BCBT1738 • Triclopyr: [(3,5,6-trichlor-2-pyridinyl)oxy]acetic acid; 5535-06-3; ≥ 98%; Batch: BCBW3270		
Preparation of toxicant stock	Stock solutions (100-10,000 mg L^{-1}) of imazapic and haloxyfop were prepared in Milli-Q® water. Imazapic was dissolved using the carrier solvent methanol (< 0.01% (v/v) in exposure treatments). Haloxyfop was dissolved in the carrier solvent acetone (≤ 0.01% (v/v) in exposure treatments). Triclopyr was weighed directly into treatment solutions with no carrier solvent.		
Exposure type	Static		
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).		
Reference toxicant	Nil		
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal models, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA) for all herbicides except triclopyr stem length. Triclopyr stem length was fitted with a Weibull model using R (Ritz & Streibig, 2005; R Development Core Team, 2015). see Figure A1.		
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR relative to controls (EC$_{10}$ and EC$_{50}$, respectively) were calculated from concentration-response curves (4-parameter sigmoidal models) fitted to the percent inhibition and measured herbicide concentrations for each		

	treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA). Weibull model was used for triclopyr stem length using R (Ritz & Streibig, 2005; R Development Core Team, 2015).
Data variance	95% Confidence Limits (CL) (see Tables A4 and A5)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade herbicides (≥ 98% purity) were used for preparation of all stock solutions.

Summary of results

The toxicity of three herbicides to *C. demersum* is presented in Table A4 (SGR – B), Table A5 (SGR – L), Figure A1 (SGR – B, SGR-L) and Figure A2 (SGR – L) triclopyr. The biomass response to imazapic could not be modelled due to the presence of an adhering gelatinous mucous to fronds in imazapic concentrations 690 µg L^{-1} and higher (presumed to be a response to herbicide exposure). The mucous interfered with accurate weighing of fronds at day 7. Triclopyr exhibited strong hormetic effects at lower concentrations for stem length SGR but did not exhibit the same response in biomass SGR (Figures A1 and A2).

Table A4. Modelled toxicity estimates for the inhibition of two herbicides on the biomass specific growth rate (SGR - B) of *Ceratophyllum demersum* (Fig. A1). All concentrations in µg L-1 (95% confidence limits).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Haloxyfop	207 (8.40 – 1,390)	1,190 (576 – 2,390)
Triclopyr	68.4 (18.1 – 145)	356 (252 – 467)

Table A5. Modelled toxicity estimates for inhibition of two herbicides on the stem length specific growth rate (SGR - L) of *Ceratophyllum demersum* (Fig. A1). All concentrations in µg L-1 (95% confidence limits).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Imazapic	7.25 (2.00 x 10^{-12} – 35.4)	67.8 (25.6 – 148)
Triclopyr	3,030 (246 – 5,810)	8,540 (2,640 – 14,400)
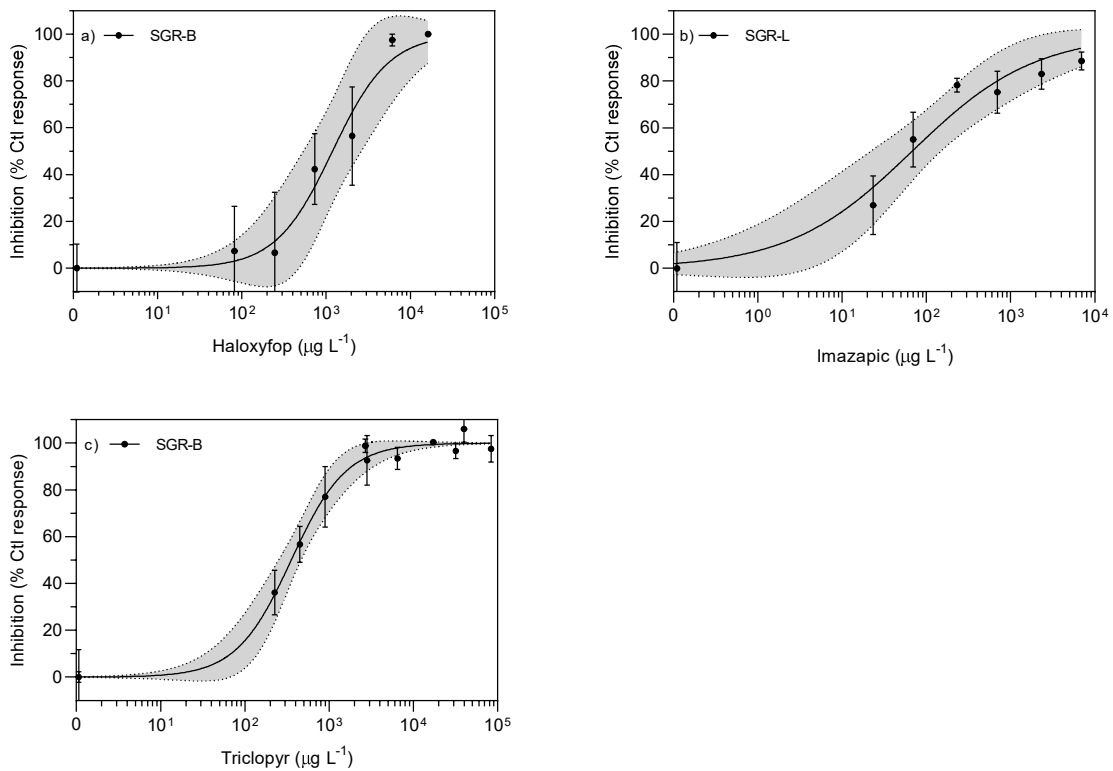
Figure A1. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 7-day biomass specific growth rate (SGR-B) or stem length specific growth rate (SGR-L) of *Ceratophyllum demersum* (mean ± SE) following herbicide exposure to a) imazapic; b) haloxyfop and c) triclopyr at increasing concentrations. All concentrations are reported in µg L-1 (n = 5 for each treatment, bars not visible are smaller than symbol).
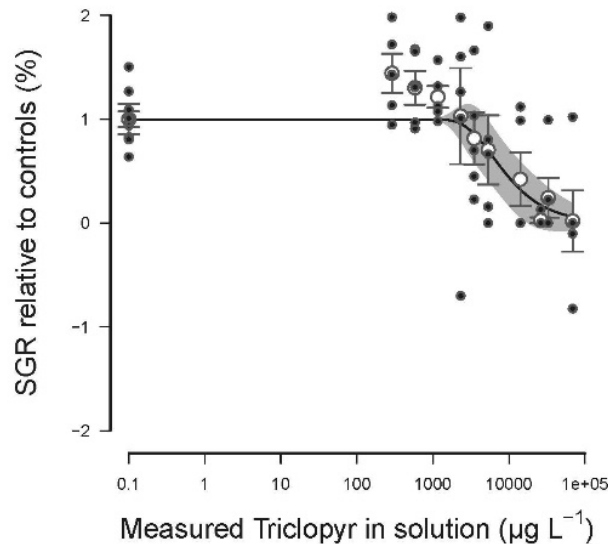
Figure A2. Weibull curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 7-day stem length specific growth rate (SGR-L) of *Ceratophyllum demersum* (mean ± SE) following exposure to triclopyr at increasing concentrations. All concentrations are reported in µg L-1 (n = 5 for each treatment, bars not visible are smaller than symbol).

Appendix L: Freshwater: *Chlorella* sp*.*

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The herbicides and their mode of action that were used in toxicity tests for this species were:
- Bromacil - PSII inhibitor
- Diuron - PSII inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Hexazinone - PSII inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Isoxaflutole - 4-hydroxyphenyl-pyruvate-dioxygenase inhibitor
- Prometryn - PSII inhibitor
- Propazine - PSII inhibitor

Test species: *Chlorella* sp. (freshwater)

Test phylum: Chlorophyta

Biological effect: Inhibition of specific growth rate and effective quantum yield

Summary

The effect of eight herbicides (bromacil, diuron, haloxyfop, hexazinone, imazapic, isoxaflutole, prometryn and propazine) were assessed on growth of the freshwater chlorophyte *Chlorella* sp. over a 72 hour exposure period. The concentrations that inhibited 10% and 50% of specific growth rate (SGR) and effective quantum yield ($\Delta F/Fm'$) of *Chlorella* sp. relative to control response (EC_{10} and EC_{50}, respectively) were calculated from 4-parameter sigmoidal model concentration-response curves. The toxicity thresholds for SGR (EC_{10}; EC_{50} in µg L^{-1}) were as follows: bromacil (14.6; 26.3), diuron (11.2; 24.7), haloxyfop (2,180; 7,810), hexazinone (22.8; 51.3), imazapic (38,100; >190,000), prometryn (5.29; 22.0), propazine (72.4; 178). No effects on SGR were observed for isoxaflutole at the highest concentrations tested. The inhibition of $\Delta F/Fm'$ (EC_{10}; EC_{50} in µg L^{-1}) were as follows: bromacil (11.0; 21.4), diuron (2.32; 8.73), hexazinone (29.5; 34.0), prometryn (1.19; 15.6), propazine (29.7; 138). No effects on $\Delta F/Fm'$ were observed for isoxaflutole at the highest concentrations tested. Haloxyfop and imazapic were not assessed for $\Delta F/Fm'$.

Methods

The inhibition of the specific growth rate in *Chlorella* sp. by bromacil, diuron, haloxyfop, hexazinone, imazapic, isoxaflutole, prometryn and propazine was tested in static 72 hr exposure periods (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$) in static 72 hr exposure period was assessed also. Details of the experimental methods are provided in Tables A1 to A3.

Table A1. Source of *Chlorella sp,* its culturing and test conditions.

Source of tests species	James Cook University in-house culture, parental stock supplied by Supervising Scientist, Dept of Environment and Energy, Darwin, NT.	
Maintenance conditions of test species (culture conditions, light, temp, etc)	Cultures were maintained in 100 mL of MBL medium (Riethmuller et al., 2003; Pease et al., 2016) in 250 mL Erlenmeyer flasks on an orbital shaker at 26 ± 2 °C, under a 12:12 hr light:dark cycle (91 ± 12 µmol photons m^{-2} s^{-1}).	
Test endpoint	Inhibition of specific growth rate (SGR) of culture in log growth phase	Inhibition of effective quantum yield (ΔF/Fm′, proportional to photosynthetic efficiency)
Test duration	72 hr	
Test chambers	100 mL glass conical flasks	
Test volume	50 mL	
Starting density	3.0 - 3.1x10^4 cells mL^{-1}	
Counting of cells, calculation of SGR; chlorophyll *a* fluorescence determination	Cells automatically counted from photographs using ImageJ (Rueden & Eliceiri, 2019) and / or manually counted using haemocytometer. SGR calculated as per OECD test 201 (OECD, 2011).	Pulse amplitude modulated fluorometer (mini-PAM).

Table A2. Measured physico-chemical parameters of test media. Original data including SGR and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/e90d967a-846a-4d05-8782-ff774257c01f.

Light intensity (mean ± SD, n = 7 measurements across chamber)	190 ± 14 µmol photons m^{-2}s^{-1} over a 12:12 hr L:D cycle
Chamber Temperature (mean ± SD, logged at 15 min intervals)	26.6 ± 0.5 °C
pH (mean ± SD, averaged 0 and 72 hr, n = 136)	7.14 ± 0.1
Electrical Conductivity (mean ± SD, averaged 0 and 72 hr, n = 136)	316 ± 14 µS cm^{-1}
Test Media Temperature (mean ± SD, averaged 0 and 72 hr, n = 136)	25.4 ± 0.8 °C

Table A3. Test criteria for specific growth rate and effective quantum yield of *Chlorella sp*.

Exposure duration	72 hr		
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase of biomass over 72 hr (OECD, 2011).	Inhibition of the effective quantum yield (ΔF/Fm') which is proportional to photosynthetic efficiency for a given light intensity	
Biological endpoint definition	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce SGR (or ΔF/Fm') by 10% and 50%, respectively, in comparison to control treatments.		
Controls used	Imazapic was dissolved in the carrier solvent methanol (final concentration ≤ 0.01% (v/v)). All other herbicides except propazine were dissolved in the carrier solvent acetone (final concentration ≤ 0.01% (v/v)). No carrier solvent was used for propazine. A separate control treatment with no solvent was included for each experiment.		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Bromacil	1	8	3
Diuron	1	7	3
Haloxyfop	1	8	3
Hexazinone	1	8	3

	1	8	3
Imazapic	1	8	3
Isoxaflutole	2	14	3
Prometryn	2	14	3
Propazine	1	8	3

Test acceptability criteria	• Control SGR ≥ 0.92 day^{-1} as per (OECD, 2011). Observed mean control SGR: 1.13 ± 0.05 day^{-1} (mean ± SD, n = 48) • The coefficient of variation (CV) of mean SGR in controls ≤ 10% as per (OECD, 2011). Observed control CV: ≤ 5%
Characteristics of the test organism	4-7 day old culture in exponential growth phase, starting density 3.0- 3.1x10^4 cells mL^{-1}
Type of test media	MBL culture media (0.5x strength) (Riethmuller et al., 2003)
Toxicant (common name; IUPAC Name; CAS no.; purity)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Bromacil: (*RS*)-5-bromo-3-sec-butyl-6-methyluracil ; 314-40-9; ≥ 98%. Batch:SZBF139XV • Diuron (DCMU): 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98%; Batch: BCBS1743 • Haloxyfop-p-methyl: methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98%; Batch: BCBT1738 • Hexazinone: 3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione; 51235-04-2; ≥ 98%. Batch: BCBT6090 • Imazapic: 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%. Batch: BCBZ6821 • Isoxaflutole: (5-cyclopropyl-1,2-oxazol-4-yl)(α,α,α-trifluoro-2-mesyl-p-tolyl)methanone; 141112-29-0; ≥ 98%. Batch: BCBT2782 • Prometryn: *N*2,*N*4-diisopropyl-6-methylthio-1,3,5-triazine-2,4-diamine; 7287-19-6; ≥ 98%. Batch: BCBV7467 • Propazine: 6-chloro-*N*2,*N*4-diisopropyl-1,3,5-triazine-2,4-diamine; 139-40-2; ≥ 98%. Batch: BCBX0853
Preparation of toxicant stock	10 – 1,000 mg L^{-1} bromacil, diuron, haloxyfop, hexazinone, isoxaflutole and prometryn were dissolved using the carrier solvent acetone (final concentration ≤ 0.01% (v/v) in all exposure treatments). Imazapic was dissolved in the carrier solvent methanol (final concentration ≤ 0.01% (v/v) in all exposure treatments). Propazine stock solution (5 mg L^{-1}) was prepared with no carrier solvent.
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Diuron experiment conducted as a reference test for this species
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR (or ΔF/Fm') relative to controls (EC$_{10}$ and EC$_{50,}$ respectively) were calculated from

	concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Table A4)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade herbicides (≥ 98% purity) was used for preparation of stock solution. Analytical grade chemicals were used for preparation of test and culture media.
Randomisation	Daily randomisation and flasks shaken by hand 3 x daily

Summary of results

The toxicity of eight herbicides to *Chlorella* sp. is presented in Table A4, Table A5 and Figure A1. Toxicity was assessed relative to control and/or solvent control responses. The non-PSII herbicide isoxaflutole did not inhibit SGR or photosynthetic efficiency (ΔF/Fm′) in *Chlorella* sp. at the highest concentration (2,570 µg L^{-1}) tested. 95% confidence intervals could not be determined for photosynthetic efficiency (ΔF/Fm′) for hexazinone. Haloxyfop and imazapic were not assessed for photosynthetic efficiency (ΔF/Fm′).

Table A4. Modelled toxicity estimates for the inhibition of eight herbicides on the specific growth rate (SGR) of *Chlorella sp.* (Figure A1). All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Bromacil	14.6 (12.8 – 16.7)	26.3 (24.9 – 27.8)
Diuron	11.2 (9.87 – 12.8)	24.7 (23.1 – 26.4)
Haloxyfop	2,180 (1,630 – 2,930)	7,810 (6,960 – 9,160)
Hexazinone	22.8 (20.1 – 25.5)	51.3 (48.7 – 54.0)
Imazapic	38,100 (21,800 – 57,900)	>190,000
Isoxaflutole	>2,570	>2,570
Prometryn	5.29 (2.20 – 10.9)	22.0 (16.1 – 29.4)
Propazine	72.4 (61.7 – 83.3)	178 (168 – 189)

Table A5. Modelled toxicity estimates for inhibition of eight on the photosynthetic efficiency (ΔF/Fm′) of *Chlorella sp.* (Figure A1). N/A = Not Assessed. N.D. – Not able to be determined. All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Bromacil	11.0 (8.80 – 13.1)	21.4 (19.6 – 23.5)
Diuron	2.32 (1.99 – 2.68)	8.73 (8.16 – 9.33)
Haloxyfop	N/A	N/A
Hexazinone	29.5 (N.D.)	34.0 (N.D.)
Imazapic	N/A	N/A
Isoxaflutole	>2,570	>2,570
Prometryn	1.19 (0.182 – 3.11)	15.6 (9.98 – 24.1)
Propazine	29.7 (20.9 – 39.9)	138 (122 – 155)
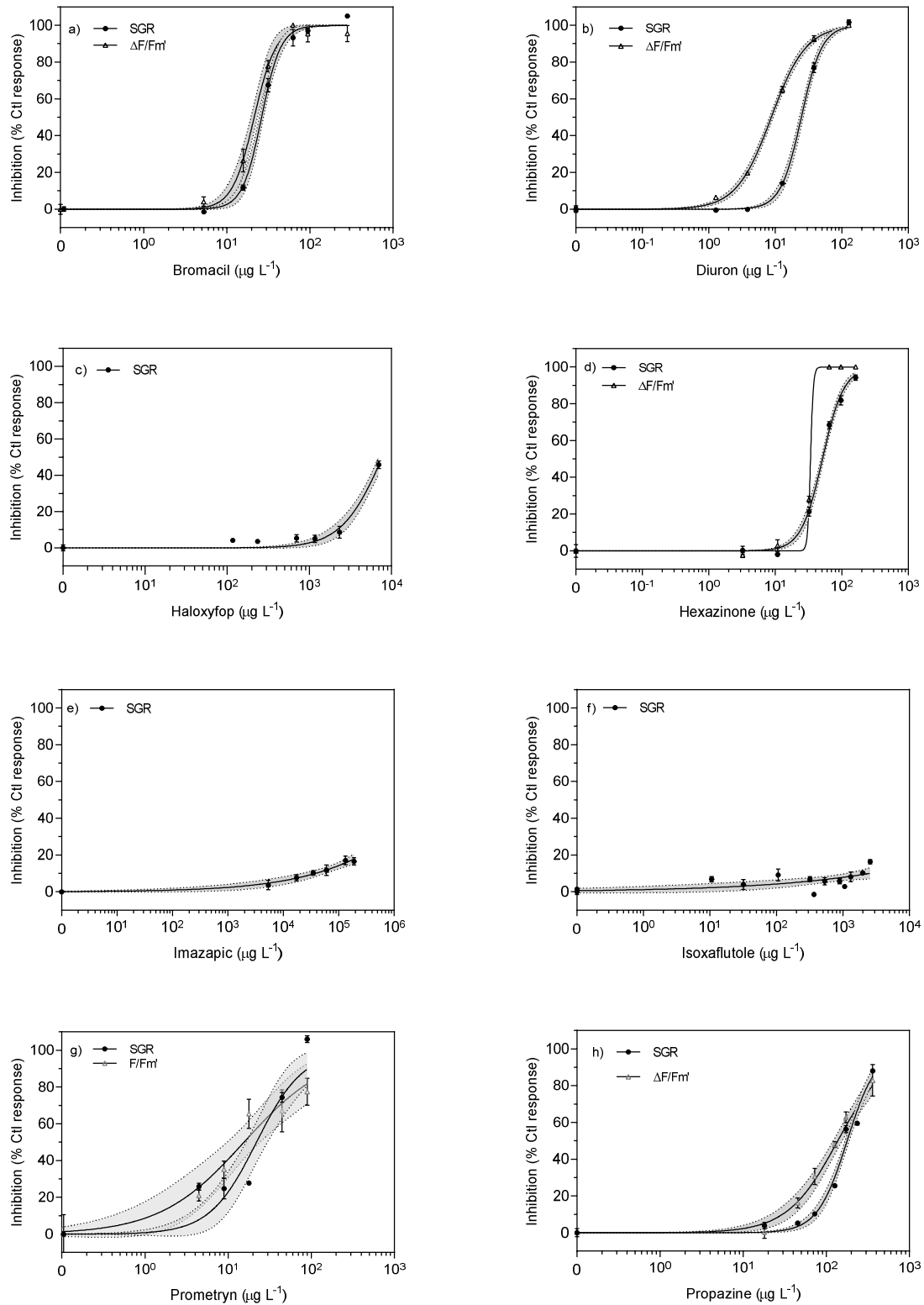
Figure 1a-h. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 3-day specific growth rate (SGR) and effective quantum yield (ΔF/Fm′) of *Chlorella sp.* (mean ± SEM) following herbicide exposure to a) bromacil; b) diuron; c) haloxyfop; d) hexazinone; e) imazapic; f) isoxaflutole; g) prometryn and h) propazine at increasing concentrations. All concentrations are reported in µg L-1 (n = 3 for each treatment, bars not visible are smaller than symbol).

Appendix M: Freshwater: *Desmodesmus asymmetricus*

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Contributing authors: Mulama, V., Templeman M.A., McKenzie M., Williams C.D. and Elisei, G.

The herbicides and their mode of action that were used in toxicity tests for this species were:
- Bromacil – PSII Inhibitor
- Diuron - PSII inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Hexazinone – PSII Inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Isoxaflutole - 4-hydroxyphenyl-pyruvate-dioxygenase inhibitor
- Propazine - PSII Inhibitor

Test species: *Desmodesmus asymmetricus* (freshwater)

Test phylum: Chlorophyta

Biological effect: Inhibition of specific growth rate and effective quantum yield

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Contributing authors: Mulama, V., Templeman M.A., McKenzie M., Williams C.D. and Elisei, G.

Summary
The effect of seven herbicides (bromacil, diuron, haloxyfop, hexazinone, imazapic, isoxaflutole and propazine) were assessed on growth of the freshwater chlorophyta *Desmodesmus asymmetricus* over a 72 hour exposure period. The concentrations that inhibited 10% and 50% of *D. asymmetricus* specific growth rate (SGR) and effective quantum yield ($\Delta F/Fm'$) relative to control response (EC_{10} and EC_{50}, respectively) were calculated from 4-parameter sigmoidal model concentration-response curves. The toxicity thresholds (EC_{10}; EC_{50} in $\mu g \ L^{-1}$) were bromacil (12.9; 36.8), diuron (6.13; 28.4), haloxyfop (311; 921), hexazinone (12.6; 52.0) and propazine (54.4; 153). No effects on SGR were observed for imazapic and isoxaflutole at the highest concentrations tested. The inhibition of photosynthetic efficiency ($\Delta F/Fm'$) relative to control response (EC_{10}; EC_{50} in $\mu g \ L^{-1}$) were bromacil (37.8; 43.8), diuron (1.94; 14.5), hexazinone (5.85; 22.6) and propazine (11.7; 69.3). Haloxyfop, imazapic and isoxaflutole were not assessed for photosynthetic efficiency.

Methods

The inhibition of the specific growth rate in *Desmodesmus asymmetricus* by bromacil, diuron, haloxyfop, hexazinone, imazapic, isoxaflutole and propazine was tested in static 72 hr exposure periods (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$) was tested in static 72 hr exposure period also (chronic). Details of the experimental methods used in the *Desmodesmus asymmetricus* toxicity tests are provided in Tables A1 to A3. Original data including SGR and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/589e0f48-5a9b-4957-bd47-9fd6c6cc0bfb.

Table A1. Test species and test conditions.

Source of tests species	James Cook University in-house culture (strain CS-905/9), purchased from Australian National Algae Supply Service, Hobart.	
Maintenance conditions of test species (culture conditions, light, temp, etc)	Cultures were maintained in 100 mL of MLA medium in 250 mL Erlenmeyer flasks on an orbital shaker at 26 ± 2 °C, under a 12:12 hr light:dark cycle (91 ± 12 µmol photons m^{-2} s^{-1}).	
Test endpoint	Inhibition of specific growth rate (SGR) of culture in log growth phase	Inhibition of effective quantum yield (ΔF/Fm′, proportional to photosynthetic efficiency)
Test duration	72 hr	
Test chambers	100 mL glass conical flasks	
Test Volume	50 mL	
Starting density	3.0 - 3.1x10^4 cells mL^{-1}	
Counting of cells, calculation of SGR; chlorophyll *a* fluorescence determination	Cells automatically counted from photographs using ImageJ (Rueden & Eliceiri, 2019) and / or manually counted using haemocytometer. SGR calculated as per OECD test 201 (OECD, 2011).	Pulse amplitude modulated fluorometer (mini-PAM)

Table A2. Measured physico-chemical parameters of test media for *Desmodesmus asymmetricus*.

Light intensity (mean ± SD, n = 7 measurements across chamber)	190 ± 14 µmol photons $m^{-2}s^{-1}$ over a 12:12 hr L:D cycle
Chamber Temperature (mean ± SD, logged at 15 min intervals)	26.6 ± 0.5 °C
pH (mean ± SD, averaged 0 and 72 hr, n = 189)	7.61 ± 0.2
Electrical Conductivity (mean ± SD, averaged 0 and 72 hr, n = 189)	397 ± 49 µS cm^{-1}
Test Media Temperature (mean ± SD, averaged 0 and 72 hr, n = 189)	25.8 ± 0.5 °C

Table A3. Test criteria for specific growth rate for *Desmodesmus asymmetricus*.

Exposure duration	72 hr		
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase of biomass over 72 hr (OECD, 2011).	Inhibition of the effective quantum yield (ΔF/Fm') which is proportional to photosynthetic efficiency for a given light intensity	
Biological endpoint definition	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce SGR or ΔF/Fm' by 10% and 50%, respectively, in comparison to control treatments.		
Controls used	Imazapic was dissolved in the carrier solvent methanol. Bromacil, diuron, haloxyfop, hexazinone, and isoxaflutole were dissolved in the carrier solvent acetone (final concentration 0.01 % v/v). No carrier solvent was used for propazine. A separate control treatment with no solvent was included for each experiment.		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Bromacil	2	14	3
Haloxyfop	1	8	3
Hexazinone	2	15	3
Imazapic	3	22	3
Isoxaflutole	2	16	3
Propazine	2	13	3
Test acceptability criteria	• Control SGR ≥ 0.92 day^{-1} as per (OECD, 2011). Observed mean control and/or solvent control SGR: 1.10 ± 0.09 day^{-1} (mean ± SD, n = 66)		

	• The coefficient of variation (CV) of mean SGR in controls ≤ 10% as per (OECD, 2011). Observed control CV in any one test: <7%
Characteristics of the test organism	4-7 day old culture in exponential growth phase, starting density 3.0 - 3.1x10^4 cells mL^{-1}
Type of test media	MLA culture media (0.5x strength)
Toxicant (common name; IUPAC Name; CAS no.; purity; batch)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Bromacil: (*RS*)-5-bromo-3-sec-butyl-6-methyluracil ; 314-40-9; ≥98%. Batch:SZBF139XV • Diuron (DCMU): 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98%; Batch: BCBS1743 • Haloxyfop-p-methyl: methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98%; Batch: BCBT1738 • Hexazinone: 3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione; 51235-04-2; ≥ 98%. Batch: BCBT6090 • Imazapic: 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%. Batch: BCBZ6821 • Isoxaflutole: (5-cyclopropyl-1,2-oxazol-4-yl)(α,α,α-trifluoro-2-mesyl-p-tolyl)methanone; 141112-29-0; ≥98%. Batch: BCBT2782 • Propazine: 6-chloro-*N*2,*N*4-diisopropyl-1,3,5-triazine-2,4-diamine; 139-40-2; ≥98%. Batch: BCBX0853
Preparation of toxicant stock	20 – 1,000 mg L^{-1} bromacil, diuron, haloxyfop, hexazinone and isoxaflutole were dissolved using the carrier solvent acetone (final concentration < 0.01 % v/v in all exposure treatments). 100-1,000 mg L^{-1} imazapic was dissolved in the carrier solvent methanol (final concentration < 0.01 % v/v in all exposure treatments). 5 mg L^{-1} propazine was prepared directly in Milli-Q.
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Diuron experiment conducted as a reference test for this species
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR or ΔF/Fm' relative to controls (EC$_{10}$ and EC$_{50,}$ respectively) were calculated from concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Table A4)
Test solutions, blanks and/or controls tested for	Controls were tested for contamination. Analytical grade herbicides (≥ 98% purity) was used for preparation of stock solution.

| contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Analytical grade chemicals used for preparation of test and culture media.
Randomisation	Daily randomisation and flasks shaken by hand 3 x daily

Summary of results

The toxicity of seven herbicides to *Desmodesmus asymmetricus* is presented in Table A4, Table A5 and Figure A1. Toxicity was assessed relative to control and/or solvent control responses. The non-PSII herbicides imazapic and isoxaflutole at the maximum concentrations of 198,000 µg L^{-1} and 798 µg L^{-1}. Haloxyfop, imazapic and isoxaflutole were not assessed for photosynthetic efficiency ($\Delta F/Fm'$).

Table A4. Modelled toxicity estimates for the inhibition of seven herbicides on the specific growth rate (SGR) of *Desmodesmus asymmetricus* (Figure A1). All concentrations in µg L-1 (95% confidence intervals).

	EC_{10}	EC_{50}
Bromacil	12.9 (10.1 – 16.6)	36.8 (33.1 – 40.6)
Diuron	6.13 (3.86 – 9.20)	28.4 (23.3 – 34.7)
Haloxyfop	311 (190 – 486)	921 (771 – 1120)
Hexazinone	12.6 (7.45 – 19.4)	52.0 (42.8 – 62.6)
Imazapic	>198,000	>198,000
Isoxaflutole	>798	>798
Propazine	54.4 (43.8 – 66.4)	153 (140 – 167)

Table A5. Modelled toxicity estimates for inhibition of seven herbicides on the photosynthetic efficiency ($\Delta F/Fm'$) of *Desmodesmus asymmetricus* (Figure A1). N/A = Not Assessed. All concentrations in µg L-1 (95% confidence intervals).

	EC_{10}	EC_{50}
Bromacil	37.8 (31.6 –45.2)	43.8 (42.0 – 45.8)
Diuron	1.94 (0.938 – 1.28)	14.5 (12.4 – 17.0)
Haloxyfop	N/A	N/A
Hexazinone	5.85 (4.07 – 7.97)	22.6 (19.7 – 25.7)
Imazapic	N/A	N/A
Isoxaflutole	N/A	N/A
Propazine	11.7 (5.91 – 20.3)	69.3 (53.5 – 90.2)
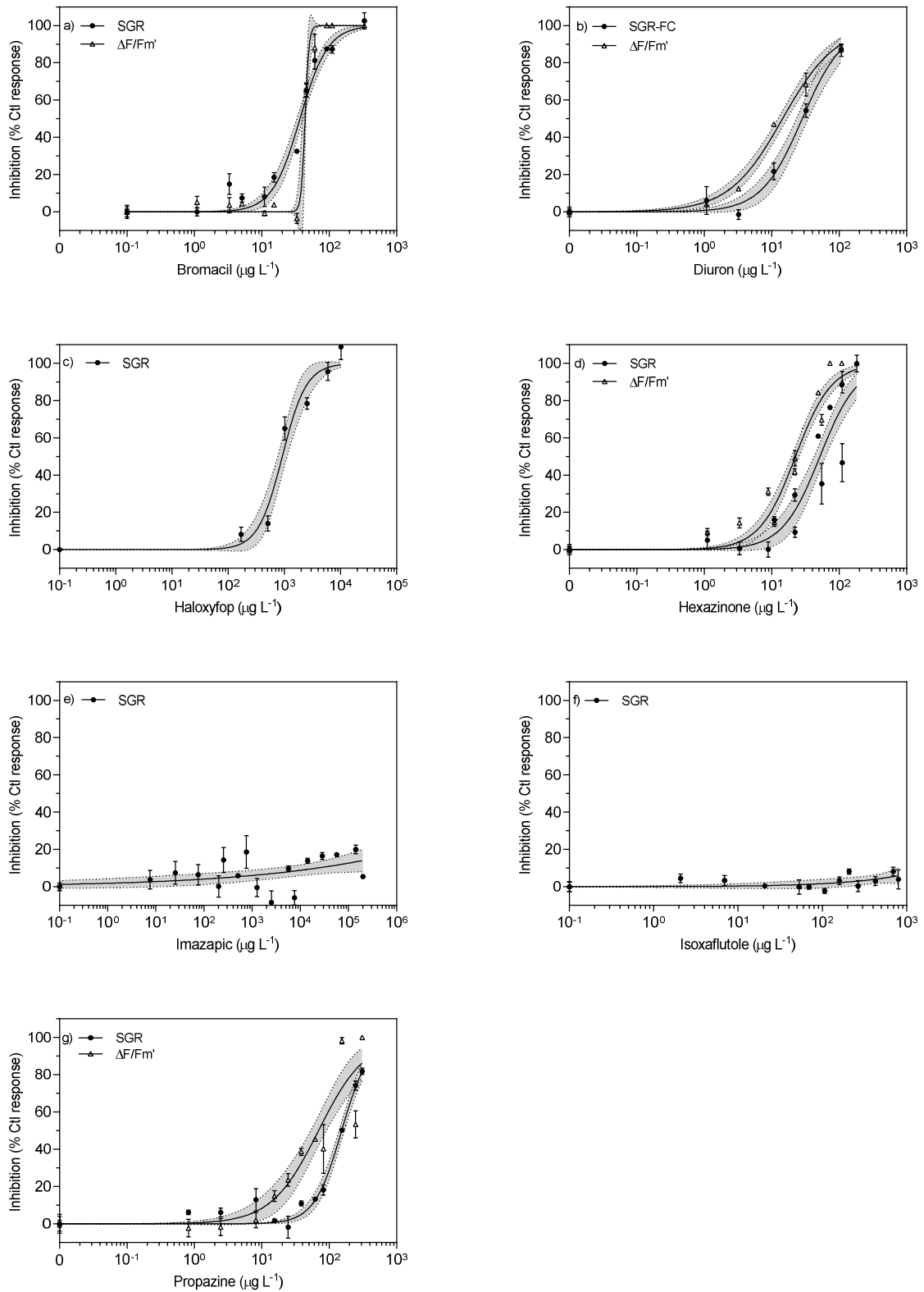
Figure 1 a-g. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 3-day specific growth rate (SGR) and photosynthetic efficiency (ΔF/Fm') for *Desmodesmus asymmetricus* (mean ± SEM) following exposure to a) bromacil, b) diuron, c) haloxyfop, d) hexazinone, e) imazapic, f) isoxaflutole and g) propazine at increasing concentrations. All concentrations are reported in µg L-1 (n = 3 for each treatment, bars not visible are smaller than symbol).

Appendix N: Freshwater: *Lemna aequinoctialis*

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Contributing authors: Templeman, M.A., McKenzie, M.R., Williams, C.D., Smythe, V.

The herbicides and their mode of action that were used in toxicity tests for this species were:
- Bromacil - PSII inhibitor
- Diuron - PSII inhibitor
- Fluroxypyr – auxin mimic
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor
- Hexazinone - PSII inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Isoxaflutole - 4-hydroxyphenyl-pyruvate-dioxygenase inhibitor
- Prometryn - PSII inhibitor
- Propazine - PSII inhibitor
- Triclopyr – auxin mimic

Test species: *Lemna aequinoctialis* (freshwater)

Test phylum: Tracheophyta – Liliopsida (moncotyledon) (Beentje & Lansdown, 2018)

Biological effect: Inhibition of specific growth rate – frond count, specific growth rate – surface area and effective quantum yield

Summary

The effect of ten herbicides (bromacil, diuron, fluroxypyr, haloxyfop, hexazinone, imazapic, isoxaflutole, prometryn, propazine and triclopyr) were assessed on growth of the freshwater macrophyte *Lemna aequinoctialis* over 96 hour exposures. The concentrations that inhibited 10% and 50% of specific growth rate (SGR) as frond number (SGR-FC) or surface area (SGR-SA) and effective quantum yield ($\Delta F/Fm'$) of *L. aequinoctialis* relative to control response (EC_{10} and EC_{50}, respectively) were calculated from 4-parameter sigmoidal model concentration-response curves. The toxicity thresholds for SGR-FC (EC_{10}; EC_{50} in µg L^{-1}) were as follows: bromacil (17.3; 63.9), diuron (6.00; 23.7), fluroxypyr (5,380; 19,500), haloxyfop (282; 2,380), hexazinone (33.9; 110), imazapic (60.7; 254), isoxaflutole (0.721; 4.87), prometryn (10.7; 38.8), propazine (32.5; 171) and triclopyr (8,540; 33,900). The toxicity thresholds for SGR-SA (EC_{10}; EC_{50} in µg L^{-1}) were as follows: bromacil (14.2; 51.8), diuron (3.73; 24.1), fluroxypyr (4,730; 18,100), haloxyfop (223; 1,450), imazapic (29.2; 298), isoxaflutole (0.766; 2.57), prometryn (7.75; 30.9), propazine (27.0; 171) and triclopyr (12,200; 31,400). No effects on SGR-SA could be determined for hexazinone. The inhibition of $\Delta F/Fm'$ (EC_{10}; EC_{50} in µg L^{-1}) were as follows: bromacil (4.34; 19.4), diuron (1.24; 7.03), hexazinone (4.27; 31.0), isoxaflutole (10.6; 129), prometryn (2.01; 12.1) and propazine (11.0; 77.1). No effects on $\Delta F/Fm'$ were observed for imazapic at the highest concentrations tested. Fluroxypyr, haloxyfop and triclopyr were not assessed for $\Delta F/Fm'$.

Methods

The inhibition of the frond number specific growth rate (SGR-FC) and surface area specific growth rate (SGR-SA) in *Lemna aequinoctialis* by bromacil, diuron, fluroxypyr, haloxyfop, hexazinone, imazapic, isoxaflutole, prometryn, propazine and triclopyr were tested in static 96 hr exposure periods. The inhibition of effective quantum yield ($\Delta F/Fm'$) was also assessed in static 96 hr exposure periods. Details of the experimental methods are provided in Tables A1 to A3. Original data including SGR and physico-

chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/88457f1b-21b1-451a-8ac8-70db31fa53e8.

Table A1. Source of *Lemna aequinoctialis*, its culturing conditions and test conditions.

Source of tests species	James Cook University in-house culture, parental stock supplied by Supervising Scientist, Dept of Environment and Energy, Darwin, NT.		
Maintenance conditions of test species (culture conditions, light, temp, etc)	Cultures were maintained in 100 mL of 0.5 CAAC medium (Pease et al., 2016) in 250 mL Erlenmeyer flasks at 26 ± 2 °C, under a 12:12 hr light:dark cycle (41 ± 5 µmol photons m^{-2} s^{-1}).		
Test endpoints	Inhibition of frond number specific growth rate (SGR - FC)	Inhibition of surface area specific growth rate (SGR-SA)	Inhibition of effective quantum yield ($\Delta F/Fm'$, proportional to photosynthetic efficiency)
Test duration	96 hr		
Test chambers	250 mL glass or plastic jars		
Test volume	100 mL		
Starting density	12 – 14 fronds per replicate		
Calculation of SGR	• Frond number and surface area automatically assessed from photographs using ImageJ (Rueden & Eliceiri, 2019) or manually counted and SGR calculated as per OECD test 221 (OECD, 2011). • Effective quantum yield was assessed via pulse amplitude modulation fluorometer (mini-PAM; WALZ, Germany).		

Table A2. Measured physico-chemical parameters of test media for *Lemna aequinoctialis*.

Light intensity (mean ± SD, n = 14 measurements across chamber)	110 ± 13 µmol photons $m^{-2}s^{-1}$ over a 12:12 hr L:D cycle
Synthetic soft water test media (SSW) pH (mean ± SD, averaged 0 and 96 hr, n = 56) 0.25CAAC test media pH (mean ± SD, averaged 0 and 96 hr, n = 242)	6.52 ± 0.1 6.43 ± 0.3
SSW Electrical Conductivity (mean ± SD, averaged 0 and 96 hr, n = 56) 0.25CAAC Electrical Conductivity (mean ± SD, averaged 0 and 96 hr, n = 244)	18.8 ± 2.5 µS cm^{-1} 780 ± 21 µS cm^{-1}
SSW Media Temperature (mean ± SD, averaged 0 and 96 hr, n = 864) 0.25CAAC Media Temperature (mean ± SD, averaged 0 and 96 hr, n = 236)	26.7 ± 0.8 °C 25.7 ± 0.7 °C

Table A3. Test criteria for specific growth rate (frond number and surface area) and effective quantum yield for *Lemna aequinoctialis*.

Exposure duration	96 hr		
Biological effect metric	Inhibition of the mean specific growth rate (SGR-FC) - the logarithmic increase of frond number over 96 hr (OECD, 2011).	Inhibition of the mean specific growth rate (SGR-SA) - the logarithmic increase in surface area over 96 hr (OECD, 2011).	Inhibition of the effective quantum yield ($\Delta F/Fm'$) which is proportional to photosynthetic efficiency for a given light intensity
Biological endpoint definition	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce SGR-FC, SGR-SA or $\Delta F/Fm'$ by 10% and 50%, respectively, in comparison to control and / or solvent control treatments.		
Controls used	Imazapic and fluroxypyr were dissolved in the carrier solvent methanol (final concentration ≤ 0.01% (v/v)). All other herbicides except bromacil, propazine and triclopyr were dissolved in the carrier solvent acetone (final concentration ≤ 0.01% (v/v)). No carrier solvent was used for bromacil, propazine or triclopyr. A separate control treatment with no solvent was included for each experiment.		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Bromacil	3	21	3
Diuron	2	14	3
Fluroxypyr	1	8	3
Haloxyfop	3	21	3
Hexazinone	2	14	3
Imazapic	2	14	3
Isoxaflutole	2	13	3
Prometryn	3	22	3
Propazine	2	13	3
Triclopyr	2	12	3
Test acceptability criteria	• Control SGR - FC ≥ 0.325 day^{-1} as per (OECD, 2011). Observed mean control SGR: 0.384 ± 0.05 day^{-1} (mean ± SD, n = 93) • The coefficient of variation (CV) of mean SGR in controls ≤ 10% of each experiment as per (OECD, 2011). Observed control CV: < 10%	• Control SGR - SA ≥ 0.305 day^{-1} as per (OECD, 2011). Observed mean control SGR: 0.397 ± 0.05 day^{-1} (mean ± SD, n = 80) • The coefficient of variation (CV) of mean SGR in controls ≤ 10% of each experiment as per (OECD, 2011). Observed control CV: < 10%	
Characteristics of the test organism	Actively growing culture free of overt disease and deformity. Starting density four triplicate frond colonies.		
Type of test media	CAAC culture media (no sucrose) - 0.25x strength for all tests **except** hexazinone and imazapic (Riethmuller et al., 2003). Hexazinone and imazapic – synthetic soft water (Pease et al., 2016).		
Toxicant (common name; IUPAC Name; CAS no.; purity; batch)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Bromacil: (*RS*)-5-bromo-3-sec-butyl-6-methyluracil; 314-40-9; ≥ 98%. Batch:SZBF139XV • Diuron (DCMU): 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98%; Batch: BCBS1743 • Fluroxypyr: 4-amino-3,5-dichloro-6-fluoro-2-pyridyloxyacetic acid; 69377-81-7; ≥ 98%. Batch: SZBF100XV		

	• Haloxyfop-p-methyl: methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98%; Batch: BCBT1738 • Hexazinone: 3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione; 51235-04-2; ≥ 98%. Batch: BCBT6090 • Imazapic: 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%. Batch: BCBZ6821 • Isoxaflutole: (5-cyclopropyl-1,2-oxazol-4-yl)(α,α,α-trifluoro-2-mesyl-p-tolyl)methanone; 141112-29-0; ≥ 98%. Batch: BCBT2782 • Prometryn: *N*2,*N*4-diisopropyl-6-methylthio-1,3,5-triazine-2,4-diamine; 7287-19-6; ≥ 98%. Batch: BCBV7467 • Propazine: 6-chloro-*N*2,*N*4-diisopropyl-1,3,5-triazine-2,4-diamine; 139-40-2; ≥ 98%. Batch: BCBX0853 • Triclopyr: [(3,5,6-trichlor-2-pyridinyl)oxy]acetic acid; 5535-06-3; ≥ 98%; Batch: BCBW3270
Preparation of toxicant stock	Diuron, haloxyfop, hexazinone, isoxaflutole and prometryn (10 – 20,000 mg L^{-1}) were dissolved using the carrier solvent acetone (final concentration < 0.01% v/v in all exposure treatments). Imazapic (100 mg L^{-1}) was dissolved in the carrier solvent methanol (final concentration < 0.01% v/v in all exposure treatments). Bromacil, propazine and triclopyr (5 - 200 mg L^{-1}) were dissolved directly into Milli-Q water® with no solvent carrier. Fluroxypyr was dissolved in carrier solvent methanol to final concentration < 0.01% v/v in all exposure treatments. Fluroxypyr and triclopyr were weighed and added directly to test media.
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Diuron experiments conducted as a reference tests for this species
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR relative to controls (EC$_{10}$ and EC$_{50}$, respectively) were calculated from concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Table A4)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity	Controls were tested for contamination. Analytical grade herbicides (≥ 98% purity) was used for preparation of stock solution. Analytical grade chemicals were used for preparation of test and culture media.

chemicals used for the experiment	
Randomisation	Daily randomisation

Summary of results

The toxicity of ten herbicides to *Lemna aequinoctialis* is presented in Table A4 (SGR-FC), Table A5 (SGR-SA), Table A6 (ΔF/Fm') and Figure A1. Toxicity was assessed relative to control and/or solvent control responses. SGR-SA was not able to be determined for hexazinone. Imazapic had no effect on ΔF/Fm' at the maximum concentration of 915 µg L^{-1}. Fluroxypyr, haloxyfop and triclopyr were not assessed for ΔF/Fm'.

Table A4. Modelled toxicity estimates for the inhibition of ten herbicides on the frond number specific growth rate (SGR - FC) of *Lemna aequinoctialis* (Figure A1). All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Bromacil	17.3 (14.0 – 21.0)	63.9 (58.6 – 69.7)
Diuron	6.00 (4.83 – 7.36)	23.7 (21.4 – 26.1)
Fluroxypyr	5,380 (4,020 – 7,020)	19,500 (17,500 – 21,700)
Haloxyfop	282 (179 – 440)	2,380 (1,950 – 3,020)
Hexazinone	33.9 (27.1 – 41.4)	110 (101 – 120)
Imazapic	60.7 (39.7 – 86.1)	254 (220 – 292)
Isoxaflutole	0.721 (0.241 – 1.55)	4.87 (3.21 – 7.64)
Prometryn	10.7 (8.86 – 12.7)	38.8 (35.5 – 42.4)
Propazine	32.5 (25.9 – 39.9)	171 (158 - 186)
Triclopyr	8,540 (5,940 – 11,300)	33,900 (29,500 – 40,800)

Table A5. Modelled toxicity estimates for the inhibition of ten herbicides on the surface area specific growth rate (SGR - SA) of *Lemna aequinoctialis* (Figure A1). N.D. – Not able to be determined. All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Bromacil	14.2 (11.5 – 17.3)	51.8 (47.1 – 57.0)
Diuron	3.73 (2.94 – 4.65)	24.1 (21.8 – 26.8)
Fluroxypyr	4,730 (4,080 – 5,440)	18,100 (16,900 – 19,300)
Haloxyfop	223 (158 – 311)	1,450 (1,200 – 1,770)
Hexazinone	N.D.	N.D.
Imazapic	29.2 (11.0 – 65)	298 (206 – 581)
Isoxaflutole	0.766 (0.443 – 1.13)	2.57 (2.07 – 3.26)
Prometryn	7.75 (6.00 – 9.85)	30.9 (27.5 – 34.7)
Propazine	27.0 (23.2 – 31.2)	171 (161 – 182)
Triclopyr	12,200 (10,100 – 14,600)	31,400 (28,700 – 34,600)

Table A6. Modelled toxicity estimates for inhibition of ten herbicides on the photosynthetic efficiency (ΔF/Fm′) of *Lemna aequinoctialis* (Figure A1). N/A = Not Assessed. All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Bromacil	4.34 (3.68 – 5.07)	19.4 (18.2 – 20.6)
Diuron	1.24 (0.995 – 1.40)	7.03 (6.53 – 7.58)
Fluroxypyr	N/A	N/A
Haloxyfop	N/A	N/A
Hexazinone	4.27 (3.27 – 5.50)	31.0 (27.8 – 34.4)
Imazapic	> 915	> 915
Isoxaflutole	10.6 (5.44 – 20.7)	129 (93.3 – 204)
Prometryn	2.01 (1.79 – 2.44)	12.1 (11.3 – 13.0)
Propazine	11.0 (8.04 – 14.4)	77.1 (68.7 – 86.6)
Triclopyr	N/A	N/A
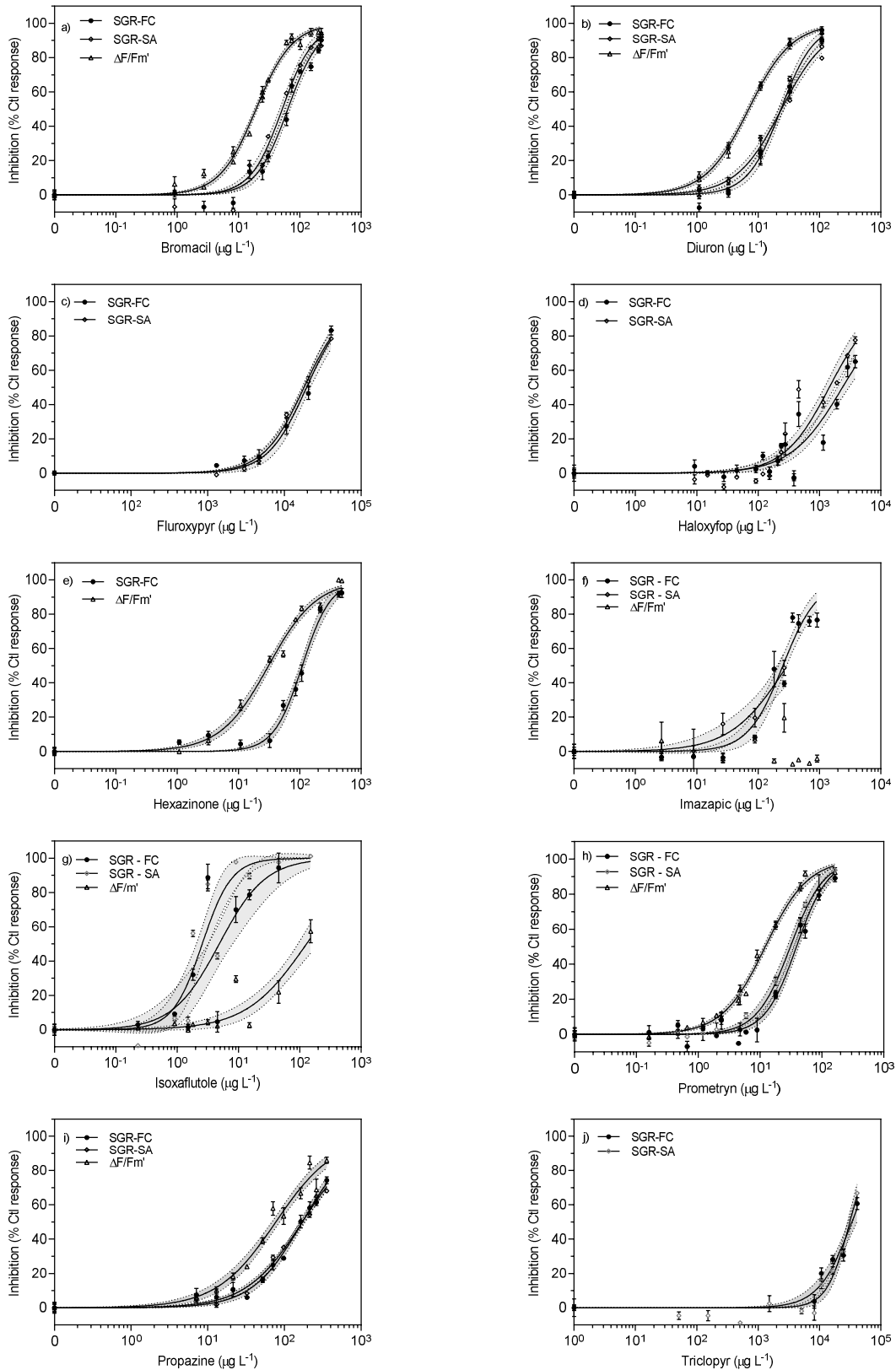
Figure 1a-j. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 4-day specific growth rate frond number (SGR-FC), specific growth rate surface area (SGR-SA) and effective quantum yield (ΔF/Fm') of *Lemna aequinoctialis* (mean ± SEM) following herbicide exposure to a) bromacil; b) diuron; c) fluroxypyr; d) haloxyfop; e) hexazinone; f) imazapic; g) isoxaflutole; h) prometryn; i) propazine and j) triclopyr at increasing concentrations. All concentrations are reported in μg L-1 (n = 3 for each treatment, bars not visible are smaller than symbol).

Appendix O: Freshwater: *Microcystis aeruginosa*

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Contributing authors: Templeman M.A.

The herbicide and its mode of action that was used in the toxicity test for this species was:

- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor

Test species: *Microcystis aeruginosa* (freshwater)

Test phylum: Cyanophyta

Biological effect: Inhibition of specific growth rate

Summary of test results

The effect of the herbicide Imazapic was assessed on growth of the freshwater cyanobacterium *Microcystis aeruginosa* over a 72 hour exposure period. The concentrations that inhibited 10% and 50% of specific growth rate (SGR) of *M. aeruginosa* relative to control response (EC_{10} and EC_{50}, respectively) were calculated from a 4-parameter sigmoidal model concentration-response curve. The SGR EC_{10} and EC_{50} were 9,370 µg L^{-1} and 102,000 µg L^{-1} imazapic, respectively.

Methods

The inhibition of the specific growth rate in *Microcystis aeruginosa* by imazapic was tested in a static 72 hr exposure period (chronic). Details of the experimental methods are provided in Tables A1 to A3. Original data including SGR and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/a39156dc-2037-46f1-8072-b38cd809c546.

Table A1. Source of *Microcystis aeruginosa*, its culturing and test conditions.

Source of tests species	James Cook University in-house culture (strain CS338/01), purchased from Australian National Algae Supply Service, Hobart.
Maintenance conditions of test species (culture conditions, light, temp, etc)	Cultures were maintained in 100 mL of MLA medium in 250 mL Erlenmeyer flasks on an orbital shaker at 26 ± 2 °C, under a 12:12 hr light:dark cycle (91 ± 12 µmol photons m^{-2} s^{-1}).
Test endpoint	Inhibition of specific growth rate (SGR) of culture in log growth phase
Test duration	72 hr
Test chambers	100 mL glass conical flasks
Test volume	50 mL
Starting density	3.1x10^4 cells mL^{-1}
Counting of cells, calculation of SGR	Replicate treatments sonicated for 60 s to disperse clumps as per Voltolina (1991) and Wang (2015). Cells manually counted using haemocytometer and SGR calculated as per OECD test 201 (OECD, 2011).

Table A2. Measured physico-chemical parameters of test media for *Microcystis aeruginosa*.

Light intensity (mean ± SD, n = 7 measurements across chamber)	59 ± 9.7 µmol photons $m^{-2}s^{-1}$ over a 12:12 hr L:D cycle
Chamber temperature (mean ± SD, logged at 15 min intervals)	26.6 ± 0.5 °C
pH (mean ± SD, averaged 0 and 72 hr, n = 16)	6.9 ± 0.4
Electrical conductivity (mean ± SD, averaged 0 and 72 hr, n = 16)	312 ± 13.8 µS cm^{-1}
Test media temperature (mean ± SD, averaged 0 and 72 hr, n = 16)	26.0 ± 0.2 °C

Table A3. Test criteria for specific growth rate for *Microcystis aeruginosa*.

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Exposure duration	72 hr
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase of biomass over 72 hr (OECD, 2011).
Biological endpoint definition	Effect concentrations, EC_{10} and EC_{50}, are the concentrations that reduce SGR by 10% and 50%, respectively, in comparison to control treatments.
Controls used	Imazapic was dissolved in the carrier solvent methanol (final concentration 0.01% v/v in exposures). A separate control treatment with no solvent was included for the experiment.
Replication	One definitive test contributed to the concentration-response curve. There were 8 treatment concentrations which each had 3 replicates.
Test acceptability criteria	• Control SGR ≥ 0.92 day^{-1} as per (OECD, 2011). Observed mean solvent control SGR: 1.04 ± 0.04 day^{-1} (mean ± SD, n = 3) • The coefficient of variation (CV) of mean SGR in solvent control ≤ 10% as per (OECD, 2011). Observed control CV: < 5%
Characteristics of the test organism	4-7 day old culture in exponential growth phase, starting density 3.1×10^4 cells mL^{-1}
Type of test media	MLA culture media (0.5x strength)
Toxicant (common name; IUPAC Name; CAS no.; purity)	Imazapic was analytical grade and purchased from Sigma-Aldrich. • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%. Batch No: BCBZ6821
Preparation of toxicant stock	A stock solution (100 mg L^{-1}) of imazapic was prepared in Milli-Q® water for the lower concentrations (1 – 9 mg L^{-1} nominal concentration) using < 0.01% methanol as a carrier solvent. Imazapic was weighed directly into treatment solutions for nominal concentrations 20-60 mg L^{-1} using methanol as a carrier solvent (< 0.01% v/v final concentration in all treatments).
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Nil
Concentration-response relationship	• EC_x: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.

Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR relative to controls (EC_{10} and EC_{50}, respectively) were calculated from concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Table A4)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade imazapic (≥ 98% purity) was used for preparation of stock solution.
Randomisation	Daily randomisation and flasks shaken by hand 3 x daily

Summary of results

The toxicity of the herbicide imazapic to *M. aeruginosa* is presented in Table A4 and Figure A1. Toxicity was assessed relative to solvent control response. The presence of methanol as a carrier solvent stimulated higher growth rates in the solvent control relative to the media control only (see e-Atlas data).

Table A4. Modelled toxicity estimates for the inhibition of imazapic on the specific growth rate (SGR) of *Microcystis aeruginosa* (Figure A1). All concentrations in µg L-1 (95% confidence intervals).

	EC_{10} (95% CI)	EC_{50} (95% CI)
Imazapic	9,370 (5,090-15,600)	102,000 (84,500-127,000)
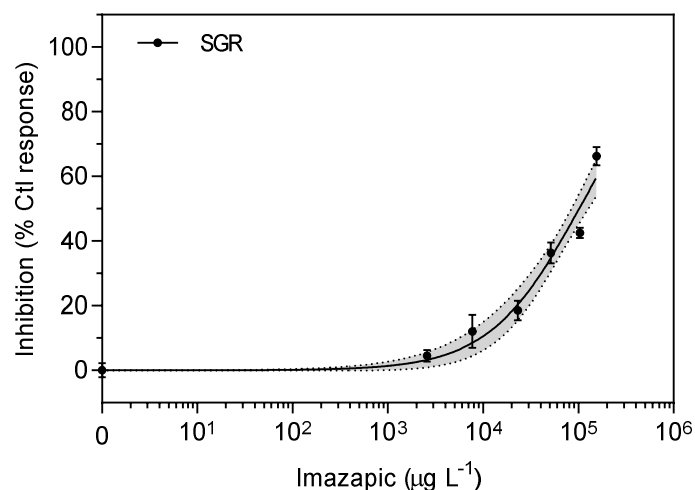
Figure A1. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 3-day specific growth rate (SGR) *Microcystis aeruginosa* (mean ± SEM) following exposure to imazapic at increasing concentrations. All concentrations are reported in µg L-1 (n = 3 for each treatment, bars not visible are smaller than symbol).

Appendix P: Freshwater: *Raphidocelis subcapitata*

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Contributing authors: Mulama, V., McKenzie, M.R., Templeman, M.A., Williams, C.D.

The herbicides and their mode of action that were used in toxicity tests for this species were:
- Diuron - PSII inhibitor
- Imazapic - acetohydroxyacid synthase (AHAS) inhibitor
- Haloxyfop - acetyl-CoA carboxylase (ACCase) inhibitor

Test species: *Raphidocelis subcapitata* (freshwater)

Test phylum: Chlorophyta

Biological effect: Inhibition of specific growth rate and effective quantum yield

Summary

The effect of three herbicides (diuron, imazapic and haloxyfop) were assessed on growth of the freshwater chlorophyta *Raphidocelis subcapitata* over a 72 hour exposure period. The concentrations that inhibited 10% and 50% specific growth rate (SGR) and effective quantum yield ($\Delta F/Fm'$) of *R. subcapitata* relative to control response (EC_{10} and EC_{50}, respectively) were calculated from 4-parameter sigmoidal model concentration-response curves. The toxicity thresholds (EC_{10}; EC_{50} in $\mu g\ L^{-1}$) were diuron (5.32; 20.6), imazapic (27,500; 432,000). No effects on SGR were observed for haloxyfop at the highest concentration tested (10,200 $\mu g\ L^{-1}$). The inhibition of photosynthetic efficiency ($\Delta F/Fm'$) for diuron (EC_{10}; EC_{50} in $\mu g\ L^{-1}$) was 2.66 and 9.21, respectively. Imazapic and haloxyfop were not assessed for $\Delta F/Fm'$.

Methods

The inhibition of the specific growth rate in *Raphidocelis subcapitata* by diuron, haloxyfop and imazapic was tested in static 72 hr exposure periods (chronic). The inhibition of effective quantum yield ($\Delta F/Fm'$) by diuron was also tested in static 72 hr exposure periods (chronic). Details of the experimental methods used in the *Raphidocelis subcapitata* toxicity tests are provided in Tables A1 to A3. Original data including SGR and physico-chemical data can be found in e-Atlas Link: https://eatlas.org.au/data/uuid/c27340dc-c06d-405a-818b-39d7a9e4e596.

Table A1. Source of *Raphidocelis subcapitata*, its culturing and test conditions.

Source of tests species	James Cook University in-house culture (strain CS-327), purchased from Australian National Algae Supply Service, Hobart.	
Maintenance conditions of test species (culture conditions, light, temp, etc)	Cultures were maintained in 100 mL of MLA medium in 250 mL Erlenmeyer flasks on an orbital shaker at 26 ± 2 °C, under a 12:12 hr light:dark cycle (91 ± 12 μmol photons m^{-2} s^{-1}).	
Test endpoint	Inhibition of specific growth rate (SGR) of culture in log growth phase	Inhibition of effective quantum yield (ΔF/Fm′, proportional to photosynthetic efficiency)
Test duration	72 hr	
Test chambers	100 mL glass conical flasks	
Test volume	50 mL	
Starting density	3.0 - 3.1x10^4 cells mL^{-1}	
Counting of cells, calculation of SGR; chlorophyll *a* fluorescence determination	Cells automatically counted from photographs using ImageJ (Rueden & Eliceiri, 2019) and / or manually counted using haemocytometer and SGR calculated as per OECD test 201 (OECD, 2011).	Pulse amplitude modulated fluorometer (mini-PAM).

Table A2. Measured physico-chemical parameters of test media for *Raphidocelis subcapitata*.

Light intensity (mean ± SD, n = 7 measurements across chamber)	190 ± 14 μmol photons m^{-2}s^{-1} over a 12:12 hr L:D cycle
Chamber temperature (mean ± SD, logged at 15 min intervals)	26.6 ± 0.5 °C
pH (mean ± SD, averaged 0 and 72 hr, n = 46)	7.53 ± 0.3
Electrical conductivity (mean ± SD, averaged 0 and 72 hr, n = 46)	378 ± 30 μS cm^{-1}
Test media temperature (mean ± SD, averaged 0 and 72 hr, n = 46)	25.3 ± 0.6 °C

Table A3. Test criteria for specific growth rate and effective quantum yield of *Raphidocelis subcapitata*.

Exposure duration	72 hr		
Biological effect metric	Inhibition of the mean specific growth rate - the logarithmic increase of biomass over 72 hr (OECD, 2011).	Inhibition of the effective quantum yield (ΔF/Fm') which is proportional to photosynthetic efficiency for a given light intensity	
Biological endpoint definition	Effect concentrations, EC$_{10}$ and EC$_{50}$, are the concentrations that reduce SGR or ΔF/Fm' by 10% and 50%, respectively, in comparison to control treatments.		
Controls used	Imazapic was dissolved in the carrier solvent methanol, haloxyfop and diuron were dissolved in the carrier solvent acetone (final concentration 0.01 % v/v). A separate control treatment with no solvent was included for each experiment.		
Test, treatment and replicate numbers	Tests in final concentration-response curve	Concentrations in final concentration-response curve	Replicates per concentration
Diuron	1	7	3
Haloxyfop	1	8	3
Imazapic	1	8	3
Test acceptability criteria	• Control SGR ≥ 0.92 day^{-1} as per (OECD, 2011). Observed mean solvent control SGR: 1.18 ± 0.09 day^{-1} (mean ± SD, n = 18) • The coefficient of variation (CV) of mean SGR in controls ≤ 7% as per (OECD, 2011). Observed control CV in any one test: <5%		

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Characteristics of the test organism	4-7 day old culture in exponential growth phase, starting density 3.0 - 3.1x10^4 cells mL^{-1}
Type of test media	MLA culture media (0.5x strength)
Toxicant (common name; IUPAC Name; CAS no.; purity)	All chemicals were analytical grade and purchased from Sigma-Aldrich. • Diuron (DCMU); 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 330-54-1; > 98%; Batch: BCBS1743 • Haloxyfop-p-methyl; methyl (2R)-2-[4-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate; 72619-32-0; ≥ 98%; Batch: BCBT1738 • Imazapic; 5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; 104098-48-8; ≥ 98%. Batch: BCBZ6821
Preparation of toxicant stock	20 – 1,000 mg L^{-1} diuron and haloxyfop were dissolved using the carrier solvent acetone (final concentration < 0.01 % v/v in all exposure treatments). 1,000 mg L^{-1} imazapic was dissolved in the carrier solvent methanol (final concentration < 0.01 % v/v in all exposure treatments).
Exposure type	Static
Measured contaminant concentrations	Herbicide concentrations (2-3 per pesticide) were measured at initiation and termination of test. The measured concentrations for all treatments were calculated based on the linear relationship between nominal and the time weighted average of measured concentrations. All herbicide analyses were performed at the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland using HPLC-MS/MS (SCIEX Triple QuadTM 6500 QTRAP® mass spectrometer Shimadzu Nexera X2 uHPLC system) (Mercurio et al., 2015; Mercurio, 2016).
Reference toxicant	Diuron experiment conducted as a reference test for this species
Concentration-response relationship	• EC$_x$: 4-parameter sigmoidal model, fitted to the percent inhibition and measured herbicide concentrations using the program GraphPad Prism (v 8.1.0, San Diego, CA, USA). see Figure A1.
Statistical method or model used to determine effect of toxicant on test species	• Regression analysis following prescribed procedures (OECD, 2006a). The concentrations of herbicide that inhibited 10% and 50% of the SGR or ΔF/Fm' relative to controls (EC$_{10}$ and EC$_{50,}$ respectively) were calculated from concentration-response curves (4-parameter sigmoidal model) fitted to the percent inhibition and measured herbicide concentrations for each treatment using the program GraphPad Prism (V 8.1.0, San Diego, CA, USA).
Data variance	All results reported with 95% Confidence Limits (CL) (see Tables A4 and A5)
Test solutions, blanks and/or controls tested for contamination *or* analytical reagent grade chemicals or the highest possible purity chemicals used for the experiment	Controls were tested for contamination. Analytical grade herbicides (≥ 98% purity) was used for preparation of stock solution. Analytical grade chemicals used for preparation of test and culture media.
Randomisation	Daily randomisation and flasks shaken by hand 3 x daily

Summary of results

The toxicity of three herbicides to *R. subcapitata* is presented in Table A4, Table A5 and Figure 1. Toxicity was assessed relative to combined control /solvent control responses. The non-PSII herbicide haloxyfop did not inhibit SGR in *Raphidocelis subcapitata* at the highest concentration (10,200 µg L^{-1}) tested. Haloxyfop and imazapic were not assessed for photosynthetic efficiency (ΔF/Fm′).

Table A4. Modelled toxicity estimates for the inhibition of diuron, haloxyfop and imazapic on the specific growth rate (SGR) of *Raphidocelis subcapitata* (Figure A1). All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	5.32 (4.31 – 6.47)	20.6 (18.5 – 22.8)
Haloxyfop	>10,200	>10,200
Imazapic	27,500 (16,800 – 41,700)	432,000 (282,000 – 855,000)

Table A5. Modelled toxicity estimates for inhibition of diuron on the photosynthetic efficiency (ΔF/Fm′) of *Raphidocelis subcapitata* (Figure A1). N/A = Not Assessed. All concentrations in µg L-1 (95% confidence intervals).

	EC$_{10}$ (95% CI)	EC$_{50}$ (95% CI)
Diuron	2.66 (1.71 – 4.10)	9.21 (7.96 – 10.6)
Imazapic	N/A	N/A
Haloxyfop	N/A	N/A
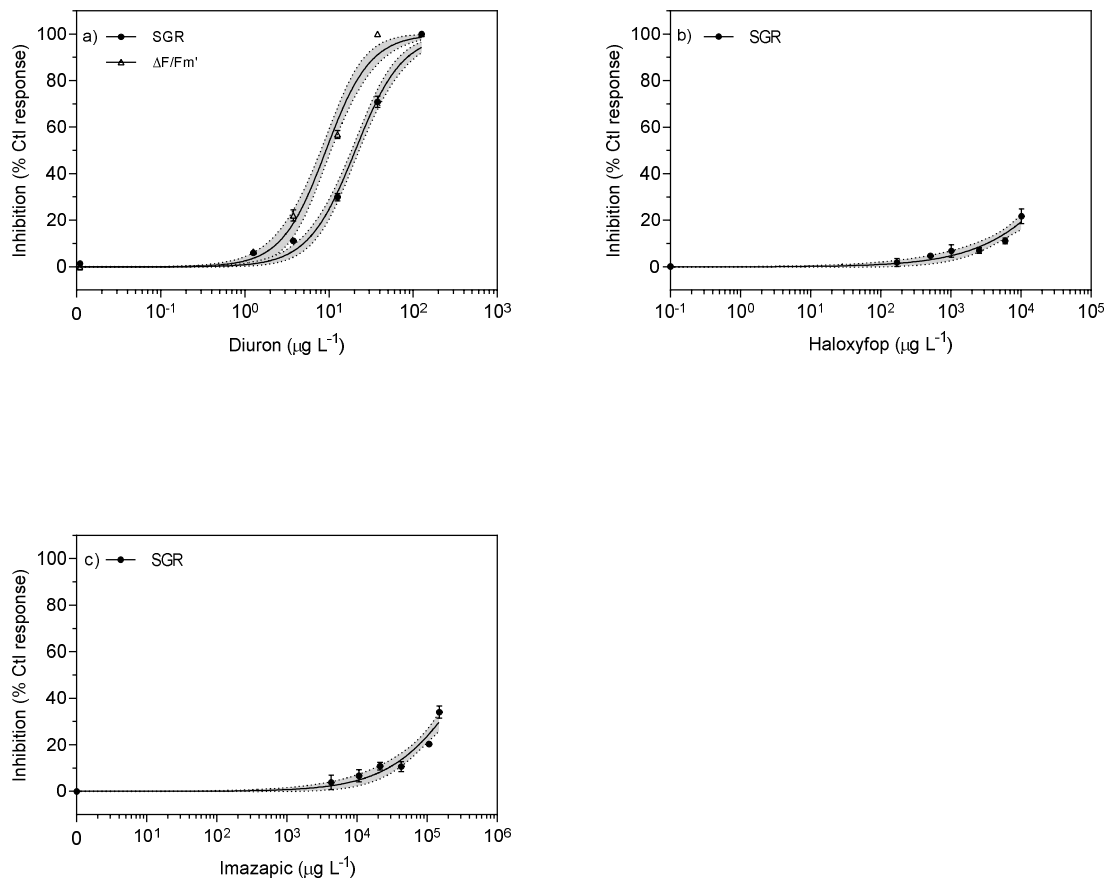
Figure A1 a-c. Sigmoidal, 4-parameter curve fit and 95% confidence intervals (shaded area) on the relative percent inhibition of 3-day specific growth rate (SGR) and photosynthetic efficiency (ΔF/Fm′) for *Raphidocelis subcapitata* (mean ± SEM) following exposure to a) diuron, b) haloxyfop and c) imazapic at increasing concentrations. All concentrations are reported in µg L-1 (n = 3 for each treatment, bars not visible are smaller than symbol).

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