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Community-Level Analysis of *psbA* Gene Sequences and Irgarol Tolerance in Marine Periphyton▽

K. M. Eriksson,[1]* A. K. Clarke,[1] L.-G. Franzen,[2] M. Kuylenstierna,[3] K. Martinez,[4] and H. Blanck[1]

Department of Plant and Environmental Sciences, University of Gothenburg, Box 461, SE-405 30 Göteborg, Sweden[1]; Halmstad University, Box 823, SE-301 18 Halmstad, Sweden[2]; Department of Marine Ecology, University of Gothenburg, Sven Lovén Centre for Marine Sciences Kristineberg, SE-450 34 Fiskebäckskil, Sweden[3]; and Spanish National Research Council—IIQAB-CSIC, Department of Ecotechnology, Josep Pascual Vila, Jordi Girona 18-26, 08034 Barcelona, Spain[4]

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This study analyzes *psbA* gene sequences, predicted D1 protein sequences, species relative abundance, and pollution-induced community tolerance in marine periphyton communities exposed to the antifouling compound Irgarol 1051. The mechanism of action of Irgarol is the inhibition of photosynthetic electron transport at photosystem II by binding to the D1 protein. The metagenome of the communities was used to produce clone libraries containing fragments of the *psbA* gene encoding the D1 protein. Community tolerance was quantified with a short-term test for the inhibition of photosynthesis. The communities were established in a continuous flow of natural seawater through microcosms with or without added Irgarol. The selection pressure from Irgarol resulted in an altered species composition and an inducted community tolerance to Irgarol. Moreover, there was a very high diversity in the *psbA* gene sequences in the periphyton, and the composition of *psbA* and D1 fragments within the communities was dramatically altered by increased Irgarol exposure. Even though tolerance to this type of compound in land plants often depends on a single amino acid substitution (Ser_{264}→Gly) in the D1 protein, this was not the case for marine periphyton species. Instead, the tolerance mechanism likely involves increased degradation of D1. When we compared sequences from low and high Irgarol exposure, differences in nonconserved amino acids were found only in the so-called PEST region of D1, which is involved in regulating its degradation. Our results suggest that environmental contamination with Irgarol has led to selection for high-turnover D1 proteins in marine periphyton communities at the west coast of Sweden.

Development of tolerance, or resistance, to anthropogenic toxicants released into the environment is an issue of increasing importance. Reports of tolerant organisms are increasing, and tolerance toward a variety of compounds has been found (22, 44, 46, 100). This is essentially evolution in action and shows that toxicants can act as selective pressures in the environment. Even though high concentrations of toxicants can be released in episodes or pulses, the overall environmental concentrations of toxicants are often quite low, which implies that adaptation and nonlethal selection are more common than acute and/or lethal effects.

Since sensitivities to a given toxicant differ within species and even more so between species (12), a toxicant-induced succession (TIS) will occur in toxicant-exposed communities, where sensitive species, individuals, or genotypes are replaced by more tolerant ones, giving an increase in average tolerance in the community. This chain of events is fundamental for the pollution-induced community tolerance (PICT) concept (14). PICT studies can be performed in natural or model ecosystems and have the important advantage of a causal link between exposure and effect. The concept has been used to demonstrate long-term selection pressure from toxicants on several types of communities, as reviewed by Blanck (10) and Boivin et al. (15). Periphyton communities (110) are attached to submerged surfaces and consist of a food web of interacting organisms. Our focus in this paper is on the eukaryotic and prokaryotic oxygenic primary producers, microalgae and cyanobacteria.

Toxicants used as antifouling compounds are made to prevent the growth of organisms on ship hulls and underwater installations. Irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) is used to eliminate algae and cyanobacteria, and since it acts as a triazine-type photosystem II (PS II) inhibitor, it is less toxic to heterotrophic organisms (42). These inhibitors replace the native plastoquinone at the Q_B-binding niche in the D1 protein within PS II (29, 72, 86, 105). This results in various toxic effects like inhibition of photosynthetic electron transport, which in turn leads to reduced ATP and NADPH production (25, 29) and oxidative stress due to the accumulation of reactive oxygen species (ROS) at PS II (29, 31, 85, 89, 91).

Several authors have previously reported increased community tolerance to Irgarol in freshwater communities (8, 79) and to other PS II inhibitors in both freshwater (23, 40, 70, 92) and marine (7, 80, 96) communities. It should be noted, however, that the magnitudes of the PICT responses were quite small in some of these studies. Moreover, none of the above-mentioned studies tried to identify any tolerance mechanism active in the communities. Despite this, in terrestrial weed species tolerant

* Corresponding author. Mailing address: Sven Lovén Centre for Marine Sciences, Kristineberg 566, SE-450 34 Fiskebäckskil, Sweden. Phone: 46 523 18538. Fax: 46 523 18502. E-mail: martin.eriksson@dpes.gu.se.

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to PS II inhibitors, there are two common tolerance mechanisms. One mechanism is increased detoxification of the compound by dealkylation or by hydroxylation, followed by conjugation (3, 17, 38, 41, 67). The other mechanism is alteration of the target site by mutations in the *psbA* gene coding for D1. This gene is found in genomes of plastids, cyanobacteria, and cyanophages and is evolutionarily very conserved (99, 103, 113). Mutations in *psbA* have emerged in plants growing in environments contaminated by PS II inhibitors as a consequence of their selection pressures (reviewed in references 24 and 81). The mutations result in amino acid substitutions in or close to the herbicide-binding region of D1 in either of two positions, Ser_{264} and Val_{219} (9, 51, 53, 55, 62, 68), which are thought to give tolerance through a reduced affinity of the herbicide to the Q_B-binding niche (45, 97). Whereas eukaryotes have only one or two copies of *psbA* per chloroplast genome (26, 27), cyanobacteria have from two to five paralogous *psbA* genes (19, 73, 107). These genes encode two different forms of D1 proteins, which gives different functional characteristics to PS II and are means for light adaptation in cyanobacteria (16). The flexibility of interchanging between high- and low-light forms of D1 could be a selective trait during Irgarol exposure.

In this work, we have studied the highly conserved region of D1 protein predicted from the metagenome of periphyton communities exposed to low and elevated concentrations of Irgarol. The region from amino acids 197 to 291 contains most parts of helices four and five and the herbicide-binding niche between these helices. Important sites within this region are the before-mentioned amino acids Ser_{264} and Val_{219}, as well as the initial cleavage site (39, 69) and the so-called PEST region (from Arg_{225} to Arg_{238}) (39). PEST regions are typically rich in Pro, Glu, Ser, and Thr and are often found in proteins with high turnover rates (88, 90). Although this region is not the actual cleavage site of D1, it is thought to regulate the degradation of the protein (64). Apart from this metagenomic approach, this study is very similar to the study by Dahl and Blanck (20) performed in 1994, when Irgarol was new on the Swedish antifouling market. At that time, only a weak TIS and no clear PICT signal were observed for Irgarol. Field studies of community tolerance to Irgarol conducted repeatedly between 1994 and 2004 (11) indicated that PICT developed slowly over the years, probably through a successive enrichment of algae and cyanobacteria with more efficient tolerance mechanisms. It has been argued (10) that highly conserved target proteins like D1 would require a strong and persistent selection pressure to establish tolerance-conveying mutations in an algal community. Although many tolerance-conveying mutations have been described for algal and cyanobacterial strains (24, 81), it is not known how competitive they would be in a contaminated natural environment. Therefore, it is advantageous to use natural communities with many competing species to single out those mechanisms that have the greatest fitness under environmental (or environment-like) conditions in the field or in a microcosm system. The ecological relevance can then be evaluated. This information has so far been lacking for aquatic environments, although tolerance-conveying mutations at Ser_{264} or Val_{219} have been reported to be viable in agricultural environments.

Here we show that these mutations (Ser_{264} or Val_{219}) were not present in Irgarol-tolerant periphyton communities. Instead, the tolerance mechanism seems to be coupled to the turnover of D1, since there were systematic differences between the amino acid sequences of the PEST region of the periphyton exposed to the background concentration and those exposed to the elevated concentration of Irgarol. To our knowledge, this study is the first attempt to find an ecotoxicogenomic mechanism behind a PICT response.

MATERIALS AND METHODS

Microcosm system. A flowthrough microcosm system (13) was used to establish periphyton communities. The experiment was performed indoors at Sven Lovén Centre for Marine Sciences, Kristineberg, on the west coast of Sweden during July and August in 2001. Seawater, with its indigenous microbiota, was continuously pumped with an air-driven Teflon membrane pump (Dominator; Maskin AB, Sweden) from a depth of 3 m in the Gullmar fjord into the microcosm system. The system was composed of a water delivery system, similar to the one used by Granmo and Kollberg (37) and modified by Molander et al. (71), a toxicant delivery system, and four 22-liter aquaria. A nylon net (1-mm mesh) was used to prevent larger organisms from entering the system. The seawater flow rate was approximately 220 ml/min through each aquarium, and the mean residence time of the water was about 100 min. The Irgarol water solutions were delivered from the start of the periphyton colonization at a flow rate of 2 ml/min using a peristaltic pump (Ismatech IPN 26; Ismatech AG, Switzerland). These water solutions were renewed every third day. Irgarol stock solutions were made in acetone at concentrations of 2.38, 1.19, and 0.59 mM. Irgarol water solutions were made by adding 10 ml stock solution to 10 liters of deionized water. Equal amounts of acetone and deionized water were added to the untreated microcosm. Flow rates of the seawater and Irgarol water solutions were checked daily and adjusted when they deviated by more than 5% from the desired values. Each aquarium had two fluorescent tubes (Osram Lumilux Daylight L18W/12) as the light source, giving a photon flux density of ca. 120 μmol photons $\cdot m^{-2} \cdot s^{-1}$ at the water surface and set to a light/dark regimen corresponding to the light/dark cycle in Sweden at this time of year. A stirring device in each aquarium ensured thorough mixing of the water.

Periphyton were sampled by colonization of small glass discs (1.5 cm^2). These discs were mounted in polyethylene holders and placed along the sides of each aquarium. Before being submerged into the aquaria, the discs were boiled for 10 min in concentrated nitric acid, rinsed in deionized water, and rinsed again in 70% ethanol.

Measurements of photosynthetic activity. Photosynthetic activity was measured by the incorporation of ^{14}C-labeled sodium bicarbonate and/or carbon dioxide into acid-stable compounds. The solution of ^{14}C-labeled sodium bicarbonate was prepared by diluting a stock solution of 1 mCi ml^{-1} (DHI Lab Products, Hoersholm, Denmark) in filtered seawater (filtered with a glass microfiber filter, grade GF/F; Whatman), giving a concentration of 1.48 MBq ml^{-1} and a final activity of 0.074 MBq in each sample. The samples were incubated at the current in situ temperature in the aquaria. Fluorescent tubes (Osram Lumilux Daylight L18W/11) with a photon flux density of ca. 125 μmol photons m^{-2} s^{-1} were used as the light source. The preincubation time, i.e., before addition of 50 μl of the radioactive solution to each sample, was 30 min, and the subsequent incubation time was 15 min. Carbon fixation activity was terminated by adding 50 μl of formaldehyde (37%) to each sample. In order to estimate the amount of abiotic ^{14}C fixation, biotic activity was inhibited by adding 50 μl formaldehyde in three samples before the incubation. The samples were acidified with 1 ml acetic acid and dried at 60°C under a gentle stream of air. To each sample, 1 ml dimethyl sulfoxide and 8 ml of Ready gel scintillation cocktail (Beckman Inc.) were added, and the samples were thoroughly mixed. The radioactivity of the samples was measured using a liquid scintillation spectrometer (LS 5000 TD; Beckman Inc.). Disintegrations per minute were calculated from counts per min based on the correction factors for the sample quench characteristics and the machine efficiency.

Analysis of the relative abundance of morphologically distinct taxa. The analyses of abundance of algal and cyanobacterial taxa were made according to the method described by Dahl and Blanck (20). Periphyton was sampled at the end of the experiment and stored in 70% ethanol at 4°C in darkness until analyzed. Three glass discs per microcosm and 50 randomly chosen fields (diameter of 252 μm) per disc were analyzed using a phase-contrast microscope at ×1,000 magnification. The relative abundance of each taxa was estimated as the number of fields where the species was observed, thus giving a relative abundance scale from 0 to 50.

Chemical analysis of Irgarol and the Irgarol metabolite M1. Solid-phase extraction cartridges (Isolute ENV+) were conditioned with 6 ml of methanol (Lichrosolv) and 10 ml of Milli-Q water. Approximately 500 ml of water from each microcosm was filtered (glass microfiber filter, grade GF/F; Whatman) and passed through the cartridges at a flow rate of 10 ml/min. The cartridges were dried under vacuum for 10 min and stored at −20°C. Cartridges were eluted and analyzed at CSIC, Barcelona, Spain, according to the method described by Martinez et al. (61).

DNA extraction, *psbA* amplification, cloning, and sequencing. The periphyton community was carefully scraped off the glass discs with a scalpel into filter-sterilized seawater and centrifuged at 6,500 × *g* for 10 min. Microscopy inspection of scraped discs showed high efficiency in the removal of attached life forms. The pellets were snap-frozen in liquid nitrogen and stored at −80°C. DNA extraction was made according to the protocol of plant DNAzol reagent (Invitrogen, Carlsbad, CA).

PCR was performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Uppsala, Sweden), with 3 ng of template DNA and 4 and 4.6 μmol of forward and reverse primers, respectively, in each 50-μl volume of reaction mixture. Degenerate primers were used to amplify a 285-bp region of the *psbA* gene. The forward and reverse primer sequences were 5′GTIT TYCARGCIGARCAYAAYATIYTIATGCAYCC3′ and 5′CCRTTIARRTT RAAIGCCATIGT3′, respectively. PCR started with an initial 2-min period at 95°C, followed by 45 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 30 s and then a final extension period of 5 min at 72 C°. Amplification of the correctly sized DNA fragment was verified by agarose gel electrophoresis, with the excised fragment then purified using a Wizard PCR Preps DNA purification system (Promega, Madison, WI). The *psbA* fragments were ligated into the pGEM-T Easy vector system II (Promega, Madison, WI) and transformed into competent *Escherichia coli* (JM109) cells. After overnight growth on agar plates, cells from randomly chosen bacterial colonies were collected with a toothpick and transferred to 150 μl of 0.1× Tris-EDTA buffer and lysed in a microwave oven for 1 to 2 min. The lysed *E. coli* cells were transported in sealed 96-well plates on dry ice to Genomic Ecology, Lunds University, Sweden, for sequencing. In total, 288 clones were investigated by DNA sequencing. The bacterial lysates (1 μl in a total reaction mixture volume of 10 μl) were used directly as templates for PCR amplification of cloned fragments, using standard PCR conditions and the universal primers M13 forward (5′-CCCAGTCACGACGTTGTAAAACG) and M13 reverse (5′-AGCGGATAACAATTTCACACAGG). After PCR, amplicons were purified by standard isopropanol precipitation and then redissolved in 10 μl of H_2O. PCR products (2 μl in a total reaction mixture volume of 10 μl) were used for DNA sequencing in both directions, using either the M13 forward or the M13 reverse primer and a BigDye kit (Applied Biosystem), followed by analyses on an ABI3100 instrument (Applied Biosystem) according to the manufacturer.

Sequence analysis. Many authors have shown that artifacts (polymerase errors or chimera formation) and biases can be associated with PCR-produced clone libraries (1, 48, 57, 60, 87, 106, 108, 109). These reports have been concerned mainly with 16S rDNA libraries, probably because 16S rDNA is the predominant gene used in clone libraries and tools have also been developed for detecting 16S rDNA chimeras (e.g., Chimera Check software at the ribosomal database project II, Mallard software, ChimeraBuster, Bellerophon, and Ccode software). However, for other genes and especially for shorter fragments (<300 bp), to our knowledge no such tools have been developed. Since rRNA forms secondary structures as part of its function in ribosomes, the formation of chimeric sequences during PCR is likely to be more common when amplifying 16S rDNA than functional genes such as *psbA*. Still, a strategy was developed in order to minimize the problem of PCR artifacts within our libraries. Sequencing errors were removed by excluding clones with noncomplementary forward and reverse sequences from further analysis. In order to avoid artifacts from polymerase errors, we excluded sequences that differed by only one nucleotide from any other sequence and that occurred only once within each library. Moreover, we manually identified possible recombination points in all sequences in each library and checked whether any sequence could originate from other sequences within the library, i.e., be of chimeric origin. This strategy gave a reduction in the number of unique haplotypes from 95 to 72 but also resulted in a conservative library with a very low probability of containing chimeric sequences.

Sequences were aligned using Mafft version 5.64 software (49, 50) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The settings for Mafft were FFT-NS-i (slow, iterative refinement method), a gap opening penalty of 1.53, and an offset value of 0.123. The similarity of sequences was analyzed using the sequence identity matrix incorporated in Bioedit software (43).

TABLE 1. Concentrations of Irgarols and the Irgarol degradation product M1 in the microcosms

Sampling date (yr-mo-day)[a]	Nominal Irgarol concn (nM)[b]	Analyzed Irgarol concn (nM)	Analyzed M1 concn (nM)[c]
2001-07-31		0.02	0
	5	2.9	0
	10	5.5	0.008
	20	10	0.008
2001-08-16		0.02	0
	5	2.9	0.008
	10	5.6	0.008
	20	9.5	0.016

[a] Samples were taken on the two dates indicated during the experiment.
[b] Values shown are the experimentally intended concentrations. The background concentration of Irgarol in the incoming water was 0.02 nM.
[c] Degradation product of Irgarol (2-methylthio-3-*tert*-butylamino-6-amino-*S*-triazine), also known as GS26575.

Nucleotide sequence accession numbers. The *psbA* sequences were deposited in the EMBL-EBI database library under accession numbers AM933675 to AM933749. Additional sequences, which were excluded from further analysis and not deposited in EMBL-EBI due to their unproven nonchimeric origin, can be obtained from the corresponding author.

RESULTS

Analyzed concentrations of Irgarol. The seawater in the control microcosm contained Irgarol contamination from the environment. Therefore, this microcosm could not be viewed as a true zero-concentration control but rather as one with the current background concentration of Irgarol (0.02 nM). In the following discussion, we therefore use the terms "background" and "Irgarol" to couple sequences or taxa to the exposure of periphyton either to the background or to the experimentally elevated Irgarol concentrations. The analyzed concentrations in the microcosms dosed with Irgarol were approximately half of those of the experimentally intended, nominal concentrations (Table 1). In all figures and tables, the analyzed concentrations are used. Low concentrations of the Irgarol degradation product M1 were also found in Irgarol microcosms (Table 1).

PICT, TIS, and relative abundance of morphologically distinct taxa. We clearly detected both TIS and PICT to Irgarol. The PICT signals are shown in Fig. 1 as higher 50% effective concentration (EC_{50}) values at higher exposure levels. In the 10 nM Irgarol treatment, the detected EC_{50} value was 78 nM, which is a very high value for photosynthesis in periphyton communities. This value is five times higher than the EC_{50} value detected in the background exposure microcosm (16 nM). However, here it is also important to note that the community tolerance level in the background community is relatively high compared to that in sensitive periphyton communities (3 to 4 nM) sampled in a less contaminated area nearby in the same year (11). The PICT signal was accompanied by TIS in the form of clear shifts in the distribution of morphologically distinct taxa (Fig. 1). In total, we identified 32 algal and cyanobacterial taxa in the four microcosms (Table 2). In the background and the 10 nM Irgarol communities, 16 and 14 species were found, respectively (Fig. 2D). These numbers, however, are low compared to the number of haplotypes (i.e., the num-

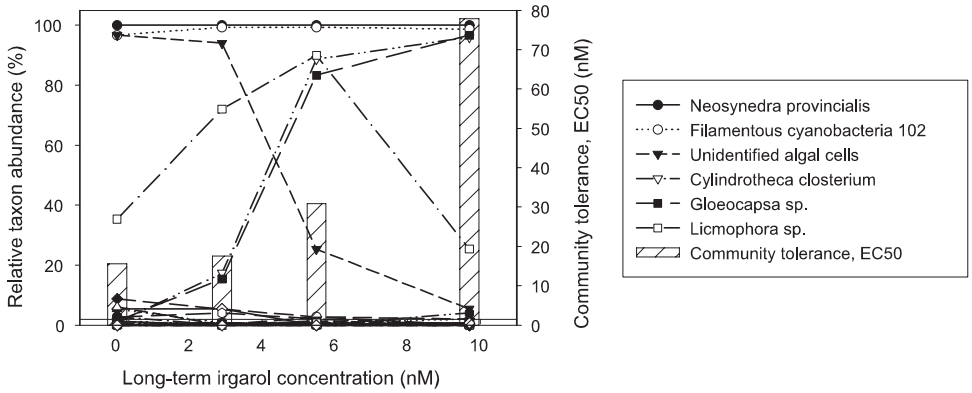

FIG. 1. Response patterns of all identified, morphologically distinct taxa and community tolerance in the communities. The response patterns of the different taxa are given as relative abundance, i.e., the percent fraction of microscopy fields in which a species was found, as shown on the left *y* axis. The identity of the dominant species is shown in the legend. The identities of all species are given in Table 2. Community tolerance, detected as EC_{50} values from the short-term tests, are shown as hatched bars and given on the right *y* axis.

ber of unique nucleotide sequences) found in the clone libraries.

Nucleotide sequences. Clone libraries were built only for the background community and the 10 nM Irgarol community. After some sequences were excluded in order to minimize PCR artifacts (see Materials and Methods), there was 68% efficiency in producing high-quality sequences. The total number of high-quality *psbA* fragments was 89 for the background community and 108 for the Irgarol community. Despite the *psbA* gene being overall highly conserved within phototrophic organisms and the fact that the region sequenced is one of the most conserved within the gene itself, we found as many as 72 unique *psbA* haplotypes in total. Surprisingly, only three haplotypes were found in both communities. Thus, the distributions of *psbA* haplotypes were drastically different, with 34 haplotypes found only in the background community and 35 haplotypes found only in the Irgarol community (Fig. 2A). Note that none of the previously described tolerance-conferring mutations, resulting in amino acid substitutions at Ser_{264} and Val_{219} (9, 51, 53, 55, 62, 68), was found in any of the *psbA* haplotypes.

Predicted protein sequences. In spite of degeneracy in the genetic code, a high level of diversity was maintained at the protein level, with 40 different predicted D1 protein fragments in total. The clear differences between the background and Irgarol communities remained after translation of the nucleotide sequences. There were 4 protein sequences present in both communities, whereas 19 were found only in the background community and 17 were found only in the Irgarol community (Fig. 2B).

As mentioned above, no protein sequences contained the amino acid substitutions (e.g., Ser_{264} or Val_{219}) previously shown to confer tolerance to PS II inhibitors. However, we also searched for other differences typical for Irgarol or background protein sequences. In particular, nonconserved amino acid changes were examined, since these are more likely to affect the function of a protein than conserved amino acid substitutions. We found only one region, located between amino acids 228 and 238, where background and Irgarol sequences had nonconserved amino acid differences; this region corresponded to the PEST region of D1 (39). In both communities, we found 14 different PEST region sequence types. Five of those types were found in both communities, whereas six and three types were found exclusively in the background and the Irgarol communities, respectively (Fig. 2C). A greater diversity in PEST regions occurred in the background than in the Irgarol community, in which only three types dominated (Fig. 2C). This indicates that there is a strong Irgarol selection pressure in this region of D1 and that its amino acid composition is causative for the increased community tolerance to Irgarol. The amino acid sequences of the PEST sequence types are shown in Fig. 3.

DISCUSSION

There are several significant findings from this study that deserve further attention. (i) None of the mutations known to convey tolerance to PS II inhibitors in higher plants, algae, and cyanobacteria was present in the communities. (ii) Despite this, Irgarol caused a clear difference in the composition of *psbA* haplotypes and D1 sequence types, which implies that Irgarol exerted a strong selection pressure on this gene. (iii) A new putative tolerance mechanism was discovered, involving the amino acid sequence in the PEST region of D1 that is likely to affect the rate of D1 degradation. (iv) Although *psbA* has been reported to be relatively conserved, the variability and diversity of this gene within periphyton species were surprisingly high.

The absence of those mutations that are regularly found in terrestrial environments contaminated by PS II inhibitors (e.g., resulting in the amino acid substitution Ser_{264}→Gly) in the Irgarol community implies that these mutations are not functional in marine periphyton species, in spite of a strong Irgarol selection pressure. The reason for this remains to be clarified. A similar result within a marine environment was also found by Galgani et al. (33) when *psbA* was sequenced from the red macroalga *Porphyra linearis* in environments with different atrazine concentrations. Despite this, the amino acid sequence of D1 still appears very important during Irgarol selection, since there were such clear differences between the backgrounds of the *psbA* haplotypes and the D1 protein types and Irgarol communities, with regard to both (Fig. 2A and B).

TABLE 2. Relative abundance of morphologically distinct algal and cyanobacterial taxa in periphyton[a]

Morphologically distinct taxon (strain no.)[b]	Relative abundance (%) of analyzed Irgarol concn in microcosms[c]			
	0.02 nM (background)	2.9 nM	5.5 nM	9.7 nM (Irgarol)
Neosynedra provincialis	100	100	100	100
Filamentous cyanobacterium (102)	97	99	99	99
Unidentified green algal cells	97	94	25	5, 3
Cylindrotheca closterium	0.7	17	89	96
Gloeocapsa sp.	1.3	15	83	97
Licmophora sp. (102)	35	72	90	25
Filamentous cyanobacterium (105)	8.7	5.3	2.7	2.0
Unidentified algal cells	6.0			
Anabaena sp.	5.3	5.3		
Licmophora sp. (101)	5.3	0.7	0.7	2.0
Spirulina sp.	2.7	4.0	2.0	
Navicula sp. (110)		0.7		4.0
Sprout of brown alga	2.7			
Gomphonemopsis sp.			2.7	
Berkeleya sp.	1.3			0.7
Filamentous alga	1.3			
Amphora sp. (104)			1.3	
Amphora hyalina		0.7	0.7	
Licmophora abbreviata	0.7	0.7		
Prorocentrum lima			0.7	0.7
Alga (103)			0.7	
Amphora sp. (148)				0.7
Chaetoceros sp.			0.7	
Filamentous cyanobacterium (101)		0.7		
Filamentous cyanobacterium (107)	0.7			
Erythrotrichia sp.				0.7
Diatom (125)		0.7		
Leptolyngbya sp.		0.7		
Licmophora cf. *communis*[d]			0.7	
Nitzschia sp. (124)		0.7		
Nitzschia sp. (113)				0.7
Pleurosigma sp.			0.7	

[a] Taxa are sorted according to their average dominance in the communities.
[b] Morphologically distinct taxa were listed according to species level when possible. Some algal and cyanobacterial cells were identified according to our reference files (M. Kuylenstierna, Göteborg University) using a different set of characteristics (e.g., shape, size, morphology). Numbers in parentheses indicate distinct morphs which are also referred to as taxa.
[c] See Materials and Methods for a description of the calculation of relative abundance.
[d] A *Licmophora* sp. that looks like *Licmophora communis*.

However, we cannot at this stage exclude the possibility that the differences in the distributions of *psbA* haplotypes and D1 protein types are a result of a changed species distribution, which in turn could be caused by other tolerance-conferring traits within the community. This would imply selection pressure on a gene(s) or possibly on a region(s) of D1 other than those analyzed in this study. For cyanobacteria, there might also be a selection for more efficient use of their paralogous *psbA* genes. Despite such possibilities, however, the distributions of PEST sequences between the different communities (see below) does suggest that this D1 domain is an important factor in conferring Irgarol tolerance.

The strong discrimination by Irgarol between amino acid sequences in the PEST region (Fig. 2C, compare upper and lower panels) indicates that D1 degradation is particularly important in periphyton species during Irgarol exposure. Only three PEST sequence types seem to be functional under selection of Irgarol, since they make up the majority of all PEST sequences (Fig. 2C, lower panel). Thus, the PEST region of D1 is a putative Irgarol tolerance-regulating region in marine periphyton species. It should be stressed that this represents a novel discovery of a tolerance mechanism that is functional under environmental (or environment-like) conditions, i.e., in a multispecies system with continuous immigration from the natural environment, ecological interactions like competition and predation present, and naturally fluctuating nutrient and light history conditions. This finding is in contrast to those of many studies in which tolerance to PS II inhibitors has been studied in artificial systems using site-directed mutants of standard laboratory species. It is also important to point out that most of the Irgarol-selected PEST sequence types were already present in the background community. This is consistent with the observations that there was Irgarol contamination (Table 1) and Irgarol selection (detected as a comparatively high EC_{50} value) in the experimentally untreated microcosm (Fig. 1).

D1 is naturally a high-turnover protein (32, 65), and its turnover is involved in regulating electron flow and activating/inactivating PS II as a mechanism of light adaptation, e.g., during photoinhibition (18, 83, 93, 94). Importantly, PS II inhibitors have been shown to block D1 turnover (32, 47, 54, 56, 63, 76). This occurs when the inhibitor binds to D1 and either blocks its proteolytic site or induces a conformational change which reduces the accessibility of this site (25, 65, 102). This means that there are at least three toxic effects of PS II inhibitors: (i) inhibition of photosynthetic electron transport, leading to reduced ATP and NADPH production; (ii) increased oxidative stress due to accumulation of ROS at PS II; and, importantly, (iii) blockage of D1 turnover. Since D1 turnover is vital for continued photosynthesis, it is evident that its impairment will be an ecologically relevant effect of PS II inhibitors. In fact, the amino acid substitutions Ser_{264} and Val_{219}, thought to confer tolerance through a reduced affinity of the herbicide to the Q_B-binding niche, also alter the turnover rate of D1. Both increases (82; C. Sundby, S. McCaffery, W. S. Chow, and J. M. Anderson, presented at the 9th International Congress on Photosynthesis, Nagoya, Japan, 1992) and decreases (21, 95) in turnover have been described, and in the former cases, the tolerance could also originate from a less-affected D1 turnover rate during exposure to PS II inhibitors.

There are three conditions that must be met for the PEST region to be a regulator for Irgarol tolerance. First, the amino acid sequence of the PEST region must be able to modulate the degradation rate of D1. Support for this condition comes from studies showing that point mutations in the PEST region result in both higher (75) and lower (104) rates of D1 degradation. Since several studies have shown that deletion mutants with no PEST domains retain the ability to degrade D1 (6, 74, 75, 78), the complete PEST signal does not seem to be necessary for D1 degradation. Although the PEST signal was removed from these mutants, the regulatory role of this part of D1 persisted, since the mutants gave either higher (74, 75, 78) or lower (6) turnover rates. The mechanism by which the PEST region regulates D1 degradation could be via interaction with other PS II

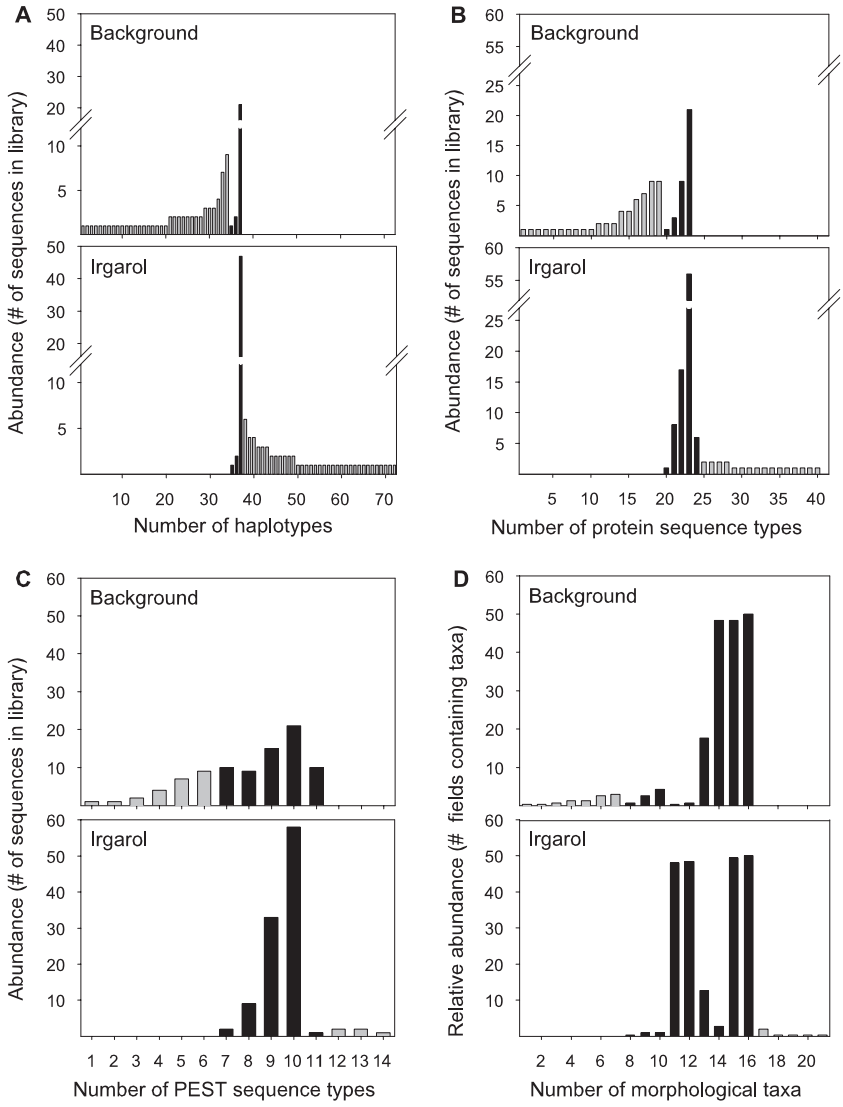

FIG. 2. (A) Distributions of different *psbA* haplotypes in background (top panel) and Irgarol (bottom panel) communities. (B) Distributions of different protein sequences, i.e., all the unique amino acid sequences produced when translating the *psbA* haplotypes, in background (top) and Irgarol (bottom) communities. (C) Distributions of different PEST sequences in background (top) and Irgarol (bottom) communities. (D) Distributions of all morphologically distinct taxa detected in background (top) and Irgarol (bottom) communities. In all graphs, the *x* axis shows the number of unique types of the different variables: haplotypes (A), protein sequences (B), PEST sequences (C), and morphologically distinct taxa (D); and the *y* axis shows the abundance of each unique type for the different variables. Black bars indicate types or taxa common to both the background and the Irgarol community, whereas gray bars indicate types or taxa present in either one of the background or the Irgarol community.

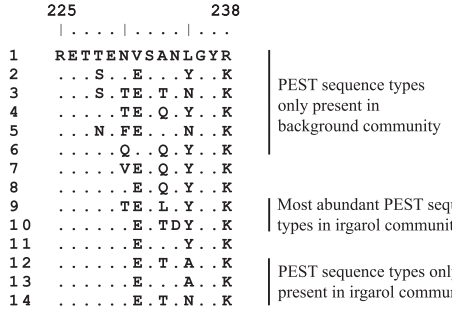

FIG. 3. Alignment of all periphyton PEST sequence types. The PEST region is defined as amino acid numbers 225 to 238 (39).

components. For example, it has been proposed that when amino acids in the PEST region of D1 are oxidized (e.g., by ROS), intermolecular cross-links to the D2 protein are formed and that this conformational change can act as a signal for D1 protein degradation (69). It should also be kept in mind that the rate of D1 degradation is dependent on light intensity and that a mutation giving a higher turnover rate at low light (40 μmol photons m^{-2} s^{-1}) can produce a lower turnover rate in high light (1,500 μmol photons m^{-2} s^{-1}) (71). This finding is important in an ecological context, since the environmental light regimen is often well in excess of 40 μmol photons m^{-2} s^{-1} and fluctuates constantly.

The second condition is that D1 degradation must be rate

limiting for its overall turnover. This is again a premise supported by many studies that have shown that the turnover of D1 is indeed limited by its degradation (63, 66, 77, 98). The final condition is that increased or optimized turnover of D1 should result in increased tolerance. Pace et al. (84) provide experimental support for the hypothesis that increased D1 turnover is a mechanism of tolerance to triazine PS II inhibitors (atrazine) in a moderately tolerant species (pea). Furthermore, Trebst et al. (101, 102) and Draber et al. (25) have argued that the inhibition of D1 turnover by PS II-inhibiting herbicides, and thereby the interference of key regulatory processes in photosynthesis, might be much more devastating than the mere inhibition of electron transport. Consequently, any mechanism that maintains the D1 turnover rate closer to that of a nonexposed situation would give tolerance. Also, in a study by Kless et al. (52), moderate tolerance was detected in PEST deletion mutants of *Synechocystis* sp. strain PCC 6803. Even though that study did not directly couple the tolerance to increased turnover of D1, it shows that alterations in the PEST region affect tolerance to PS II inhibitors. Interestingly, these moderately tolerant mutants showed no reduction in photosynthesis (as measured by O_2 evolution) or in growth rate. Those authors also point out that the PEST region may be the rate-limiting factor of D1 degradation under conditions of stress.

The toxic effects of acute Irgarol exposure (e.g., impaired electron transport, increased production of ROS, and blockage of D1 turnover) are interrelated, producing complex cause-effect relationships. Proteins damaged by increased ROS production, or by UV/light radiation, need to be replaced. However, replacement of D1 has to be preceded by its degradation (2, 4, 5, 112), and since Irgarol blocks the degradation of D1, this repair mechanism is prevented, resulting in entire PS II complexes becoming nonfunctional. This situation is similar to, although more severe than, that of photoinhibition, in which the rate of photodamage to the D1 protein by high irradiation exceeds the rate of repair. Interestingly, it has been shown that in *Synechocystis* mutants with amino acid substitutions in the PEST region, photoinhibition that is reversible in the wild type is irreversible, dependent on de novo D1 synthesis (59). Also, Mulo et al. (74) showed that the reversible and irreversible components of photoinhibition in the same cyanobacterium were affected differently by deletions in the PEST region. Collectively, this also implies that the amino acid sequence in the PEST region is one factor that influences the light regulation of photosynthesis, its susceptibility to high excitation pressures, as occurs during photoinhibition, and its tolerance to PS II inhibitors.

Some studies have also described increased D1 turnover as a physiological stress-compensating mechanism. Increased D1 turnover occurs in response to fumigation with ozone (36) or with mixtures of air pollutants (O_3, NO_2, and SO_2) (58), as well as during potassium deficiency (58), cadmium exposure (30, 34), and drought (35). It has been proposed that D1 turnover acts as a general adaptive response to environmental extremes (35), which gives further support to our hypothesis of increased D1 turnover as an Irgarol tolerance mechanism.

When the occurrences of PEST sequence types in background and Irgarol communities were analyzed (Fig. 2C), all non-PEST amino acids were excluded from the sequences. This analysis does not reveal whether PEST sequence types, selected for by Irgarol, also occur in background D1 proteins. We therefore analyzed the distribution of the two most abundant Irgarol-selected PEST sequences (Fig. 2C, types 9 and 10) in the D1 protein sequence types. PEST types 9 and 10 were found in 14 out of the 40 D1 protein types. Most interesting was the finding that the occurrence patterns of the two PEST types are different (Fig. 4). PEST type 10 was present only in one type of D1 protein in the background community, namely, protein type 1 (Fig. 4, upper panel). In the Irgarol community, not only was protein type 1 more abundant but the PEST type number 10 was also present in two additional D1 protein sequence types found exclusively in the Irgarol community, namely, protein sequence types 2 and 3 (Fig. 4, lower panel). Thus, PEST type 10 is favored by Irgarol both in terms of its abundance in the community and its occurrence in different types of Irgarol-selected D1 proteins. PEST type 9 was also more abundant in the Irgarol than in the background community (Fig. 2C, compare upper and lower panels), but, in contrast, this PEST type occurred in some protein types which were found exclusively in the background community (Fig. 4, protein types 11 to 14, upper panel). Consequently, PEST sequence type 9 is less likely than type 10 to contribute to tolerance in the Irgarol community.

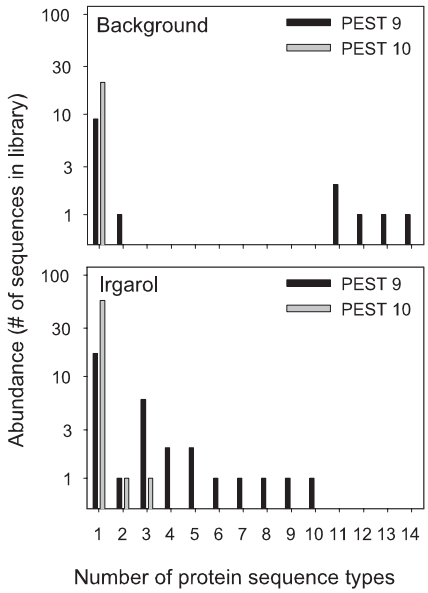

FIG. 4. Distribution of D1 protein sequence types containing PEST region 9 (black bars) and 10 (gray bars) in background (upper panel) and Irgarol (lower panel) communities. Note the logarithmic scales of the *y* axes of both panels. Note also the PEST region 9 containing protein sequence types 11 to 14 in the background community (black bars at the right in the upper panel).

In spite of the evolutionary conservation of *psbA* and the fact that only a small part of the gene was sequenced, we found as many as 72 unique haplotypes within periphyton communities from both microcosms. This number of periphyton haplotypes can be compared to the numbers found in marine picophytoplankton communities, which were 4, 12, and 28, in the Mediterranean Sea, the Red Sea, and the Pacific Ocean around

Hawaii, respectively (111). Even though the sampling of picophytoplankton in this study excluded cells of >3 μm, it seems as though the phototrophic part of periphyton communities is very diverse. The number of periphyton haplotypes can also be compared to the 21 morphologically distinct species found in these microcosms (Fig. 2D; Table 2). The corresponding value at the protein level is 40, which still is almost twice the number of morphologically distinct species. This is not so surprising, since identification of a species by microscopy does not necessarily reflect the genetic identity of that species. Moreover, visual inspection by microscopy cannot reliably detect very small photosynthetic species, even at the highest magnifications used. Since the identities of the morphologically distinct species are uncoupled from the identities of the *psbA* haplotypes, a phylogenetic analysis of the haplotypes has been performed and will soon be submitted for publication elsewhere.

The different outcomes in terms of PICT induction between this study and that of Dahl and Blanck (20) are probably due to the environmental contamination of Irgarol between the years 1994 and 2001. This is further supported by the study of Blanck et al. (11) which shows that PICT to Irgarol has slowly developed in environmental periphyton communities in this area between 1994 and 2004. The spatial and temporal PICT pattern reported in that study also shows that community tolerance in waters near the microcosm inlet is relatively high during midsummer, when this study was performed. This is again consistent with the elevated EC_{50} value in the background exposure microcosm and means that the indigenous community in the fjord, continuously sampled for this study, already contained Irgarol-tolerant components. The experimental exposure then further selected for increased tolerance. This is also consistent with the fact that the PEST sequences selected for in the Irgarol treatment were already present in the background exposure microcosm. Thus, there is a three-step selection process behind the observed PICT response, namely, a long-term selection over the years, a selection over the season, and the experimental selection in the microcosm. The first two steps are documented in the studies by Dahl and Blanck (20) and Blanck et al. (11), while the higher community tolerance observed here also involves an experimental selection step and, thus, is dependent on all three steps.

In conclusion, this study shows that PICT to Irgarol can now be clearly induced in periphyton communities. The mechanism of tolerance is not the previously identified mutations in the *psbA* gene, resulting in amino acid substitutions at Ser_{264} or Val_{219}, which are known to convey tolerance to PS II inhibitors. Instead, it seems to be related to the degradation of D1 as a result of nonneutral amino acid differences in the PEST region of D1 being more prevalent in the microcosm with elevated Irgarol exposure. This is consistent with findings from biochemical studies of photosynthesis over the last 2 decades, and we can now add that it is only modifications in the PEST region that are functional under environmental (or environment-like) conditions in the Irgarol-contaminated marine periphyton habitat on the west coast of Sweden.

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