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The Enzymatic Detoxifying System of a Native Mediterranean Scorpio Fish Is Affected by *Caulerpa taxifolia* in Its Environment

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The accidental introduction and extremely rapid growth of the tropical alga *Caulerpa taxifolia* in the northern coastal waters of the Mediterranean sea represents an adaptive challenge for indigenous animal and plant species. Since first being detected near Monaco, it has spread to many areas, sometimes hundreds of kilometers away from the initial site of implantation. This alga is known to produce some repulsive toxic compounds such as caulerpenyne derivatives. In this study, specimens of *Scorpaena porcus* (a typical mediterranean fish) were maintained under laboratory conditions in the presence of this alga, and hepatic glutathione S-transferase and cytochrome P450 monooxygenases activities of fish were examined. The collective data indicate that the presence of *Caulerpa taxifolia* in the environment of these fish modifies the hydroxylation stereospecificity of hepatic microsomal cytochromes P450 toward steroid substrates. These results reflect changes in the relative levels of P450 isoforms in the liver and thus demonstrate the influence of *Caulerpa taxifolia* on the physiology of fish sharing space with this new alga. In addition, this phenomenon was found to be dependent on the contact time between the fish and the alga.

Introduction

The tropical green seaweed *Caulerpa taxifolia* (Vahl) C. Agardh is rapidly growing along the northwestern coast of the Mediterranean sea after its likely accidental introduction (*1, 2)*. Since first being detected near Monaco, it has spread to many areas, sometimes hundreds of kilometers away at an expansion rate (0.03 sq kilometers in 1990 and >15 in 1994) that is surprisingly rapid at this latitude. The presence in the marine environment of a new species constitutes a gamble for resident species because of competition for space and resources. The behavior, the number, and the varieties of fish were affected by the presence of *Caulerpa taxifolia* colonies (*3*). In addition, this originally tropical alga is known to contain high amounts of terpenoid compounds having various biological effects (*4*), some of these being strongly toxic to mammals (*5*). Thus, the expansion of *C. taxifolia* leads us to anticipate effects on filter feeders and grazers as well as the possible contamination of the marine food chain via these animals. In addition, a recent publication (*6*) reports on a possible link between food poisoning of humans by fish consumption and the presence of *C. taxifolia* near the fishing area.

In animals, exogenous molecules must be rapidly transformed or excreted in order to avoid concentration in the body. This process is of particular importance for toxic compounds that are inactivated by numerous enzymatic systems in the liver, kidney, and skin. These processes are classified as phase I (functionalization) and phase II (conjugation) reactions (*7*). Typical enzymes for the phase I reactions, cytochrome P450 monooxygenases (P450s), are a large group of heme containing enzymes. The basic reaction they catalyze is the insertion of an oxygen atom into an organic molecule via the activation of molecular dioxygen leading to aliphatic and aromatic hydroxylations (*8*). These systems are remarkably versatile in the type of chemical reactions catalyzed and in their choice of substrates. The broad specificity in substrates is, with the large number of isoforms (*9*), an essential feature of the cytochrome P450 superfamily. Glutathione-S-transferase enzymes (GST), classified as phase II, catalyze the nucleophilic attack of glutathione on electrophilic centers in a wide variety of organic molecules, some of them results of phase I oxidative metabolism. A prominent feature of GSTs in an organism is the existence of isoenzymes (*10*).

The relative levels of individual P450 and GST forms in the liver are under genetic regulation involving positive (induction) or negative (repression) effects exerted by foreign (xenobiotic) or endogenous compounds (*10, 11*).

While extensively studied in mammals, these two enzyme systems have been also investigated in fish (*12*−*17*). The influence of various pollutants, particularly, polycyclic aromatic hydrocarbons (PAHs), on their expression is ongoing research (*18*−*21*). One of the other aspects of these studies is that induction in fish liver or kidney of various enzymatic activities, typically those of P450s, provides an early warning signal of exposure to chemicals. Thus P450 dependent activities are checked to evaluate the level of contamination by various pollutants (*22*−*26*).

In this work, we have simulated representative conditions resulting from the modification of the marine environment by the presence of *C. taxifolia* in an aquarium in which a test group of *Scorpaena porcus* (typical mediterranean fish) were caged or by feeding the fish with mussels containing *C. taxifolia* homogenate. Variations in the stereospecificity of the P450 dependent activities toward typical substrates are clearly demonstrated. These observations suggest that the proliferation of *C. taxifolia* in the Mediterranean sea modifies important xenobiotic detoxifying enzymes in the local fish population. In addition, the demonstration of this effect on a nonherbivorous fish species indicates that a product secreted by the algae is responsible for the observed phenomenon and numerous living species could be concerned by the presence of *C. taxifolia*.

Materials and Methods

Caulerpa taxifolia were collected near Cap Martin (Alpes Maritime, France) at a depth of −7 to −8 m. Samples were

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TABLE 1: Glutathione S-Transferase (in μmol·min^{-1}·mg^{-1} Protein) Activity Measured in Fish Liver Cytosols After the Fish Were Fed with Treated Mussels (FD), Caged in the Presence of *Caulerpa Taxifolia* for Four Weeks (CL) or in Standard Conditions (UT)

treatment to fish	none (UT)	fed with treated mussels (FD)	presence of *Caulerpa* in the aquarium (CL)
glutathione-S-transferase activity	1.63 (1.46−2.05)	1.79 (1.41−2.02)	2.79 (1.75−4.15)

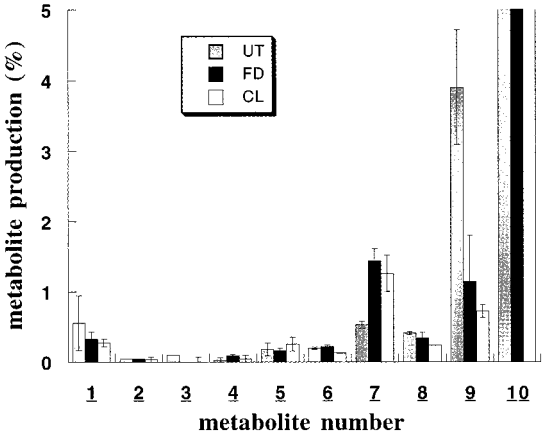

FIGURE 1. Schematic representation of the progesterone metabolites produced by fish liver microsomes. Values are calculated as the percentage (±SE) of individual metabolite relative to the concentration of the total progesterone derivatives concentration: UT, untreated fish; FD, fish fed with treated mussels; and CL, fish in the presence of the caulerpa for a month. product 10 corresponds to the excess of progesterone substrate.

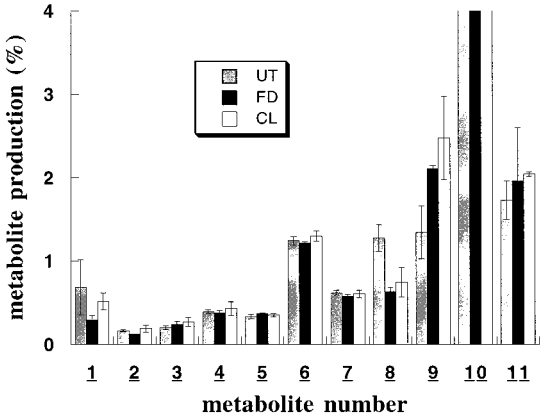

FIGURE 2. Schematic representation of the testosterone metabolites produced by fish liver microsomes. Values are calculated as the percentage (±SE) of individual metabolite relative to the concentration of the total testosterone derivatives: UT, untreated fish; FD, fish fed with treated mussels; and CL, fish in the presence of the *Caulerpa* efor a month. Products 10 and 11 were identified to testosterone and androstenedione, respectively.

immediately transported to the laboratory in aerated aquaria maintained at average seawater temperature (17 °C ± 2).

Scorpion fish, *Scorpaena porcus*, were caught by fishing net in Cannes (Golfe de La Napoule, France) and brought to the laboratory. Recent under sea observations demonstrated that *Caulerpa taxifolia* did not exist in that area (*27*). Fish used in the experiments were 11−23 cm length and 20−280 g weight, with an average of 15.6 cm and 95.7 g, respectively. The fish were acclimatized in natural seawater maintained at ambient seawater temperature (17 °C ± 2) for 15−21 days, salinity was 35 ‰ ± 1, pH was 8.3 ± 0.1. The scorpion fish were protected from direct light and fed with frozen shrimp and mussels. The water in each aquarium was recycled, filtered through sand, and renewed every 2 weeks.

Experimental Exposure. The scorpion fish were divided into three groups (Table 1), each containing 5−6 individuals (control, 12 individuals). One group was kept untreated (control), the second was exposed to *Caulerpa taxifolia* in the aquarium for four weeks, and the third group was fed with mussels which had received a single 0.3 mL injection of crude homogenate of *Caulerpa taxifolia*.

Preparation of Liver Subcellular Fractions. The fish were killed by cervical dislocation, and their livers were removed, weighed, and homogenized individually in the solution of 50 mM potassium phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) using a Potter Teflon-glass homogenizer. The homogenates were subsequently centrifuged at 9000g for 20 min in a Beckman centrifuge. The pellet resulting from the 9000g centrifugation was resuspended in the same buffer and centrifuged again at 9000g for 20 min. These supernatants were pooled and centrifuged at 105 000g for 1 h. Resulting supernatants were stored at − 80 °C in small aliquots for further analysis of cytosolic glutathione-S-transferase activity. Pellets 105 000g, mainly constituting microsomes, were resuspended in 1 mL of 50 mM phosphate buffer pH 7.4, 1 mM EDTA, 0.1 mM DTT, containing 20% glycerol. The microsomal suspensions were sampled and stored at −80 °C.

Biochemical Assays. Microsomal and cytosolic protein concentrations were determined using the BCA protein assay reagent (Pierce) with bovine serum albumin (BSA) as standard. When possible, P450 concentrations were determined spectrophotometrically by reduction and carbon monoxide binding according to the method of Omura and Sato (*28*). The microsomal P450 activity was tested using ^{14}C-(4)-progesterone and ^{14}C-(4)-testosterone as substrates as previously described (*29*). The substrate concentration was adjusted to 100 μM, and the reaction was initiated by the addition of 1 mM NADPH to the microsomal suspension adjusted to 1 mg protein per milliliter. After 10−30 min incubation at 25 °C, the reaction was stopped, and the steroidal products (metabolites plus the remaining substrate) extracted by addition of 2 mL of dichloromethane and vigorous shaking. The organic phase was recovered, concentrated to 20−50 μL under a stream of dry nitrogen, and spotted onto a Merck 60F254 TLC plate. Plates were developed twice in chloroform:ethyl acetate:ethanol (4:1:0.2) for the progesterone assay. When testosterone was used as substrate, the metabolites were separated by development in dichloromethane/acetone (4:1), followed by a development in chloroform:ethyl acetate:ethanol (4:1:0.7). The TLC plates were then subjected to autoradiography (1−3 days exposure) with Kodak X-AR films. Visualized metabolites were numbered according to increasing polarity (see Figure 4). For quantitative analysis the radioactive spots were cut out from the TLC plates and placed in a toluene scintillator (Packard) for counting. Alternatively, the TLC plates were kept in contact with a storage phosphor screen for 12 to 48 h and then the screen was read on a phosphor-imager system 445Si (Molecular Dynamics).

Total glutathione S-transferase activity was assayed on the cytosolic fraction with CDNB (1-chloro-2,4-dinitrobenzene) as substrate (*30*). GST activities and P450 spectra were recorded on a Uvikon 931 spectrophotometer (Kontron Instruments).

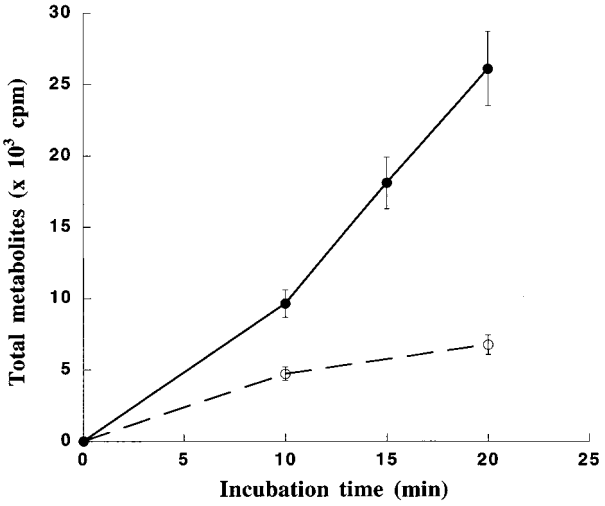

FIGURE 3. Inhibition of total progesterone hydroxylase activity by 12.5% carbon monoxide (open circles) compared with the reference activity (closed circles).

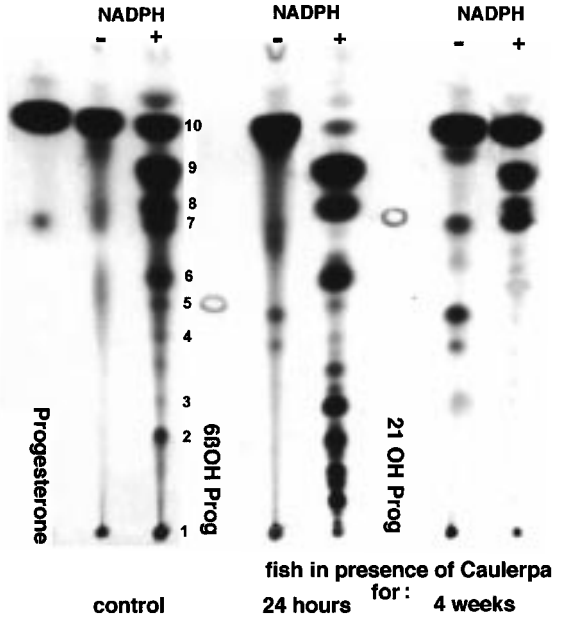

FIGURE 4. Autoradiogram of the TLC of progesterone metabolites. TLC plate was developed twice with chloroform/ethyl acetate/ethanol (4:1:0.2): progesterone substrate alone (lane 1); NADPH dependent metabolism of progesterone by liver microsomes prepared from: control fish (lanes 2 and 3); fish caged 24 h in the presence of *Caulerpa taxifolia* (lanes 5 and 6); fish caged 4 weeks in the presence of *Caulerpa taxifolia* (lanes 8 and 9); authentic 6β-hydroxy- and 21-hydroxyprogesterone derivatives (lanes 4 and 7) localized under UV illumination.

Results and Discussion

The hydroxylase activities of fish liver microsomes were evaluated using two typical steroidal substrates: progesterone and testosterone. These substrates have numerous sites for hydroxylation on their backbone, some of them being specific for particular P450 isoforms (*29*, *31*). Thus, alterations in either the concentration or the nature (isoforms) of the cytochrome P450 content of the liver microsomes is revealed by a significant modification of the steroid metabolite pattern (*30*, *31*). The oxidative metabolism of progesterone by microsomes from fish liver is presented in Figure 1. The relative amounts of progesterone metabolites (percent of the substrate concentration) are compared for three populations of fish. The presence of *C. taxifolia* in the aquarium as well as feeding fish with treated mussels results in a modification of the metabolite pattern. We observe a 4-fold decrease in the production of metabolite **9**, which is the major product in microsomes from untreated fish. In contrast, the production of **7** has increased 2.5-fold. By comparison with authentic standards, we tentatively assign metabolite **9** to the 2α-hydroxy derivative of progesterone; metabolite metabolite **7** remains unidentified.

Liver microsomal testosterone hydroxylase activities were evaluated; the pattern of metabolites generated are illustrate in Figure 2. In contrast with what we observed for progesterone, these activities were not strongly affected by the presence of *C. taxifolia*. However, the decrease in the production of metabolite **8** and the induction of metabolite **9** reflects some changes in the stereospecificity of the testosterone hydroxylations.

In liver microsomes, the metabolism of steroids could be mediated by two classes of enzymes, the oxido-reductases and cytochrome P450 systems. To identify which of these systems is responsible for the production of progesterone metabolites **7** and **9**, we repeated the experiments described above in the presence of 12.5% carbon monoxide (a P450 inhibitor) dissolved in the buffer. The result was unambiguous since the production of metabolites was strongly inhibited by this low carbon monoxide concentration (Figure 3). Note that we have chosen to work with a low CO concentration to ensure that enough oxygen was present for other possible oxy-enzymes. We can thus conclude that the production of these metabolites is P450 dependent and that these activities in fish liver are affected by the presence of the alga *Caulerpa taxifolia* in their environment. Due to the central role played by this enzymatic system in the metabolism of both endogenous and exogenous compounds this finding is of interest for future studies on the influence of environmental changes on local fish populations.

The glutathione S-transferase activity was assayed on the liver cytosols of the same fish populations (Table 1). GST activity tended to increase was in the presence of the alga, but due to the high interindividual variability the effects were not statistically significant. A striking observation was that the most affected group was the one which was in the aquarium containing the algae and not the one which was fed with *C. taxifolia* extract. This phenomenon was also observed for the steroid hydroxylase activity.

This last observation suggests that, in addition to herbivores, a multitude of marine species could be affected by this kind of environmental modification. To a possible effect via the food chain, these results add the possibility of a direct effect via a toxin dissolved in the water.

In our attempts to characterize cytochromes P450 in fish liver microsomes, we tried to record the characteristic spectra of these enzymes in the presence of carbon monoxide (*28*). The results were unreliable. Surprisingly, the only spectra we detected came from microsomes of fish killed only 24 h after their introduction in the aquarium containing the algae. This observation prompted us to study the influence of a short exposure to the algae on progesterone metabolism. Results presented in Figure 4 confirmed that progesterone hydroxylase activity is higher after 24 h than after 4 weeks exposure to the algae. Under these conditions, all the progesterone substrate was oxidized, and many unidentified polar metabolites were produced (Figure 4) which likely result from multiple hydroxylations on the same steroid molecule (*29*). The reason for this striking increase in activity is unknown and currently under study.

Various terpenes have been identified in the mediterranean *Caulerpa taxifolia*, the most abundant of them being the caulerpenyne (Cyn). The presence of this product in the water of aquaria was clearly demonstrated (*34*), and its effects on marine species was studied (*5*, *35*). Based on the structural

characteristics of this compound and particularly the presence of an acetylenic triple bond, our working hypothesis is that Cyn (or a parent derivative) could work first as a P450 inducer then as a suicide substrate acting via its acetylenic triple bound. This behavior has been demonstrated for other P450 substrates sharing this structural characteristic (*36*). It is generally assumed that the activation of the acetylenic function leads to the formation of a reactive intermediate that destroys the P450 structure (*8*, *37*).

The effects of authentic caulerpenyne on microsomes and purified P450 are now under investigation. For now, the nature of the compounds responsible for modifying the P450 dependent activities remains speculative. We demonstrate in this work that monitoring P450 activities can be used as a biosensor not only for the survey of external pollution (*38*, *39*) but also for evaluating subtle aquatic ecosystem modifications.

Acknowledgments

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