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Rapid toxicity assessment of six antifouling booster biocides using a microplate-based chlorophyll fluorescence in *Undaria pinnatifida* gametophytes

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Abstract

Biocides of antifouling agents can cause problems in marine ecosystems by damaging to non-target algal species. Aquatic bioassays are important means of assessing the quality of water containing mixtures of contaminants and of providing a safety standard for water management in an ecological context. In this study, a rapid, sensitive and inexpensive test method was developed using free-living male and female gametophytes of the brown macroalga *Undaria pinnatifida*. A conventional fluorometer was employed to evaluate the acute (48 h) toxic effects of six antifouling biocides: 4,5-Dichloro-2-octyl-isothiazolone (DCOIT), diuron, irgarol, medetomidine, tolylfluanid, zinc pyrithione (ZnPT). The decreasing toxicity in male and female gametophytes as estimated by EC_{50} (effective concentration at which 50% inhibition occurs) values was: diuron (0.037 and 0.128 mg l^{-1}, respectively) > irgarol (0.096 and 0.172 mg l^{-1}, respectively) > tolylfluanid (0.238 and 1.028 mg l^{-1}, respectively) > DCOIT (1.015 and 0.890 mg l^{-1}, respectively) > medetomidine (12.032 and 12.763 mg l^{-1}, respectively). For ZnPT, 50% fluorescence inhibition of *U. pinnatifida* gametophytes occurred at concentrations above 0.4 mg l^{-1}. The *Undaria* method is rapid, simple, practical, and cost-effective for the detection of photosynthesis-inhibiting biocides, thus making a useful tool for testing the toxicity of antifouling agents in marine environments.

Keywords Antifouling biocides · Fluorescence · Bioassay · *Undaria pinnatifida* · Gametophytes

Introduction

Many chemical compounds derived from industry and other human activities have adverse effects on the marine environment. Marine shipping is one source of harmful chemicals, including petroleum hydrocarbons, metals, organic compounds and antifouling agents (Moro et al. 2018; Sun et al. 2016; Talvitie et al. 2015). Among these pollutants, antifouling agents can cause problems in marine ecosystems because they contain biocides which, when released into the surrounding water, are damaging to non-target algal species (Braithwaite and Fletcher 2005; Park et al. 2017; Wendt et al. 2013). Algicidal booster herbicides are well-known to inhibit algal photosynthesis, growth and development (Kottuparambil et al. 2013; Molino and Wetherbee 2008; Voulvoulis et al. 1999). In particular, irgarol and diuron have been used worldwide as active ingredients of antifouling treatments (Gatidou et al. 2007), and act as inhibitors of photosystem II (PSII), leading to reduced CO_2 fixation and growth (Voulvoulis et al. 1999). These pollutants also have a detrimental effect on the environment of semi-enclosed marine systems and populated coastal areas (Karlsson et al. 2010; Turner 2010; Voulvoulis et al. 1999). The biocides DCOIT and tolylfluanid exert their effects via cytotoxic modes of action that can potentially affect many different organisms. DCOIT causes an oxidative stress once it has diffused through the cell membrane (Arning et al. 2009) and is also an inhibitor of the PSII electron transport (Guardiola et al. 2012). Tolylfluanid inhibits the thiol-containing enzymes by forming disulfide bridges, thus

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affecting photosynthetic processes through inhibition of the carbon-concentrating mechanism (Johansson et al. 2012). The action mechanism of medetomidine is not clearly established for algae, but Ohlauson et al. (2012) described its effects on basic physiological functions, such as photosynthesis and protein synthesis. ZnPT acts on the iron-sulfur proteins in algae (Feng et al. 2017).

To avoid degradation and maintain the integrity of marine systems, it is necessary to establish environmental protection strategies which include effective monitoring and regulation. Aquatic bioassay is an important means of assessing the quality of water containing mixtures of contaminants (some of which may be unknown) and of providing a safety standard for water management in an ecological context (Seery et al. 2006). The choice of a suitable model organism for toxicity testing is dependent on sensitivity to specific pollutants (Thursby et al. 1985). Model species used for marine ecotoxicity testing include seven primary producers (i.e. macroalgae *Ceramium tenuicorne* (Kützing) Waern, *Champia parvula* (C. Agardh) Harvey, *Fucus edentatus* Bachelot de la Pylaie, *Laminaria saccharina* (Linnaeus) Lamouroux, and *Macrocystis pyrifera* (Linnaeus) Agardh; microalgae *Phaeodactylum tricornutum* Bohlin, and *Skeletonema costatum* (Greville) Cleve, forty-two consumers (i.e. amphipods *Monocorophium acherusicum* Costa, and *Mandibulophoxus mai* Jo; copepod *Amphiascus tenuiremis* Brady and Robertson; mysid *Mysidopsis bahia* Molenock; bivalve *Mytilus edulis* Linnaeus and so on and one decomposer (i.e. bacterium *Vibrio fischeri* Beijerinck) (ASTM 2020; EPA 2003; ISO 2020; OECD 1994; USEPA 2020).

Marine macroalgae are major primary producers and play an important ecological role in marine food webs. They support diverse communities by providing food for herbivores, physical structures for shelter and protection from predators, and they also act as nurseries for many marine animals (Bos et al. 2007; Jones et al. 1994, 1997). Seaweeds have long been a valuable ecological and economic resource, especially in Asia, but there has been little interest in their potential as test species for assessing the effect of aquatic pollutants. To date, few standardized ecotoxicity tests have been reported using marine macroalgae except for *Ceramium* (rhodophyte) growth test, *Macrocystis* (phaeophyte) and *Hormosira* (phaeophyte) germination test (Eklund 1998; Gully et al. 1999; Gunthorpe et al. 1995). It is notable that one method based on reproductive inhibition in *Ulva pertusa* Kjellman has recently been proposed as an international standard (Han and Choi 2005). More research is needed to develop other seaweed-based toxicity tests using a wider range of native species (Park et al. 2016). Establishing a standardized testing method is also important as it can be an objective indicator, in that testing can be administered and conducted in the same way regardless of the location, time, or nationality. Standardized testing methods ensure objectivity, effectiveness, and consistency in environmental management.

The brown alga *Undaria pinnatifida* (Harvey) Suringar, a species of kelp, grows throughout the year and is distributed widely in Asia, the Mediterranean, the Atlantic coast of Europe, Australia, Argentina and Mexico (Campbell and Burridge 1998; Curiel et al. 1998; Fletcher and Manfredi 1995; Hay and Luckens 1987; Martin and Cuevas 2006; Salinas et al. 1996; Silva et al. 2002) The life cycle of *U. pinnatifida* is typical for brown kelp species. The heteromorphic life cycle consists of an alternation of a macroscopic sporophyte with a microscopic filamentous gametophyte. Gametophytes can delay their development, thus forming a 'gametophyte bank' analogous to the 'seed bank' in terrestrial plants (Carney and Edwards 2006).

Bioassays have been developed using giant brown seaweeds such as *Macrocystis pyrifera* (L.) Agardh, *Hormosira banksii* (Turner) Decaisne, *Ecklonia radiata* Agardh and Agardh, and *U. pinnatifida* (Anderson and Hunt 1988; Bidwell et al. 1998; Gully et al. 1999; Gunthorpe et al. 1995; Kevekordes 2001; Park et al. 2016). These methods employ endpoints such as germination and germ tube formation, which occur in the early stages of the life cycle. However, use of these endpoints is constrained by the availability of spores since the adult plants reproduce for only a limited period each year (Lee et al. 2019b; Park et al. 2016). Thus, the tests can be conducted only during the period when mature and fertile adult plants are available. This limitation must be overcome if these early developmental stages are to be used for routine toxicity testing. The use of free-living gametophytes would allow for the possibility of conducting year-round testing.

The overall aim of this study was to establish a rapid, sensitive and inexpensive test method with free-living gametophytes of the brown macroalga *U. pinnatifida*, using a conventional fluorometer which allows easy, rapid and sensitive assessment of the potential damage to photosystem efficiency (Eullaffroy and Vernet 2003). The following six common ingredients used in antifouling paints were tested: diuron, 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT), irgarol, medetomidine, tolylfluanid and zinc pyrithione (ZnPT).

Materials and methods

Gametophyte preparation and culture

U. pinnatifida has an annual heteromorphic diplohaplontic life cycle that comprises separate macroscopic and microscopic phases. A macroscopic plant (diploid, 2n) is the spore-producing sporophyte (diploid, 2n) and the other

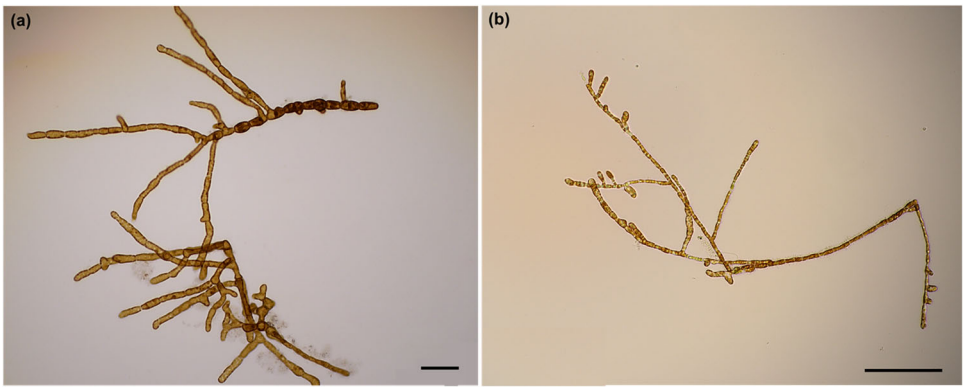

Fig. 1 *Undaria pinnatifida* gametophytes used in this study. Female (**a**) and male (**b**) gametophytes. Scale bar, 100 µm

phase encompasses the microscopic zoospores and male/female gametophytes (haploid, n). The zoospores are released by the sporophyll (reproductive part of the sporophyte) and germinate into microscopic gametophytes (Mandrekar 2018; Murphy et al. 2016, 2017). The dioecious gametophytes may undergo a dormant period (Vilà et al. 2009). They are capable of surviving adverse conditions and may act as seed banks that could persist a vegetative states, especially in low light (Carney and Edwards 2006; Hewitt et al. 2005; Lüning 1981; Thornber et al. 2004).

U. pinnatifida gametophytes (Fig. 1) were acquired from the National Institute of Fisheries Science (Haenam, Republic of Korea) and maintained in axenic batch cultures in 500 ml round-bottom flasks filled with 450 ml of the Provasoli-Enriched Seawater (PES) (Provasoli 1968). Cultures were incubated at 10 °C under 10–20 µmol photons m^{-2} s^{-1} of red light provided by a white fluorescent lamp (FL20SS; Philipps, Eindhoven, the Netherlands) with a broadband filter (106; Lee Filters, Andover, UK) on a 12:12 h light:dark cycle.

Determination of optimal test sample density for fluorescence measurements

To determine the optimal sample density, F684/F735 ratio was measured using a Gemini EM plate reader (Molecular Devices, San Jose, California, USA) with a xenon flash lamp at the sample densities of 0.025, 0.05, 0.1, 0.2, 0.4 and 0.6 mg ml^{-1} wet weight of *U. pinnatifida* gametophytes. Spectra were corrected for the wavelength-dependent response of the photomultiplier.

The F684 to F735 ratio with a conventional fluorometer is based on the fact that two maxima (F684 and F735) are apparent in the chlorophyll fluorescence spectra of leaves: one in the red region near 690 nm and the other in the far-red region near 735 nm (Eullaffroy and Vernet 2003; Fai et al. 2007). As the F684/F735 fluorescence ratio has long been used as tool to effectively assess performance of the photosynthetic apparatus reflecting the PSII and PSI activities (Eullaffroy and Vernet 2003), we have simply used this ratio to determine the appropriate test sample density prior to the main experimentation. However, all subsequent tests were conducted based on the emission at 677 nm.

To establish the emission and excitation spectra for a microplate-based fluorescence assay, fluorescence emission spectra were recorded at excitation peaks of 438 nm (chlorophyll *a* absorption peak), 444 nm (chlorophyll c_1 absorption peak), and 449 nm (chlorophyll c_2 absorption peak), using 9 nm excitation and emission slits (Lüning 1990).

Toxicity testing

The toxicity test was conducted by dispensing 1 ml PES into one well as control and five different concentrations of 1 ml test solution into other wells in 48-well plates (30048; SPL, Korea). The test sample density was 0.2 mg ml^{-1}. Test solutions in the absence of algal samples were used as blanks. Static cultures were established at 4°C in the dark for 48 h.

Table 1 lists key information on the six tested biocides. Stock solutions of diuron, DCOIT, irgarol, tolylfluanid and ZnPT were prepared in dimethyl sulfoxide (DMSO, ≥99.9%; CAS No. 67-68-5; Sigma-Aldrich, Co., USA), while medetomidine was dissolved in deionized water due to its limited solubility in DMSO. Biocide stock solutions made up in DMSO did not exceed 0.1% (v/v) concentration. Stock solutions were then diluted in five test concentrations with PES medium.

As there was no analytical control (i.e. solubility, adsorption, and half-time), no information was provided on the solubility, adsorption to well walls, and half-time during the exposure. However, OECD (2000) states that the potential loss of toxicant due to adsorption to test vessels is expected with high hydrophobicities and an octanol/water partition coefficient (log K_{ow}) > 4. The six antifouling biocides used in the present study are not volatile and have a K_{ow} of 2.8, 2.82, 2.85, 2.6, 3.9, and 0.9 (for irgarol, diuron, DCOIT, medetomidine, tolylfluanid, and ZnPT, respectively). Therefore, adsorption of the used toxicants to the

Table 1 Information on the six antifouling biocides used for the *Undaria pinnatifida* toxicity testing

Biocides	Concentrations (mg l^{-1})	CAS no.	Manufacturer
4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT)	0.0625–1.0	64359-81-5	Tokyo Chemical Industry, Japan
Diuron	0.025–0.4	330-54-1	Sigma Aldrich, USA
Irgarol	0.0625–1.0	28159-98-0	Sigma Aldrich, USA
Medetomidine	1.25–20	86347-15-1	Cayman Chemical Company, USA
Tolylfluanid	0.0625–1.0	731-27-1	Sigma Aldrich, USA
Zinc pyrithione (ZnPT)	0.025–0.4	13463-41-7	Sigma Aldrich, USA

test vessels was unlikely, and nominal concentrations were deemed accurate for the chosen exposure regimes (Logan 2002; Martins et al. 2018; Thomas and Brooks 2010).

It is possible for antifouling biocides to degrade, such as through hydrolysis, photolysis, or biodegradation. DCOIT is considered as a readily biodegradable with a reported half-life in natural seawater from less than 24 h to 3 d (Callow and Willingham 1996; Jacobson and Willingham 2000; Thomas et al. 2003). Some studies have found contradicting results regarding its degradability. Slower biodegradative kinetics have been observed for DCOIT in natural seawater samples in several different regions of the world, such as 2.6 d in Denmark (Larsen et al. 2003), >4 d in Hong Kong (Chen et al. 2015), 10 d in Japan (Harino et al. 2005), 8.5 d in UK (Callow and Willingham 1996), and 13.1 d in Greece (Sakkas et al. 2002). For ZnPT, the half-life in sterile seawater was 7–8 min under the influence of sunlight, but more than 48 h in dark conditions (Dahllöf 2005; Maraldo and Dahllöf 2004; Marcheselli et al. 2010; Zepp and Cline 1977).

Additionally, the half-life of diuron and irgarol in seawater exceeds 31.4 d (Martins et al. 2018) and 100 d (Hall et al. 1999; Scarlett et al. 1999; Thomas et al. 2002), respectively. Tolylfluanid degrades within 2 weeks (Logan 2002). Hydrolysis and aqueous photo-degradation studies conducted for medetomidine demonstrated that it was hydrolytically and photolytically stable at all environmentally relevant pH and temperatures (ECA 2014). Thus, the toxicity loss through hydrolysis, photolysis, or biological degradation of the six antifouling biocides during 48 h of exposure should be minimal.

Fluorescence emission spectra

After 48 h exposure, room temperature fluorescence emission spectra were recorded. The samples were excited at 438, 444 and 449 nm, respectively. The PSII contribution at 677 nm was subtracted from the fluorescence emission spectra. Percent relative fluorescence (%RF) was then calculated by dividing the relative chlorophyll fluorescence measured from each concentration of toxicants by the mean chlorophyll fluorescence value of the control, using the following equation:

$$\text{Percent relative fluorescence}(\%) = \frac{\text{Relative chlorophyll fluorescence (a.u)}}{\text{Mean of chlorophyll fluorescence of control (a.u)}} \times 100$$

Statistical analysis

One-way analyses of variance (ANOVAs), followed by least significant difference *post-hoc* tests at $P < 0.05$, were carried out to test differences among treatments. Results have been reported as EC_{10} and EC_{50} (i.e. effective concentration at which 10 and 50% inhibition occurs, respectively) values with 95% confidence intervals, that were estimated using the linear interpolation method (Toxicalc 5.0; Tidepool Science, McKinleyville, California, USA).

The linear interpolation method was used to calculate a point estimate of the effluent or other toxicant concentration that caused a given percent reduction (e.g., 10, 25, 50%, etc.) in the test organisms (Klemm et al. 1992; OECD 2002). The method assumes a linear response from one concentration to the next. Thus, the ECp is estimated by linear interpolation between two concentrations whose responses encompass the response of interest, the percent (p) reduction from the control.

Use of the linear interpolation method is based on the assumptions that the responses (1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration); (2) follow a piece-wise linear response function; and (3) are from a random, independent, and representative sample of test data. The assumption for a piece-wise linear response cannot be tested statistically, and no defined statistical procedure is provided to test the assumption for monotonicity. Where the observed means are not strictly monotonic by an examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. The linear

interpolation estimate was calculated following the method reported by Klemm et al. (1992).

The coefficient of variation (CV), which is the standard deviation expressed as a percentage of the mean, was calculated to estimate the precision of the tests.

A Student's t-test has performed for comparison of the sensitivity based on the EC_{50} values of both gametophytes.

Results and discussion

The fluorescence emission spectra of male and female gametophytes of *U. pinnatifida* exhibited a clear red maximum near 677 nm (Fig. 2a). The relative intensity of the F677 increased with gametophyte density.

Excitation of one single wavelength can produce distinct peaks in the scanning mode of a spectrofluorimeter. At a gametophyte density of 0.025 mg ml^{-1}, the intensity of the red fluorescence maximum was relatively low, while the red F677 showed distinct peaks at densities of 0.4 and 0.6 mg ml^{-1}.

The F684/F735 fluorescence ratio as a tool to assess performance of the photosynthetic apparatus reflecting the PSII and PSI activities (Eullaffroy and Vernet 2003) rose rapidly with increasing male and female gametophyte densities in the range of 0.025–0.6 mg ml^{-1} when excitation was performed at 438 nm and 444 nm. By contrast, at 449 nm excitation, the F684/F735 ratio increased at 0.2 mg ml^{-1} and then saturated above 0.4 mg ml^{-1} in both sexes. Self-shading effects or fluorescence emission re-absorption by pigments may explain the signal saturation above 0.4 mg ml^{-1} of the gametophytes. The optimal test sample density was therefore determined to be 0.2 mg ml^{-1} for both gametophyte sexes, and all subsequent emission spectra were recorded at 677 nm with excitation at 449 nm.

EC_{10} and EC_{50} values for male and female gametophytes exposed to the six biocides are shown in Table 2. All antifouling biocides had significant negative effects on chlorophyll fluorescence of male and female gametophytes. Diuron was found to be the most toxic of the tested biocides, with EC_{50} values of 0.037 mg l^{-1} for male gametophytes and 0.128 mg l^{-1} for females. Little as 0.006 mg l^{-1} was sufficient to induce a 10% decrease in %RF of male gametophytes. Medetomidine was the least toxic of the tested biocides, with EC_{50} values of 12.032 mg l^{-1} for male gametophytes and 12.763 mg l^{-1} for females. The CV of EC_{10} and EC_{50} for the six antifouling biocides (2.683–38.055 and 5.588–31.366%, respectively) lay within the recommended levels of precision (<40%) (Environment Canada 1990).

A comparison of the relative sensitivity of male and female gametophytes to the six antifouling biocides is presented in Fig. 3. Data points falling on the line (i.e., slope = 1) indicate equivalent sensitivity of both sexes to a given toxicant. Data points to the right and left of the line indicate a higher sensitivity of the male and female gametophytes, respectively ($P < 0.05$). We found two data points (diuron and tolylfluanid) to the right and no data to the left. There is little information on comparative physiological and biochemical differences between different sexes of algae. However, some large brown algal species exhibit gender-based differences in temperature tolerance (Bolton and Lüning 1982; Lee and Brinkhuis 1988; Thomas and Kirst 1991).

Table 2 compares the EC_{50} values obtained from *U. pinnatifida* gametophytes with results obtained from other marine macroalgae (using a variety of test methods). For DCOIT, EC_{50} values of *U. pinnatifida* gametophytes were similar to those of the green alga *U. pertusa* (96 h exposure $EC_{50} = 0.664–0.902$ mg l^{-1} for reproduction; Lee et al. 2020), but higher than the values reported for *Ulva intestinalis* L., 1753, *Ulva lactuca* L., 1753, *Fucus serratus* L., 1753, *H. banksii* and *Saccharina latissima* (L.) Lane et al., which range from 0.019 to 0.621 mg l^{-1} (Braithwaite and Fletcher 2005; Johansson et al. 2012; Myers et al. 2006; Wendt et al. 2013; Willingham and Jacobson 1996) (Table 2). DCOIT causes an oxidative stress and inhibits PSII electron transport (Arning et al. 2009; Guardiola et al. 2012). Acute toxic effects have also been reported in a wide range of other algae (Johansson et al. 2012; Wendt et al. 2013).

Male gametophytes of *U. pinnatifida* were about three times more sensitive to diuron than female gametophytes, with EC_{50} values of 0.037 mg l^{-1} and 0.128 mg l^{-1}, respectively. Diuron has been shown to be highly toxic to plant and algal photosynthesis by binding with high affinity at the Q_B-binding site of the PSII photosynthetic complex (Amara et al. 2018). Both gametophyte sexes showed lower sensitivity to diuron than the green algae *U. intestinalis* (96 h exposure $EC_{50} = 0.0035$ mg l^{-1} for growth; Girling et al. 2015), *U. pertusa* (96 h exposure $EC_{50} = 0.069$ mg l^{-1} for reproduction; Lee et al. 2019a) and the red algae *Ceramium tenuicorne* (Kützing) Waern (7 d exposure $EC_{50} = 0.003$ mg l^{-1} for growth; Karlsson et al. 2006), *Gracilaria tenuistipitata* Chang and Xia (96 h exposure $EC_{50} = 0.020$ mg l^{-1} for growth; Haglund et al. 1996). By contrast, *U. pinnatifida* gametophytes showed higher sensitivity to diuron than the brown alga *H. banksii* (48–72 h exposure $EC_{50} = 4.650–6.820$ mg l^{-1} and 6.750–7.330 mg l^{-1} for germination and rhizoid growth, respectively; Myers et al. 2006). It is also notable that *U. pinnatifida* male gametophytes showed EC_{50} values similar to, or lower than that of *Saccharina japonica* (Areschoug) Lane et al. (14 d exposure $EC_{50} > 0.40$ mg l^{-1} for area growth; Kumar et al. 2010), despite the difference in exposure times.

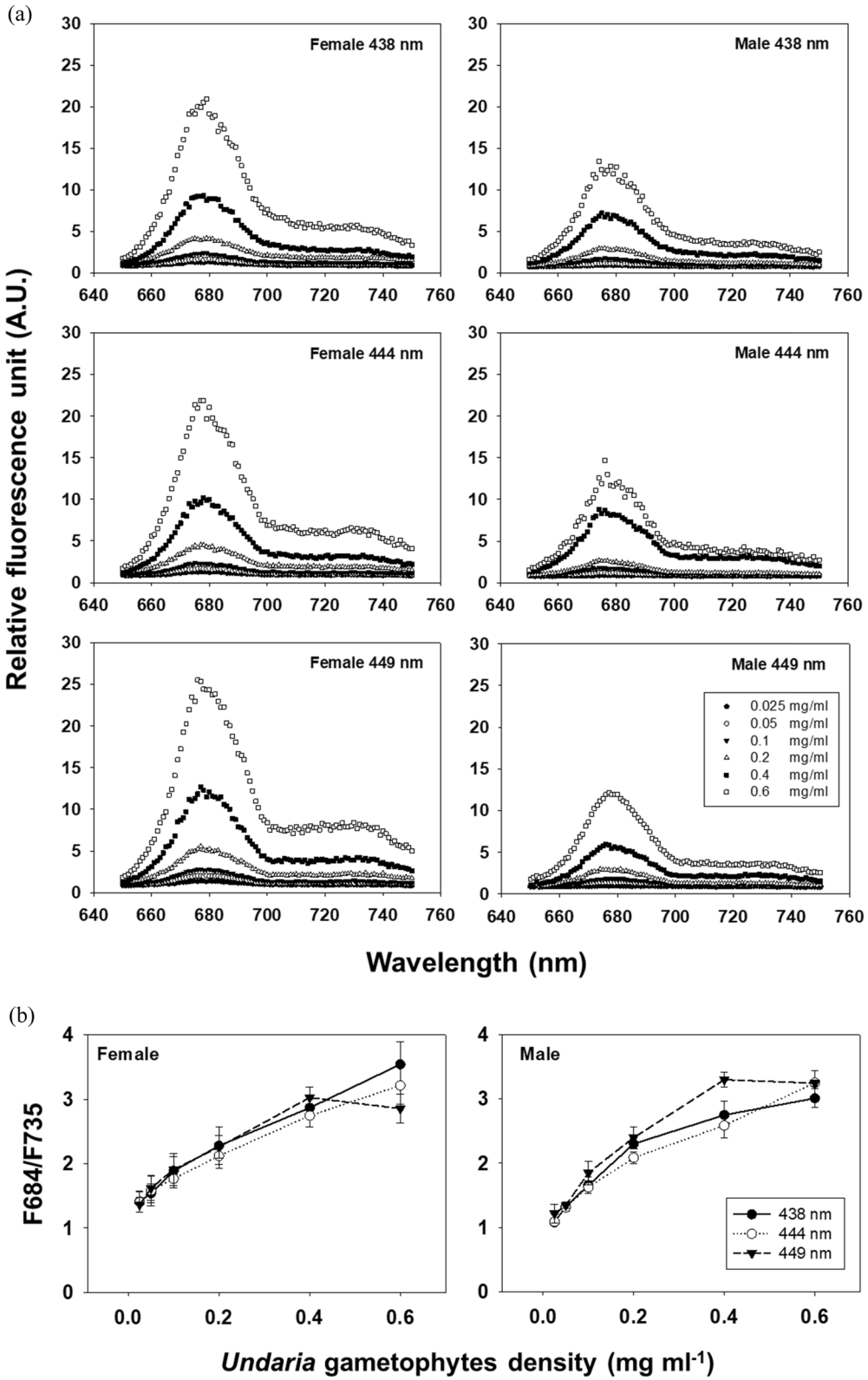

Fig. 2 **a** Chlorophyll (Chl) fluorescence emission spectra of male and female *Undaria* gametophytes excited at 438, 444 and 449 nm at different gametophyte densities: 0.025, 0.05, 0.1, 0.2, 0.4 and 0.6 mg ml^{-1}. **b** Relationship between the *Undaria* gametophyte density and the Chl fluorescence ratio F684/F735 obtained after excitation at 438, 444 and 449 nm

Table 2 Summary comparison of EC_{10}, EC_{50} and coefficients of variation (CV) values obtained from this study and various macroalgal toxicity tests

Toxicants	Test species	Endpoints	Test period	EC_{10} (95% CI range; mg l^{-1})	CV(%)	EC_{50} (95% CI range; mg l^{-1})	CV(%)	References
DCOIT	*Ulva intestinalis*	ST	120 h	–	–	0.460	–	Willingham and Jacobson (1996)
	U. lactuca	ST, GR	72 h	0.008 (0.006–0.010)	–	0.023 (0.021–0.026)	–	Wendt et al. (2013)
	U. pertusa	RP	96 h	–	–	0.783 (0.664–0.902)	13.424	Lee et al. (2020)
	Fucus serratus	ZG	24 h	–	–	0.019	–	Braithwaite and Fletcher (2005)
	Hormosira banksii	G	48 h	–	–	0.340 (0.280–0.440)	19	Myers et al. (2006)
			72 h	–	–	0.420 (0.290–0.550)	24	
		GR	48 h	–	–	0.430 (0.290–0.650)	31	
			72 h	–	–	0.460 (0.340–0.540)	22	
	Saccharina latissima	^{14}C-incorporation	3.25 h	–	–	0.621 (0.223–1.044)	–	Johansson et al. (2012)
	Undaria pinnatifida (F)	RF	48 h	0.129 (0.107–0.150)	14.720	0.890 (0.833–0.946)	5.588	This study
	U. pinnatifida (M)	RF	48 h	0.130 (0.104–0.156)	17.569	1.015 (0.819–1.211)	19.729	
Diuron	*Ulva intestinalis*	GR	96 h	–	–	0.0035	–	Girling et al. (2015)
	U. pertusa	RP	96 h	0.010 (0.007–0.013)	29.47	0.069 (0.064–0.075)	7.70	Lee et al. (2019a, 2019b)
	Ceramium tenuicorne	GR	7 d	–	–	0.003 (0.003–0.004)	–	Karlsson et al. (2006)
	Gracilaria tenuistipitata	GR	96 h	–	–	0.020	–	Haglund et al. (1996)
	Fucus spiralis	GR	96 h	–	–	0.020	–	Girling et al. (2015)
	Saccharina japonica	GR	14 d	0.0023	–	0.087	–	Kumar et al. (2010)
		GR (area)	14 d	0.004 (0.003–0.009)	–	>0.40	–	
	S. latissima	^{14}C-incorporation	3.25 h	–	–	0.007 (0.004–0.011)	–	Johansson et al. (2012)
	Hormosira banksii	ΔF/Fm'	2 h	–	–	0.0016	19.4	Seery et al. (2006)
		G	48 h	–	–	4.650	40.3	
		G	48 h	–	–	6.290 (5.930–6.830)	6	Myers et al. (2006)
		G	72 h	–	–	6.820 (5.980–7.760)	10	
		RG	48 h	–	–	6.750 (5.930–7.590)	9	
		RG	72 h	–	–	7.330 (6.350–7.520)	12	
	Undaria pinnatifida (F)	**RF**	48 h	0.013 (0.012–0.014)	6.706	0.128 (0.101–0.154)	18.469	This study
	U. pinnatifida (M)	**RF**	48 h	0.006 (0.006–0.007)	3.387	0.037 (0.034–0.040)	7.196	
Irgarol	*Ulva intestinalis*	GR	96 h	–	–	0.0005	–	Girling et al. (2015)
	U. pertusa	RP	96 h	–	–	0.048 (0.033–0.066)	15.370	Lee et al. (2020)
	Ceramium tenuicorne	GR	168 h	–	–	0.001 (0.0005–0.001)	–	Karlsson et al. (2006)
	Pyropia yezoensis	GR	96 h	–	–	0.0006 (0.0001–0.001)	83.333	Okamura et al. (2000)
		G	96 h	–	–	0.004 (0.002–0.006)	34.146	
	Eisenia bicyclis	GR	96 h	–	–	0.006 (0.003–0.007)	–	
	Gracilaria tenuistipitata	GR	96 h	–	–	0.002	–	Haglund et al. (1996)

Table 2 (continued)

Toxicants	Test species	Endpoints	Test period	EC_{10} (95% CI range; mg l^{-1})	CV(%)	EC_{50} (95% CI range; mg l^{-1})	CV(%)	References
	Fucus spiralis	GR	96 h	–	–	0.020	–	Girling et al. (2015)
	Saccharina latissima	^{14}C-incorporation	3.25 h	–	–	0.010 (0.007–0.013)	–	Johansson et al. (2012)
	Hormosira banksii	ΔF/Fm'	2 h	–	–	0.0002	21.2	Seery et al. (2006)
		G	48 h	–	–	3.540	22.1	
	Undaria pinnatifida (F)	RF	48 h	0.017 (0.016–0.017)	2.683	0.172 (0.111–0.232)	31.366	This study
	U. pinnatifida (M)	RF	48 h	0.019 (0.015–0.023)	18.113	0.096 (0.090–0.103)	5.830	
Medetomidine	*Ulva lactuca*	GR	72 h			>20.030		Wendt et al. (2013)
	U. pertusa	RP	96 h			11.600 (9.331–13.867)	17.277	Lee et al. (2020)
	Undaria pinnatifida (F)	RF	48 h	2.663 (1.603–3.724)	35.180	12.763 (11.701–13.825)	7.354	This study
	U. pinnatifida (M)	RF	48 h	2.371 (2.201–2.540)	6.317	14.311 (10.641–17.981)	22.665	
Tolylfluanid	*Ulva lactuca*	ST	72 h	0.012 (0.007–0.025)	–	0.030 (0.020–0.038)	–	Wendt et al. (2013)
	Saccharina latissima	^{14}C-incorporation	3.25 h	–	–	>3.472	–	Johansson et al. (2012)
	Undaria pinnatifida (F)	RF	48 h	0.051 (0.031–0.071)	35.271	1.028 (0.725–1.331)	26.076	This study
	U. pinnatifida (M)	RF	48 h	0.037 (0.022–0.053)	36.915	0.238 (0.162–0.313)	28.094	
Zinc pyrithion	*Ulva pertusa*	RP	96 h	–	–	3.556 (2.555–5.147)	20.182	Lee et al. (2020)
	Ceramium tenuicorne	GR	168 h	0.0026(0.0015–0.0035)	–	0.006 (0.005–0.007)	–	Karlsson and Eklund (2004)
	Saccharina latissima	^{14}C-incorporation	3.25 h	–	–	>0.318	–	Johansson et al. (2012)
	Hormosira banksii	G	48 h	–	–	0.210 (0.160–0.250)	18	Myers et al. (2006)
			72 h	–	–	0.190 (0.120–0.280)	30	
		GR	48 h	–	–	0.310 (0.210–0.380)	23	
			72 h	–	–	0.240 (0.150–0.360)	32	
	Undaria pinnatifida (F)	RF	48 h	0.110 (0.087–0.133)	18.697	>0.40	–	This study
	U. pinnatifida (M)	RF	48 h	0.068 (0.039–0.098)	38.055	>0.40	–	

Mean values with 95% confidence intervals (CIs) are shown

SP Sporulation, *SR* Spore release, *RF* Relative fluorescence, *RP* Reproduction, *ST* Settlement, *GR* Growth, *ZG* Zygote germination, *G* Germination, *NRU* Neutral Red Uptake, D Development, *F* Fertilization, *FC* Function, *R* Respiration

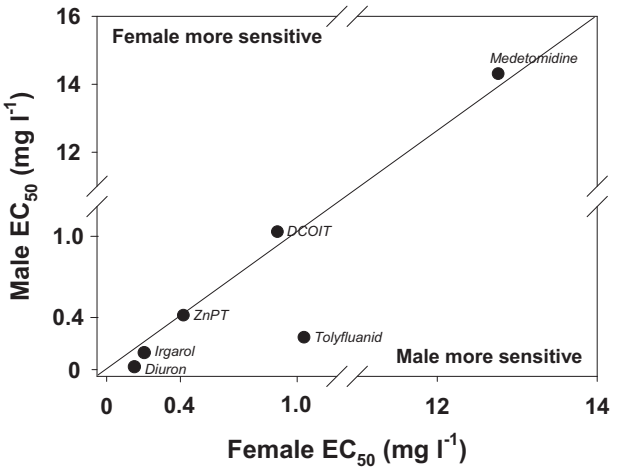

Fig. 3 A comparison of the relative sensitivity of male and female gametophytes to the six antifouling biocides. All units are in mg l^{-1}. Data points falling on the line (i.e., slope = 1) indicate equivalent sensitivity of both sexes to a given toxicant. Data points to the right and left of the line indicate higher sensitivity of male and female gametophytes, respectively

Irgarol belongs to the S-triazine group of compounds, which act as PSII inhibitors by interfering with the photosynthetic electron capture in chloroplasts (Koutsaftis and Aoyama 2006). Although *U. pinnatifida* gametophytes were less sensitive to irgarol than the ISO standard macroalgal species *C. tenuicorne* (7 d exposure $EC_{50} = 0.001$ mg l^{-1} for growth; Karlsson et al. 2006), both seaweeds showed lower sensitivity than two other ISO standard microalgae *Phaeodactylum tricornutum* Bohlin (4.5 h exposure $EC_{50} = 0.0003$ mg l^{-1} for electron transport activity; Sjollema et al. 2014) and *Skeletonema costatum* (Greville) Cleve (96 h exposure $EC_{50} = 0.00057$ mg l^{-1} for growth; Bao et al. 2011).

Inhibition of the carbon-concentrating mechanism by tolylfluanid has been reported to affect the photosynthesis process of macroalga *S. latissima* (Johansson et al. 2012). The action mechanism of medetomidine is not clearly established for algae, but effects on photosynthesis have been reported in phytoplankton community (Ohlauson et al. 2012). *U. pinnatifida* gametophytes are more sensitive to medetomidine than the green macroalga *U. lactuca* (72 h exposure $EC_{50} > 20.030$ mg l^{-1} for growth; Wendt et al. 2013) and to tolylfluanid than the brown macroalga *S. latissima* (3.25 h exposure $EC_{50} > 3.472$ mg l^{-1} for ^{14}C-incorporation; Johansson et al. 2012).

It was reported that pyrithiones interfere with the proton motility in target organisms (Martins et al. 2018). High toxicity of ZnPT was reported for the maximum quantum yield (F_v/F_m) of phytoplankton *Emiliania huxleyi* (Lohmann) Hay and Mohler and *Synechococcus* sp. (Devilla et al. 2005). The 50% fluorescence inhibition of *U. pinnatifida* gametophytes after exposure to ZnPT occurred at concentrations above 0.4 mg l^{-1}.

Conclusions

This study describes the use of a chlorophyll fluorescence-based bioassay using *U. pinnatifida* gametophytes to assess biocidal effects on aquatic ecosystems. Delayed development of *U. pinnatifida* gametophytes allows formation of a 'gametophyte bank' (analogous to a 'seed bank'), removing the need to maintain live stocks of the test species. Cryopreservation may be another way of making the assay possible regardless of the time and location, and, recently, successful cryopreservation has been performed with *U. pinnatifida* and other brown algal gametophytes (Visch et al. 2019; Wang et al. 2011). The assay can therefore be applied year-round using stored gametophytes. The current chlorophyll fluorescence measurements are made using a conventional fluorometer. Performance is very rapid and precise, with a complete test taking less than 3 min, making this method a simple, easy and rapid assessment of the effects of antifouling biocides.

The ubiquitous presence of *U. pinnatifida* in coastal marine environments, and its important role in the marine food web, confer an ecological significance to its use in ecotoxicity testing. In some cases, *U. pinnatifida* is also considered as an invasive species, thus, possibly interfering with its use as a standardized test from a viewpoint of the local regulators.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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