Ecotoxicology and Environmental Safety 94 (2013) 37–44

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Application of a fluorometric microplate algal toxicity assay for riverine periphytic algal species

Takashi Nagai [a,*], Kiyoshi Taya [a], Hirochica Annoh [b], Satoru Ishihara [c]

[a] National Institute for Agro-Environmental Sciences, Kannondai 3-1-3, Tsukuba, Ibaraki 305-8604, Japan
[b] ESCO Cooperation, Tomitake 173-2, Nagano, Nagano 381-0006, Japan
[c] Food and Agricultural Materials Inspection Center, Agricultural Chemicals Inspection Station, Suzukicho 2-772, Kodaira, Tokyo 187-0011, Japan

ARTICLE INFO

Article history:
Received 12 December 2012
Received in revised form
28 March 2013
Accepted 23 April 2013
Available online 21 May 2013

Keywords:
Attached algae
In-vivo fluorescence
Pesticides
Species sensitivity distribution

ABSTRACT

Although riverine periphytic algae attached to riverbed gravel are dominant species in flowing rivers, there is limited toxicity data on them because of the difficulty in cell culture and assays. Moreover, it is well known that sensitivity to pesticides differ markedly among species, and therefore the toxicity data for multiple species need to be efficiently obtained. In this study, we investigated the use of fluorometric microplate toxicity assay for testing periphytic algal species. We selected five candidate test algal species *Desmodesmus subspicatus*, *Achnanthidium minutissimum*, *Navicula pelliculosa*, *Nitzschia palea*, and *Pseudanabaena galeata*. The selected species are dominant in the river, include a wide range of taxon, and represent actual species composition. Other additional species were also used to compare the sensitivity and suitability of the microplate assay. A 96-well microplate was used as a test chamber and algal growth was measured by in-vivo fluorescence. Assay conditions using microplate and fluorometric measurement were established, and sensitivities of 3,5-dichlorophenol as a reference substance were assayed. The 50 percent effect concentrations (EC_{50}s) obtained by fluorometric microplate assay and those obtained by conventional Erlenmeyer flask assay conducted in this study were consistent. Moreover, the EC_{50} values of 3,5-dichlorophenol were within the reported confidence intervals in literature. These results supported the validity of our microplate assay. Species sensitivity distribution (SSD) analysis was conducted using the EC_{50}s of five species. The SSD was found to be similar to the SSD obtained using additional tested species, suggesting that SSD using the five species largely represents algal sensitivity. Our results provide a useful and efficient method for high-tier probabilistic ecological risk assessment of pesticides.

1. Introduction

Paddy fields occupy more than half of the total agricultural land in Japan, and various herbicides are used for weed prevention in these fields. Up to 50 percent of the applied herbicides are removed as run-off, and they directly flow into rivers through drainage channels (Watanabe et al., 2008). Generally, algae is a sensitive taxonomic group to herbicides (van den Brink et al., 2006). Moreover, herbicide effects on the species composition and community structure of benthic algal assemblage were found in natural aquatic ecosystems (Sabater et al., 2007; Ricart et al., 2010). Therefore, an important concern of the herbicidal effect on non-target organisms is algae in the river ecosystems.

Riverine periphytic algae attached to riverbed gravel plays an important role in ecological function as the primary producers and as food for invertebrates and fish (Finlay et al., 2002). Especially, diatoms are the most dominant algal group in terms of species number and biomass (Round et al., 1990). For example, *Navicula* sp. is extremely common and occurs in almost all periphyton samples containing diatoms (Biggs and Kilroy, 2000). Further, *Achnanthidium minutissimum* is common and widespread in a range of ecological conditions but does best in clean, low conductivity streams. *Nitzschia palea* is widespread, common, and well known as a pollution tolerant species (Biggs and Kilroy, 2000). Besides diatoms, green algae and cyanobacteria are also common periphytons. For example, *Desmodesmus* (*Scenedesmus*) sp. can be extremely common in the periphyton of low to moderately enriched streams and *Pseudanabaena* (*Phormidium*) sp. is often very abundant in high-conductivity streams (Biggs and Kilroy, 2000; ESCO, 2006).

However, in the conventional ecological effect assessment, the effects of pesticides on algae are mostly assessed by using a single standard species, the green alga *Pseudokirchneriella subcapitata*, which is a planktonic algae and not the dominant species in river

* Corresponding author. Fax: +81 29 838 8199.
E-mail address: nagait@affrc.go.jp (T. Nagai).

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http://dx.doi.org/10.1016/j.ecoenv.2013.04.020

ecosystems (Kasai, 2003). Thus, only a few data are available for the pesticide toxicity on riverine periphytic algal species (for example Larras et al., 2012). Moreover, it is well known that the sensitivity to pesticides differs markedly among species (van den Brink et al., 2006; Nagai et al., 2011), and a single specific indicator algal species cannot be representative of the whole algal assemblage (Freemark et al., 1990; Swanson et al., 1991). Therefore, toxicity data for multiple species should be obtained efficiently.

On the other hand, species sensitivity distribution (SSD) analysis has been developed to statistically deal with multiple species toxicity data in ecological risk assessment (Posthuma et al., 2002). The SSD is a statistical distribution (often a log-normal distribution) describing the variance among a set of species in their sensitivity to toxicants, for example, the 50 percent effect concentrations (EC_{50}s) or no observed effect concentrations (NOECs) of a certain chemical. The SSD has been used for high-tier ecological risk assessment of pesticides as a probabilistic method. The fifth percentile of a distribution (called the hazardous concentration for five percent of the species, HC_5) has been chosen as a safe concentration that protects most species in a community (Posthuma et al., 2002). However, more data are required for the analysis, and in particular toxicity data from more than five algal species is desired for SSD analysis (OECD, 1995; TenBrook et al., 2008).

The purpose of this study was to demonstrate the application of an efficient and economical high-throughput algal toxicity assay using five riverine periphytic species. The effects of pesticides on algae are generally analyzed by standard test methods (OECD, 2006), which require considerable labor for testing multiple species and is not suitable for periphytic species because of algal attachment to the surface of Erlenmeyer flasks (Swanson et al., 1991). Instead, Ishiahara et al. (2006) showed the use of a microplate assay in which periphytic algae are attached to the bottom of a microplate. Microplate algal toxicity assay have been widely utilized as rapid and economical screening assay (Blaise and Vasseur, 2005). Moreover, this test method is employed officially for testing algal toxicity in Canada (Environment Canada, 2007). The advantages of microplate-based toxicity assay are summarized as follows: (1) small sample volume requirement; (2) incubator space economy; (3) disposable microplate and pipette tips; and (4) increased bioanalytical output (Blaise and Vasseur, 2005). Moreover, Eisentraeger et al. (2003) showed that fluorometric measurement of algal growth has high measurement sensitivity. Therefore, we optimized fluorometric microplate toxicity assay for testing multiple periphytic species.

In this study, five candidate test algal species were selected considering not only their suitability for microplate assay but also ecological relevance. Assay conditions using microplate and fluorometric measurements were then established, and the sensitivities of 3,5-dichlorophenol (DCP) were assayed and compared among species and among assay methods. Moreover, the validity of candidate species selection was discussed based on the obtained results.

2. Materials and Methods

2.1. Test organisms and maintenance

We selected candidate algal species by considering not only the above ease in assay handling but also the following ecological relevance: (1) widely distributed and frequently observed species in river ecosystems in Japan; (2) including a wide range of taxonomic groups (green algae, diatoms, cyanobacteria), and reflecting the actual species composition in Japanese rivers (diatoms are dominant followed by green algal and cyanobacteria); and (3) selection from several environmental conditions, including saproxenous species (adapted to clean water), saprophilic species (adapted to organically polluted water), and eurysaprobic species (Watanabe et al., 1986). From the results of previous investigations on riverine periphytons in Japanese rivers (Watanabe, 2005; ESCO, 2006), we selected a green alga (*Desmodesmus* sp.), three diatoms (*Achnanthidium* sp., *Nitzschia* sp., and *Navicula* sp.), and a cyanobacteria (*Pseudanabaena* sp.) as representatives of riverine periphytic algal genera. Moreover, other additional species were used for toxicity tests to verify the selection.

The algal strains used for the toxicity assay (eight species with eleven strains) are listed in Table 1. NIES strains were obtained from the National Institute for Environmental Studies, Japan, Microbial Culture Collection (Kasai et al., 2004). UTEX strain was obtained from UTEX The Culture Collection of Algae at The University of Texas at Austin. Although *Navicula pelliculosa* was renamed as *Fistulifera pelliculosa* (Lange-Bertalot, 1997), original registration name by UTEX was used in this paper. *Mayamaea atomus* strain NIAES K11-11, *N. palea* strain NIAES PD3, and *N. palea* strain NIAES U3-3 were collected and isolated from the Sakura River, paddy fields, and Saka River, Tsukuba city, Japan, respectively.

Stock cultures were maintained in the conditions listed in Table 1. Culture mediums, C, CT, CB, and CSi (Kasai et al., 2004), were prepared using Milli-Q water (resistance 18.3 MΩ; Millipore, Billerica, MA, USA). Light irradiation was continuous by using a daylight fluorescent lamp (color temperature 6500 K). Routine culture maintenance was by using both aqueous and solid agar medium, but using solid agar medium was impossible with two of the cyanobacterial strains and the use of an aqueous medium resulted in unstable growth for the two *Nitzschia* strains (Table 1).

2.2. Algal toxicity assay using microplate

Fluorometric algal toxicity assay were conducted using 96-well microplates, according to the standardized algal growth inhibition test (OECD, 2006). DCP, which was purchased from Wako Pure Chemical Inc. Ltd. (Osaka, Japan), was used for the toxicity assay as a reference substance for investigating the validity of the toxicity test and sensitivity of the tested species (OECD, 2006). Stock solutions of DCP were prepared in dimethyl sulfoxide (DMSO; Wako, Osaka, Japan). The final concentration of DMSO was less than 0.1 percent (v/v;≈1 g l^{-1}), a concentration at which no adverse effects have been observed (Jay, 1996). In addition, the effect of

Table 1
Test species, strains, and their culture conditions, including growth medium, applicability of solid and aqueous medium (○ indicates suitable and × indicates unsuitable), light intensity used for maintenance or toxicity assay, and temperature.

Species	Strain	Taxonomic group	Medium	Solid medium	Aqueous medium	Light intensity (maintenance), (lux)	Light intensity (toxicity assay), (lux)	Temp. (°C)
Pseudokirchneriella subcapitata	NIES-35	Green algae	C	○	○	1000	5000	23
**Desmodesmus subspicatus*	NIES-797	Green algae	C	○	○	1000	2000	23
Mayamaea atomus	NIAES K11-11	Diatom	CSi	○	○	1000	2000	20
Nitzschia palea	NIAES PD3	Diatom	CSi	○	×	1000	2000	20
Nitzschia palea	NIAES U3-3	Diatom	CSi	○	×	1000	2000	20
**Nitzschia palea*	NIES-487	Diatom	CSi	○	○	1000	2000	20
**Achnanthidium minutissimum*	NIES-71	Diatom	CSi	○	○	1000	2000	20
Achnanthidium minutissimum	NIES-414	Diatom	CSi	○	○	1000	2000	20
**Navicula pelliculosa*	UTEX-B673	Diatom	CSi	○	○	1000	2000	20
**Pseudanabaena galeata*	NIES-512	Cyanobacteria	CT	×	○	500	2000	23
Anabaena flos-aquae	NIES-73	Cyanobacteria	CB	×	○	1000	2000	23

* Candidate species.