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Microalgae response to petroleum spill: An experimental model analysing physiological and genetic response of *Dunaliella tertiolecta* (Chlorophyceae) to oil samples from the tanker *Prestige*

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ABSTRACT

In November 2002, the oil tanker *Prestige* sank off the northwestern coast of Spain, spilling more than 50,000 tons of petroleum with disastrous ecological and economical consequences. In order to analyse the harmful consequences of the oil spill on marine microalgae, short- and long-term effects of oil samples from the *Prestige* spill were studied using laboratory cultures of *Dunaliella tertiolecta* (strain Dt1Lwt). Significant inhibition of photosynthesis (assessed by F_v/F_m, ETR_{max} and α estimations) was observed after only 1 h of oil exposure with clear concentration dependency. Three days later, photosynthetic activity remained inhibited although cell survival was only slightly effected. In cultures exposed to the lowest oil concentration, mitotic rates and percentage of motile cells were 17–33% and 12–42% of the controls, respectively. After 1 month, neither dividing nor motile cells were observed at the highest oil concentrations. However, after further incubation, occasionally the growth of rare cells resistant to oil was found. A fluctuation analysis was carried out to distinguish between resistant cells arising from rare spontaneous mutations and resistant cells arising from physiological or other mechanisms of adaptation. The existence of rapid evolution as result of preselective mutations from petroleum sensitivity to petroleum resistance was observed. Resistant cells arose by rare spontaneous mutations prior to the addition of oil, with a mutation rate of 2.76×10^{-5} oil-resistant mutants per cell division. Apparently, rare spontaneous preselective mutations are able to assure the survival of microalgae in oil-polluted environments.

1. Introduction

In November 13th, 2002, the 26-year-old tanker *Prestige*, which was carrying more than 77,000 tons of oil cargo from Latvia to Singapore, was damaged during a gale 45 km off the northwestern coast of Spain. Almost immediately, more than 6000 tons of heavy oil leaked from the damaged vessel. Six days later, the *Prestige* broke in half and sunk in the Northeast Atlantic Ocean (42°11′N, 12°02′W). As the ship was breaking the central oil tanks were affected and almost 50,000 tons of oil spilled into the sea, giving rise to a huge oil slick that washed ashore and polluted about 1000 km of sensitive Spanish coastline. From the sinking onward, more than 6000 additional tons of oil have spilled from the broken tanks of the *Prestige*.

The affected coastal zone is considered one of the most important ecological area of Spain, not only because is a stopover for migratory birds and a nesting place for resident seabirds, but also for its richness and abundance in marine flora and fauna. In addition, this region is among the main shellfish supplier for the rest of Europe (Fraga et al., 1984). Both ecological and economic consequences of the disaster have been dramatic as the oil slick brought about the oiling of thousands of seabirds, the damage of the intertidal flora and fauna of the affected area, and the breakdown of the shellfish industry and fisheries of that region. To date, more than 79,000 tons of residues have been collected from the affected area. Six years after its sinking, images of the *Prestige* remain etched in our memory. Never before in Spain had a disaster of this nature had such media, social and political impact. The oil reached the coast in several waves, affecting Galicia, Asturias, Cantabria, the Basque Country, and to a lesser extent, France and Portugal. Fishermen, shellfish gatherers, and thousands of volunteers took it upon themselves to prevent the worst consequences of the slick (Vinas, 2009). Unfortunately, environmental damages caused by hydrocarbons have increased over the last years due to both deliberate and accidental oil spills.

Effects of oil spills on aquatic ecosystems have been investigated *in situ* by short- and long-term studies. Short-term observations, mainly centred on both photosynthetic and invertebrate aquatic communities, usually show a shift of species composition and abundance after an oil spill due to the replacement of sensitive

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species by resistant ones (e.g., Lytle and Peckarsky, 2001; Marshall and Edgar, 2003). Long-term studies usually report cascades of late, indirect impacts on coastal communities due to chronic exposures to environment-sequestered petroleum products that delayed ecosystem recovery for years after an oil spill (reviewed by Peterson et al., 2003). Results seem to indicate that the ecological impact following an oil spill depends on the volume spilled, oil type, geographical location of the spill, the characteristics of the receiving water and its biota (i.e., sensitivity of organisms), and duration of contact with oil (Gin et al., 2001).

On the other hand, short-term laboratory experiments, using laboratory-tolerant taxa and model experimental designs, have also been conducted in order to evaluate more specifically the harmful effects of different petroleum products. Although most of these studies have been performed with zooplankton, some of them have been done using microscopic aquatic photosynthesizers as experimental organisms. Since phytoplankton is the base of the food web of aquatic ecosystems, it is very important to evaluate the effects of oil on them in order to understand and predict the long-term effects of oil spills on. In this sense, it has been suggested that perturbations of food web at the phytoplankton trophic level may have great impact upon marine ecosystems, depending on the magnitude of changes to phytoplankton, the group involved, and the physical characteristics of the water, such as concentrations of dissolved organic compounds, temperature, salinity currents, redox potential and nutrient loading (Daly and Smith, 1993; Pollumaa et al., 2001). Results from laboratory assays indicate that, in general, responses of microscopic photosynthesizers to oil are diverse (e.g., Fábregas et al., 1984; Megharaj et al., 2000; Singh and Gaur, 1990), and even this impact may be overestimated when comparing results with *in situ* field experiments (Mancinnis-Ng and Ralph, 2003). Despite this, the adaptation of phytoplankton to sublethal oil exposure is still poorly understood, and direct and indirect impacts remain scarcely quantified (Banks, 2003).

The aim of the present work was to assess the short- and long-term toxic effects of oil samples from the *Prestige* spill on marine phytoplankton using laboratory cultures of *Dunaliella tertiolecta* Butcher (Chlorophyceae). In addition, we also evaluated the adaptation of *D. tertiolecta* to oil spill from an evolutionary point of view. In general, organisms should be able to survive in contaminated environments as a result of two alternative processes: physiological acclimation resulting from modifications of gene expression or genetic adaptation due to mutations that confer resistance (Sniegowski and Lenski, 1995; Belfiore and Anderson, 2001). For this purpose, we performed a fluctuation analysis (Luria and Delbrück, 1943) using oil samples from the *Prestige* spill as selective agent. This experimental model is suitable to discriminate between cells that become resistant from acquired specific adaptation in response to oil samples from the *Prestige* (physiological acclimation) and resistant cells arising from rare spontaneous mutations that occur randomly prior to the oil samples exposure.

2. Materials and methods

2.1. Experimental organism

Experiments were performed with haploid vegetative cells of the marine microalgae *Dunaliella tertiolecta* (strain DtL1 wt) from the algal culture collection at the Laboratory of Genetics, School of Veterinary Sciences, Complutense University of Madrid (Spain). Prior to the experiments, cells were grown in cell-culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of filtered seawater (0.22 μm, Stericup presterilised, Millipore Corporation, Bedford, USA) enriched with f/2 growth medium (Sigma–Aldrich Química, Spain), at 25 °C and 25 μmol m^{-2} s^{-1} PAR, provided by white fluorescence tubes, under continuous light. Cultures were maintained, as axenically as possible, in balanced growth (acclimated, free-running and mid-log exponential growth) (Cooper, 1991) by serial transfers of an inoculum to fresh medium once a month. The absence of bacteria was tested periodically by direct examination using an epifluorescence microscope (Zeiss Axiovert) after staining with acridine orange. Only cultures lacking of detectable bacteria were used in the experiments.

2.2. Oil sample collection

Two different sampling sites were selected on the western coast of Galicia (Spain): Santa Cristina beach (NW Galicia), and San Miguel de Oia beach (SW Galicia). Oil samples from Santa Cristina and San Miguel de Oia were collected on the 12th of December 2002 (19 days after the *Prestige* sinking) and on the 31st of January 2003 (79 days after the *Prestige* sinking), respectively. Three oil samples were collected at each site and kept in plastic bottles in cold and dark conditions. Before the experiments, samples from each site were homogeneously mixed to yield two different samples: sample 1, from Santa Cristina beach, and sample 2, from San Miguel de Oia beach. The chemical composition of the Prestige oil is detailed in CSIC (2003).

2.3. Preparation of exposure media and experimental cultures

Since in oil spills part of the oil appears dispersed in the seawater while other part remained long time settled on the sea bottom, two experimental models were used in order to evaluate the effects of the oil samples on *D. tertiolecta* (a schematic diagram is represented in Fig. 1). In the so-called "planktonic model", sample 1, oil samples were sonicated and homogeneously mixed with f/2 growth medium. The obtained mixture was introduced in sterile cell-culture flasks, which were placed in a suspensor mixer (model 802, Lucham) and maintained in continuous moderate agitation during the experiments. In the "benthonic model", sample 2, oil samples were homogeneously spread at the bottom of sterile flasks and no agitation was applied. For both planktonic and benthonic models culture flasks were filled up to 20 ml of f/2 growth medium and approximately 5×10^5 *D. tertiolecta* cells from mid-log exponentially growing cultures were introduced. Cultures were kept at 25 °C and 25 μmol m^{-2} s^{-1} PAR during the experiments.

The experiment was performed in a concentration-response manner, hence oil samples were introduced into the growth medium at concentrations of 1:1000, 1:100 and 1:20 (fresh weight of oil:volume of culture medium). A concentration of 1:10 was also employed (but it was lethal). Experiments with both oil samples, 1 and 2, were conducted separately using three replicates for each oil concentration and six unexposed controls for each model.

2.4. Exposure studies

The effects of the different concentrations of the two oil samples were studied on several biological variables (photosynthesis, cell survival, mitotic rate and cell motility) and at three different times (1 h, 72 h and 30 days).

Photosynthetic activity was estimated by measuring variable chlorophyll *a* fluorescence of PSII with a pulse amplitude modulated PAM-2000 fluorometer (Walz, Effeltrich, Germany). After both 1 h and 72 h of oil exposure, the optimal quantum yield, defined as the ratio of variable (F_v) to maximal fluorescence (F_m), F_v/F_m, of the dark-adapted cultures (15 min dark guarantees an oxidised electron transport chain) was assessed according to Schreiber et al. (1986). In this ratio, F_v is calculated as $F_v = F_m - F_0$, in which F_0 is the initial fluorescence (when all PSII reaction centers are active or "open") and F_m is the maximal fluorescence (when all reaction centers of PSII are "closed").