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ARTÍCULO

A fascinating example of microalgal adaptation to extreme crude oil contamination in a natural spill in Arroyo Minero, Río Negro, Argentina

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ABSTRACT

Nowadays, accidental spills of crude oil are one of the most worrisome environmental problems. Usually, the crude oil spills rapidly inhibited photosynthesis of microalgae (the main primary producers of aquatic ecosystems) causing a severe damage to inland waters ad marine ecosystems. In order to add knowledge about microalgal response to crude oil spill, here we study a fascinating example of extreme contamination by crude oil spills continuously (at least since 1915) in Arroyo Minero, Nirihuan de Arriba, Río Negro, Argentina. This study is changing our pre-conceived ideas on the adaptation of microalgae to crude oil. Astonishingly, a high biomass of microalgae proliferates in contact with crude oil. In contrast with the paradox of the plankton (which predict that more than 30 microalgal species would coexist in the same water body) only four species were detected in the crude oil spill area. They are cosmopolitan mesophile species and not extremophile ones (as would be expected). The most abundant species was the

Chlorophyta *Scenedesmus obtusus*. Other abundant species seems to be a new *Scenedesmus* species. The other two species *Symploca dubia* Cyanobacteria and *Chlamydomonas dinobryonis* Chlorophyta are new records for flora of Argentina. These species were isolated maintained in clonal laboratory cultures and characterised. They are resistant to crude oil of Arroyo Minero and to analytical petroleum special standard. In contrast similar species isolated from areas without crude oil contamination were destroyed by petroleum.

Key Words: Adaptation; Crude oil spill; Microalgae; *Chlamydomonas dinobryonis*; *Scenedesmus obtusus*; *Symploca dubia*.

RESUMEN

Un fascinante ejemplo de adaptación de microalgas a una contaminación extrema de petróleo en un arroyo natural en Arroyo Minero, Río Negro, Argentina

En la actualidad los vertidos accidentales de petróleo constituyen uno de los más preocupantes problemas ambientales. Los vertidos de petróleo inhiben rápidamente la fotosíntesis de las microalgas (principales productores primarios en ecosistemas acuáticos) y causan un daño severo en ecosistemas continentales y marinos. Para aumentar nuestro conocimiento sobre los efectos de los vertidos de petróleo en las microalgas, estudiamos aquí un ejemplo fascinante de una contaminación extrema por un vertido continuo de petróleo que empezó al menos en 1915 en Arroyo Minero, Nirihuan de Arriba, Río Negro, Argentina. Este estudio está cambiando muchas de nuestras ideas preconcebidas sobre la adaptación de microalgas al petróleo. Sorprendentemente, una elevada biomasa algal prolifera en contacto con el petróleo. En contraste con la paradoja del plancton (que predice que más de 30 especies de microalgas deberían coexistir en la misma masa de agua) sólo se detectaron cuatro especies en al área de vertido de petróleo. Se trata de especies mesófilas cosmopolitas y no de especies extremófilas (como cabría esperar). La especie más abundante fue la Chlorophyta *Scenedesmus obtusus*. Otra especie abundante parece ser una nueva especie de *Scenedesmus*. Las otras dos especies, *Symploca dubia* Cyanobacteria y *Chlamydo-*

monas dinobryonis Chlorophyta, son nuevos registros para la flora de Argentina. Estas especies se aislaron, clonaron, mantuvieron en cultivos clónicos en el laboratorio y caracterizaron. Estos cultivos resultaron ser resistentes al petróleo del Arroyo Minero y al petróleo de estándares analíticos. En contraste, el petróleo destruyó a especies semejantes aisladas de áreas no contaminadas.

Palabras clave: Adaptación; Vertidos de petróleo; Microalgas; *Chlamydomonas dinobryonis*; *Scenedesmus obtusus*; *Symploca dubia*.

1. INTRODUCTION

Nowadays, we are living in a geological instant in which global extinction rates are 500 times background and are increasing due to those human activities that are contaminating biosphere. It is supposed that several million populations and 300 to 30.000 species go extinct annually from a total of > 10 million species (1). Distinctive features of biosphere future could include a proliferation of opportunistic species resistant to anthropogenic contaminants (2). The biodiversity crisis is reasonably understood for animals and plants, but is less predictable in microbes that succumb to anthropogenic toxins (1).

The occurrence of crude oil spills is one of the most worrisome environmental problems since the World's energy dependence on petroleum. Crude oil is a highly toxic mixture of more than 10.000 different hydrocarbons (with approx. 55% naphtenes, 20% aromatic compounds and 20% paraffin) and variable quantities of sulphur and others. Accidental spills of crude oil in environment cause severe contamination of marine and continental ecosystems. Contamination due to spill of processed petroleum derivates (especially diesel and fuel) is an important problem in continental waters (3). Crude oil spills are between the worst environmental catastrophes (i.e. Exxon Valdes, Prestige).

In aquatic ecosystems, the tolerance of microalgae and cyanobacteria to environmental stress consequence of water contamination is very relevant because these organisms are the principal primary producers of aquatic ecosystems (4). Consequently, survival and growth of phytoplankton living in contaminated environments is an interesting topic from an ecological point of view (5). There is a growing interest to study effects of crude oils and oil components on microalgae (for example, see references 6-14). Usually, crude oil spills rapidly inhibits photosynthesis and growth of these primary producers (10, 15). However, the effects of crude oil on phytoplankton are very variable (16-19), influence of dispersants (20). Recent experiments suggest that different species show a different response against crude oil contamination (15). Unfortunately, little is known about algal response to crude oil contamination.

In order to add knowledge about microalgal response to crude oil spill, we study a fascinating example of the extreme contamination by crude oil in Arroyo Minero, Nirihuan de Arriba, Río Negro, Argentina. A natural spill of crude oil has been pouring perhaps during hundreds of years. Around 50 m of natural spill there is and artificial spill from a tasting held made in 1915 for a possible exploitation of the crude. Both spills are composed of crude oil mixed with gas and fresh water. Both spill fall into the Arroyo Minero River after flowed slowly a few meters.

Surprisingly, the crude oil spill area of Arroyo Minero River has an abundant microalgal biomass living in contact with crude oil. The main aim of this study was to describe the first known community of microalgae living in permanently crude oil contaminated environment.

2. MATERIALS AND METHODS

Sampling of water and phytoplankton was carried out in Arroyo Minero, Nirihuan de Arriba, Río Negro, Argentina, on April 4th, 2008. Two samples points were studied: i) the spring of the natural oil spill, and ii) Arroyo Minero river about 25 meters down water from the petroleum spill.

In each point physicochemical characterization of the water were carried out using an YSI- 6820 Multi-parameter Water Quality Monitors Sonde (1700/1725 Brannum Lane. Yellow Springs Ohio 45387, USA). In addition water samples of 1 L (+ crude oil) were collected in dark glass (amber type) for chemical analysis. Phytoplankton was identified *in situ* directly after collection using a McArthur portable microscope (Kirk Technology, England), as well as in laboratory on fresh and a 4% PBS-buffered formalin samples using a Zeiss microscope with phase contrast and Nomarski (Oberkochen, Germany). Identification of algae was carried out in accordance with Bourrelly (21), Cox (22), Zalocar *et al.* (23), Mirande & Tracanna (24, 25), Mirande *et al.* (26). Cell densities of phytoplankton were estimated on 4% PBS-buffered formalin fixed samples in settling chambers using an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany).

In addition, we isolated microalgae from the crude spill area of Arroyo Minero to be cultured in laboratory. Sampling was performed using 13 ml polystyrene culture sterile tubes (Sarstedt, Aktiengesellschaft & Co., D-51588 Nümbrecht, Germany). The samples were maintained at 20 ºC until we arrive to laboratory.

Two methods were used for isolation: i) direct isolation using micropipettes with a Zeiss-Eppendorf micromanipulator connected to inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany), and procedures of successive dilutions in polystyrene culture sterile tubes [as previously described in Costas *et al.* (27) and Brand (28)].

Once isolated the strains were re-cloned by isolating a single cell. The strains were grown in 100 ml cell culture flasks with aerator cap (Greiner Bio-One Inc Longwood, NJ, USA), in 20 ml of culture medium BG-11 (Sigma Aldrich Chemist, Taufkirchen, Germany), under continuous light of 60-mmol m^{-2} s^{-1} over the waveband 400-700 nm provided by daylight fluorescent tubes (Phillips TLD 36W/33, France), at 20 °C in growth chambers (Cámaras de Crecimiento, Modelo AGP, Ing. Climas, C/ Industria 498-500, Badalona 08918, Barcelona, Spain). BG-11 culture medium was prepared with distilled water from the distiller (Elix 3uv Millipore) and filtered through sterile-cup (Express Plus membrane, 250 ml) with filter of 0.22 mm. Cultures were maintained axenically in mid-log exponential growth phase by serial transfers of subcultures of a small inoculum (1-3 ml) to new culture medium (20 ml) once every 30 days. The strains were added to the algal culture collection of at Genetics Laboratory, School of Veterinary Medicine, Complutense University of Madrid.

The damage caused by crude oil on these Arroyo Minero strains was measured using a toxicity test. We also used the *Scenedesmus intermedius SiD1* wild-type strain (from algal collection of Genetics Laboratory, School of Veterinary Medicine, Complutense University of Madrid) as a control. *SiD1* strain was isolated from a pristine lake in Doñana National Park (Huelva, Spain), which never has been exposed neither to crude oil nor petroleum derivates. Crude oil collected in Arroyo Minero as well as a standard of petroleum (Fluka Analytical Petroleum special standard. Sigma-Aldrich Chemie, GmbH, Ch-9471, Buchs, Germany) were used at concentration of 12% v/v diluted in BG-11 medium. The mixing of petroleum and culture medium, was obtained by sonication with Vibra Cell (Sonics & Materials Inc Danbury CT, USA), maintaining the tubes within a bucket of crushed ice during the process. The pattern for sonication was of 4 pulses of 10 seconds each, with a power of 40 watts and a frequency of 16 KHz. Measuring crude oil effect on photosynthetic performance we performed the toxicity test. The change in the effective quantum yield of photosystem II (FPSII) was measured on three replicates of both exposed and control cultures using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany). The measures were performed after 48 hours of petroleum exposure. Effective quantum yield was calculated as follows:

$$\Phi PSII = (F'm - Ft) / F'm$$

where F'm and Ft are the maximum and the steady-state fluorescence of light-adapted cells, respectively (29).

3. RESULTS

Apparently, the natural spill of crude oil of Arroyo Minero is a complex and variable mix of crude oil, gas and water (Figure 1a, 1b). Total hydrocarbons concentration in the spill area was 11,7% v/v. Water in the spill area was at 19 ºC, pH 8.1 and 694 µS conductivity. There is a strong evaporation of the more volatile parts of the crude oil with an intense odour to hydrocarbons in all the area.

Dense patches of fuel remain in the natural emergence of spill and adjacent areas (Figure 1b). Astonishingly, all the area of petroleum spill of Arroyo Minero has important microalgal concentrations even in contact with crude oil (Figure 1c). This microalgal biomass seems to be enough to sustain a short trophy chain of few zooplankton and micro-invertebrate species. Crude oil reaches the Arroyo Minero and dilutes progressively (Figure 1d, 1e). Arroyo Minero waters 25 meters down water of crude oil spill was at 16 ºC, pH 8.3 and 517 µS conductivity. In this area total concentration of crude oil was 1,5% v/v.

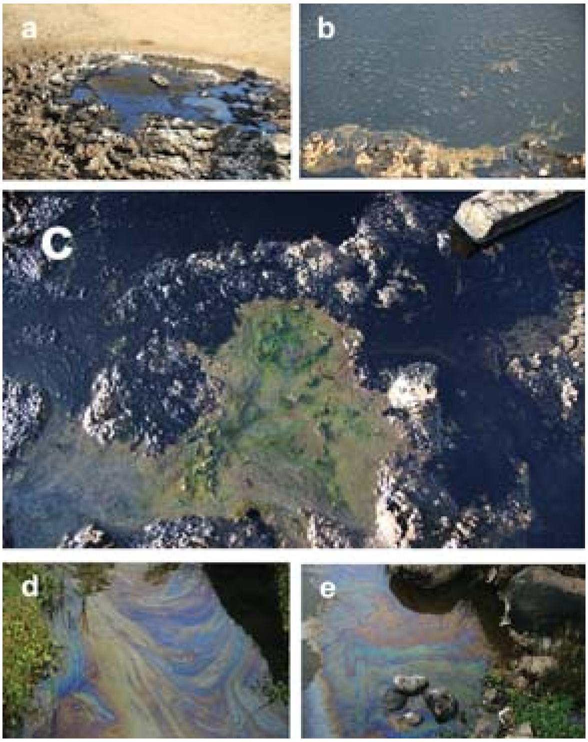

Figure 1. **a)** Natural spill of crude oil of Arroyo Minero. **b)** Crude oil with gas bubbles and water. **c)** Microalgal concentrations in contact with crude oil. **d)** Crude oil reaches Arroyo Minero. **e)** Crude oil dilutes progressively in Arroyo Minero.

However, the great microalgal biomass inhabiting the natural emergence of crude oil of Arroyo Minero is constituted only by very few species. We only found four cosmopolitan mesophile species of phytoplankters: three Chlorophyceans and one Cyanobacteria species. They are:

Symploca dubia (Nägeli, 1849) Gomont, 1892. Cyanobacteria, Oscillatoriales, Phormidiaceae. Fascicles of filaments parallel oriented to the surface, without branched, and anastomosed. The filaments are more 1 mm of wide, and without heterocysts (Figure 2a). Cell density this species was of 98 ± 16 filaments ml^{-1}.

Chlamydomonas dinobryonis G. M. Smith, 1920. Chlorophyceae, Volvocales, Chlamydomonadaceae. The motile cells are pyriforms, and it presents a small parietal pyrenoid. 5.5 µm in diameter. The palmoid form presented three-dimensional without branched, and without spines (Figure 2b). Cell density this species was of 2100 ± 200 cells ml^{-1}.

Scenedesmus obtusus Meyen, 1829. Chlorophyceae, Chlorococcales, Oocystaceae. Cenoby usually in 2-4-cells (rarely 8 or more) retained within persistent mother cell wall. Cells of the more common 4-celled colonies laying in the same plane, however, cell can be displaced in the axis. Cells oval 7.5 µm diameter major and 6.5 µm in diameter minor (Figure 2d). Cell density this species was of 9.200 ± 700 cells ml^{-1}.

Finally, Chlorophyceae, Chlorococcales, Oocystaceae a species with cenoby usually in 2-4-cells cells, 4-celled colonies laying in the same plane, however, cell can be displaced in the axis. Cells almost spherical (only lightly oval) around 5.5 µm diameter major and around 5.0 µm in diameter minor (Figure 2c). After consult several taxonomic expertise, this species seem to be a *Scenedesmus*, perhaps a new species (*Scenedesmus rapoporti*). To confirm this, a molecular genetic characterization (DNA sequenciation, immunological and lectin binding patterns) is in process. Cell density this species was of 7.200 ± 300 cells ml^{-1}.

Clonal cultures of these four species were established in laboratory. The four strains were able to grow in BG-11 medium in absence of petroleum. The strains were propagated in cultures exclusively by asexual reproduction. The two species of *Scenedesmus* grow rapidly around one doubling every two days. *Symploca dubia*

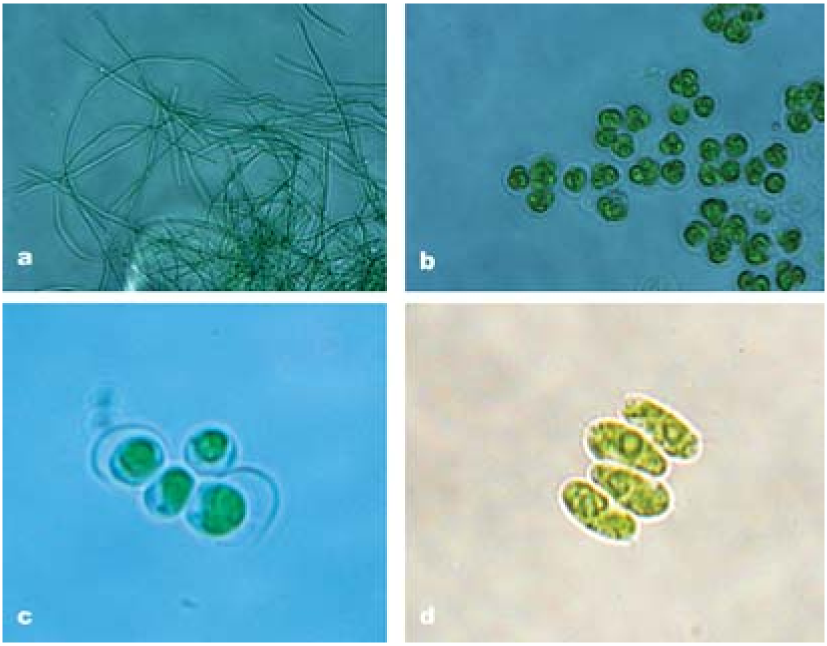

Figure 2. **a)** *Symploca dubia*. **b)** *Chlamydomonas dinobryonis*. **c)** *Scenedesmus sp* (sp nov.?). **d)** *Scenedesmus obtusus*.

and *Chlamydomonas dinobryonis* strains grow more slowly (about one doubling in three days).

The toxicity test shows that neither crude oil from Arroyo Minero, nor Fluka Analytical Petroleum Special Standard were toxic for the species isolated from the crude oil spill of Arroyo Minero (Table 1). Photosynthesis performance was not inhibited by concentrations of 12% v/v crude oil (11,7% v/v crude oil was maximum dose detected in Arroyo Minero) after 48 hours under crude oil exposure (a complete cell cycle in *Scenedesmus* species). In contrast, both crude oil from Arroyo Minero as well as Fluka Analytical Petroleum Special Standard cause inhibition of more than 50% of effective quantum yield of photosystem II of the control species (*Scenedesmus intermedius*) isolated from Doñana National Park, after 48h of 12% v/v petroleum exposure.

Table 1. **Toxicity test. Inhibition of photosynthetic performance (effective quantum yield of photosystem II; ΦPSII) of species isolated from the crude oil spill of Arroyo Minero as well as the control isolated from Doñana National Park, after 48 h. of petroleum exposure (10% v/v in BG-11 medium)**

		Inhibition of photosynthesis by crude oil of Arroyo Minero	**Inhibition of photosynthesis by Fluka Analytical Petroleum Special Standard**
Species from crude oil spill	*Symploca dubia*	Undetectable	Undetectable
	Chlamydomonas dinobryonis	Undetectable	Undetectable
	Scenedesmus sp	Undetectable	Undetectable
	Scenedesmus obtusus	Undetectable	Undetectable
Species from pristine environment	*Scenedesmus intermedius*	> 50%	> 52%

4. DISCUSSION

The crude oil spill of Arroyo Minero is a natural laboratory to study adaptation of phytoplankton to extreme petroleum contamination, because at least crude oil spills continuously at least since 1915. A series of astonishing finding in Arroyo Minero are changing our pre-conceived ideas on the adaptation of microalgae to crude oil in this fascinating example of the extremely contaminated freshwater ecosystem.

First of all, a surprisingly high biomass of microalgae proliferates even in the more contaminated areas of Arroyo Minero. Microalgae have been able to grow in contact with recalcitrant contamination by fuel patches. These algae are the base for a few species of zooplankton and micro-invertebrates.

The coexistence of more than 30 species of microalgae in the same water body is a usual characteristic of all inland freshwater and marine ecosystems that has been called «the Paradox of the Plankton» (30). After thousand studies on the most diverse aquatic ecosystems, the Paradox of the Plankton is a well-established fact (31, 32). Even in red tide events there several species coexist (33). In contrast, only four species were detected in the crude oil spill area of Arroyo Minero. However, many phytoplankton species inhabits Arroyo Minero River previously to the crude oil spill area (we identify 39 microalgal species of Cyanobacteria, Bacillariophyta, Chlorophyta, Dinophyta and others). Apparently, adaptation to crude oil contamination is not easy and most of microalgal species are unable to adaptation at toxic effect of petroleum.

Extreme environments (characterized by extreme values of pH, toxic minerals, temperature, and other factors) frequently support unusual communities of phytoplankters (34). It is frequently assumed that extremophile species more than mesophile species inhabit the extreme environments. However, the 4 species living in the crude oil spill area of Arroyo Minero are mesophile species. Only species of Chlorophyta and Cyanobacteria Division were able to proliferate in the crude oil spill area.

Recent works suggest only a few mesophile species (mainly Chlorophyta) are able to proliferate in extreme environments. This pattern was observed by first time in the Guadiamar River (S, Spain) after the toxic heavy metal spill of Aznalcollar mine (35) and confirmed in the heavy metal contaminated environments of Aguas Agrias, Spain (36), Mynydd Parys, Wales, UK (37), and Rio Tinto, Spain (38). In la Hedionda, Spain, an example of extreme sulphureus waters, also proliferate a few mesophile Chlorophyta species (39).

This fact seems to be the pattern followed by microalgae in extreme geothermal waters of Eolias islands, Italia and Argentina (38, 40).

The most abundant species in the crude oil area was the cosmopolite mesophile Chlorophyta *Scenedesmus obtusus*. This species is able to grow rapidly under the extreme crude oil contamination of Arroyo Minero as well as under a Fluka Analytical Petroleum Special Standard. However, crude oil of Arroyo Minero and Petroleum Special Standard caused inhibition of more than 50% of effective quantum yield of photosystem II of *Scenedesmus intermedius*, with died afterwards. This species closely related with *Scenedesmus* was isolated from a pristine lagoon in Doñana National Park, which never was exposed to petroleum prior to the experiment. This fact suggest the four species proliferating in the spill area of Arroyo Minero are the result of some kind of genetic evolutionary change occurs in the past, which allows the adaptation of phytoplankton to extreme crude oil contamination.

In extreme environments characterized by values of environmental contaminants exceeding the physiological limits of organisms, survival of microbes depends exclusively of selection on pre-existing genetic variability that occurs spontaneously by rare spontaneous mutation [i.e. mutations occur exclusively by chance and independently of the selective agent; reviewed by Sniegowski (41)]. However, some microbiologist debates this neo-Darwinian theory suggesting an alternative explanation called adaptationist process [reviewed by Creager (42)]. In some cases, adaptive mutation mutations occur in bacteria as a direct and specific response to the selective agent (43, 44). However a lot of works on adaptation of algae to antibiotics, toxic substances and extreme environments have find that adaptation depends exclusively of selection on rare spontaneous mutants that occurs prior to exposure at selective agent (45-52).

Finally, although several studies recorded phytoplankton flora from N Argentina (i.e. 23-26, 53), the knowledge of the flora of phytoplankters of Patagonia Argentina is very scant. We identify two species from crude oil spill of Arroyo Minero (*Symploca dubia* and *Chlamydomonas dinobryonis*) that are new records for flora of Argentina. Other species could be a new species (*Scenedesmus* *rapoporti*). However, Mirande *et al.* (26) previously recorded *S. obtusus* from Tucamán province (N Argentina).

5. CONCLUSION

A high microalgal biomass proliferates in contact with crude oil patches in Arroyo Minero, Nirihuan de Arriba, Río Negro, Argentina, an area extremely contaminated by continuous spills of crude oil at least since 1915. Only four species of phytoplankton are able to grow (the Chlorophyta *Scenedesmus obtusus*, other *Scenedesmus* species (perhaps a new species), the Chlorophyta *Chlamydomonas dinobryonis* and the Cyanobacteria *Symploca dubia*). They are cosmopolitan mesophile species and not extremophile ones (as would be expected). These species (isolated and maintained in clonal laboratory cultures) are resistant to crude oil of Arroyo Minero as well as to analytical petroleum special standard.

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