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Toxicity testing of cosmetic ingredients using gametophyte beads of the brown alga *Undaria pinnatifida* (Laminariales, Phaeophyta)

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Abstract

A 6-h toxicity test of cosmetic ingredients (methylparaben, 2-phenoxyethanol, sodium dodecyl sulfate, triethanolamine) was developed, based on the photosynthetic maximum quantum yield (F_v/F_m) of immobilized gametophytes of the brown macroalga *Undaria pinnatifida*. From calculated EC_{50} values, the toxicity ranking of the tested ingredients is: SDS (0.0060%) > MP (0.0634%) > 2-PE (0.2418%) > TEA (3.7023%). Compared to the results from conventional endpoints with other ecotoxicity test organisms, measurements of F_v/F_m is a more sensitive indicator of the toxic effects of cosmetic ingredients. The present technique is simple, rapid, practical, accurate, and requires little space to carry out. This novel method will be a useful tool for assessing the toxicity of a wide range of cosmetic ingredients once the respective sensitivities are fully established.

Keywords Phaeophyceae · *Undaria pinnatifida* · Immobilized gametophytes · Alginate bead · Toxicity test · Cosmetic ingredients

Introduction

Despite the global economic downturn, the market for cosmetics continues to grow according to a recent industry analysis (Hsiao et al. 2017). The global cosmetic market was US$ 46 billion in 2014 and it will increase to US$ 67 billion in 2020 (Hsiao et al. 2017). Cosmetics contain various chemical mixtures including emulsifiers, preservatives, dyes, fragrances, and sunscreen, some of which are natural products while others are synthetic chemicals. Some are known to have harmful effects on the human body, but the extent of the potential impacts remains unclear (Vinardell 2015). In the 1930s, products containing thallium was reported to cause hair loss, and were sometimes fatal (Mulkey and Oehme 1993). Photoallergic reactions caused by cosmetics that contained salicylanilide were reported from the UK in 1958 and 1959, and long-term inflammatory allergic reactions to deodorants containing zirconium were identified in the 1950s and 1960s (Shelley and Hurley 1958; Kleinhans and Knoth 1976). With ingredients potentially damaging to children, the elderly, and pregnant women, long-term safety considerations have become paramount, and in many countries, ingredient safety assessment guidelines and re-evaluation are enforced to ensure the protection and health of citizens through improvements in quality assurance and safety standards of cosmetics.

The first in vivo testing standards for cosmetics applied to the skin and eyes were established in 1940 (Draize et al. 1944), while in vivo sensitization performance tests, phototoxicity tests, and photosensitizer functions/clinical animal safety assessments were not developed until the 1960s and 1970s. After enactment of the European Animal Welfare Law 86/609/EC in 1986, researchers attempted to develop "alternative methods to materialize the reduction and replacements of laboratory animal experiments through biomedical research/testing or education." The guiding principles for performing more humane animal testing, known as the 3Rs, i.e., Replacement, Reduction and Refinement, was established in 1959 by Russell and Burch (Worth and Balls 2002). Under the guidance of European Union, regulatory authorities (Registration,

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Evaluation, Authorization and Restriction of Chemicals [REACH]) and the European Chemical Industry Council (CEFIC; Chemical Industry Council) were established to improve the protection of human and the environment from the risks posed by chemicals. The EU and its member states have successfully reduced the use of animals, developed test and non-testing methods to minimize the number of experiments, and have introduced alternative chemical methods (European Central Bank 2006, 2008). However, assessment of cosmetic toxins by chemical analysis alone does not provide information on potential toxicity to humans and other organisms (Mallick and Rai 2002). Therefore, to address this problem, biological analysis techniques employing microorganisms, invertebrates, fish, and birds have been developed (Rotini et al. 2015; Lee et al. 2017; Vita et al. 2018). Some such bioassays are difficult to conduct and do not always provide a quick response. Moreover, some tests with fish and vertebrates are considered ethically objectionable.

Alternative procedures, for example, using mammalian cell lines can be expensive for culture maintenance (Browne and Al-Rubeai 2007), and although bioassays using fish cells are useful indicators of chronic toxicity, due to their long life cycle, they have some disadvantages such as low sensitivity long testing periods and requirements for specialized equipment and expertise (Farré and Barceló 2003). While bioassays are a pragmatic way of testing the harmful effects of chemicals to living organisms, the ease of maintaining test species under laboratory conditions also needs to be considered.

Seaweeds species belonging to the order Laminariales (hereafter referred to as kelps) represent good candidates for toxicity tests as they grow throughout the year and have a large geographical distribution, including along the Mediterranean coast, the Atlantic coast of Europe, Australia, Argentina, and Mexico (Bolton 2010). The endpoints applied in bioassays using kelps include the initial step of the life cycle, germ tube length, gametophytes growth, and production of young sporophytes (Fang et al. 1982, Lee et al. 1989; Anderson et al. 1997; Lee and Kang 2002; Verlaque 2001; Burridge and Bidwell 2002; Myers et al. 2006; Seery et al. 2006; Selivanova et al. 2007; Han et al. 2011; Park et al. 2016). In particular, the early life cycle of brown algae is considered to be excellent for evaluation because this stage is sensitive to a variety of toxic materials and the analysis requires relatively simple technical procedures (Burridge and Bidwell 2002).

However, one drawback of the methodology is the limitation of possible spore procurement due to spore-bearing thalli being available for limited periods of time. This factor must be overcome if these early developmental stages are to be used for routine toxicity testing.

Recent studies using microalgae such as the green alga *Scenedesmus subspicatus* (Sphaeropleales, Chlorophyta) have highlighted the potential of using immobilization technology to overcome issues associated with maintaining laboratory cultures (Awasthi and Rai 2005; Corrêa et al. 2009). These techniques have been used in toxicity bioassays mainly using green microalgae. Zhang et al. (2012) evaluated a toxicity of sediments, spiked with Cu or diuron, using immobilized green microalga *Pseudokirchneriella subcapitata* (72 h growth inhibition), Wang et al. (2013) reported the effects of combined mixed polycyclic aromatic hydrocarbons (PAHs) and heavy metals on growth and antioxidant responses of immobilized *Selenastrum capricornutum* and Peña-Vázquez et al. (2010) described a protocol for testing the toxic effects of Cu on chlorophyll *a* fluorescence of immobilized *Dictyosphaerium chlorelloides*.

As an indicator of the environmental stress of plants and algae, chlorophyll *a* fluorescence is becoming more and more popular (Seery et al. 2006; Jianrong and Qiran 2009; Kumar et al. 2009; Peña-Vázquez et al. 2010; Kottuparambil et al. 2013; Kumar et al. 2014). A variety of fluorescence parameters are used as ecotoxicological endpoints, including F_v/F_m (maximum quantum yield of photosystem II (PSII)), F_v'/F_m' (effective quantum yield of PSII), rETR (the relative electron transport rate), NPQ (non-photochemical quenching), and so on. The F_v/F_m ratio represents the maximum quantum yield of the photochemical process of the photosystem, i.e., the relative efficiency of PSII capture of light energy and is one of the most common parameter of stress in algae and plants.

This study presents a new method testing the toxicity of some of the primary cosmetic ingredients including methylparaben (MP), sodium dodecyl sulfate (SDS), triethanolamine (TEA), and 2-phenoxyethanol (2-PE) by using gametophytes of *Undaria pinnatifida* (Harvey) Suringar (Laminariales, Phaeophyta) immobilized in beads composed of calcium alginate, a polysaccharide extracted from brown seaweeds. The test endpoint is photosynthetic performance, as determined from measurements of the maximum quantum yield of PSII by variable chlorophyll *a* fluorescence (Chl *a*).

Traditional test organisms for toxicity test of these cosmetic compounds are protozoa (*Tetrahymena thermophila*, cell enumeration, 24–28 h exposure), invertebrate (*Daphnia magna* and *Artemia franciscana*, immobilization, 24–48 h), bacteria (*Vibrio fisheri* and *Photobacterium leiognathi*, luminescence, 15–30 min), fish (*Pimephales promelas*, survival, 48 h), and algae (*Pseudokirchneriella subcapitata* and *Phaeodactylum tricornutum*, growth inhibition, 72 ± 2 h; Van der Plassche and Balk 1997; Van Dijk 1997; Libralato et al. 2010; Brausch and Rand 2011; Yamamoto et al. 2011).

Materials and methods

Gametophyte preparation and culture

Gametophytes of *Undaria pinnatifida* were acquired from the National Institute of Fisheries Science (Busan, Republic of

Korea) and maintained in axenic batch cultures in 500 mL round-bottom flasks filled with 300 mL Provasoli enriched seawater (PES; Provasoli 1968). The cultures were incubated in a temperature-controlled chamber (10 °C) under 12:12 h L/D cycle using fluorescent lamps with a light intensity of 10 μmol photons m^{-2} s^{-1}.

Immobilization of *Undaria* gametophytes

Gametophytes were immobilized in beads of calcium alginate. Sodium alginate (Sigma-Aldrich, CAS number 9005-38-3) was dissolved with PES medium to form a 5% (*w*/*v*) solution, which was autoclaved and cooled to room temperature. Then, the alginate solution was mixed with an algal suspension of known cell density (0.08 g fresh weight (FW) mL^{-1}). After thorough mixing, aliquots of this mixture were transferred into a 50-mL burette and extruded dropwise into a 0.1 M calcium chloride solution, from a height of approximately 15 cm at a rate of one drop per second (Fig. 1). The burette was kept full of the alginate-algae mixture to ensure constant flow and homogeneity of bead sizes (3 mm diameter). The beads were stirred in calcium chloride solution for a minimum of 60 min to allow gel hardening to occur. Then, the beads were washed with distilled water, stored in PES medium in the dark at 4 °C, and used within 1 day of preparation.

Determination of optimal culture conditions

The algal beads were distributed into 24-well plates (SPL Life Sciences, Republic of Korea) with fresh medium and cultured under different environmental conditions for 6 h, including irradiance (0–120 μmol photons m^{-2} s^{-1}), pH (4–9), salinity (0–65), and temperature (5–25 °C).

Toxicity testing procedure

The algal beads were placed in test cell plates with different toxicant concentrations. Static cultures were established at 10 °C under dark condition. The controls consisted of the artificial seawater medium without toxicants. The toxicity of four cosmetic ingredients (MP (Duksan, CAS number 99-76-3), SDS (Sigma-Aldrich, CAS number 151-21-3), TEA (Samchun Chemicals, CAS number 102-71-6), and 2-PE (Junsei Chemical, CAS number 122-99-6)) was investigated. Appropriate volumes of stock solution were added to obtain the final nominal concentrations (wt/vol): 0.4% MP, 1% 2-PE, 5% TEA, and 1% SDS. After 30, 60, 90, 120, 240, and 360 min of exposure, the maximum quantum yield of the gametophytes was measured with an imaging pulse-amplitude-modulated fluorometer (Imaging-PAM; Walz, Germany). Toxicity tests were conducted with a concentration series generated by dilution with the artificial seawater medium to produce the desired concentration range (1, 0.5, 0.25, 0.125, and 0.0625% (vol/vol) of the original solution). The pH and salinity of the test solutions were 6.9–8.3 and 30–35 PSU, respectively, at the start and end of the exposure period.

PAM fluorometry

The PAM measurement was initiated by exposing a dark-adapted sample to modulated light (1 μmol photons m^{-2} s^{-1}) for 15 min to obtain the minimal level of fluorescence (F_0). Then a saturating flash of light (3598 μmol photons m^{-2} s^{-1} for 0.2 s) was applied. This flash resulted in a reduction of all primary electron-acceptor sites, which allowed measurement of the maximal fluorescence yield (F_m). The F_0 and F_m measurements permitted evaluation of the variable fluorescence (F_v), which enabled calculation of the maximum quantum yield (Φ_m).

$$\Phi_m = F_v/F_m = (F_m - F_0)/F_m \quad (1)$$

This yield is a measure of the maximum photochemical efficiency of PSII.

Data analysis

The effect of the different exposure concentrations of the beads on the maximal quantum yield of the immobilized *U. pinnatifida* was evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's multi-comparison test. Dunnett's test was used to compare the means of each treatment with the mean of the control group. This procedure uses simulations to analyze the power and significance level of multiple-comparison procedures by performing two-sided hypothesis tests of each treatment group mean versus the control group mean. For the cosmetic toxicity experiment, the maximum quantum yield was compared using one-way ANOVA. Statistical significance was established at $p \leq 0.05$. The results are reported as the EC_{10} and EC_{50} (effective concentrations at which 10 and 50% inhibition occurs, respectively), NOEC (concentration with no observed), and LOEC (concentration with lowest observed effect) values with 95% confidence intervals estimated by the linear interpolation method (ToxCalc 5.0, Tidepool Science, USA). 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.

Results and discussion

Before conducting the toxicity tests, the optimal conditions (irradiance, pH, salinity, and temperature) for culturing the immobilized gametophytes of *U. pinnatifida* were established (Fig. 1). The highest value of the maximum quantum yield

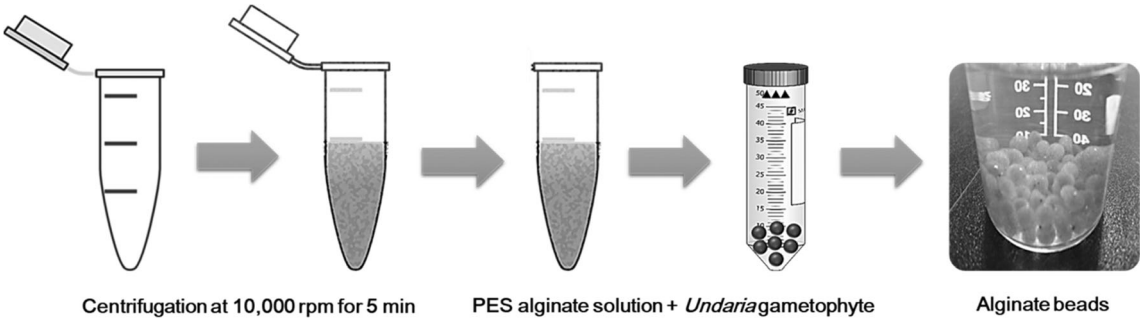

Fig. 1 A schematic diagram showing the preparation of immobilized gametophyte cells of *Undaria pinnatifida* in alginate beads

(F_v/F_m) of *U. pinnatifida* was found in the dark condition. The F_v/F_m decreased when the irradiance increased from 10 to 120 μmol photons m^{-2} s^{-1}. When the photosynthetic performance of *U. pinnatifida* was measured at six different irradiances, the highest F_v/F_m value (0.573, 95% CI) was found in the dark condition. *Undaria pinnatifida* grew well in the irradiance range 10 to 80 μmol photons m^{-2} s^{-1}. However, the maximum quantum yield of *U. pinnatifida* algal beads in static cultures was not affected by different irradiances over the range of 10–120 μmol photons m^{-2} s^{-1}, or even by the presence and/or absence of light. In macroalgal species, early development stages appear to often occur in the dark (Han et al. 2011). This physiological trait may be related to the long-term (several months to years) survival of gametophytic stages in the dark (Lüning 1990).

The F_v/F_m values did not vary significantly under the five different pH conditions (pH 5–9) tested (Fig. 2). Gibbon and Kropf (1991) reported that a low pH inhibited rhizoid elongation in the brown alga *Pelvetia fastigiata*, the pH-induced inhibition was assumed to occur as a result of trans-cellular pH gradients in the medium surrounding the developing *Pelvetia* zygotes and the alkalization of the future rhizoid end of the cell. In the pH experiments, only the photosynthetic performance was measured, the photosynthetic ability of *U. pinnatifida* was as follows: pH 4 was inhibitory, whereas there was no inhibition at pH 5–9. Similarly, there was no significant difference in F_v/F_m over a broad range of salinities (0–65 PSU). Salinity is one of the primary factors that determine the growth, reproduction, and distribution of seaweeds (Connan and Stengel 2011). However, the salinity tolerance of kelp gametophytes is not well known; only one early study reported the salinity necessary for the growth of adult sporophytes of *U. pinnatifida* (Saito 1975).

The highest F_v/F_m was recorded at 5 °C (0.56; 95% CI) and values significantly decreased with increasing temperature up to 25 °C (Fig. 1). This result contrasts with previous studies that report *U. pinnatifida* as having a broad range of temperature tolerance, e.g., for germination occurring (13–25 °C), growth of filamentous gametophytes (10–25 °C), and growth of sporophytes (3–20 °C) (Sinner et al. 2000; Morita et al. 2003; Gao et al. 2013). In the chlorophyll fluorescence analysis, the decrease in the dark-adapted F_v/F_m value indicated the occurrence of photoinhibitory damage in response to environmental stress. In many experiments, the maximum photochemical efficiency (F_v/F_m) has been used as an indicator to monitor a healthy photosynthetic condition. Under the six different irradiances conditions included in the present study, a significant decrease in F_v/F_m was observed for irradiances higher than darkness. These results reveal that irradiances higher than darkness are not suitable for toxicity test with our materials.

The photosynthetic yield of immobilized gametophyte cultures under controlled laboratory conditions demonstrated the suitability of using *U. pinnatifida* cells encapsulated in calcium alginate beads for cosmetic toxicity testing. Determination of the basic requirements and properties of the ideal matrix for the immobilized algae requires many experiments using the entrapped cells, including assessments of the viability, photosynthetic ability, impact of high cell density, growth stability, continuous productivity ability, and proof of immobilization superiority, compared to free cells. Algal cells maintain their respiratory and photosynthetic activities during immobilization.

In an early study, Klaine and Lewis (1995) introduced six different types of immobilization methods such as entrapment, affinity immobilization, adsorption, confinement in liquid-liquid emulsion, capture behind semipermeable membrane, and covalent coupling. The first report involving a study of immobilized algae used chemically fixed *Chlorella* cells to measure the Hill reaction (Ku et al. 1974). Subsequently, Hallier and Park (1969) showed that glutaraldehyde immobilized *Anacystis nidulans*, *Porphyridium cruentum*, and *Chlorella pyrenoidosa* could perform light-dependent O_2 production in the presence of suitable electron acceptors. Uses of immobilized algae include culture for metabolite production, improvement of culture collection handling, obtaining energy (via H_2 or electricity power), nutrients, metal or organic pollutant removal from aquatic media, measurements of toxicity, and co-immobilization system production for different purposes (Table 1).

Microalgae have been found to be sensitive organisms to different pollutants (León et al. 2001; Miazek et al. 2015) in

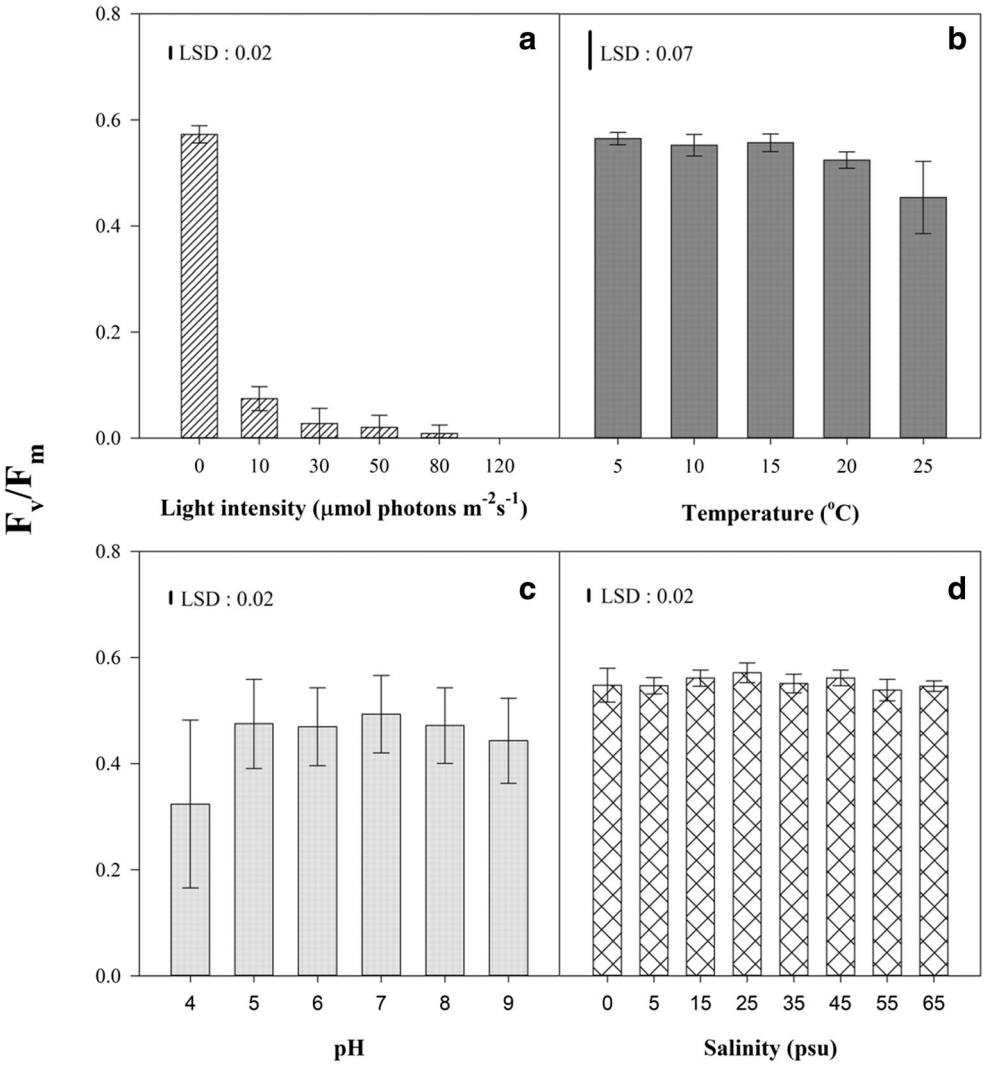

Fig. 2 Effects of light intensity (**a**), pH (**b**), salinity (**c**), and temperature (**d**) on the maximum quantum yields of immobilized gametophytes of *Undaria pinnatifida*. The bar denotes the least significant difference (LSD) at the 5% level, and each error bar indicates the 95% confidence interval

toxicity bioassays, possibly due to their high surface/volume ratio. Their key role in freshwater and marine aquatic trophic nets indicates the necessity of developing suitable toxicity tests for inclusion as efficient tools for researchers and authorities when required. In situ experiments have been designed to increase the environmental relevance of toxicity tests (Santos et al. 2002; Santos et al. 2004; Connon et al. 2012), including avoidance of manipulation of samples carried to the laboratory and maintenance of natural light, temperature, or pH fluctuations. In a pioneering work, Bozeman et al. (1989) compared the toxicity of seven pollutants of different origins (cadmium, copper, glyphosate, hydrothol, paraquat, pentachlorophenol, and SDS) to free and immobilized cells of the green microalga *Selenastrum capricornutum* (currently *Pseudokirchneriella subcapitata*) and, suggested the possibility of using of immobilized systems for in situ toxicity experiments (Bozeman et al. 1989). Following that study, differences in toxicity for free and immobilized algae have been found to vary from no significant differences for copper and pentachlorophenol, to nearly four times more sensitivity for free cells in the case of glyphosate or paraquat. Admiraal et al. (1999) experimented with sand and natural glass-attached microbenthic assemblages of algae and bacteria in a metal-polluted stream in the Dommel River (Belgium). The authors investigated the sensitivity of those assemblages to zinc and found different sensitivities in the function of the origin of the assemblages (i.e., the most polluted origin had a lower sensitivity). Protection against toxicity in immobilized cells has been reported in different works. For instance, Awasthi and Rai (2005) demonstrated lower inhibition of nitrate uptake in immobilized *S. quadricauda* than in free cells when exposed to Ni, Zn, or Cd, and Perullini et al. (2014) showed that the immobilization technique protects the cyanobacteria by preventing direct contact of toxic solvents.

This study did not perform metal measurements in media. The simplest explanation is the removal of a portion of the metals by the entrapping matrix, which makes less metal available for the cell. However, the removal of toxicants by fixed matrixes would not account for all cases of reduced toxicity in immobilized cells. Surfactants are not selectively

Table 1 Summary of immobilized algae and their use

Algal genera	Immobilizing matrix	Use		Reference
Haematococcus pluvialis (Chlorophyta)	Ca-alginate	Culturing for metabolite production	Biotransformation of phenyl propanoid compounds	Tripathi et al. (2002)
Chlamydomonas reindharti (Chlorophyta)	Ba-alginate		Photoproduction of ammonium	Santos-Rosa et al. (1989)
Porphyridium cruentum (Rhodophyta)	Urethane pre-polymer		Production of polysaccharides	Thepenier et al. (1985)
Scenedesmus quadricauda (Chlorophyta)	Ca-alginate	Culture collection handling	3 years of storage	Chen (2001)
Isochrysis galbana (Haptophyta)	Ca-alginate		1 year of storage	Chen (2003)
Phaeodactylum tricornutum (Heterokontophyta)	Ca-alginate		1 year of storage	Hertzberg and Jensen (1989)
Cyanophytes and eukaryotic algae	2% agar		32 months of storage	Lukavsky (1988)
Dunaliella bardawil, *Chlorella minutissima*, *Pavlova lutheri*, *Haematococcus pluvialis* (Chlorophyta)	Ca-alginate		Highly dense cultures	Joo et al. (2001)
Chlorella vulgaris (Chlorophyta)	Ca-alginate	Nutrient removal	Nitrate and phosphate	Mallick and Rai (1993)
Nannochloris sp. (Chlorophyta)	Ca-alginate		Removal of macronutrients	Jiménez-Pérez et al. (2004)
Dunaliella salina (Chlorophyta)	Ca-alginate		Nitrate, ammonium, phosphate	Thakur and Kumar (1999)
Ascophyllum nodosum (Heterokontophyta)	Hypol pre-polymer	Metal removal	Copper	Alhakawati and Banks (2004)
No use	Ca-alginate		Cu, Co	Jang et al. (1995b, c)
No use	Ca-alginate		Cu	Nestle and Kimmich (1996)
Microcystis sp. (Cyanobacteria)	Ca-alginate		Cu	Jang et al. (1995a)
Cuscuta salina (Chlorophyta)	Ca-alginate		Co, Mn, Zn	Garnham et al. (1992)
C. vulgaris (Chlorophyta)	Ca-alginate, agarose immobilized		Biosorption of Cu	Aksu et al. (1998)
Nannochloropsis gaditana (Heterokontophyta)	Ca-alginate		Cu, Zn	Moreno-Garrido et al. (2002)
Chlorella sorokiniana (Chlorophyta)	Loofa sponge		Ni	Akhtar et al. (2004)
Tetraselmis chuii (Chlorophyta)	Ca-alginate		Cu, Cd	Moreno-Garrido et al. (2005)
C. vulgaris (Chlorophyta)	Ca-alginate		Au	Gee and Dudeney (1987)
C. sorokiniana (Chlorophyta)	Sodium salicylate	Organic pollutants removal	Cu(II) biosorption	Munoz et al. (2006), Munoz and Guieysse (2006)
Pseudokirchneriella subcapitata (Chlorophyta)	Ca-alginate	Measuring toxicity	Seven pollutants of different origin (Cd, Cu, glyphosate, hydrothol, paraquat, penta chlorophenol, and sodium dodecyl sulfate)	Bozeman et al. (1989)
Scenedesmus quadricauda (Chlorophyta)	Ca-alginate		Ni, Zn or Cd	Awasthi and Rai (2005)
Phaeodactylum tricornutum (Heterokontophyta)	Ca-alginate		Lineal alkylbenzene sulfonate (LAS)	Moreno-Garrido et al. (2007)
Scenedesmus subspicatus (Chlorophyta)	Ca-alginate		Eutrophication in surface waters	Twist et al. (1997)
Selenastrum capricornutum (Chlorophyta)	2% agar		Cr^{6+} toxicity	Lukavský and Maršálek (1997)
S. subspicatus (Chlorophyta)	Alginate		Pollutants in water and soil extracts	Frense et al. (1998)
C. vulgaris (Chlorophyta)	Glass micro fiber filter		Atrazine, simazine and diuron	Naessens et al. (2000)

adsorbed by Ca-alginates. It was found that the immobilized cells of *P. tricornutum* were less affected than free cells when exposed to sediments containing surfactant linear alkylbenzen sulfonate (LAS) (Moreno-Garrido et al. 2007). The low diffusion of toxic substances in beads can partially explain the low toxicity to immobilized cells (Jang 1994). Twist et al. (1997) developed a Ca-alginate immobilized biomonitoring technique using *S. subspicatus* for the evaluation of eutrophication. The advantage of this technique is that local flora can be applied to the biomonitoring. However, Ca-alginate of beads might be degraded within a few days or a couple of weeks in marine and freshwater environment, respectively (Lukavský and Maršálek 1997).

Several types of biosensors related to microalgae cells were designed to detect environmental contaminants. Lukavský and Maršálek (1997) evaluated Cr^{6+} toxicity using immobilized *S. capricornutum*. The sensitivity was similar to that reported in other bioassays, including growth inhibition tests. *Chlorella vulgaris* has also been used in optical biosensors to determine the toxicity of herbicides, such as atrazine, simazine, and diuron, which are commonly used in cereal cultures (Naessens et al. 2000). The limitation of toxicity testing using free or immobilized microalgae is restriction to toxicants that affect structures present in algal cells. Thus, pollutants affecting bone development or the nervous system will not be easily detected using microalgal-based bioassays. However, toxicants affecting photosynthesis (such as copper ions or herbicides) will be more appropriately detected with algal toxicity bioassays.

Seery et al. (2006) used two parameters (photosynthetic effective quantum yield and germination) for testing antifouling substances (diuron and irgarol 1051) using brown macroalga *Hormosira banksii* gametes. Whereas *H. banksii* gamete germination was inhibited in 48 h, the 50% inhibition on photosynthetic effective quantum yield occurs in 2 h.

The protocol for the bioassay method used here based on the photosynthetic yield of the brown macroalga *U. pinnatifida* is summarized in Table 2. The derived toxicity values (EC_{10}, EC_{50}, NOEC, and LOEC) are listed in Table 3. Clearly, the test algal beads showed different responses to the different cosmetic ingredients. Response curves showing the effects of cosmetic ingredients on the F_v/F_m of the immobilized beads are illustrated in Fig. 3. The EC_{10}, EC_{50}, NOEC, and LOEC values under the MP and 2-PE treatments were consistent throughout the exposure period. For SDS, the EC_{10}, EC_{50}, NOEC, and LOEC values decreased with the increased exposure time. Interestingly, the EC_{10} and EC_{50} values for the TEA treatment decreased as the exposure time increased (> 5% at 15 min to 1.4195% at 360 min for EC_{10}, and > 5% [15 to 120 min] to 3.7023% at [360 min] for EC_{50}), whereas the NOEC (1.25%) and LOEC (2.5%) values were consistent over the exposure time.

Table 2 Summary of *Undaria* gametophyte bead test conditions

Test type	Static, non-renewal
Test endpoint	Optimal quantum yield
Temperature	5 °C
Salinity	30–35 PSU
Irradiance	0 μmol photons m^{-2} s^{-1}
Test vessel	24-well cell plate
Test solution volume	2.5 mL
Number of algal bead/vessel	1
Dilution/sample	10
Dilution water	Artificial seawater
Test duration	6 h
Renewal of test solution	None
Aeration	None
Culture media	Provasoli enriched seawater (PES)
Number of replicates	$n = 6$

Parabens, which have a broad antibacterial function, have been widely used as preservatives in cosmetics, food, and medicines over the last 50 years. Because in vivo and in vitro risks of MP have been shown in humans (Frederiksen et al. 2011; Kang et al. 2013; Watkins et al. 2015) and animals (Soni et al. 2002; Popa et al. 2011), the Cosmetic, Toiletry, and Fragrance Association (CTFA) permits parabens up to 0.8% based on a mixture and 0.4% of a single component; in general cosmetics, the limited concentration of parabens has been reported to be 1.776% in adults and 0.0378% in early childhood (CTFA 2005). MP is one of the homologous series of the methyl ester of p-hydroxybenzoic acid, and parabens (methyl, ethyl, butyl, hexyl, and benzyl paraben) are the most commonly used preservatives in cosmetics (Mowad 2000). These compounds hydrolyze in warm/cold water and are colorless, odorless, resistant (available sterile), stable over a pH range, non-volatile, and antibacterial. According to the US Food & Drug Administration (FDA), MP is widely used in 8786 different products. The EC_{50s} (with 95% CI) of MP toxicity obtained from this study were 0.0634% (0.0576–0.0697), which is less sensitive than an invertebrate *Daphnia magna* (0.001–0.006% for immobilization, 48 h) (Terasaki et al. 2009; Yamamoto et al. 2011; Lee et al. 2017) and the *P. subcapitata* (0.002–0.009% for growth inhibition, 72 h) (Yamamoto et al. 2011; European Chemicals Agency 2016). However, when compared to the LD_{50} (0.84%, oral, 6 h) of mice with the same exposure time (Bionetics 1974), *U. pinnatifida* beads showed about 13 times higher sensitivity.

2-PE is a fragrance ingredient used in many products, including advanced perfume, shampoo, soap, and other toiletries, and can be easily found a flavor ingredient in daily products, such as cosmetics, household detergents, and surfactants. 2-PE is a type of viscous oily liquid with a faint rose scent, is mainly found in plants such as avocado and mango and can be found in abundance in nature (VCF 1963). The allowed 2-PE

Table 3 EC_{10}, EC_{50}, NOEC, and LOEC values for inhibition of F_v/F_m in gametophyte beads of *Undaria pinnatifida* exposed to four different cosmetic ingredients for 6 h

Toxicants		Exposed time					
		15 min	30 min	60 min	120 min	240 min	360 min
Methyl paraben (%)	EC_{10} (95% CI)	0.0614 (0.0505–0.0709)	0.0233 (0.0198–0.0278)	0.0164 (0.0111–0.0213)	0.0123 (0.0098–0.0163)	0.129 (0.0100–0.0158)	0.0122 (0.0085–0.0155)
	EC_{50} (95% CI)	0.3102 (0.2798–0.3315)	0.1648 (0.1451–0.1897)	0.1158 (0.1038–0.1254)	0.0910 (0.0723–0.1136)	0.0741 (0.0657–0.0872)	0.0634 (0.0576–0.0697)
	NOEC	0.05	0.0125	0.0125	0.0125	0.0125	0.0125
	LOEC	0.1	0.025	0.025	0.025	0.025	0.025
	CV (%)	3.27	5.74	3.58	9.87	5.75	3.83
2-Phenoxy ethanol (%)	EC_{10} (95% CI)	0.1105 (0.0398–0.1780)	0.0673 (0.0381–0.0833)	0.0753 (0.0596–0.0865)	0.0741 (0.0587–0.0856)	0.0683 (0.0372–0.0875)	0.0738 (0.0175–0.0873)
	EC_{50} (95% CI)	0.7626 (0.6595–0.8413)	0.3996 (0.2819–0.4595)	0.3101 (0.2329–0.3669)	0.2747 (0.2348–0.3018)	0.2511 (0.2139–0.2974)	0.2418 (0.1955–0.2785)
	NOEC	0.0625	0.03125	0.0625	0.0625	0.015625	0.0625
	LOEC	0.125	0.0625	0.125	0.125	0.03125	0.125
	CV (%)	4.42	8.83	8.64	5.21	6.59	7.57
Sodium dodecyl sulfate (%)	EC_{10} (95% CI)	0.0575 (0.0174–0.1547)	0.0009 (0.0005–0.0209)	0.0010 (0.0006–0.0062)	0.0044 (0.0028–0.0053)	0.0039 (0–0.0043)	0.0027 (0–0.0045)
	EC_{50} (95% CI)	0.5474 (0.3169–0.8432)	0.2691 (0.0932–0.4021)	0.0602 (0.0206–0.0795)	0.0129 (0.0098–0.0171)	0.0067 (0.0057–0.0085)	0.0060 (0.0055–0.0067)
	NOEC	0.125	< 0.0009	0.0039	0.0039	0.0019	0.0019
	LOEC	0.25	0.0009	0.0078	0.0078	0.0039	0.0039
	CV (%)	23.62	20.14	22.32	12.44	7.57	3.87
Triethanol amine (%)	EC_{10} (95% CI)	> 5	> 5	2.7174 (0–3.4624)	1.7430 (0.0371–2.9697)	1.6668 (1.1540–1.9674)	1.4195 (0.3128–1.6487)
	EC_{50} (95% CI)	> 5	> 5	> 5	> 5	4.3739 (4.1383–4.5296)	3.7023 (3.1815–3.9876)
	NOEC	2.5	2.5	0.078125	0.3125	1.25	1.25
	LOEC	5	5	0.15625	0.625	2.5	2.5
	CV (%)	–	–	26.47	37.12	1.80	4.12

concentration in the fine fragrance consumer product is 4.09%, and in the formulation of cosmetics is 1% (IFRA 2004). Regarding EC_{50} values of PE, the result based on F_v/F_m (0.242%) in this study was less sensitive than the growth inhibition of *P. subcapitata* (< 0.013%, Tamura et al. 2012). SDS is an anionic surfactant that is often used in household cleaning products (laundry detergents, spray cleaners, detergents, and dishwasher detergents), emulsifying agents, and cleaning agents. Generally, the SDS concentration ranges from 0.01 to 50% in cosmetics and from 1 to 30% in cleaning products (CIR 2005). Consumers are easily exposed to products containing SDS, particularly cleaning products, and are usually exposed one to two times on average, depending on the frequency of cleaning. There is a potential risk of dermal infections (skin and eyes) or inhalation by abuse of products and through exposure to some surfactants and detergents, although not by direct contact or absorption. Consumer concerns about the safety and toxicity of SDS began in the early 1990s. In an evaluation of SDS toxicity using weights and deposition of shells with *Physa heterostropha* and *Lymnaea vulgaris* as indicators, the EC_{50} value of weight was clearly reduced after 6 days of exposure to SDS (Tarazona and Nuñez 1987). The EC_{50} of SDS was reported: 0.0107% (0.00532–0.01434) for sporulation inhibition of the green alga *Ulva pertusa* for 96 h (Han and Choi 2005), 0.0002–0.0004% for germination inhibition of *U. fasciata*, 0.0002–0.0009% and 0.0002–0.0007% for fertilization inhibition and embryo development inhibition of sea urchin *Echinoidea* sp., respectively (Hooten and Carr 1998). In this study, the EC_{50} value (95% CI) obtained was 0.0060% (0.0055–0.0067).

TEA is used as a neutralizing agent or pH adjuster with other surfactants in various cosmetics, including skin lotion, eye cream, moisture cream, and shampoo (Kim et al. 2003; Gottschalck and Bailey 2010). The TEA concentration usually ranges between 0.0002 and 19% (Fiume et al. 2013). In the case of the use of trialkanolamine as a salt form, the allowed concentration of the formulation is defined as 2.5%, but there is no particular permitted standard for the TEA formulation

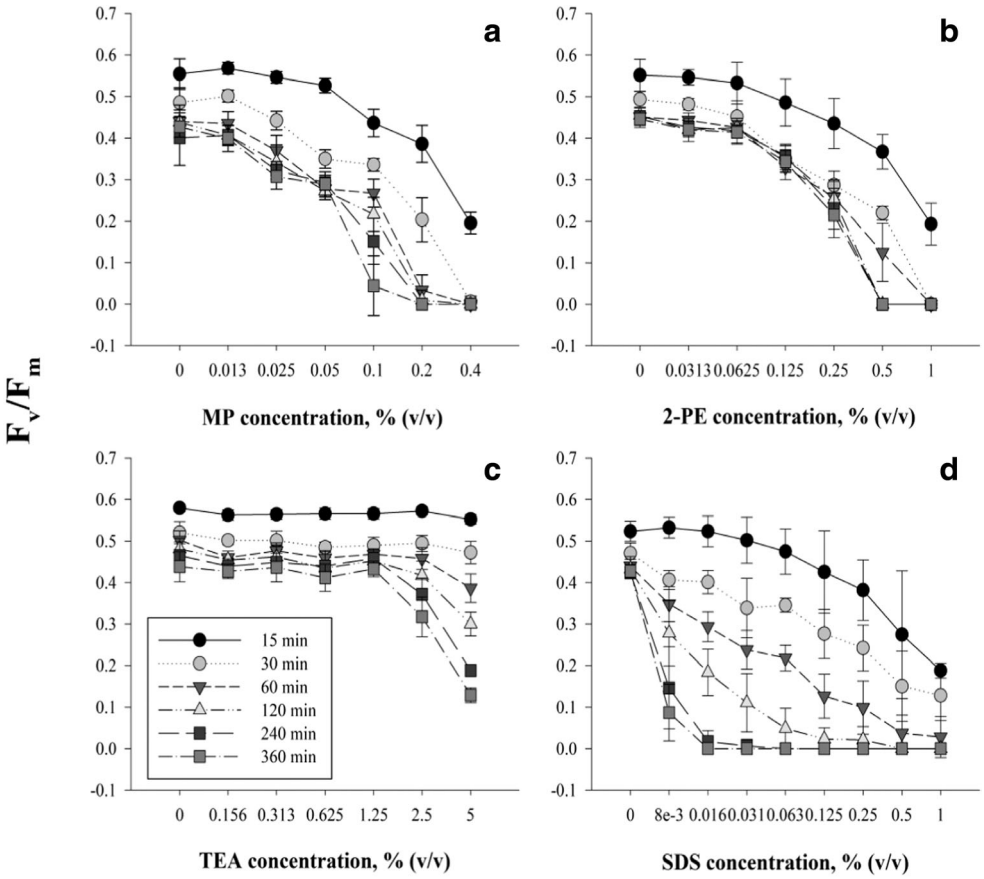

Fig. 3 Dose-response of the maximum quantum yield in immobilized gametophytes of *Undaria pinnatifida* to four cosmetic ingredients. **a** MP, **b** 2-PE, **c** TEA, **d** SDS. Data are presented as mean values and standard deviations (SD, $n = 6$). MP, methylparaben; 2-PE, 2-phenoxyethanol; TEA, triethanolamine; SDS, sodium dodecyl sulfate

(Fiume et al. 2013). According to the Ministry of Health and Welfare Canada, the concentrations of TEA used in some cosmetics range between 10 and 30%, and some allowable concentration standards range between 30 and 100% (Health Canada, personal communication). To determine the toxicity of TEA, acute blood toxicity experiments with rabbits used as the test method reported no fatal toxicity, whereas only slight stimulations were caused by 6 h of exposure (Gamer et al. 2008). Conversely, skin absorption and inflammation were reported to occur, which affected the lungs, liver, and kidneys, and even some cancers were reported in chronic toxicity tests (Konish et al. 1992; DePass et al. 1995). EC_{50}s (with 95% CI) of TEA were 3.7023% (3.1815–3.9876% in this study. In a study on the luminescent bacterial toxicity test with *V. fisheri*, EC_{50} derived from inhibition of luminescent was 0.011% (Gordon 1992) and in the alga *P. tricornutum* the EC_{50}s showed 0.011–0.030% for growth inhibition (Libralato et al. 2010).

Conclusions

Many ingredients used in personal care products are becoming of increasing environmental concern since a significant amount of these compounds enter and are persistent in the aquatic environment (Vita et al. 2018). On the other hand, there has been little research assessing the environmental effects of cosmetic compounds even if they are used in greater amount than pharmaceutical compounds (Brausch and Rand 2011).

The animal test was the most commonly employed test in toxicological studies of cosmeceutical compounds. However, the use of animal tests for assessment of human and environmental risks has been completely banned in Europe since 2013 (Boxall et al. 2012).

When developing innovative and standardized test procedures, several important criteria must be met. The test method (1) should be sensitive to toxicants and simple to conduct, (2) should be suitable for general laboratories with limited space and equipment, (3) should exhibit little variability in the test results, and (4) biological samples should be available throughout the year.

We propose a simple, efficient, and reliable alternative method to assess the toxicity of cosmetic ingredients using gametophytes of the brown seaweed *U. pinnatifida*. Our study demonstrates the high sensitivity of *U. pinnatifida* gametophytes to a panel of four cosmetic ingredients. The total working time required for preparing and conducting the test is 6–7 h for one person, and this test requires only 1–2 m^2 of working space. Importantly, dark incubation was introduced for this testing instead of light incubation, which removed the absolute requirement of using culture chambers with proper

lighting systems. Thus, simply leaving cell plates containing test sample sets in a closed space with no light or wrapped in foil is sufficiently provided that the temperature can be maintained at 10 °C for 24 h prior to taking measurements. In addition, *U. pinnatifida* represents an important component of marine ecosystems and is commercially cultivated in large areas with aquaculture beds in China, Japan, and Korea. Moreover, this species is an invasive seaweed in many coastal locations over a broad geographic range, which assures the availability of samples.

The application of immobilization technology to maintain *U. pinnatifida* gametophytes in a bead state would also guarantee both the possibility of conducting year-round testing and a maintenance-free system. Toxicity assays using *Undaria pinnatifida* gametophyte beads are ecologically meaningful and reliable and can be used to assess the biological effects of cosmetic ingredients using a reproducible toxicological protocol.

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