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FACULTAD DE VETERINARIA
Departamento de Producción Animal

ADAPTACIÓN DE MICROORGANISMOS FOTOSINTÉTICOS DEL PLACTON DE AGUAS CONTINENTALES AL CAMBIO AMBIENTAL BRUSCO: CONTAMINANTES DE ORIGEN ANTROPOGENÉTICO Y CONDICIONES NATURALES EXTREMAS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Fernando Marvá Ruiz

Bajo la dirección de los doctores
Eduardo Costas Costas y Victoria López Rodas

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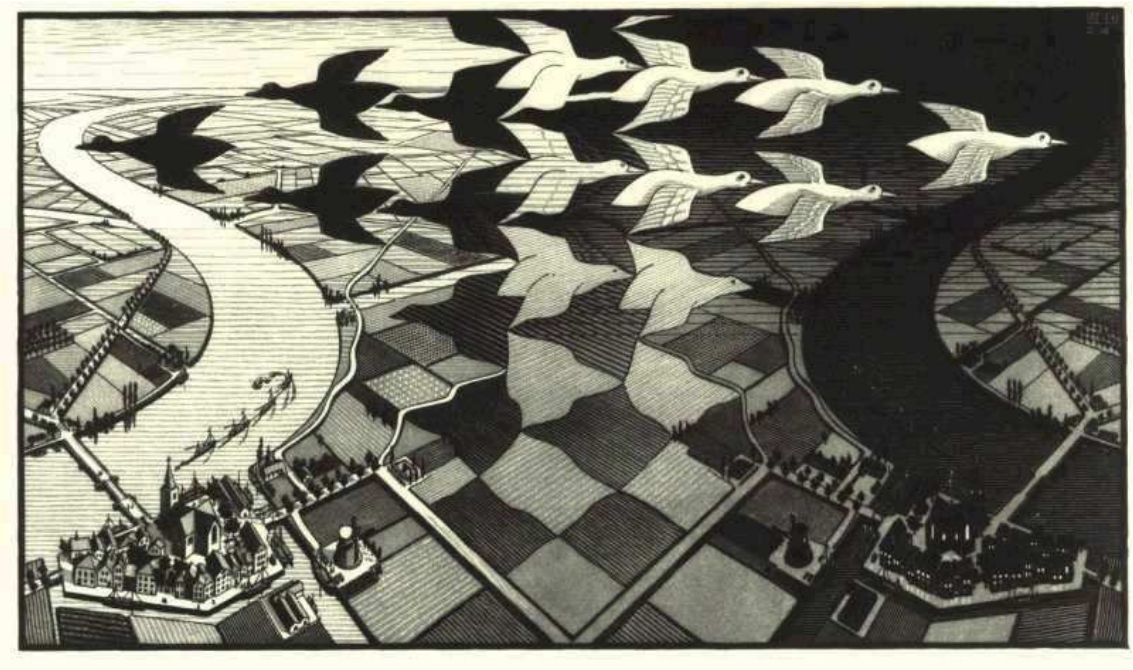

TESIS DOCTORAL

Fernando Marvá Ruiz
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El Dr. Eduardo Costas Costas, Catedrático de Genética del Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid y la Dra. Victoria López Rodas, Profesor Titular de Genética del Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid.

INFORMAN:

Que el trabajo de Tesis titulado: "**Adaptación de microorganismos fotosintéticos del plancton de aguas continentales al cambio ambiental brusco: contaminantes de origen antropogénico y condiciones naturales extremas**", ha sido realizado en el Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid bajo nuestra Dirección.

Y para que así conste lo firmamos en Madrid a cinco de mayo de dos mil ocho.

Dr. Eduardo Costas Costas
Catedrático de Genética.
Dpto. de Producción Animal
Facultad de Veterinaria
Universidad Complutense de Madrid

Dra. Victoria López Roda
Profesor Titular de Genética
Dpto. de Producción Animal
Facultad de Veterinaria
Universidad Complutense de Madrid

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Facultad de Veterinaria
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Adaptación de microorganismos fotosintéticos del plancton de aguas continentales al cambio ambiental brusco: contaminantes de origen antropogénico y condiciones naturales extremas

Memoria presentada por
Fernando Marvá Ruiz
para optar al grado de
Doctor

A mi padre.

“Hay hombres que luchan un día, y son buenos.
Hay otros que luchan un año, y son mejores.
Hay quienes luchan muchos años, y son muy buenos.
Pero hay los que luchan toda la vida: esos son los imprescindibles”

Bertolt Brecht

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Esta tesis se ha hecho realidad gracias al apoyo de mi familia. Raquel, más de la mitad de esta tesis te pertenece. Gracias por esos años en nuestra mini-house. En ti especialmente pienso cuando digo que no se puede agradecer con palabras toda la ayuda prestada. Padres, Marcos, Eva, Nacho, Dorotea, Marina. Gracias por constante apoyo y por hacer posible esta tesis. Mi más sincero agradecimiento a la fundación MAR-ES por su firme y solidaria apuesta por los jóvenes investigadores. A mi tío Andrés por estar siempre ahí dispuesto a echar una mano. A mis tías Val y Carmina, por los viajes aprovechados a la Terreta.

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RESUMEN

Los microorganismos fotosintéticos del plancton tienen un papel muy importante en el correcto funcionamiento de los ecosistemas, puesto que son los principales productores primarios en medios acuáticos. Son responsables directos de aproximadamente la mitad de la producción primaria de la biosfera a través de la fotosíntesis oxigénica. Las cianobacterias, además, son capaces de fijar el nitrógeno atmosférico en moléculas orgánicas nitrogenadas, cerrando el ciclo del nitrógeno. Por tanto, aunque la tolerancia de estos microorganismos a contaminantes de origen antropogénico es importante desde un punto de vista ecológico, aún no se conoce lo suficiente sobre su capacidad de adaptación a estos nuevos ambientes.

En esta tesis analizamos un aspecto muy poco estudiado de la genética de los microorganismos fotosintéticos del plancton: los mecanismos genéticos de adaptación a contaminantes de origen antropogénico presentes en el medio a concentraciones superiores a los límites de aclimatación fisiológica. Los contaminantes de origen antropogénico estudiados en esta tesis pertenecen a familias de: herbicidas (glifosate, simazina y diquat), antibioticos (cloranfenicol), químicos industriales (formaldehído) y metales pesados (Cr(VI)). Los resultados indican que el fitoplancton es capaz de adaptarse a los contaminantes de origen antropogénico estudiados gracias a mutaciones preadaptativas que sucede al azar con unas frecuencias comprendidas entre $3{,}1 \times 10^{-7}$ y $5{,}3 \times 10^{-5}$ mutaciones por división celular. Esta adaptación conlleva un coste para las células resistentes, puesto que estas tienen una tasa de división menor que las sensibles en condiciones no selectivas. Además estudiamos la progresiva adaptación de las células resistentes al agente selectivo.

Por otro lado investigamos sobre la capacidad de adaptación de los microorganismos fotosintéticos del plancton de aguas continentales a ambientes naturales extremos. Los resultados sugieren que la aparición de mutaciones espontáneas preadaptativas es un mecanismo capaz de asegurar la adaptación instantánea de algunos microorganismos fotosintéticos mesófilos a ambientes naturales extremos. Los ambientes estudiados fueron: por un lado

aguas con sustancias tóxicas de origen volcánico procedente la Isla de Vulcano (Italia), y por otro aguas ácidas con una elevada concentración de metales pesados procedentes de antiguas explotaciones mineras: Arroyo de Aguas Agrias en Tharsis (Huelva) y Mynyyd Parys (País de Gales). Las clorofíceas (eucariotas) fueron capaces de adaptase a estos medios, mientras que en el caso de las cianobacterias (procariotas) no se dio la adaptación.

Por último, en esta tesis proponemos la aplicación de cepas de la clorofícea *Dictiosphaerium chlorelloides* sensibles y resistentes al cromo (VI), para la construcción de biosensores sensibles y específicos. Además estudiamos la sensibilidad de diferentes especies de clorofíceas al amonio cuaternario DEAB. Proponemos que la respuesta diferencial del rendimiento cuántico de la fotosíntesis y de la producción de oxígeno, y dependiente de la concentración del tóxico, podría utilizarse como mecanismo para detectar aguas contaminadas con este amonio cuaternario.

SUMMARY

Phytoplankton plays an important ecological role in aquatic ecosystems as primary producers. Approximately phytoplankton produces almost a half of the total atmosphere oxygen. Some cyanobacteria fix nitrogen and assimilate it as an organic nitrogen. Thus, the tolerance of these microorganisms to anthropogenic water pollutants is very relevant from an ecological point of view. Unfortunately little is know about the mechanisms allowing phytoplankton adaptation to such conditions.

First of all, we analysed the ability of phytoplankton for adaptation to anthropogenic water pollutants through genetic mechanisms. The anthropogenic substances tested were herbicides (glifosate, simazine and diquat), antibiotic (chloramphenicol), chemicals pollutants (formaldehyde and DEAB) and a heavy metal (Cr(VI)). The results suggest that phytoplankton is able to adapt to polluted waters through pre-selective spontaneous mutation that appear before the exposure to the pollutant. The mutation rates were estimated into the range from $3.1x10^{-7}$ to $5.3x10^{-5}$ mutants per cell generation. However, this adaptation has an energetic cost. The resistant cells have a lower fitness than sensitive ones under non selective conditions. We also evaluated the evolution of pollutant-resistant cells under polluted environments.

We also studied if pre-selective rare spontaneous mutations are enough to ensure adaptation of mesophilic phytoplanktonic microorganisms to natural extreme environments. The extreme environments studied were sulphureous, acidic (pH 3.1) water from the largest geothermal pound on Vulcano Island (S Italy), and two environments of acidic and high concentration of heavy metals waters, from two different abandoned mines: Aguas Agrias in Thasis (Huelva, SE Spain) and Mynyyd Parys (N Wales). In these cases, the chlorophita *Dictyosphaerium chlorelloides* was able to adapt to these natural extreme environments, whereas the cyanobacteria was not able to adapt to.

Finally, we tentatively propose that the different response respect to photosynthetic activity observed between sensitive and resistant cells of

D.chlorelloides in the presence of Cr(VI), could be used to obtain a chromium-specific microalgal biosensors. We also studied the sensibility of two different strains of chlorophiceae to the quaternary ammonium (DEAB). We propose that concentration-dependent toxic response of photosynthesis performance and oxygen production could be used to detect presence of quaternary ammonium pollutants in freshwater.

ESTRUCTURA GENERAL DE LA TESIS DOCTORAL

La presente memoria está basada en artículos científicos publicados (o en trámites de publicación) en revistas científicas incluidas en el *Science Citation Index*.

Los resultados se presentan divididos en cuatro capítulos:

Capitulo 1. Adaptación del fitoplancton a contaminantes de origen antropogénico:

López-Rodas, V., Flores-Moya , A., Maneiro, E., Perdigones, N., **Marvá**, F., García, M.E., Costas, E. 2006. Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of pre-selective mutations. **Evolutionary Ecology**. 21:537-547. DOI: 10.1007/s10682-006-9134-8.

Marvá, F., López-Rodas, V., Rouco, M., Navarro M. C., Toro, F. J., Flores- Moya, A. and Costas, E. 2008. Human-synthesized pollutants drive emergence of evolutionary novelties: pre-selective mutations confer resistance to the herbicides simazine and diquat in green microalgae. **Environmental Science and Pollution Research.** (under review)

López-Rodas, V., Perdigones, N., **Marvá, F**., Rouco, M. and García-Cabrera, J.A. 2007. Adaptation of phytoplankton to novel residual materials of water pollution: An experimental model analysing the evolution of an experimental microalgae population under formaldehyde contamination. **Bulletin of Environmental Contamination & Toxicology**. 21:535-547. DOI: 10.1007/s00128-007-9336-y.

Marvá et al. Experimental models to analyze adaptation of algae to anthropogenic contamination: 1. Pre-selective mutation confers antibiotic resistance in green microalgae. (en preparación)

Capítulo 2. ¿Qué sucede tras la primera mutación que permite la adaptación?:

Marvá et al. Experimental models to analyze adaptation of algae to anthropogenic contamination: 2. Adaptive change by mutation-selection increase fitness in antibiotic-resistant green microalgae. (en preparación)

Capítulo 3. Adaptación de microorganismos fotosintéticos del plancton a ambientes naturales extremos:

López-Rodas, V., Costas, E., Maneiro, E., **Marvá**, **F.,** Rouco, M., Delgado, A. and Flores-Moya, A. 2007. Living in vulcan´s forge: algae adaptation to stressful

geothermal ponds on Vulcano Island (S Italy) as result of pre-selective mutations. **Phycological Research**. (under review)

López-Rodas, V., **Marvá, F**., Costas, E., Flores-Moya, A. 2007. Microalgal adaptation to a stressful environment (acidic, metal-rich mine waters) could be due to selection of pre-selective mutants originating in non-extreme environments. **Environmental and Experimental Botany**. (in press). DOI: 10.1016/j.envexpbot.2008.01.001.

Costas, E., Flores-Moya, A., **Marvá, F**., Rouco, M., López-Rodas, V. 2008. Adaptation of the chlorophycean Dictyosphaerium chlorelloides to stressful acidic, mine metal-rich waters as result of pre-selective mutations mutations. **Chemosphere**. (in press) DOI: 10.16/ j.chenoschere.2008.04.009

Capítulo 4. Aplicación: biosensores microalgales sensibles y específicos:

Sánchez-Fortún, S., **Marvá, F**., D'Ors, A., Costas, E. 2007. Inhibition of growth and photosynthesis of selected green microalgae as tools to evaluate toxicity of dodecylethyldimethyl-ammonium bromide. **Ecotoxicology.** (in press). DOI: 10.1007/s10646-007-0189-2.

Sánchez-Fortún, S., **Marvá, F.**, Rouco, M., Haigh-Florez, D., López-Rodas, V. and Costas, E. 2008. Resistance of Phytoplankton to Chromium Contamination: Physiologicalacclimation *versus* Genetic Adaptation. **Water Research**. (under review)

Los cuatro capítulos están precedidos de una introducción general y un breve resumen de la metodología empleada. A continuación se plantean cuatro objetivos, cada uno de los cuales se aborda en cada uno de los capítulos a través de artículos científicos. La memoria acaba con una discusión general y las conclusiones.

1. INTRODUCCIÓN.

1.1. La importancia ecológica de los microorganismos fotosintéticos del plancton.

Los microorganismos fotosintéticos del plancton son un conjunto polifilético de microorganismos que poseen la característica común de realizar la fotosíntesis oxigénica. Dentro de este grupo se engloban procariotas (cianobacterias), protistas (dinoflagelados) y eucariotas (clorofíceas). Forman parte de todos los ecosistemas acuáticos del planeta, de manera ubicua, tanto de los ecosistemas acuáticos de aguas marinas y continentales (Kirk, 1994), como de los ecosistemas edáficos (McCann y Cullimore, 1979).

Dentro de los ecosistemas acuáticos estos microorganismos juegan un papel ecológico muy importante. En primer lugar son los principales productores primarios. A través de la fotosíntesis oxigénica reducen carbono inorgánico (CO_2 y carbonatos) a carbono orgánico (azúcares). En este proceso se desprende oxígeno, imprescindible para el resto de organismos que realizan la respiración aerobia en la biosfera. Se estima que los microorganismos fotosintéticos del plancton son responsables de, aproximadamente, la mitad de la producción primaria total en el planeta, y por tanto de la mitad del oxígeno atmosférico y de la materia orgánica presente en la tierra (Falkowski *et* al., 1998). Por otro lado, juegan un papel importante en los ciclos biogeoquímicos de elementos como el nitrógeno y el fósforo. Las cianobacterias, son uno de los pocos grupos taxonómicos capaces de fijar el nitrógeno atmosférico en formas asimilables por el resto de seres vivos. Por tanto, el fitoplancton es una pieza clave en el mantenimiento de los ecosistemas acuáticos como base de la cadena trófica (Nyholm & Peterson, 1997; Western, 2001). Estos microorganismos también están presentes en los medios edáficos, cumpliendo exactamente el mismo papel ecológico que en los ecosistemas acuáticos. Además, ayudan a mantener la estructura del suelo impidiendo su degradación (McCann y Cullimore, 1979).

Dentro del fitoplancton algunas especies pueden comportarse como microorganismos extremófilos, ocupando nichos ecológicos con altas concentraciones de metales pesados, valores de pH extremos, etc… (Fogg, 2001)

1.2. Retos adaptativos: Ambientes naturales extremos y contaminantes ambientales de origen antropogénico.

Desde la Revolución Industrial a finales del siglo XVIII el hombre ha desarrollado la capacidad de modificar su entorno con un elevado coste ecológico (Palumbi, 2001). Actualmente, y probablemente debido a la acción del ser humano, estamos inmersos en la sexta gran extinción masiva que afronta la biosfera desde el origen de la vida sobre la tierra. Se estima que, hoy en día, la tasa de extinción global de especies es entre 100 y 1000 veces superior a la que existía antes de la aparición del *Homo sapiens* (Chapin III *et al*., 2000). Los efectos de las actividades humanas repercuten en el entorno modificando los ciclos biogeoquímicos, el clima, y disminuyendo la biodiversidad de las biotas, entre otras cosas (Myers y Knoll, 2001). Esta modificación del entorno, de manera directa o indirecta, produce presiones ambientales tan fuertes que aproximadamente entre 3.000 y 30.000 especies se extinguen anualmente (Hughes *et al.*, 1997).

Una de las grandes presiones ambientales que produce el ser humano sobre el resto de especies son los vertidos masivos de contaminantes procedentes de grandes urbes, industria, y producciones intensivas agrícolas y ganaderas. La aparición de vertidos de sustancias contaminantes de nueva síntesis está poniendo en jaque el correcto funcionamiento de los ecosistemas de todo el planeta (Vitousek *et* al., 1997; Wu, 1999; Islam y Tanaka, 2004). Entre estos contaminantes vertidos masivamente al medio, cabe destacar vertidos industriales de metales pesados (Whitton, 1984; Macnair, 1997; Islam y Tanaka, 2004), así como el uso indiscriminado de herbicidas, insecticidas y pesticidas en el tratamiento de plagas (Boyle, 1984; Bérard y Pelte, 1999; Palumbi, 2001). Se estima que la industria química produce cada año alrededor

de 100.000 Gg de productos químicos que son liberados al medio y cuyo impacto ambiental se desconoce en la mayoría de los casos (Vitousek *et* al., 1997).

Todas estas sustancias representan en la actualidad un reto adaptativo para la mayoría de especies que habitan el planeta, siendo las de menor plasticidad adaptativa las que se extinguen más rápidamente.

Por otro lado, los ambientes naturales extremos (aguas con valores extremos de pH, concentraciones de metales pesados, compuestos tóxicos orgánicos, etc…) también supusieron un reto adaptativo para los organismos que los habitan. En estos medios contaminados de forma natural, encontramos variantes extremófilas de especies de fitoplancton habitualmente mesófilas (Amaral Zettler, 2002). Por tanto estos ambientes son un excelente "laboratorio natural" para estudiar los mecanismos biológicos que permiten la adaptación.

1.3. Citotoxicidad de los contaminantes sobre los microorganismos fotosintéticos del plancton.

Los contaminantes ambientales actúan de formas muy diversas sobre los microorganismos fotosintéticos del plancton. En general la mayoría de especies de fitoplancton son muy sensibles a contaminantes, por este motivo, desde principios del siglo XX han sido utilizados como bioindicadores de la calidad del agua (Nyholm y Peterson, 1997).

Los contaminantes ambientales de origen antropogénico comprenden sustancias tan variadas como herbicidas, fungicidas, antibióticos, metales pesados, etc…y por tanto el modo de acción sobre el fitoplancton es muy variado. Así, los herbicidas actúan inhibiendo los procesos fotosintéticos. Por ejemplo, El DCMU (3-(3,4-dicloro-fenil)-1,1-dimetil-urea) o la simazina (2-cloro-4,6-bis[etilamino]-s-triazina) actúan inhibiendo la cadena transportadora de electrones en la membrana tilacoidal (Quimby et al. 1978). Otros contaminantes como los antibióticos se clasifican en función de su mecanismo de acción, pudiendo interferir en diferentes puntos metabólicos críticos, como la síntesis

proteica o la acción específica de determinadas enzimas (Russell et al. 1995). Los metales pesados a elevadas concentraciones también tienen un efecto tóxico sobre el fitoplancton. Están descritos, entre otros, los efectos del mercurio (Juneau et al., 2001), el plomo (Bajguz y Godlewska-Zylkiewicz, 2004), el cobre (Abd-El-Monem et al.,1998; Devriese et al., 2001; Bossuyt y Janssen, 2004), el níquel (Fargasová et al., 1999) y el zinc (Abd-El-Monem et al., 1998; Devriese et al., 2001) o el cadmio (Devriese et al., 2001).

Dadas las diferentes dianas moleculares de los tóxicos, el efecto nocivo de los contaminantes sobre el fitoplancton se determina midiendo parámetros implicados en el correcto funcionamiento general de las células. Los parámetros elegidos habitualmente son la producción de oxígeno y la emisión de fluorescencia del fotosistema II (PSII) (Dyer et al., 1992; Brack and Frank, 1996; Danilov and Ekelund, 2001; Ma et al., 2001). Estos dos parámetros, junto con el crecimiento (medido como parámetro malthusiano de fitness), son indicadores de la “salud” de estos microorganismos y cualquier alteración se reflejará en estas medidas.

1.4. Adaptación de microorganismos a nuevos ambientes

Los organismos que ocupan de manera estable un nicho ecológico están en condiciones de equilibrio con su entorno, y están adaptados a este medio de manera que pueden aprovechar todos los recursos para mantener su eficacia biológica y así sobrevivir. Cuando un determinado ambiente cambia bruscamente, de manera que se compromete la eficacia biológica de los organismos, éstos tienen dos opciones: adaptarse o morir.

Dentro de un determinado rango de variación ambiental, la modificación de la expresión génica puede ser suficiente para que las poblaciones de fitoplancton se adapten al nuevo ambiente (Bradshaw and Hardwick, 1989; Bradshaw and Hardwick, 1989; Fogg, 2001). A este fenómeno se le denomina adaptación fisiológica o aclimatación. Cuando la variación ambiental supera los límites de adaptación fisiológica, solamente la variación genética por mutación

puede crear nuevos genotipos capaces de afrontar el cambio ambiental (Belfiore and Anderson, 2001; Sniegowski and Lenski, 1995).

En microorganismos de reproducción asexual, las mutaciones son consideradas el motor de la evolución, ya que en estas poblaciones constituyen la única fuente de variabilidad genética. Los organismos se adaptarían a nuevos ambientes mediante la selección de la variabilidad genética existente en sus poblaciones (originada en último término como resultado de mutaciones preadaptativas, que tienen lugar espontáneamente antes del cambio ambiental) (Luria & Delbrück 1943; Sniegowsky y Lensky, 1995). Sin embargo en los últimos veinte años, se han realizado numerosos estudios evolutivos en bacterias (con un gran número de generaciones), que parecen sugerir que en estos organismos, podrían existir mutaciones adaptativas (adaptive mutation) en respuesta a agentes específicos que recuerdan a la teoría lamarckista (Cairns et al., 1988; Hall, 1988; Foster, 1999; Roth et al 2006).

1.5. Evolución de microorganismos en ambientes contaminados tras la primera mutación que permite la adaptación

La selección natural actúa sobre la variabilidad genética de las poblaciones. En los organismos de reproducción estrictamente asexual al no disponer de recombinación genética durante la meiosis, la única fuente de variabilidad es la mutación (Ridley, 1993). Pero, ¿que sucede tras la mutación que permite la adaptación al agente selectivo?, ¿son los microorganismos resistentes capaces de mejorar en presencia del agente selectivo?, y si lo son, ¿Qué mecanismos evolutivos actúan?

El mecanismo evolutivo básico propuesto por la corriente neodarwinista es la selección natural. El cambio evolutivo en las poblaciones es fruto de la adaptación de modo que las diferencias fenotípicas siempre tienen un valor adaptativo. (Ridley 1993; Gould 2002). Sin embargo, otras escuelas evolutivas matizan esta teoría proponiendo que el azar juega un papel muy importante en la evolución. Este es el caso del neutralismo, que se debe científico japonés Kimura (Kimura, 1983). Para esta escuela cualquier mutación es válida para

una población, pues las condiciones ambientales varían al azar. Las mutaciones no tienen por que proporcionar ventajas adaptativas. En este caso se les denomina mutaciones neutras.

A través de los diseños experimentales realizados por Travisano en *E. coli* (Travisano et al. 1995) nos planteamos que sucede tras la primera mutación que permite la adaptación al agente selectivo. Estudiamos los mecanismos que permiten la evolución de las poblaciones resistentes en medios con el agente selectivo, y que importancia tienen el azar y la selección en la evolución del parámetro malthusiano de fitness y del rendimiento fotosintético.

1.6. Aplicación: detección de toxicidad mediante genotipos sensibles y resistentes.

En la década de los sesenta se definía biosensor como cualquier sonda basada en un componente biológico que daba una señal cuantificable. Los primeros biosensores consistían en la unión de electrodos de pH u oxígeno con enzimas inmovilizadas. Hoy en día estos métodos son práctica habitual para detectar y cuantificar diferentes sustancias. Actualmente se considera que un biosensor es una herramienta o sistema analítico compuesto por un material biológico inmovilizado (enzima, anticuerpo, célula entera, orgánulo o combinaciones de los mismos), acoplados a un sistema transductor adecuado que convertirá la señal biológica en una señal cuantificable (D'Souza, 2001; Jei et al., 2006).

En la actualidad la necesidad de sistemas de alerta temprana para la detección de contaminantes ha hecho proliferar el uso de biosensores, muchos de los cuales se basan en microrganismos fotosintéticos (Naessens et al. 2000). Estos biosensores presentan ventajas sobre los clásicos sistemas analíticos, pues son más rápidos y sencillos de usar y de menor coste. Así, en los últimos años se han desarrollado y comercializado numerosos biosensores, basados en organismos fotosintéticos para detectar herbicidas (Merz et al. 1996; Frense et al. 1998, Naessens et al. 2000; Védrine et al. 2003)

compuestos orgánicos volátiles (Naessens and Tra-Minh, 1998), e incluso agentes de guerra química (Sanders et al. 2001). Los microorganismos fotosintéticos son idoneos para construir biosensores, debido a que permiten medir fácil y rápidamente tanto variaciones de fluorescencia de su fotosistema II (PSII) como la producción de oxígeno, los cuales son indicadores de su estado fisiológico.

Hasta el presente, la investigación de biosensores de microorganismos fotosintéticos ha sido enfocada para incrementar el número de contaminantes que se pueden detectar y para mejorar la señal o los métodos de inmovilización (Frense et al. 1998, Naessens et al. 2000). Estos biosensores son suficientemente sensibles. Sin embargo presentan una importante limitación: no son lo bastante específicos, pues muchos tóxicos diferentes pueden dar falsos positivos al inhibir la fluorescencia. Esta carencia de especificidad representa el punto más débil de los actuales biosensores (D'Souza, 2001). Las alternativas propuestas para incrementar la especificidad de los biosensores se concretan en modificaciones genéticas, ninguna de las cuales tuvo éxito en microorganismos fotosintéticos (Horsburg et al., 2002).

La aparición de mutantes resistentes a contaminantes de nueva síntesis como resultado de mutaciones espontáneas (Costas et al., 2001, López-Rodas et al. 2001, Baos et al., 2002), aporta un nuevo método para conseguir especificidad en biosensores. El biosensor se basa en dos clones diferentes, el primero de ellos sensible al contaminante (que aporta la sensibilidad) y el segundo resistente a ese contaminante (que aporta la especificidad) (Figura 1). Empleando un clon sensible a Cr(VI) y otro resistente, desarrollamos las bases del primer biosensor basado en células integras de microorganismos fotosintéticos, específico para el metal pesado tóxico Cr(VI).

Tabla 1. Bases de la señal diferencial entre células resistentes y sensibles en biosensores sensibles y específicos.

	Rendimiento del PSII	
Cepa	sensible	resistente
No hay tóxico	+	+
Tóxico diferente	-	-
Tóxico específico del biosensor	-	+

2. OBJETIVOS

1. Analizar la adaptación genética del fitoplancton a contaminantes de origen antropogénico, desarrollando un modelo general utilizando herbicidas, antibióticos y biocidas.

2. Profundizar en los fenómenos evolutivos que podrían tener lugar tras la primera mutación. Como modelo utilizaremos la clorofícea *Scenedesmus intermedius* y el antibiótico cloranfenicol.

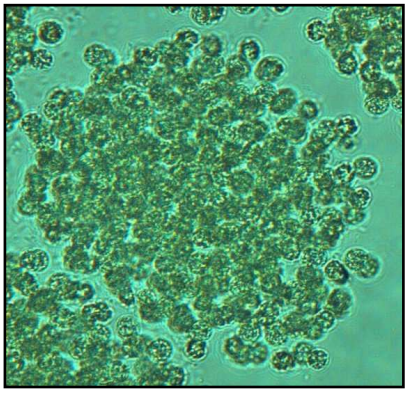
3. Estudiar la capacidad de las poblaciones de fitoplancton para adaptarse a ambientes naturales extremos.

4. Utilizar cepas de fitoplancton sensibles y resistentes como biosensores sensibles y específicos.

3. MATERIAL Y MÉTODOS

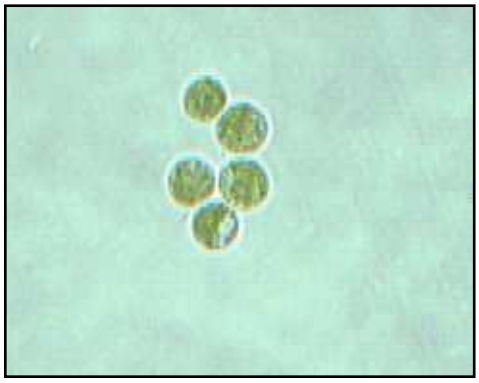
3.1. Microorganismos experimentales y sustancias tóxicas

Los microorganismos fotosintéticos del plancton elegidos para la realización de los experimentos fueron la cianobacteria (procariota) *Microcystis aeruginosa* (Kützing) Kützing, y dos especies de Clorofíceas (eucariotas): *Scenedesmus sp.* y *Dyctiosphaerium chlorelloides* (Naum.) Komárek and Perkam. Todas las cepas pertenecen a la colección de fitoplancton del Laboratorio de Genética de la Facultad de Veterinaria de la Universidad Complutense de Madrid. Una característica importante que tienen en común los microorganismos fotosintéticos del plancton escogidos es que solo se reproducen asexualmente (no tienen recombinación meiótica), por lo tanto la única fuente de variabilidad genética es la mutación. Además, son especies con tamaños de poblaciones ingentes, ubicuas y cosmopolitas en las aguas continentales de todo el mundo.



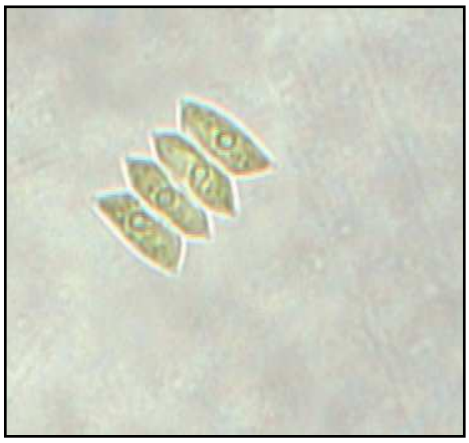
Microcystis aeruginosa

Microcystis aeruginosa (Kützing, Lemmerman) es una cianobacteria (procariota) clasificada dentro del grupo de eubacterias Gram-negativas. Pertenece a la División Cyanophyta, al Orden Chroococcales y a la Familia Cyanophyceae. Las células de *Microcystis aeruginosa* son esféricas y de un tamaño comprendido entre 3 y 7 µm. En el citoplasma tienen vesículas de gas que confieren mayor o menor grado de flotabilidad. Las células pueden estar agrupadas en colonias de contornos imprecisos mediante una envoltura de mucílago. Además es una de las especies que con más frecuencia forma blooms tóxicos en ecosistemas de aguas continentales (revisado por Skulberg *et al*., 1993).



Dictyosphaerium chlorelloides

Dyctiosphaerium chlorelloides (Naum.), Komárek and Perman, pertenece a la División Chlorophyta, al Orden Chlorococcales. El tamaño de las células está comprendido entre 5 y 10 µm en la naturaleza, a veces, pueden estar unidas por mucílago formando colonias o libres, aunque en cultivos de laboratorio normalmente están en forma individual.



Scenedesmus intermedius

Scenedesmus intermedius pertenece al Phylum Chlorophyceeae, al Orden Chlorococcales y a la Familia Scenedesmaceae. Presentan un tamaño celular comprendido entre 4 y 10 µm y forman colonias normalmente de 4 células, pudiendo ser de 2, 8 o 16 células. Las células terminales pueden presentar dos espinas situadas en posiciones opuestas.

Las sustancias contaminantes de origen antropogénico ensayadas en los presentes trabajos fueron: el formaldehído (ampliamente utilizado en la industria química), los herbicidas simazina, glifosate y diquat, (ampliamente utilizados en agricultura), el antibiótico cloranfenicol, el metal pesado Cromo (VI) y el amonio cuaternario dodeciletildimetil-amonio (usado como desinfectante). Por otra parte también estudiamos los efectos de aguas tóxicas de origen natural, como son el agua procedente de emisiones volcánicas de la isla de Vulcano (Italia) y las aguas ácidas y con alta concentración de metales pesados de las antiguas minas de cobre de Tharsis (Huelva) y Mynydd Parys (Anglashey, Gales).

3.2. Análisis de fluctuación. Determinando la naturaleza de la mutación que permite la adaptación.

Desde que Luria y Delbrück (1943), en un trabajo magistral, introdujeron el análisis de fluctuación como un combinado de procedimientos estadísticos y experimentales para analizar la aparición de organismos resistentes en poblaciones bacterianas, numerosos trabajos teóricos y experimentales han perfeccionado este análisis, permitiendo aplicarlo a un alto rango de organismos, desde bacterias a células humanas (Tlsty et al. 1989, Rossman et al. 1995, Jones et al. 1994). El análisis de fluctuación es un procedimiento adecuado para determinar la naturaleza de la mutación que permite la adaptación de un microorganismo a un agente selectivo. Es decir, si la mutación que hace posible la adaptación es preadaptativa (tuvo lugar espontáneamente y al azar antes de entrar en contacto con el agente selectivo) o postadaptativa (la mutación se produce como una respuesta directa y específica al agente selectivo). Así mismo, el análisis de fluctuación permite calcular las tasas de mutación de sensibilidad a resistencia mediante el estimador P_0 entre otros (Luria and Delbrück, 1943). A pesar de los numerosos procedimientos desarrollados desde entonces para estimar la tasa de mutación (Li and Chu, 1987; Tlsty et al. 1989) el estimador P_0 sigue siendo considerado el mejor (Rosche and Foster, 2000). Por todo esto, el análisis de fluctuación de Luria y Delbrück es considerado uno de los experimentos mejor diseñados de la Historia de la Humanidad (Bunje, 1979). Recientemente se ha modificado el análisis de fluctuación para adecuarlo a cultivos líquidos de fitoplancton (Costas et al. 2001, López-Rodas et al. 2001)

El análisis de fluctuación consiste en 2 baterías de experimentos. La primera batería (Set1) se compone de 10^2 cultivos, en los que cada uno se inocula aproximadamente 10^2 células (un número lo suficientemente bajo para asegurar, estadísticamente, la ausencia de células mutantes resistentes). Estos cultivos se dejan crecer en medio no selectivo hasta que alcanzan un número aproximado de 10^5 células por cultivo. En ese momento se añade a todos los cultivos del Set 1 el agente selectivo a una concentración que, previamente, se determina como concentración letal. La segunda batería de experimentos (Set

2) se compone de 30 cultivos que contienen medio selectivo (a la misma concentración que en el Set 1) y en los que se inocula directamente 10^5 células por cultivo, procedentes de la misma población parental que las células inoculadas en el Set 1.

Según Luria y Delbrück se pueden encontrar dos resultados distintos cuando se lleva a cabo un análisis de fluctuación (Figura 1). Cada uno de ellos se interpreta como la consecuencia independiente de dos fenómenos distintos. Si las células resistentes aparecen sólo por mutaciones que ocurren de manera aleatoria antes de la selección (antes de la adición del agente selectivo), se encontrará una gran variación en cuanto al número de células resistentes por cultivo (fluctuación). Esto es debido a que, como la mutación sucede al azar, esta puede ocurrir pronto en algunos cultivos (y por tanto encontraremos muchas células resistentes) tarde en otros y no suceder en muchos. En este caso el cociente entre la varianza y la media de células resistentes encontradas en los cultivos del Set 1 será superior a 1 (Varianza/Media >> 1) (Figura 1, Set 1B). Por el contrario, si las células resistentes aparecen sólo como respuesta al agente selectivo, entonces la distribución de células resistentes entre los diferentes cultivos no mostrará ninguna fluctuación, reflejando la baja y constante probabilidad de que cada célula pueda desarrollar resistencia. En este caso, el cociente entre la varianza y la media en el número de células resistentes en los cultivos del Set 1 será aproximadamente igual a 1, y por tanto consistente con la distribución de Poisson (Varianza/Media = 1) (Figura 1, Set 1A). El Set 2 es el control experimental del análisis de fluctuación (Figura 1, Set 2). Mide la varianza de la población parental y el error experimental. Si existe una diferencia significativa en la proporción Varianza/Media entre Set 1 y 2, se confirma que las células resistentes no aparecen en respuesta a la presión ambiental, sino que aparecen por mutaciones que ocurrieron al espontáneamente antes de entrar en contacto con el agente selectivo.

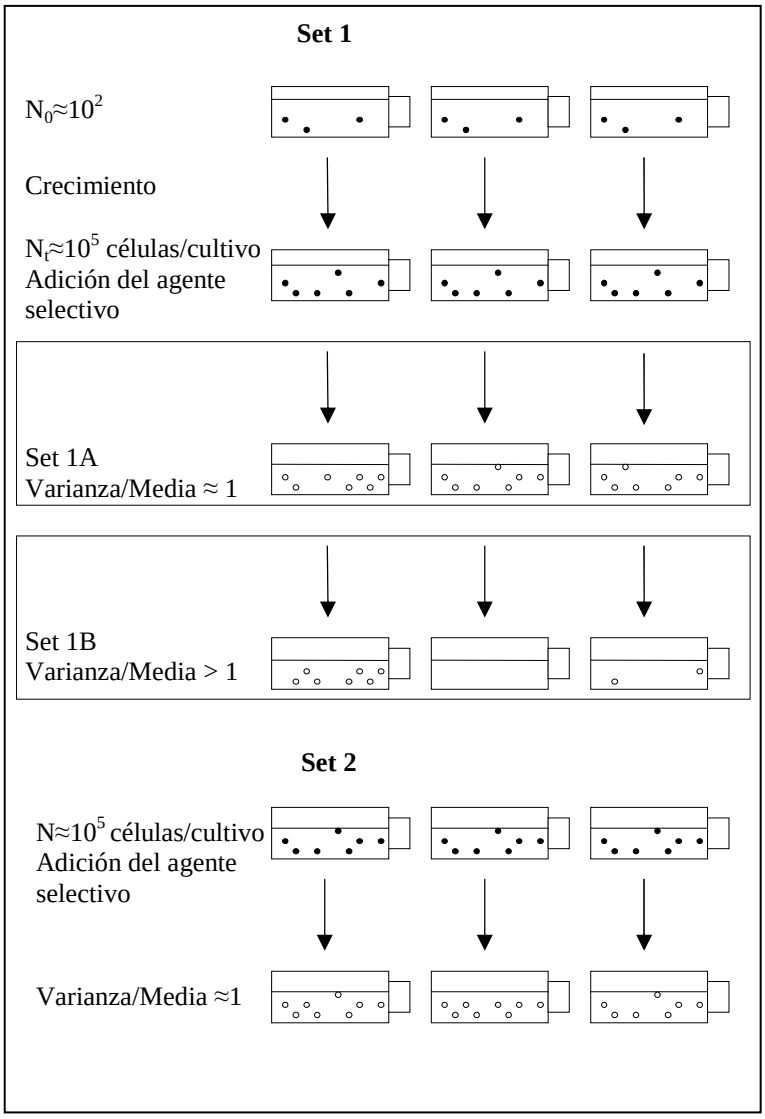


Figura1. Esquema de la modificación del análisis de fluctuación diseñado por Luria y Delbrück. Los puntos rellenos representan las células sensibles a la sustancia tóxica antes de su adición, mientras que los puntos vacíos representan las células resistentes. En el set 1, incubamos 100 cultivos en condiciones no selectivas desde $N_0 \approx 10^2$ células hasta $N_t \approx 10^5$ células. A continuación, añadimos la dosis letal del agente selectivo. Si las células resisitentes aparecen por mutaciones postadaptativas, el número de células resistentes por cultivo será similar en todos ellos (set 1A). Si por el contrario las células resistentes aparecen como consecuencia de una mutación preadaptativa, es decir, que tiene lugar al azar antes del contacto con la sustancia tóxica, el número de células resistentes por cultivo será desigual (set 1B). El set 2 es un control experimental que mide la varianza en la aparición de células resistentes de la población parental.

3.3. Experimento de evolución. Influencia de la selección y el azar.

Travisano y colaboradores diseñaron en 1995 un experimento para analizar la contribución del azar y la adaptación en la evolución de poblaciones de bacterias *E. coli* propagadas en laboratorio y expuestas a un cambio ambiental (Travisano et al. 1995). La base del experimento es el cultivo, bajo condiciones idénticas, de réplicas procedentes de un único genotipo parental en unas condiciones de cultivo nuevas, de manera que se fuerza a los microorganismos a evolucionar, a adaptarse al nuevo medio para recuperar su eficacia biológica. Al principio del experimento se determina el valor medio de determinados rasgos cuantitativos de los microorganismos (como la tasa de división o el tamaño celular) entre las réplicas fundadas. En este caso, se espera que la varianza calculada sea baja, puesto que corresponde al error de las medidas realizadas. A continuación, se incuban las diferentes réplicas bajo idénticas condiciones de cambio ambiental durante un largo periodo de tiempo. Al final del experimento, se calcula de nuevo el valor medio de los mismos rasgos cuantitativos y su varianza. Las diferencias entre los valores iniciales y finales (de la media y de la varianza) se explican como resultado del efecto de la adaptación, azar, o ambos. Si no hay una diferencia estadísticamente significativa entre la media inicial y final de las medidas de un determinado rasgo, podría interpretarse que la población de microorganismos derivada no ha evolucionado respecto a la ancestral (Figura 2A). Si la media aumenta y la varianza (determinada como medida de dispersión de los valores medios entre las diferentes réplicas) son similares al inicio y al final del experimento ($V_0 \approx V_t$) podría interpretarse que la evolución de este rasgo está fuertemente influido por la selección natural (Figura 1B). Sin embargo si la varianza es significativamente mayor al final del experimento ($V_o < V_t$), esto indicaría que los caracteres no han evolucionado por igual en cada una de las réplicas, y por tanto que el azar también juega un papel importante en la evolución de ese rasgo (Figura 1D).

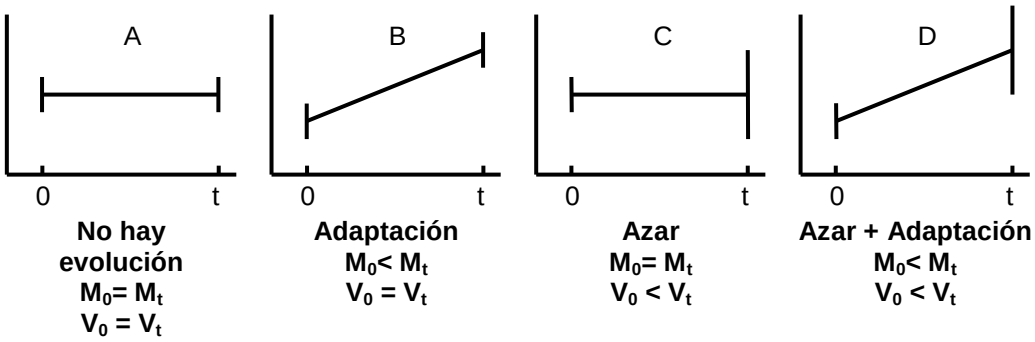


Figura 2. Representación esquemática de los efectos debidos a la adaptación (B), azar (B) y a la adaptación más azar (D). Las diferencias entre la Media (M) y la Varianza (V) de las medidas de un determinado rasgo específico en diferentes réplicas, determinadas al inicio (0) y al final (t) del experimento, se interpretan como consecuencia de la influencia del azar, la adaptación o ambas en su evolución.

4. RESULTADOS

CAPITULO I

ADAPTACION DEL FITOPLANCTON A CONTAMINANTES DE ORIGEN ANTROPOGÉNICO

Introducción

Actualmente estamos inmersos en la sexta gran extinción masiva de especies que afronta la Tierra a lo largo de su historia (al menos 4500 millones de años). La tasa global de extinción se sitúa en valores entre 100 y 1000 veces superior a la existente antes de la aparición del ser humano (Chapin III *et al*., 2000). La capacidad del *Homo sapiens* para alterar los procesos naturales de la biosfera tiene como consecuencia que entre 3000 y 30000 especies se extinguen anualmente (Woodruff, 2001). El ser humano es responsable en gran parte del denominado Cambio Global que sufre la biosfera. Aunque el componente más conocido de este cambio global es el llamado Cambio Climático, en realidad también engloba sucesos como la extinción masiva, la homogenización de las biotas, la proliferación de especies oportunistas, etc… (Myers and Knoll, 2001). Además otra de las causas del cambio global es la contaminación antropogénica en forma de vertidos de sustancias contaminantes que se liberan a los ecosistemas (Islam y Tanaka, 2004). La capacidad del ser humano para sintetizar y producir industrialmente grandes cantidades de sustancias tóxicas y xenobióticos constituyen un nuevo reto adaptativo para la mayoría de microorganismos que habitan la Tierra.

En este capítulo estudiamos la capacidad de adaptación de especies procariotas y eucariotas del fitoplancton de aguas continentales a tres de los herbicidas más usados, al antibiótico cloranfenicol y a un contaminante ambiental modelo (el formol). Este último es un compuesto tóxico que aunque se encuentra en el medio de forma natural, es producido en grandes cantidades por el ser humano para la elaboración de multitud de compuestos químicos (EPA, 1988).

Los herbicidas son biocidas muy abundantes generados y liberados al medio por los humanos, y por tanto importantes contaminantes de medios acuáticos (Koening, 2001). El uso intensivo de estas sustancias a lo largo de estas últimas décadas tiene consecuencias evolutivas debido a la fuerte presión selectiva que causa en numerosas especies. (Belfiore and Anderson, 2001; Palumbi, 2001). Concretamente, los herbicidas utilizados en estos estudios de adaptación fueron el glifosate, la simazina y el diquat. El glifosate actúa inhibiendo dos enzimas que intervienen en la síntesis del ácido coríasmico, precursor de tres aminoácidos que solamente se sintetizan en eucariotas fotosintéticos (Cruz, 1990). Este herbicida es ampliamente aplicado en agricultura intensiva (Baucom and Mauricio, 2004). El herbicida triazínico simazina es un inhibidor de la fotosíntesis. Actúa como inhibidor de la cadena de transporte de electrones (Quimby *et al.* 1978). En el año 1998, se estimó que la tasa global de aplicación de los herbicidas de la familia de la simazina fue de 36 Gg por año (Carder y Hoagland, 1998). El diquat pertenece a la familia de los bipiridilos. El mecanismo de acción de este herbicida está basado en la inhibición de ciertas enzimas del ciclo de Calvin deteniendo, por tanto, la fijación de CO_2 (Martínez and Ayuela, 1998).

El primer trabajo de este capítulo estudia la capacidad de adaptación de dos cepas de la cianobacteria tóxica *Microcystis aeruginosa* a dosis letales de glifosate. Los resultados sugieren que las poblaciones de *Microcystis aeruginosa* son capaces de adaptarse al glifosate gracias a variantes resistentes que aparecen por una mutación espontánea que tiene lugar antes del contacto con el herbicida. Estos mutantes resistentes tienen menor tamaño y una tasa de división un 15 % menor que las células sensibles en ausencia del herbicida. Por tanto, la presencia persistente de glifosate en medio acuáticos puede producir una disminución en la producción primaria en estos ecosistemas.

En el segundo trabajo, abordamos la capacidad de adaptación de clorofíceas eucariotas a los herbicidas simazina y diquat. Los microorganismos experimentales empleados fueron, en el estudio de adaptación a simazina, dos

cepas de *Scenedesmus intermedius* de diferentes orígenes (una de las cepas fue aislada del Parque Nacional de Doñana, habitualmente expuesto a herbicidas, y la otra de una pequeña charca relicta en el desierto del Sahel, Mauritania, donde nunca se ha usado la simazina) y una cepa de *Dictyosphaerium chlorelloides* aislada de un lago de alta montaña en Sierra Nevada. Para el estudio de la adaptación a diquat empleamos solo una cepa de *Scenedesmus intermedius* (cepa aislada del Parque Nacional de Doñana). En todos los casos, los resultados indicaron que las clorofíceas también son capaces de adaptarse a estos herbicidas a través de mutaciones espontáneas preselectivas. Por consiguiente, tanto organismos procariotas como eucariotas parecen adaptarse por el mismo mecanismo, a través de raras mutaciones espontáneas que ocurren al azar antes de entrar en contacto con el agente selectivo.

En el siguiente trabajo experimental de este capítulo estudiamos los mecanismos de adaptación de la clorofícea eucariota *Dictyosphaerium chlorelloides* al formaldehído. Al igual que en las anteriores experiencias, los resultados indicaron que una mutación al azar que tiene lugar antes del contacto con la sustancia tóxica es la responsable de la adaptación a dosis letales. Los vertidos de formol tienen efectos negativos sobre las poblaciones de microalgas, disminuyendo la producción primaria del ecosistema afectado.

En el último trabajo de este capítulo estudiamos la adaptación de la clorofícea *Scenedesmus intermedius* al antibiótico cloranfenicol. Los resultados sugieren la posibilidad de adaptación de esta clorofícea al cloranfenicol a través de mutaciones preselectivas. Este artículo es el primero de una serie (de dos artículos) en los que se estudia los mecanismos genéticos de adaptación al cloranfenicol y la evolución en medios contaminados.

En todos los casos estudiados los microorganismos fotosintéticos del plancton fueron capaces de adaptarse a dosis letales de los biocidas escogidos a través de mutaciones espontáneas preadaptativas.

4.1.1. Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of preselective Mutations.

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RESEARCH ARTICLE

Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of pre-selective mutations

Victoria López-Rodas · Antonio Flores-Moya · Emilia Maneiro · Nieves Perdigones · Fernando Marva · Marta E. García · Eduardo Costas

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Abstract Adaptation of *Microcystis aeruginosa* (Cyanobacteria) to resist the herbicide glyphosate was analysed by using an experimental model. Growth of wild-type, glyphosate-sensitive (G^s) cells was inhibited when they were cultured with 120 ppm glyphosate, but after further incubation for several weeks, occasionally the growth of rare cells resistant (G^r) to the herbicide was found. A fluctuation analysis was carried out to distinguish between resistant cells arising from rare spontaneous mutations and resistant cells arising from other mechanisms of adaptation. Resistant cells arose by rare spontaneous mutations prior to the addition of glyphosate, with a rate ranging from 3.1×10^{-7} to 3.6×10^{-7} mutants per cell per generation in two strains of *M. aeruginosa*; the frequency of the G^r allele ranged from 6.14×10^{-4} to 6.54×10^{-4}. The G^r mutants are slightly elliptical in outline, whereas the G^s cells are spherical. Since G^r mutants have a diminished growth rate, they may be maintained in uncontaminated waters as the result of a balance between new resistants arising from spontaneous mutation and resistants eliminated by natural selection. Thus, rare spontaneous pre-selective mutations may allow the survival of *M. aeruginosa* in glyphosate-polluted waters via G^r clone selection.

Keywords Cell morphology · Glyphosate · *Microcystis* · Mutation rate · Natural selection

V. López-Rodas · E. Maneiro · N. Perdigones · F. Marva · E. Costas
Genética (Producción Animal), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain

A. Flores-Moya (✉)
Biología Vegetal (Botánica), Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain
e-mail: floresa@uma.es

M. E. García
Sanidad Animal (Microbiología), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain

Abbreviations and symbols

CF	Coefficient of form
G^r	Glyphosate-resistant cells
G^s	Glyphosate-sensitive cells
m_{G^r}	Malthusian fitness parameter from glyphosate-resistant cells
m_{G^s}	Malthusian fitness parameter from glyphosate-sensitive cells
N_0	No. of cells at the start of the experiment
N_t	No. of cells at the end of the experiment
P_0	Proportion of cultures without G^r cells in the set 1 fluctuation analysis experiment
q	Frequency of G^r allele in natural, non-exposed to glyphosate populations
s	Coefficient of selection
μ	Mutation rate

Introduction

Nowadays we are living in a geological instant in which global extinction rates are 50–500 times background and are increasing due to human activities that are altering biosphere-level processes. It has been estimated that several million populations and 300–30,000 species go extinct annually from a total of >10 million species (Woodruff 2001). Distinctive features of the future biosphere could include homogenization of biotas, proliferation of opportunistic species, a pest-and-weed ecology, and unpredictable emergent novelties (Myers and Knoll 2001). The biodiversity crisis is reasonably understood for terrestrial vertebrates and a few other groups, but little is known about organisms as abundant and important as microbes. Studies of bacteria and protists are clearly needed, because crucially important nutrient cycles may become less predictable as essential microbes succumb to anthropogenic toxins (Woodruff 2001). In particular, since microalgae and cyanobacteria are the principal primary producers of aquatic ecosystems (Kirk 1994; Falkowski and Raven 1997), the tolerance of these organisms to contaminated environments is very relevant from an ecological point of view. Herbicides are among the most significant human-synthesized pollutants in aquatic ecosystems (Koenig 2001). Unrelenting application of herbicides during recent decades has resulted in water pollution, with serious environmental implications and evolutionary consequences due to strong selection pressure on numerous species (Belfiore and Anderson 2001; Palumbi 2001). This is the case for the broad-spectrum herbicide glyphosate, which was introduced in 1974 and now constitutes a potent anthropogenic source of selection (Baucom and Mauricio 2004).

Within limits, organisms may survive in chemically-stressed environments as a result of two different processes: physiological adaptation (acclimation), usually resulting from modifications of gene expression; and, adaptation by natural selection if mutations provide the appropriate genetic variability (Belfiore and Anderson 2001). The neo-Darwinian view that evolutionary adaptation occurs by selection of pre-existing genetic variation was early accepted for multicellular organisms (Huxley 1942; Lewontin 1974; reviewed by Sniegowski and Lenski 1995). However, recent evolutionary studies in bacteria have suggested that hypothetical ''adaptive mutation'' could be a process resembling Lamarckism which, in the absence of lethal

selection, produces mutations that relieve selective pressure (Cairns et al. 1998; Foster 2000). The key to resolving this debate is to know the pre-adaptive or post-adaptive origin of new mutations. Surprisingly, there are almost no studies that have made a direct connection between the rates of origin of favoured mutants and the process of adaptation (Sniegowski 2005). The main reason for this lack of studies is the difficulty in measuring the rate of favoured mutants directly in diploid, multi-celled, sexual organisms living in well-defined populations. In contrast, most microbes (including cyanobacteria and many microalgae) are haploid, single-celled, asexual organisms, and their populations are composed of countless cells (Margulis and Schwartz 1982). Therefore, the study of genetic adaptation of cyanobacteria to extreme environmental changes derived from anthropogenic pollution is an adequate aproximation to the problem of the origin of favoured mutants and the process of adaptation.

The aim of this work was to evaluate, from an evolutionary point of view, the effect of glyphosate on the freshwater cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann. For this purpose, we performed a fluctuation analysis (Luria and Delbrück 1943) using glyphosate as selective agent. This experimental model is particularly well-suited to discriminate between cells that become resistant from acquired specific adaptation in response to glyphosate (including both physiological adaptation or acclimation, and possible mutations following glyphosate exposure; the first case is not an evolutionary event) and resistant cells arising from rare spontaneous mutations that occur randomly during propagation of cyanobacteria prior to the glyphosate exposure. Consequently, we have assessed the mechanisms (fitness and mutation-selection balance) that allow cyanobacteria to withstand increasing exposure to glyphosate. We demonstrate the existence of very rapid evolution in populations of *M. aeruginosa* as result of pre-selective mutations from sensitive (G^s) to the glyphosate resistant (G^r) cells.

Materials and methods

Experimental organism and culture conditions

Two strains of *Microcystis aeruginosa* (Kützing) Lemmermann (MaD3 and MaD7) from the Algal Culture Collection of the Universidad Complutense (Madrid), were grown axenically in 100 ml cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), at 20°C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Both strains were isolated from pristine ponds in Doñana National Park (SW Spain), where herbicides have never been used. Strains were maintained in mid-log exponential growth by serial transfers of a cell inoculum to fresh medium (details in Carrillo et al. 2003). Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures. Cultures were maintained as axenic as possible, and only cultures without detectable bacteria were used in the experiments.

Fig. 1 Schematic diagram of the experiment modified from the classic Luria-Delbrück fluctuation analysis, and the possible results. In the set 1 experiment, different cultures (each started from a small inoculum, $N_0 = 10^2$ cells) were propagated under non-selective conditions until a very high cell density ($N_t = 2.3 \times 10^5$ cells) was reached, and then supplemented with a lethal dose of the selective agent (120 ppm glyphosate). Set 1A: physiological adaptation (i.e., acclimation) or possible adaptive mutations. In this case, the number of resistant cells in all the cultures must be similar. Set 1B: adaptation by mutations occurring in the period of the propagation of cultures, i.e., before exposure to the selective agent. One mutational event occurred late in the propagation of culture 1 (therefore, the density of glyphosate-resistant cells found is low) and early in the propagation of culture 3 (thus, density of glyphosate-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. Therefore, the variance/mean ratio of the number of resistant cells per replicate must be >1. Set 2 samples the variance of parental populations (the distribution should be Poisson, with a variance ≈ mean) ▶

Toxicity test: effect of glyphosate on growth rate

The toxic effect of glyphosate on growth rate of the two wild-type strains was assessed as follows: a stock solution of glyphosate acid, N-(phosphonomethyl) glycine (Sigma-Aldrich Chemie, Taufkirchen, Germany) was prepared in BG–11 medium to obtain serial dilutions of 0, 10, 30, 60, and 110 ppm. Each experimental culture was inoculated with 5×10^6 cells from mid-log exponentially growing cultures. Four replicates of each concentration of glyphosate, as well as four unexposed controls, were prepared. The effect of the herbicide was estimated by calculating acclimated maximal growth rate (m) in mid-log exponentially growing cells, derived from the equation:

$$N_t = N_0 e^{mt}, \tag{1}$$

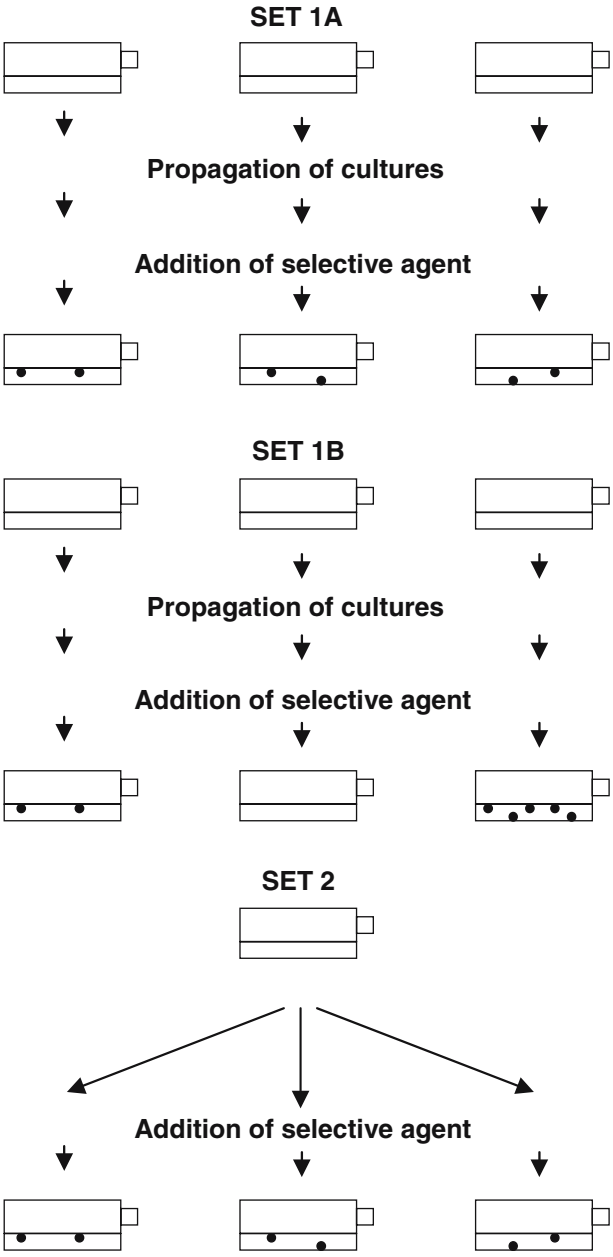
where $t = 7$ days, and N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively. Therefore, m was calculated as:

$$m = \mathrm{Log_e}\,(N_t/N_0)/7 \tag{2}$$

Acclimated maximal growth rate is the Malthusian parameter of fitness under conditions of r selection (Crow and Kimura 1970; Spiess 1989). Experimentals and controls were counted blind (i.e., the person counting the test did not know the identity of the tested sample), using a haemocytometer and an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany). The number of samples in each case was determined using the progressive mean procedure (Williams 1977), which assured a counting error <5%.

Fluctuation analysis of $G^s \rightarrow G^r$ transformation

A modified Luria–Delbrück analysis was performed as previously described (López-Rodas et al. 2001) to distinguish resistant cells that had their origin in random spontaneous pre-selective mutations (prior to glyphosate exposure) from those arising through acquired post-selective adaptation (during the exposure to glyphosate) (Fig. 1). The modification of the analysis involves the use of liquid medium containing the selective agent rather than plating on a solid medium, as was done by Luria and Delbrück (1943) with bacterial cultures. In short, two different sets of experimental cultures were prepared with both strains of *M. aeruginosa*. In the set 1 experiment, 100 culture flasks were inoculated with $N_0 = 10^2$ cells (a number small



enough to reasonably ensure the absence of pre-existing mutants in the strain). Cultures were allowed to grow until $N_t = 2.3 \times 10^5$ cells and then were supplemented with 120 ppm glyphosate acid. For the set 2 control, 45 aliquots of 2.3×10^5 cells from the same parental population were separately transferred to culture flasks containing fresh liquid medium with 120 ppm glyphosate acid. Cultures were observed for 60 days (thereby insuring that one mutant cell could generate enough

progeny to be detected), and the resistant cells in each culture (both in set 1 and set 2) were counted. The cell count was performed by at least two independent observers.

According to Luria and Delbrück (1943), two different results can be found in the set 1 experiment when conducting a fluctuation analysis, each of them being interpreted as the independent consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1A), the variance in the number of cells per culture could be found to be low if resistant cells arose by physiological adaptation or specific post-selective mutations. Because every cell is likely to had the same chance of developing resistance, interculture (flask-to-flask) variation would be consistent with the Poisson model. By contrast, if high variation in the interculture number of resistant cells is found (i.e., variance/mean > 1), it means that resistant cells appeared by random pre-selective mutations occurring before selection, and the flask-to-flask variation would not be consistent with the Poisson model. That is to say, they occurred during the time in which the cultures reached N_t from N_0 cells, before the exposure to glyphosate (Fig. 1, set 1B).

In the set 2 cultures (Fig. 1), if resistants arose by pre-selective mutations, variance is expected to be low, because set 2 samples the variance of the parental population. Thus, despite the way resistants appear, interculture variance of resistants in set 2 should be similar to the average of resistants in set 2 cultures. Because this set is the experimental control of the fluctuation analysis, if a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells appeared by acclimation or post-selective mutations, rather than by pre-selective mutations.

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. There are different approaches for accomplishing this estimation (Rosche and Foster 2000). Due to the methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of set 1 cultures showing no mutant cells after glyphosate exposure (P_0 estimator) was the parameter used to calculate the mutation rate (μ). The P_0 estimator (Luria and Delbrück 1943) is defined as follows:

$$P_0 = e^{-\mu(N_t - N_0)}, \tag{3}$$

where P_0 is the proportion of cultures showing no resistant cells. Therefore, μ was calculated as:

$$\mu = -\mathrm{Log}_e P_0/(N_t - N_0) \tag{4}$$

Reliability, reproducibility and precision of the procedure for estimating μ were determined later (British Standards Institute 1979; Thrusfield 1995). Reliability was determined based on the agreement among three iterations of the experiments; reproducibility was determined as the agreement among three sets of observations made on the same experiment by three different observers; finally, precision was calculated as the minimum variation in μ that can be detected using the procedure.

Mutation-selection equilibrium

If the $G^s \rightarrow G^r$ mutation from a normal wild-type, glyphosate-sensitive allele to a glyphosate-resistant allele is recurrent, and the glyphosate-resistant allele is

detrimental in fitness in the absence of the herbicide, then new resistant-mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance (Crow and Kimura 1970; Spiess 1989). At any one time there will be a certain number of cells that are not yet eliminated. The average number of such mutants will be determined by the balance between μ and the rate of selective elimination, in accordance with the equation:

$$q = (\mu/s)^{1/2}, \tag{5}$$

where q is the frequency of the glyphosate-resistant allele and s is the coefficient of selection (Ayala and Kiger 1980), calculated as follows:

$$s = 1 - (m_{G^r}/m_{G^s}), \tag{6}$$

where m_{G^r} and m_{G^s} are the Malthusian fitness of G^r and G^s cells measured in non-selective conditions, respectively (Crow and Kimura 1970).

Analysis of cell morphology of G^s and G^r variants

Maximum and minimum diameters, area, and perimeter of 100 cells of both G^s and G^r variants, from the MaD3 and MaD7 strains of *M. aeruginosa*, were measured directly using an image analysis system (Motic Digital Imaging 3.5, Motic, Xiamen, PRC). As a measure of the shape of the cells, the coefficient of form (CF) proposed by Renau-Piqueras et al. (1985) was calculated:

$$\mathrm{CF} = (4\pi \mathrm{A})/\mathrm{P}^2, \tag{7}$$

where A is the area and P the perimeter of the outline of the cell. According to the formulae of the area of a circle and its circumference, a CF = 1 is derived; identically, the area and the perimeter of an ellipse with semi-axes of 1 and 0.5, respectively, yield a CF = 0.8. More details are given in Goyanes et al. (1990) and Rico et al. (2006).

Results

Glyphosate-sensitive cells from both strains MaD3 and MaD7 showed similar growth rates ($m \approx 1$ doubling d^{-1}) (Fig. 2). The exposure to glyphosate had an analogous toxic effect in both clones: concentrations from 10 to 60 ppm induced a drastic decrease of fitness, and at a concentration of 110 ppm growth was totally inhibited (Fig. 2).

A high fluctuation in set 1 experiments (from 0 to more than 10^4 resistant cells per culture flask) was found in both strains of *M. aeruginosa* (Table 1). The fluctuation observed is not a consequence of experimental error in sampling G^r cells because the analyses of set 2 showed that in all cultures the number of G^r cells per flask was less than 10^3 in both clones (Table 1).

Mutation rates for $G^s \rightarrow G^r$ (estimated with high standards of reliability, reproducibility, and precision, see Table 2) were 3.6×10^{-7} and 3.1×10^{-7} in strains Ma3D and Ma7D, respectively (Table 1).

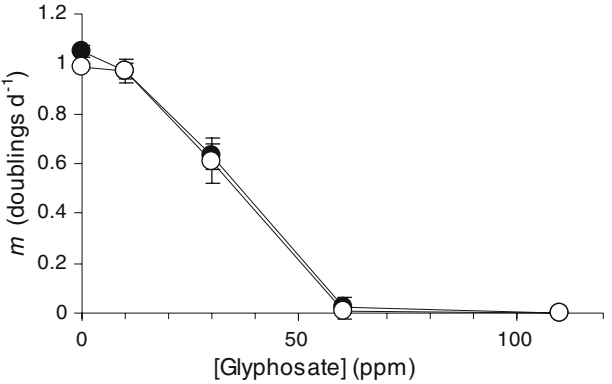


Fig. 2 Effect of glyphosate on acclimated growth rate (m; mean ± SD, $n = 4$) of *Microcystis aeruginosa* wild-type, glyphosate-sensitive Ma3D (open circles) and Ma7D (filled circles) strains

Table 1 Fluctuation analysis of G^r variants in *Microcystis aeruginosa* wild-type strains Ma3D and Ma7D

	Strain Ma3D		Strain Ma7D	
	Set 1	Set 2	Set 1	Set 2
No. of replicate cultures	100	45	100	45
No. of cultures containing the following no. of G^r cells:				
0	92	0	93	0
1–10^3	4	45	3	45
10^3–10^4	3	0	2	0
>10^4	1	0	2	0
Variance/mean (of the no. of G^r cells per replicate)	84.1	0.9	87.2	1.1
μ (mutants per cell per generation)	3.6×10^{-7}		3.1×10^{-7}	

Table 2 Reliability, reproducibility and precision of the procedure to estimate mutation rate (μ) of $G^s \rightarrow G^r$ in two strains of *Microcystis aeruginosa*

	Strain Ma3D	Strain Ma7D
Reliability of μ (%)	90	92
Reproducibility of P_0 (%)	99	97
Precision of μ (mutants per cell per generation)	0.5×10^{-7}	0.5×10^{-7}

Isolated G^r mutants growing in the absence of the selective agent, i.e., without glyphosate in the culture medium, showed growth rates only one-sixth of those found in G^s cells (Fig. 3).

The m_{G^s} and m_{G^r} values were used to compute the coefficient of selection of G^r mutants ($s = 0.84$ in strain Ma3D, and 0.82 in strain Ma7D, respectively). By using the previous values of μ and s, the frequency of glyphosate-resistant alleles was calculated; the value of q ranged from 6.14×10^{-4} in strain Ma3D, to 6.54×10^{-4} in strain Ma7D.

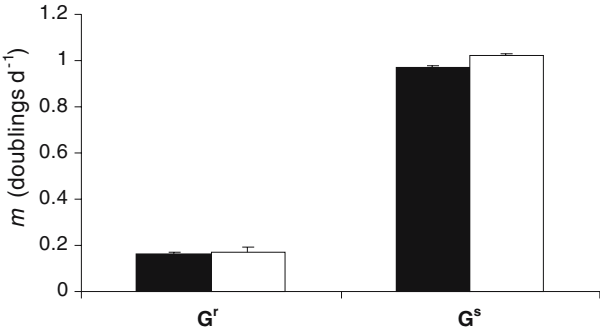


Fig. 3 Acclimated growth rate (m) of the Ma3D (open bars) and Ma7D (filled bars) strains of *Microcystis aeruginosa* wild-type, glyphosate-sensitive (G^s) and glyphosate resistant (G^r) variants. Vertical lines on bars shown SD ($n = 4$)

Table 3 Diameters and coefficient of form (CF) of G^s and G^r cells in two strains of *Microcystis aeruginosa*. Comparisons between G^s and G^r variants were carried out by Student's t-test: in all the comparisons, differences were significant at $P < 0.001$. Values are represented as overall mean ± SD ($n = 100$)

	Strain Ma3D		Strain Ma7D	
	G^s	G^r	G^s	G^r
Maximum diameter (μm)	2.40 ± 0.32	2.01 ± 0.40	2.73 ± 0.41	2.32 ± 0.36
Minimum diameter (μm)	2.35 ± 0.32	1.80 ± 0.36	2.42 ± 0.35	2.01 ± 0.41
CF (dimensionless)	1.00 ± 0.01	0.96 ± 0.01	0.99 ± 0.01	0.90 ± 0.02

Glyphosate-sensitive cells from both strains exhibited significantly greater size (t-test, $P < 0.001$, $n = 100$) than the G^r cells (Table 3). The outline of *M. aeruginosa* G^s cells is circular, with an index of roundness CF ≈ 1 in both strains, corresponding to spherical cells (Table 3). However, the G^r variants showed a significantly (t-test, $P < 0.001$, $n = 100$) lower CF value, corresponding to slightly elliptical rather than spherical cells (Table 3).

Discussion

When *M. aeruginosa* cultures were treated with a lethal dose of glyphosate, they became clear after some days due to the destruction of the sensitive cells by the toxic effect of the herbicide. However, after further incubation, some cultures became colored again, due to the growth of cells that were resistant to the effect of glyphosate. The key to understanding adaptation of cyanobacteria to survive in a glyphosate-contaminated environment seems likely to lie in characterizing the resistant cells that appear after the massive destruction of the sensitive cells. Fluctuation analysis is the appropriate procedure to discriminate between glyphosate-resistant cells arising by rare spontaneous mutations occurring randomly during propagation of organisms under nonselective conditions (i.e., prior to exposure to glyphosate) and glyphosate-resistant cells arising through acclimation or adaptive mutation in response to selection (Luria and Delbrück 1943; reviewed by Sniegowski 2005). The large fluctuation in number of

glyphosate-resistant cells observed in set 1 experiments, in contrast with the scant variation in set 2 controls, unequivocally demonstrates that resistant cells arose by rare spontaneous mutation and not through direct and specific arousal of adaptive mutations in response to glyphosate exposure. The herbicide did not stimulate the appearance of resistant cells at all. The rapid lethal effect of glyphosate seems unlikely to allow the appearance of adaptive mutations. Adaptation of cyanobacteria and algae, which are the principal primary producers of aquatic ecosystems, to environmental changes resulting from anthropogenic contamination (or even to extreme natural environments) seems to be the result of a rare event: the spontaneous mutation from sensitivity to resistance that occurs randomly prior to the cells coming into contact with the selective agent (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al. 2002, 2004; Flores-Moya et al. 2005).

The rate of mutation from $G^s \rightarrow G^r$ (from 3.1×10^{-7} to 3.6×10^{-7} mutants per cell per generation) was one to two orders of magnitude lower than the mutation rates we have described (from 2.12×10^{-5} to 1.76×10^{-6} mutants per cell per generation) for the resistance to several biocides in other cyanobacterial and microalgal species (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al., 2002, 2004), but of the same order of magnitude found for the resistance to sulphureous waters in the chlorophycean *Spirogyra insignis* (2.7×10^{-7} mutants per cell per generation) (Flores-Moya et al. 2005). Nevertheless, the pre-selective $G^s \rightarrow G^r$ mutations are sufficiently frequent in *M. aeruginosa* populations to allow them to adapt to the presence of glyphosate in culture. The presence of G^r cells in the populations of *M. aeruginosa* is regulated by the recurrent appearance of mutants and their elimination by selection, yielding an equilibrium frequency of 6–7 G^r cells per 10^4 cell divisions. This fraction of resistant mutants is presumably enough to assure the adaptation of cyanobacterial populations to catastrophic water contamination, since the natural populations of cyanobacteria are composed of countless cells. Nevertheless, mutations usually imply an energetic cost that may affect the survival of adapting populations (Coustau et al. 2000), as was demonstrated by a growth rate in G^r cells only one-sixth of that in G^s ones, in the absence of the herbicide. Thus, under a scenario of global change caused by human activies (including the appearance of biocides in ecosystems), it could be hypothesized that G^r cells from *M. aeruginosa* could develop in freshwater ecosystems polluted with glyphosate, but their contribution to primary production will be significantly lower than that occurring in pristine ecosystems with G^s cells.

Finally, in a glyphosate-contamined environment the populations could be formed of cells of smaller size and a slightly different morphology from the typical spherical cells of *M. aeruginosa* (Whitton 2002) since G^r cells showed a CF 5% lower than that of a circular outline associated with a spherical volume. It can be supposed that the mechanism linked to resistance to glyphosate is a pleiotropic gene that is also implicated in the arrangement of microtubular-protein analogues in the cells, but this point remains to be investigated. In freshwater systems located in urban or agricultural areas, cyanobacteria and microalgae are exposed to a multitude of toxicologically different biocides (Junghans et al. 2006). Therefore, it could be hypothesized that the appearance of resistant mutants can originate, simultaneously, the rise of new morphological populations driven by algicide-resistant clones.

However, this aspect remains to be investigated by using more biocides and cyanobacteria and algal species.

The origin of favoured mutants and the process of adaptation can be only achieved if appropiate genetic variability is available (Bradshaw and Hardwick 1989). In a series of preliminary studies analysing the genetic variability in populations of *M. aeruginosa*, significant variability was found for morphological and physiological (fitness and photosynthetic performance) traits, and genetic factors contributed 50–90% of the observed phenotypic variability (Bañares-España et al. 2006; López-Rodas et al. 2006; Rico et al. 2006). This kind of complementary approach can also cast some light on the ability of cyanobacteria to adapt to environmental changes, such as water pollution by herbicides, in recent years. Since *M. aeruginosa* is known to be the most important cause of toxic blooms in inland water systems (reviewed in Skulberg et al. 1993), the occurrence of herbicide-resistant cells could also be of interest to water management.

In conclusion, spontaneous pre-selective mutants, like 'hopeful monsters', are enough to assure the adaptation of cyanobacterial populations to catastrophic environmental changes.

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4.1.2. Human-synthesized pollutants drive emergence of evolutionary novelties: pre-selective mutations confer resistance to the herbicides simazine and diquat in green microalgae.

Research Article

Human-synthesized Pollutants Drive Emergence of Evolutionary Novelties: Pre-selective Mutations Confer Resistance to the Herbicides Simazine and Diquat in Green Microalgae

Fernando Marvá[1], Victoria López-Rodas[1], Mónica Rouco[1], Macarena Navarro[1], Francisco J. Toro[2], Antonio Flores- Moya[2*] and Eduardo Costas[1]

[1] Department of Animal Production (Genetics), Veterinary Faculty, Complutense University, Avda. Puerta de Hierro s/n, E-28040 Madrid, Spain

[2] Department of Plant Biology (Botany), Faculty of Sciences, University of Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain

* Corresponding author (floresa@uma.es)

SHORT RUNNING TITLE: Herbicide-resistance in green microalgae

Abstract

Background, Scope and Aim. Aquatic ecosystems located close to agricultural areas are increasingly more polluted with herbicides. This situation can cause the local extinction of phytoplankters but, simultaneously, could also promote the emergence of evolutionary novelties that have the capacity to proliferate under normally lethal doses of herbicides. We evaluated the capacity for adaptation of green microalgae to lethal concentrations of the herbicide simazine in one strain of *Dictyosphaerium chlorelloides* and two strains of *Scenedesmus intermedius*, as well as adaptation to the herbicide diquat in one of the strains of *S. intermedius*.

Methods. In all the cases, growth of wild-type, herbicide-sensitive cells of the strains initially collapsed under ostensibly lethal doses of the herbicides. Nevertheless, after further incubation for several weeks some cultures were able to grow, revealing the survival of resistant cells. A Luria-Delbrück fluctuation analysis was carried out in order to distinguish between resistant cells arising from physiological adaptation (acclimation) or post-adaptive mutation (both events occurring after the exposure to the herbicides), and adaptation due to rare, spontaneous mutations before the exposure to the herbicides.

Results and Discussion. Fluctuation tests revealed that simazine-resistant cells arose by rare spontaneous mutations before the exposure to simazine, with an estimated mutation rate of 3.0×10^{-6} mutants per cell per generation in both strains of *S. intermedius*, and of 9.2×10^{-6} mutants per cell per generation in *D. chlorelloides*. Mutants have a

diminished growth rate when compared to wild-type cells; thus, the frequency of the simazine-resistant allele, as consequence of the mutation-selection equilibrium, ranged from 11 to 30 per 10^6 wild-type cells in the three microalgal strains. Diquat-resistant cells in *S. intermedius* arose by pre-selective mutations with a rate of 17.9×10^{-6} per cell per generation, and the frequency of resistant alleles was *c*. 83 mutants per 10^6 cells.

Conclusions. Rare, pre-selective mutations may allow the survival of green microalgae in simazine- or diquat-polluted waters, via herbicide-resistant clone selection. Therefore, human-synthesized pollutants, such as the herbicides simazine and diquat, could cause the emergence of evolutionary novelties in aquatic environments.

Recommendations and Perspectives. More studies on the evolutionary effects of herbicides on non-target species must be carried out. In particular, the effects on aquatic microorganisms have been largely overlooked, yet they could be more severe than in terrestrial organisms.

Keywords: adaptation; *Dictyosphaerium chlorelloides*; diquat; fluctuation analysis; mutation; *Scenedesmus intermedius*; simazine

Introduction

At present, global extinction rates of organisms are 50-500 times background and are increasing due to human activities that are altering biosphere-level processes (Woodruff 2001). The biodiversity crisis is reasonably understood for multi-celled organisms, but little is known about organisms as abundant and ecologically important as microalgae and cyanobacteria, which are the base of trophic webs in aquatic ecosystems (Falkowski and Raven 1997). One of the causes of the present biodiversity crisis is intensive agriculture, because it is supported by the massive use of compounds with biocidal properties (Tilman 1999). The unwanted side effects of herbicides have strong evolutionary consequences due to selection pressure on non-target species (Belfiore and Anderson 2001; Palumbi 2001). Freshwater habitats close to agricultural areas are sinks for a large array of herbicides, so that phytoplankters are exposed to a multitude of these toxic compounds (Junghans et al. 2006). In fact, it is considered that herbicides are among the most significant human-synthesized pollutants in aquatic ecosystems (Koenig 2001).

Phytoplankters usually experience local extinction in herbicide-polluted waters, but they can also develop two different possibilities to survive the harmful effects of herbicides: new genetic variants originating by spontaneous mutation can be selected (genetic adaptation) (Sniegowski and Lenski 1995; Belfiore and Anderson 2001; Sniegowski 2005), or else gene expression can be modified (physiological adaptation, also called acclimation; Bradshaw and Hardwick 1989). However, some evolutionary studies in bacteria (Cairns et al. 1988; Foster 2000; Roth et al. 2006) and yeasts (Heidenreich 2007) have suggested that adaptive mutations could be a process

85 resembling Lamarckism which, in the absence of lethal selection, produces mutations
86 that relieve selective pressure. Therefore, the key to this debate is to know whether the
87 adaptation process allowing phytoplankters to survive and proliferate in herbicide-
88 polluted waters appears before or after the exposure of the cells to the herbicide. Fig. 1
89 shows the different adaptive possibilities for phytoplankton in herbicide-polluted
90 waters. In this framework, we recently demonstrated that the freshwater cyanobacterium
91 *Microcystis aeruginosa* could proliferate in algaecidal copper-polluted (García-Villada
92 et al. 2004) or the herbicide glyphosate-polluted (López-Rodas et al. 2007) waters, via
93 pollutant-resistant clone selection originating from pre-selective mutations.
94 In order to improve our understanding of the adaptation process involved in the
95 survival and proliferation of phytoplankters in herbicide-polluted waters, we addressed
96 the adaptive mechanism of freshwater chlorophyceans to lethal doses of the herbicides
97 simazine and diquat. By using the experimental procedure called fluctuation analysis
98 (Luria and Delbrück 1943), we were able to discriminate between acquired adaptations
99 in response to the herbicides (by acclimation or putative adaptive mutations) and
100 resistant cells arising from rare spontaneous mutations that appear prior to the herbicide
101 exposure. We demonstrate the occurrence of very rapid evolution in three strains of
102 chlorophyceans as result of pre-selective mutations conferring herbicide resistance. It
103 has been hypothesized that the emergence of unpredictable evolutionary novelties, such
104 as resistant-herbicide organisms, could be a distinctive feature of the future biosphere
105 (Myers and Knoll 2001). Here we present some evidence to support this hypothesis.
106
107 **1. Materials and Methods**
108

1.1 Experimental organisms and culture conditions

The experiments were performed with three different strains of chlorophyceans from the algal culture collection of the Genetics Laboratory, Veterinary Faculty, Complutense University (Madrid, Spain). The SiM strain of *Scenedesmus intermedius* Chodat was isolated from Entreuka pond in the Sahel desert (Mauritania), where herbicides have never been used, whereas the SiD strain was isolated from a lagoon in Doñana National Park (SW Spain), a place sometimes exposed to runoff from nearby agricultural areas. A strain of *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman (strain Dc) was isolated from a high-mountain, pristine lagoon in Sierra Nevada National Park (SE Spain). The strains were grown axenically in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma Aldrich Chemie, Taufkirchen, Germany), at 20ºC under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400-700 nm. Strains were maintained in mid-log exponential growth by serial transfers of a cell inoculum to fresh medium (details in Carrillo et al. 2003). Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures. All three strains were used in the study of adaptation to simazine, whereas the adaptation to diquat was exclusively studied with strain SiD.

1.2 Toxicity test: effect of simazine and diquat on growth rate

The toxic effect of simazine (2-chloro-4,6-bis[ethylamino]-*s*-triazine) and diquat (1,1'ethylene, 2,2'bipyridyl) on growth rate of the wild-type strains was assessed as

follows. A stock solution of simazine was prepared in BG-11 medium with addition of 0.5 % dimethyl sulfoxide (DMSO). Each experimental culture was inoculated with 1.5 $\times 10^5$ cells from mid-log exponentially growing cultures and exposed to concentrations from 0 to 5 ppb of simazine. A diquat stock solution was prepared in BG-11 medium to obtain serial dilutions of 10, 30, 60 and 110 ppb, and the experimental cultures were inoculated with 5×10^6 cells from mid-log exponentially growing cultures. The herbicides were purchased from Sigma Aldrich Chemie (Taufkirchen, Germany). Three replicates of each concentration of both herbicides were prepared, as well as three unexposed controls.

The toxic effect of the herbicides was estimated by calculating acclimated maximal growth rate (m) in mid-log exponentially growing cells, in the presence of different concentrations of herbicide, by using the equation of Crow and Kimura (1970):

$$m = \log_e (N_t / N_0)/t, \qquad \text{(eq. 1)}$$

where N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively, and t = 7 d, the time that cultures were exposed to different doses of simazine or diquat.

Experimental cultures and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer and an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany).

1.3 Fluctuation analysis of the transformation herbicide-sensitive → herbicide-resistant

157

158 A modified Luria-Delbrück analysis for application to liquid cultures with microalgae
159 (López-Rodas et al. 2001; Costas et al. 2001) was used to investigate the origin of
160 herbicide-resistant cells. The modification of the analysis involves the use of liquid
161 medium containing the selective agent rather than plating on a solid medium, as was
162 done by Luria and Delbrück (1943) with bacterial cultures.

163 Two different sets of experimental cultures were prepared. In the first set (set 1),
164 100-103 (for simazine) or 90 (for diquat) 20 mL culture flasks, containing 10 mL of BG
165 medium, were inoculated with $N_0 = 10^2$ cells (a number small enough to reasonably
166 ensure the absence of pre-existing mutants in the strain). In the study with simazine,
167 when each culture reached $N_t = 10^5$ cells, it was supplemented with a lethal dose
168 (determined as explained in paragraph 1.2) of 3.1 ppb simazine. The experiment with
169 diquat started with $N_t = 6.2 \times 10^5$ cells of SiD cells, then the cultures were supplemented
170 with a lethal dose (determined as explained in paragraph 1.2) of 120 ppb diquat. For set
171 2 (set control), 30 aliquots of 10^5 (for simazine) or 6.2×10^5 (for diquat) cells from the
172 same parental population were separately transferred to culture flasks containing fresh
173 liquid medium with the herbicide at the same concentration as set 1 cultures. All
174 cultures were kept under selective conditions and observed after 60 d, a period of time
175 long enough to allow resistant cells to grow. At the end of the experiments, the number
176 of resistant cells in both sets was counted. The cell count was performed by at least two
177 independent observers.

178 According to Luria and Delbrück (1943), two different results can be found in
179 set 1 when conducting a fluctuation analysis, each of them being interpreted as the
180 independent consequence of two different phenomena of adaptation. If resistant cells

arose after the exposure of the cells to the selective agent (by acclimation or specific post-selective mutations) the variance in the number of cells per culture would be low (Fig. 2, set 2A) because every cell is likely to have the same chance of developing resistance. Therefore, the coefficient of variation (CV = SD × 100/ mean) of the number of resistant-cells per flask must be relatively low. By contrast, if cells appeared by random, pre-selective mutations occurring before the exposure to the selective agent, high variation in the inter-culture number of resistant cells should be found (Fig. 2, set 2B) and, consequently, the CV should be relatively high.

Set 2 (Fig. 2) is the experimental control of the fluctuation analysis. It samples all the sources of variance associated with the experimental procedure. Thus, despite the way resistant cells appear, inter-culture (flask-to-flask) variance of resistant cells in set 2 should be similar to the average of resistant cells in set 2 cultures. Moreover, if a similar CV value is found in sets 1 and 2, it confirms that resistant cells appeared by adaptive mutations or acclimation (i.e. after the exposure to the herbicide). By contrast, if the CV from set 1 is significantly higher than the CV in set 2, it means that resistant cells aroused by spontaneous mutations prior to exposure to herbicides. The comparison of CVs was performed by the one-tailed *Z*-test according to Zar (1999).

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. There are different approaches for accomplishing this estimation (Rosche and Foster 2000). Due to methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of cultures from set 1 showing no resistant cells (P_0 estimator; Luria and Delbrück 1943) was used to calculate the mutation rate (μ) by using the equation:

$$\mu = -\log_e P_0 / (N_t - N_0) \qquad \text{(eq. 2)}$$

1.4 Mutation-selection equilibrium

If the mutation from wild-type, herbicide-sensitive allele to herbicide-resistant allele is recurrent and, in addition, the herbicide-resistant allele is detrimental in fitness in the absence of herbicides, most of these mutants are eliminated sooner or later by natural selection, if not by chance. At any one time, there will be a certain number of cells that are not yet eliminated. The average number of such mutants will be determined by the balance between μ and the rate of selective elimination, in accordance with the equation from Kimura and Maruyama (1966):

$$q = \mu / (\mu + s), \qquad \text{(eq. 3)}$$

where q is the frequency of the herbicide resistant allele and s is the coefficient of selection, calculated as follows:

$$s = 1 - (m_S^r / m_S^s), \qquad \text{(eq. 4)}$$

where m^r and m^s are the acclimated maximal growth rates of herbicide-resistance and herbicide-sensitive cells measured in non-selective conditions (i.e. in BG medium), respectively.

2. Results

2.1 Toxicity test: effects of simazine and diquat on growth rate

The exposure to simazine had a toxic effect on the three algal strains evaluated. A concentration of 1.5 ppb simazine inhibited totally the growth of the three strains and the cultures became clear after some days by massive destruction of the sensitive cells. Similarly, a concentration of 60 ppb of diquat was lethal for SiD cells. In accordance with these lethal doses figures, concentrations of 3.1 ppb simazine and 120 ppb diquat were selected for the fluctuation analysis tests.

2.2 Fluctuation analysis of the transformation herbicide-sensitive → herbicide-resistant

The fluctuation analysis culture flasks were incubated for two months. After this time, cell growth appeared in some culture flasks in the four experiments carried out. A high fluctuation in the number of resistant cells per culture flask (from 0 to $>10^5$) was found in set 1 of the three strains of chlorophyceans tested against simazine (Table 1). The CVs of the set 1 experiments were significantly ($P<0.001$) higher than those found in the respective set 2 controls (Table 1). Similarly, the number of diquat-resistant cells of SiD per flask ranged from 0 to $>10^5$ in set 1, whereas the figure ranged from 10^3-10^4 in set 2. The CV from set 1 was significantly ($P<0.001$) higher than that from set 2 (Table 2). Consequently, the high fluctuation found in set 1 cultures in the four experiments should be due to processes other than sampling error, and it could be inferred that herbicide-resistant cells arose prior to herbicides exposure by rare, spontaneous

253 mutations rather than by specific adaptation (i.e. physiological acclimation) during
254 herbicide exposure.
255 The spontaneous mutation rate (μ) of simazine sensitive cells to simazine
256 resistant cells, using the P_0 estimator, was estimated at 3.0×10^{-6} mutations per cell per
257 generation in both strains of *S. intermedius*, and at 9.2×10^{-6} mutations per cell per
258 generation in *D. chlorelloides* (Table 1). In the case of diquat, the value of μ in the
259 strain SiD was one order of magnitude higher (17.9×10^{-6} mutations per cell per
260 generation) than those found for the resistance against simazine (Tables 1,2).
261 The simazine-resistant cells, in absence of this herbicide, showed lower growth
262 rate values than simazine-sensitive cells. The coefficients of selection (*s*) were estimated
263 to be 0.109, 0.267 and 0.306 in strains SiD, SiM and Dc, respectively. The frequency
264 (*q*) of simazine-resistant alleles, in wild-type populations in the absence of simazine,
265 was calculated by using the values of *s* and μ: 27.5, 11.2 and 30.0 simazine-resistant
266 per 10^6 wild-type cells, in strains SiD, SiM, and Dc respectively. In the case of diquat,
267 the computed *q* value (by using a derived *s* value of 0.215) was of 83.2 resistant cells
268 per 10^6 wild-type SiD cells.
269
270 **3. Discussion**
271
272 In this work, we present an experimental model of evolution to analyze adaptation of
273 phytoplankters to survive in herbicide-polluted aquatic environments. In particular, the
274 possible adaptation of chlorophyceans to lethal doses of the herbicides simazine and
275 diquat was addressed. When chlorophycean cultures were exposed to the herbicides,
276 they became clear after some days due to total growth inhibition and subsequent

277 massive destruction of the cells by the lethal effect of herbicides. However, if they were
278 further incubated some cultures became green again, due to the growth of variants that
279 were resistant to the herbicides. By using the statistical and experimental approach
280 named fluctuation analysis (Luria and Delbrük 1943), we were able to discriminate
281 between simazine- or diquat-resistant cells arising by rare spontaneous mutation
282 occurring randomly during propagation of organisms under non-selective conditions,
283 and herbicide-resistant cells arising by adaptive mutations or though acclimation in
284 response to the herbicides. The high fluctuation in the number of herbicide-resistant
285 cells observed in set 1 cultures, in contrast with low fluctuation of set 2 controls, shows
286 that herbicide-resistant cells have arisen from rare, pre-selective spontaneous mutations
287 occurring randomly during replication of organisms prior to exposure to the herbicides.
288 The mutation rates from simazine-sensitivity to simazine-resistance in the three
289 stains of microalgae (3.0-9.2 $\times 10^{-6}$ mutants per cell per generation) were found to be in
290 the middle of the range of the mutations rates (from 2.1×10^{-5} to 2.7×10^{-7} mutants per
291 cell per generation) we have described in cyanobacteria and microalgae, for resistance
292 to many other biocides and extreme natural environments (Costas et al. 2001, 2007;
293 López-Rodas et al. 2001, 2007, 2008a, 2008b; Baos et al. 2002; García-Villada et al.
294 2002, 2004; Flores-Moya et al. 2005) . However, diquat-resistant cells appear
295 spontaneously in wild-populations with a frequency one order magnitude higher (17.9 $\times$
296 10^{-6}) than simazine-resistant cells.
297 Since mutation is recurrent in each generation, new mutant cells are arising
298 continuously. Herbicide-resistant mutants are impaired in growth rate and,
299 consequently, most of them are eventually eliminated by natural selection (Crow and
300 Kimura 1970; Spiess 1989). At any given time, the balance between the continuous

appearance of mutants and their selective elimination determines the number of remaining herbicide-resistant mutants in algal populations growing in the absence of herbicides. This may be the case in wild-type populations developing in non-polluted waters. Consequently, the population would be predominantly a clone line of simazine- or diquat-sensitive genotypes, accompanied by, as a very small fraction, clone lines of simazine- or diquat-resistant mutants. Moreover, recurrent exposures to herbicides could cause the rise of strains with higher selection coefficients and, consequently, enhancing the frequency of the resistance-alleles as the consequence of mutation-selection equilibrium. This is illustrated in the case of *S. intermedius*: the mutation rate from simazine-sensitivity to simazine-resistance was similar in two strains of this species. However, the frequency of the resistant allele, in the absence of simazine, was 2.5 times higher in the strain isolated from Doñana National Park (where herbicides sometime arrive in run-off) than in the strain isolated from the pristine pond Entreuka in the Sahel Desert. This difference is based on a higher selection coefficient in the strain SiM than in the strain SiD.

In conclusion, rare spontaneous mutations conferring resistance against simazine or diquat seem to be enough to assure survival of microalgae populations in simazine- or diquat-polluted waters. However, in a hypothetical future scenario with herbicide-polluted waters, the primary production supported by phytoplankters could be significantly lower that in the present, as a consequence of the diminished growth rate of resistant mutants in comparison to wild-type cells.

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Table 1. Fluctuation analysis of simazine-resistant variants in the green microalgae *Scenedesmus intermedius* (strains SiM and SiD) and *Dictyosphaerium chlorelloides* (strain Dc)

		Strain SiD		strain SiM		strain Dc	
		Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
No. of replicate cultures		100	30	102	30	103	29
No. of cultures containing the following no. of simazine-resistant cells							
	0	74	0	75	0	41	0
	$1\text{-}10^3$	0	0	1	0	1	0
	$10^3\text{-}10^4$	1	0	0	0	0	0
	$10^4\text{-}10^5$	2	0	10	0	16	0
	$>10^5$	23	30	16	30	45	30
CV of the no. simazine-resistant cell per replicate (%)							
		106.3	42.6	239.2	26.2	375.0	18.3
μ (mutants per cell per generation)		3.0×10^{-6}		3.0×10^{-6}		9.2×10^{-6}	

Table 2. Fluctuation analysis of diquat-resistant variants in the green microalga *Scenedesmus intermedius*, strain SiD

		Set 1	Set 2
No. of replicate cultures		90	30
No. of cultures containing the following no. of diquat-resistant cells			
	0	30	0
	$1\text{-}10^3$	5	0
	$10^3\text{-}10^4$	28	30
	$10^4\text{-}10^5$	21	0
	$>10^5$	6	0
CV of the no. diquat-resistant cell per replicate (%)		104.7	12.2
μ (mutants per cell per generation)		17.9×10^{-6}	

455 **Fig. 1.** Adaptation strategies of phytoplankton in herbicide-polluted waters.

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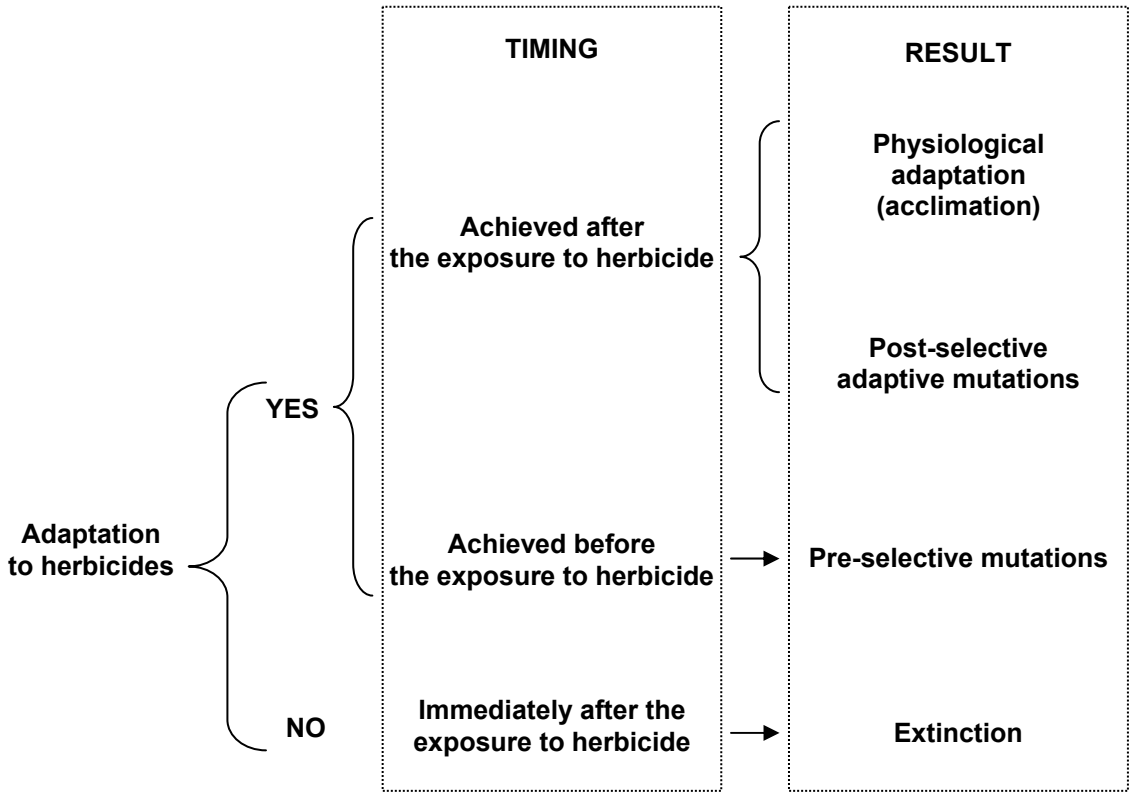

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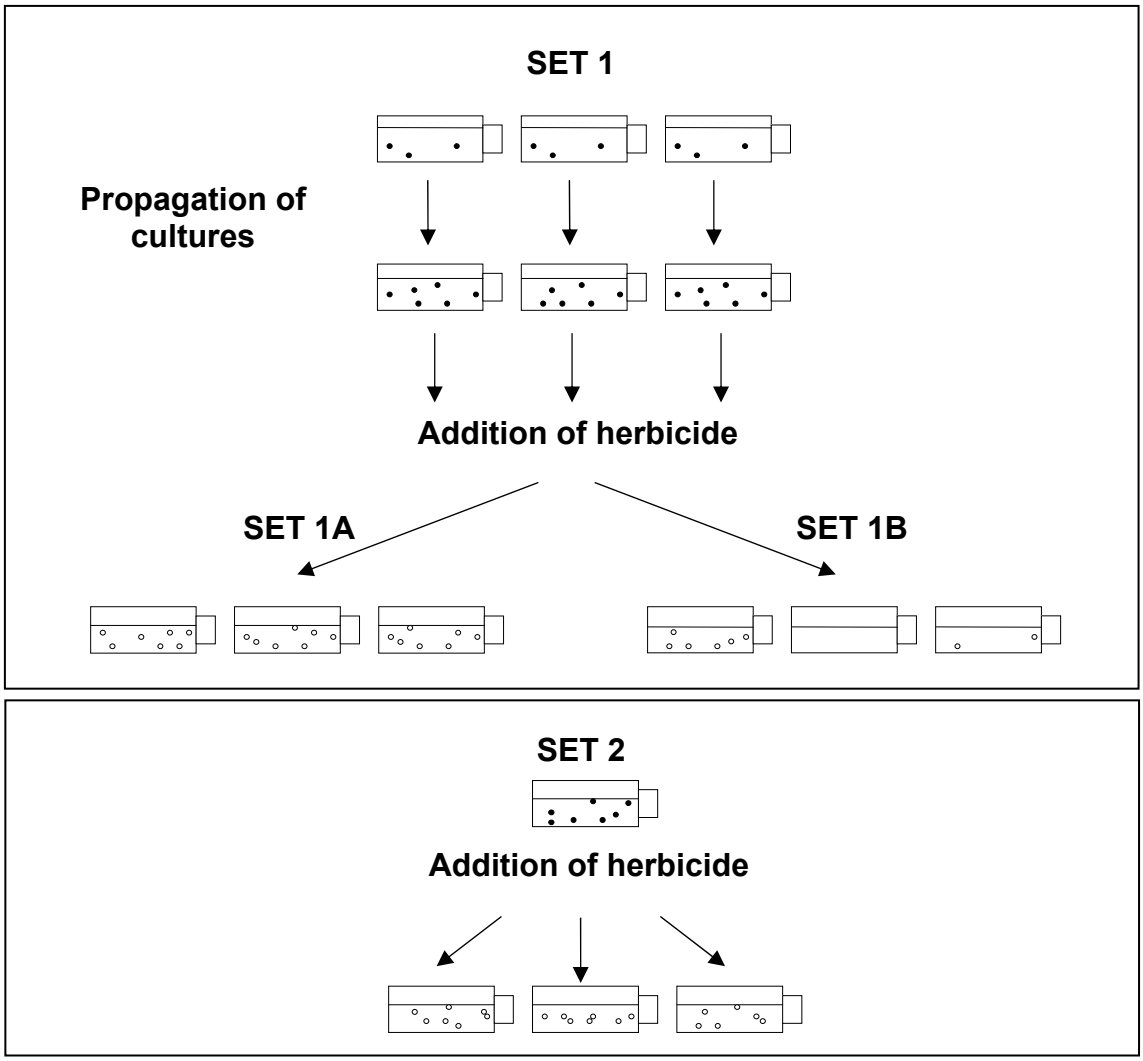
Fig. 2. Schematic diagram of the modified Luria and Delbrück (1943) fluctuation analysis. In the set 1, several cultures each inoculated with a small inoculums were propagated until a high cell density was reached, and then a lethal dose of herbicide was added. If resistant cells arose by acclimation or post-adaptive mutations the number of resistant cells in all the cultures must be similar (set 1A). If adaptation is achieved by rare mutations occurring in the period of the propagation of cultures the difference of the number of resistant cells in each culture must be huge (set 1B). Set 2 samples the variance of parental populations as an experimental control. In this case, the number of resistant cells in all the cultures must be similar. Black dots represent herbicide-sensitive cells while herbicide-resistant cells are shown by white dots.


SET 1
Propagation of cultures
Addition of herbicide
SET 1A
SET 1B
SET 2
Addition of herbicide

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4.1.3. Adaptation of phytoplankton to novel residual materials of water pollution: an experimental model analysing the evolution of an experimental microalgae population under formaldehyde contamination.

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Adaptation of Phytoplankton to Novel Residual Materials of Water Pollution: An Experimental Model Analysing the Evolution of an Experimental Microalgal Population Under Formaldehyde Contamination

Victoria López-Rodas · Nieves Perdigones · Fernando Marvá · Mónica Rouco · Jose Antonio García-Cabrera

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Abstract The adaptation mechanisms of microalgae to grow in contaminated waters were analysed using a *chlorophyta* species under formaldehyde exposure as experimental model. Cultures initially collapsed after exposure to 16 ppm formaldehyde, but occasionally resistant cells were able to grow after further incubation. Resistant cells arose by rare spontaneous mutations that appeared before the exposure to formaldehyde (mutation rate = 3.61×10^{-6}), and not as result of physiological mechanisms. Although mutations may be the mechanisms that should allow the survival of microalgae in polluted waters in a world under rapid global change, mutants have a diminished growth rate.

Keywords Adaptation · Formaldehyde · Mutation · Water pollution

Water pollution by anthropogenic substances is a problem of great magnitude that urgently needs more basic research to facilitate predictions about the future and to determine actions to mitigate this environmental crisis. In this sense, studies focused on knowing if essential microbes succumb to anthropogenic toxins are of great importance. Particularly, the tolerance of microalgae to contaminated environments is very relevant from an ecological point of view, as these organisms are the principal primary producers of aquatic ecosystems.

Among these toxics, formaldehyde has become widely used as a chemical intermediate, analytical reagent, in concrete and plaster additives, wood preservation, in agriculture, disinfectants and fumigants (EPA 1988; WHO 1989). Formaldehyde has a half-life of 24–168 h in surface waters and 48–336 h in deeper waters (Howards et al. 1991), causing acute toxicity in phytoplankton (Chiayvareesajja and Boyd 1993; Burridge et al. 1995).

In order to study adaptation of microalgae to grow and survive in formaldehyde-polluted environments, a fluctuation analysis (Luria and Delbrük 1943) was performed. Usually, formaldehyde treatment produces massive destruction of microalgae (Chiayvareesajja and Boyd 1993; Burridge et al. 1995), but some cell variants could survive in formaldehyde-contaminated environments. The fluctuation test (Luria and Delbrück 1943) provides the appropriate procedure to discriminate between adaptation by selection of rare spontaneous mutations and other procedures. Recently, fluctuation test has been conducted entirely in liquid media, growing microalgae cultures first in a benign medium and then exposing them to contaminants (López-Rodas et al. 2001, 2007; Costas et al. 2001, 2007; Baos et al. 2002; García-Villada et al. 2002; Flores-Moya et al. 2005).

Materials and Methods

Experiments were performed with a wild-type strain of *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman (Chlorophyta) isolated from a pristine lagoon (without previous formaldehyde contamination) in Sierra

V. López-Rodas (✉) · N. Perdigones · F. Marvá · M. Rouco
Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda, Puerta de Hierro s/n, 28040 Madrid, Spain
e-mail: vlrodas@vet.ucm.es

J. A. García-Cabrera
Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda, Puerta de Hierro s/n, 28040 Madrid, Spain

Nevada (SE Spain). This strain was isolated from a single cell to assure no genetic variability within it. Before the experiments, cells were grown axenically in cell-culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany) at 22°C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Cultures were axenically maintained in mid-log exponential growth by serial transfers of subcultures to fresh medium.

To determine formaldehyde toxicity, the effects of increasing doses of formaldehyde on growth and photosynthetic performance of *Dictyosphaerium chlorelloides* were measured. Experimental cultures were seeded each with 1.3×10^6 cells from mid-log exponentially growing cultures. A stock solution of about 38% formaldehyde (Sigma, Aldrich Chemie, Taufkirchen, Germany) was prepared in BG-11 medium to obtain serial dilutions of 1.60×10^{-3} % w/w (16 μg/mL), 9.94×10^{-4} % w/w (10.6 μg/mL), 6.14×10^{-4} % w/w (6.4 μg/mL), and 3.80×10^{-4} % w/w (4 μg/mL) to be used for algal exposure. Three replicate cultures of each formaldehyde concentration as well as three unexposed controls were prepared. In these cultures and controls, growth rate (m) was calculated using the equation:

$$m = \frac{Log_e \frac{N_t}{N_0}}{t}, \quad \text{(Crow and Kimura 1970)}$$

where $t = 7$ d, and N_0 and N_t are the cell numbers at the start and at the end of the experiment, respectively. Experiments and controls were counted using a spectrofluorimeter (Schimadzu RF-551S, Durisburg, Germany) relating the chlorophyll *a* fluorescence with cell density within the lineal range.

The effective quantum yield (Φ_{PSII}) was also measured in experiments and controls using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) 24 h after formaldehyde exposure. Effective quantum yield was calculated as follows:

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m}$$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al. 1986).

The fluctuation analysis (Fig. 1) was performed at 22°C and under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, and consisted of two Sets of culture flasks. Set 1 included 105 parallel cell-culture flasks, each one initially seeded with $N_0 = 125$ cells (i.e. a small number enough to assure the absence of pre-existing mutants). These cultures were allowed to grow (as previously detailed) until they reached approximately $N_t = 4.2 \times 10^5$ cells per flask, and then BG-11 medium containing formaldehyde (final concentration 1.6×10^{-3} (16 μg/ml) % w/w) was added. Control (Set 2) consisted on 25 parallel cell-culture flasks containing each 4.2×10^5 cells from the same parental population and with the same concentration of formaldehyde in BG-11 medium as Set 1. Both Sets were inoculated simultaneously. Cultures were grown for 50 days and then resistant cells in each culture were detected using a spectrofluorimeter (Schimadzu RF-551S, Durisburg, Germany). If resistant cells arose only from spontaneous mutations before selection (formaldehyde addition), then a high variance in their presence per culture (fluctuation) should be found as the chance of mutation would occur earlier in some cultures, later or even not occur in others. On the opposite, if resistant cells arose only in response to the selective medium, physiological mechanisms or post-adaptive mutation, every cell should present the same (and low) probability to adapt to the new medium. Thus, their distribution per culture should not exhibit any fluctuation at all. The control (Set 2) estimates the error in sampling resistant cells. Since this Set 2 is the experimental control of the analysis of fluctuation, a similar variance/mean ratio between Sets 1 and 2, would confirm that resistant cells arose in response to the selective medium.

The mutation rate from formaldehyde sensitive to formaldehyde-resistant cells was estimated by fluctuation analysis. The proportion of cultures from Set 1 showing no resistant cells after formaldehyde exposure was the parameter (P_0 estimator) used to calculate the mutation rate (μ). The P_0 estimator (Luria and Delbrück 1943) is defined as follows:

$$P_0 = e^{-\mu(N_t - N_0)}$$

where P_0 is the proportion of cultures showing no resistant cells, and N_0 and N_t are the initial and the final population size, respectively.

Therefore, μ (mutation rate) was calculated as:

$$\mu = \frac{-\mathrm{Log}_e P_0}{N_t - N_0}$$

The mutation from a normal wild-type formaldehyde-sensitive allele to a formaldehyde-resistant allele is recurrent. In addition, the formaldehyde-resistant allele is detrimental to fitness in the absence of formaldehyde. As a result, new resistant mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance (Crow and Kimura 1970). At each time there will be a certain number of resistant cells that are not yet eliminated. The average number of such mutants will be determined by the balance between mutation rate and selective elimination rate, in accordance with the equation:

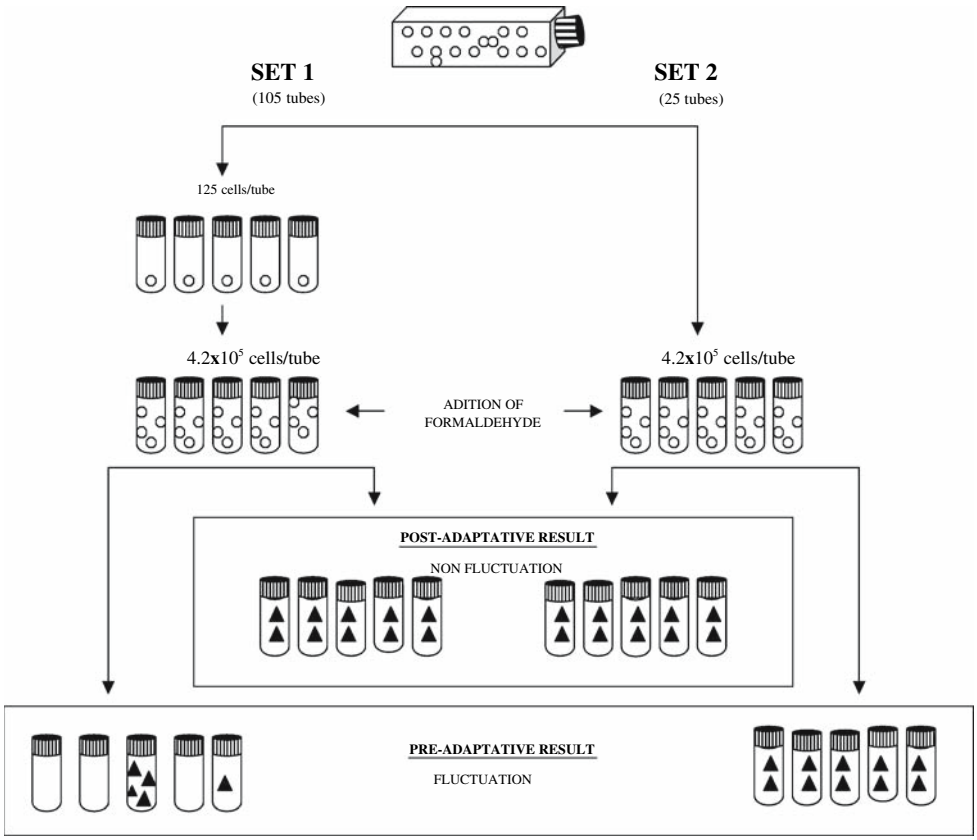

Fig. 1 Schematic diagram of the experiment modified from the classic Luria and Delbrück(1943) fluctuation analysis. Set 1 consists on 105 cultures each one containing 125 cells. They were allowed to grow, before adding the substance in study, till they reached the number of 4.2×10^5 cells. Set 2 consists on 25 tubes control with 4.2×10^5 cells that directly incorporated the studied product. If the adaptation to medium is due to the uncommon pre-selective mutations, between both Sets the existence of a huge fluctuation should be evident, as mutation appears by chance. In Set 1, some tubes would contain some mutants that had appeared early during cell division, in other tubes mutants would have appeared later, and, in the rest of them, there would be no mutants at all. On the other hand, if the resistance needs specific adaptation in response to formaldehyde exposure, both Sets 1 and 2, would be very similar, as every cell would have the same small probability to survive in that medium

$$q = \sqrt{\frac{\mu}{s+\mu}} \quad \text{(Kimura and Maruyama 1966)}$$

where q is the frequency of the formaldehyde-resistant allele, μ is the mutation rate and s is the coefficient of selection calculated as follows:

$$s = 1 - \frac{m_f^r}{m_f^s}$$

where m_f^r and m_f^s are the fitness of formaldehyde-resistant and formaldehyde-sensitive cells, respectively (Crow and Kimura 1970).

Results and Discussion

Low concentrations of formaldehyde have significant toxic effects on wild-type *D. chlorelloides* cells (Table 1). Growth rate and photosynthesis performance were severely reduced even by 6.4 µg/mL, whereas concentrations of 16 µg/mL inhibited completely growth and photosynthesis performance.

When microalgae were treated with 16 µg/mL formaldehyde in Set 1, all cultures initially collapsed due to the destruction of sensitive cells by the toxicant. But some cells were able to grow in some culture flask after 50 days, suggesting that rare formaldehyde-resistant cells occur (Table 2). A high fluctuation in the number of resistant cells per culture was observed in Set 1 (from 0 to more than 2.6×10^8 resistant cells per culture flasks). In contrast in Set 2 all the cell cultures contain formaldehyde-resistant cells showing low fluctuation (Table 2).

The mutation rate from formaldehyde susceptibility to formaldehyde resistance in *D. chlorelloides* (3.61×10^{-6} divisions) was found to be on the same order of magnitude than mutation rates we have described for resistance to many other biocides in chlorophyta (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al. 2002), significantly higher than mutation rates for

Table 1 Inhibition of growth and photosynthetic performance (effective quantum yield) of *Dictyosphaerium chlorelloides* by increasing doses of formaldehyde, calculated as percentage of untreated controls (dose-effect)

Formaldehyde concentration yield (μg mL^{-1}) (mean ± SE)	Growth rate inhibition (%) (mean ± SE)	Effective quantum inhibition (%)
0.0	0 ± 0	0 ± 0
4.0	17 ± 6	5 ± 0
6.4	61 ± 5	13 ± 1
10.6	100 ± 0	98 ± 2
16.0	100 ± 0	100 ± 0

Table 2 Fluctuation analysis of *Dictyosphaerium chlorelloides* exposed to formaldehyde (16 μg/mL)

	Set 1	Set 2
No. of culture replicates	105	25
N_0 (cells)	125	–
N_t (cells)	4.2×10^5	4.2×10^5
No. of cultures containing the following no. of formaldehyde-resistant cells:		
0	23	0
$1–10^7$	32	0
$10^7–2 \times 10^7$	11	0
$2 \times 10^7–3 \times 10^7$	8	0
$>3 \times 10^7$	31	25
Mutation rate (mutants per cell division) 3.61×10^{-6}		

sulphurous water of La Hedionda, 2.7×10^{-7} (Flores-Moya et al. 2005) and on the same order of magnitude than mutation rates for sulphurous water of Spain's Tinto River (Costas et al. 2007). Some stressful environments support populations of algal species at the extreme limits of their physiological tolerance (Fogg 2001). Algae survive in such hostile environments as a result of physiological acclimation by modifications of gene expression (Belfiore and Anderson 2001). Beyond physiological limits, adaptive evolution depends on the occurrence of new mutations that confer resistance (Belfiore and Anderson 2001).

On the opposite of formaldehyde-sensitive wild-type algae, the formaldehyde-resistant mutants isolated from Set 1 were able to grow under 16 μg/mL of formaldehyde. Furthermore, such a high formaldehyde concentration just inhibited 75% of their quantum yield. Isolated formaldehyde-resistant mutants growing in absence of formaldehyde showed a coefficient of selection of $s = 0.06$ respect to the wild-type formaldehyde-sensitive cells. Since mutation is recurrent, but mutant is usually detrimental in fitness, in each generation new mutants arise, but most of them are finally eliminated by natural selection (Crow and Kimura 1970). The frequency (q) of formaldehyde-resistant alleles in non-extreme environment was estimated, by using the values of μ and s, in 7.68 formaldehyde-resistant mutants per 10^3 cells, as the consequence of the balance between mutation and selection. Consequently, the ancestral microalgae population would be predominantly constituted by a clone line of wild-type sensitive genotype and, simultaneously, in a very small fraction, by a clone line of formaldehyde-resistant mutants. Thus, a rare spontaneous mutation from formaldehyde susceptibility to formaldehyde resistance seems to be enough to assure survival of microalgae populations in formaldehyde-contaminated environments.

Synthetic chemicals, like formaldehyde, causing water pollution could exert drastic selective pressures to facilitate rapid fixation of rare pre-adaptive mutations in natural populations of microalgae. Although some phytoplankton species could be able to rapidly adapt to new residual substances, such process usually implies a high cost for the ecosystem, as they reduce growth and photosynthetic performances.

Acknowledgments This work was supported by grants S-OSOS/AMB/0374 CAM, MAM 093/2002, CGL 2005-01938/BOS, CGL 2004-02701-HID. Thanks to Eva Salgado for her technical support.

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4.1.4. Experimental models to analyze adaptation of algae to anthropogenic contamination: 1. Pre-selective mutation confers antibiotic resistance in green microalgae.

Experimental models to analyze adaptation of algae to anthropogenic contamination: 1. Pre-selective mutation confers antibiotic resistance in green microalgae.

Fernando Marvá[1] et al.

[1]*Genética (Producción Animal), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain and*

Keywords: Chloramphenicol, *Scenedesmus intermedius*, mutation rate, fluctuation analysis.

Abstract

Modern water pollution is challenged to survival of phytoplankton population. As these microorganisms are the principal primary producers of aquatic ecosystems their tolerance to substances of water pollution is very relevant from an ecological point of view. We studied the adaptation of common chlorophyta specie (*Scenedesmus intermedius*) to inhibitory dose of chloramphenicol (an ordinary antibiotic). A fluctuation analysis demonstrated that chloramphenicol-resistant cells arise by spontaneous mutation that occurs randomly prior the antibiotic exposure. The mutation rate from chloramphenicol sensitivity to resistance was 1.01×10^{-5} mutation per cell division. Even resistant mutants exhibited a diminished fitness, until 5.8 mg L^{-1} of chloramphenicol, they were able to ensure the survival of microalgae population. In a population of *S. intermedius* in absence of chloramphenicol, the frequency of chloramphenicol-resistant allele in non-polluted environment was estimated in 5.5 chloramphenicol-resistant mutants per 10^3 sensitive-cells.

Introduction

Human activities are altering biosphere-level processes (global change) and causing biodiversity crisis (Woodruff, 2001; Myers and Knoll 2001). Waterpollution by new substances, which are producing environmental catastrophes in inland water systems, is a problem of great magnitude that urgently needs more basic research to facilitate predictions about the future, and to determine actions to mitigate this environmental crisis (Ehrlich, 2001). In this sense, studies focused on knowing if essential microbes succumb to anthropogenic toxins are very important (Woodruff, 2001) since humane activities are the greatest evolutionary force (Palumbi, 2001). Nowadays, the global extinction rates are 50-500 times background and are increasing due to those human activities that are altering biosphere-level processes. It is supposed that several million populations and 300-30,000 species go extinct annually from a total of >10 million species (Woodruff, 2001).

Particularly, the tolerance of microalgae to contaminated environments is very relevant from an ecological point of view, seeing that these organisms are the principal primary producers of aquatic ecosystems (Kirk, 1994; Falkowski and Raven, 1997).

Antibiotic production has been increasing since early 1960s (Jones, K H., 1999). As a consequence, antibiotic resistance in microorganism has been increasing (Harold, 1992) and has become one of the major threats in clinical practice (Wise et al., 1998). Overprescribing and inappropriate use of antibiotics in veterinary practice and clinical medicine is the main cause of this problem

72 (Feinman, 1999; Magee et al., 1999; Rao, 1998). Although chloramphenicol uses
73 have decreased in medicine and veterinary practice due to its serious side effects
74 as aplastic anaemia (Nagao et al., 1969), increased risk of childhood leukaemia
75 (Shu et al. 1987) and Gray baby syndrome (McIntyre et al., 2004), we used
76 chloramphenicol, that was broadly used antibiotic, in a experimental model in
77 order to understand how phytoplankton is able to adapt to this substance and the
78 ability of evolution after the adaptation.

79 Thus, the main aims of this work were to determine i) the capacity of
80 adaptation by microalgae to survive in chloramphenicol contaminated
81 environments and ii) determinate the nature of chloramphenicol-resistant cells
82 (i.e. resistant cells arising by rare spontaneous mutation prior chloramphenicol
83 exposure versus resistant cells arising by direct and specific acquired adaptation in
84 response to chloramphenicol). We also determined iii) the ability of
85 chloramphenicol resistant cells to ensure population survival in an acute pollution
86 episode with chloramphenicol.

87 This study is a complement to other works carried out in a framework
88 focused on understanding phytoplankton adaptation to grow in a contaminated
89 environment with anthropogenic water pollutants as gliphosate (López-Rodas et
90 al., 2006), DCMU (Costas et al., 2001), erythromycin (López-rodas et al. 2001),
91 formaldehyde (Lopez-Rodas et al, 2007a), copper (Garcia-Villada et al., 2004) or
92 TNT (García-Villada et al., 2002). We have also been reported rapid adaptation of
93 microalgae to extreme natural environments (Costas et al., 2007; Flores-Moya et
94 al., 2005; López-Rodas et al., 2008). In this study we applied similar procedures
95 to investigate algal adaptation to a ribosomal inhibitor antibiotic. The experiments

96 were performed with the chlorophiceae *Scenedesmus intermedius* and the broad-
97 spectrum antibiotic chloramphenicol as a selective agent.

98 Adaptive mutation depends on the occurrence of new mutation
99 (Sniegowski & Lenski, 1995). A modified fluctuation analysis from the classical
100 Luria and Delbrück experimental model (Luria and Delbrück, 1943) was
101 performed in order to determinate the first and the second aim. This is to say,
102 ability of adaptation trough mutation and the nature of chloramphenicol-resistant
103 cells. Fluctuation analysis is a well studied procedure to discriminate between
104 chloramphenicol-resistant cells arisen from rare spontaneous mutation that occur
105 randomly during propagation of microalgae prior to the chloramphenicol
106 exposure, and resistant cells that acquired specific adaptation in response to
107 chloramphenicol (as a consequence of physiological acclimation or direct and
108 specific mutation). As a summary, the figure 2 shows the different possibilities
109 which could phytoplankton experience in order to adapt to antibiotic-polluted
110 waters.

111 Chloramphenicol treatment produces inhibition in the growth of
112 microalgae, but we observed that some cell variants could survive in
113 chloramphenicol-contaminated environments. A fluctuation analysis demonstrates
114 the rapid evolution from chloramphenicol-sensitive to chloramphenicol-resistant
115 cells as a consequence of rare spontaneous mutation occurred before the addition
116 of chloramphenicol. These spontaneous pre-selective mutations keep in the
117 population as result of the equilibrium between mutation and selection, ensuring
118 the survival of the population in case of acute pollution of chloramphenicol.

119 **Materials and methods**

120

121 *Experimental organism and culture conditions*

122

123 The experiments where performed with the chloroficeae *Scenedesmus*
124 *intermedius*, isolated from a pristine lagoon in Doñana National Park (SW Spain).
125 This strain is in stock in the algal culture collection of the Universidad
126 Complutense (Madrid, Spain). *S. intermedius* grew axenically in culture flasks
127 (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium
128 (Sigma Aldrich Chemie, Taufkirchen, Germany), at 20ºC under continuous light
129 of 60 μmol m^{-2} s^{-1} over the waveband 400-700 nm. Cultures were maintained in
130 mid-log exponential growth by serial transfers of a cell inoculum to fresh medium
131 (Cooper, 1991). Prior to the experiments, the cultures were re-cloned (by isolating
132 a single cell) to avoid including any previous spontaneous mutants accumulated in
133 the cultures.

134

135 *Measure of chloramphenicol toxicity.*

136

137 To evaluate chloramphenicol toxicity, we measured the effect of
138 increasing doses of chloramphenicol on growth rate and photosynthetic
139 performance on *Scenedesmus intermedius*. A stock solution of chloramphenicol
140 (Panreac S.A., Barcelona, Spain) was prepared in BG-11 medium to obtain serial
141 dilutions of 1.3 mg L^{-1}, 2.2 mg L^{-1}, 3.6 mg L^{-1}, 5.8 mg L^{-1}, 9.8 mg L^{-1}, 15.2 mg L^{-}
142 ^{1}, 24.5 mg L^{-1} and 40 mg L^{-1}. Each experimental culture was inoculated with
143 $1.5x10^{5}$ cells from mid-log exponentially growing cultures and exposed to these

different concentrations of chloramphenicol. Three replicates of each concentration and three unexposed controls were prepared. In these cultures and controls, growth rate (*m*) was calculated using the equation:

$N_t = N_0 e^{mt}$ (Crow & Kimura 1970)

where t=7d (time that cultures were exposed to chloramphenicol), and N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively. Acclimated maximal growth rate (*m*) is the Malthusian parameter of fitness under conditions of *r* selection (Crow and Kimura, 1970; Spiess, 1989), and was calculated as:

$m = \mathrm{Log_e}\,(N_t / N_0)/t$ (Crow and Kimura 1970).

Experimental cultures and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample) using a haemocytometer and an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany).

The effective quantum yield (Φ_{PSII}) was measured in experiments and controls using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) 24 hours after chloramphenicol exposure. Effective quantum yield was calculated as follows:

$\Phi\ PSII = (F'_m - F_t) / F'_m$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al., 1986).

Fluctuation analysis.

Scenedesmus intermedius could adapt to lethal doses of chloramphenicol though selection of rare spontaneous mutations occurred prior chloramphenicol exposure or through direct and specific response to the chloramphenicol (Figure 2). A modified Luria-Delbrück fluctuation analysis (Luria and Delbrück, 1943) for application to liquid cultures of microalgae was performed in order to investigate the occurrence of chloramphenicol resistance as previously described López-Rodas *et al*. (2001) and Costas *et al*. (2001). The main aim of fluctuation analysis is distinguish between cells that have their origin in random spontaneous preselective mutations (prior to chloramphenicol exposure) from those arising through acquired postselective adaptation (during the exposure to chloramphenicol). Fluctuation analysis consist of two different sets of experimental cultures, set 1 provides the experimental result and set 2 is an experimental control. In the first set of experiment (set 1) 100 culture flasks were inoculated with $N_0 = 10^2$ wild-type cells (a number small enough to ensure the absence of pre-existing mutants). Cultures were grown in BG-11 medium until $N_t \approx 3x10^5$ cells. Afterwards they were supplemented with chloramphenicol at growth and photosynthesis inhibitory dose determinated in dose-effect experiment (Tables 1 and 2). For the set 2 (set control), 25 aliquots of $3x10^5$ cells, from the

same parental population, were separately transferred to culture flasks containing fresh liquid medium with chloramphenicol at the same concentration as set 1. Cultures were kept under selective conditions and observed after 60 days, period of time long enough to allow that one mutant cell could generate enough progeny to be detected. At the end of the experiments the number of resistant cells in each culture (both in set 1 and set 2) was counted. The cell count was performed by at least two independent observers.

In fluctuation analysis designed by Luria and Delbrück (1943) two different results can be found in the set 1, each of them is interpreted as the independent consequence of two different phenomena of adaptation. If adaptation is due to resistant cells arose by physiological acclimation or specific and direct post-selective mutations, the variance in the number of resistant cells per culture can be found to be low (Fig. 1, set 1A). In this case every cell is likely to have the same chance of developing resistance, therefore a similar number of resistant cells can be found in each culture and variation (flask-to-flask) would not be consistent with the Poisson model (i.e. $(CV)_{set1}/(CV)_{set2} \approx 1$). Coefficient of variation (CV) is estimated as variance/mean. By contrast, if cells appeared by rare pre-selective mutations occurring before selection (random mutation), high variation in the interculture number of resistant cells in set 1 is found (i.e. $(CV)_{set1}/(CV)_{set2} >1$) and the interculture resistant cells variation would be consistent with the Poisson model. In this case, the mutations that allow the resistance occurred during the time in which the cultures reached Nt from N_0 cells, prior exposure to chloramphenicol (Fig. 1, set 1B). In the culture flask that spontaneous mutation happened sooner, we find high number of resistant cells. If mutation occurs later

the number of resistant cells is expected to be low. In the cases in which the rare spontaneous mutation does not occur, we do not find any resistant cells the culture flask.

The set 2 is founded with initial inoculums big enough to ensure that, if the resistance phenomenon is possible, in every culture flask there are the same chance that the resistance appears independently on the way resistant cells occurred. This is why the set 2 is the experimental control of the analysis of fluctuation (Fig. 1. Set 2). Thus, the set 2 samples the variance of the parental population and the experimental error. Because of that if resistant cells arose by pre-selective mutations, interculture variance of chloramphenicol-resistant cells in set 1 is expected to be high. Therefore the CV of the set 1 is expected to be higher than the CV for the set 2. If a similar CV between set 1 and set 2 is found, it confirms that resistant cells appeared by direct mutations or physiological acclimation, rather than by pre-selective mutations.

Moreover Luria and Delbrück procedures allows estimate the mutation rate from chloramphenicol-sensitive to chloramphenicol-resistant cells through fluctuation analysis. The proportion of cultures from set 1 showing no resistant cells after chloramphenicol exposure (P_0 estimator) was the parameter used to calculate the mutation rate (μ). The P_0 estimator (Luria & Delbrück, 1943) is defined as follows:

$$P_0 = e^{-\mu(Nt-N0)}$$

where P_0 is the proportion of cultures showing no resistant cells. Therefore, μ was calculated as:

$$\mu = -\text{Log}P_0/ (N_t - N_0) \qquad \text{(Luria \& Delbrück, 1943)}$$

Mutation-selection equilibrium

The mutation from wild-type chloramphenicol sensitive allele to a chloramphenicol resistant allele is recurrent. Furthermore, chloramphenicol resistant allele is detrimental in fitness in the absence of the antibiotic. Luria and Delbrück fluctuation analysis showed that new resistant mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection or by chance (Crow and Kimura, 1970; Spiess, 1989). There is an equilibrium between the resistant mutants which arise and the ones which disappear in a population in non selective conditions. The average number of such mutants will be determined by the balance between mutation rate and the rate of selective elimination, in accordance with the equation:

$$q = (\mu / s)^{1/2}, \text{ (Kimura \& Maruyama, 1966)}$$

where q is the frequency of the chloramphenicol resistant allele and s is the coefficient of selection, calculated as follows:

$$s = 1- (m_C^r / m_C^s), \text{ (Ayala and Kiger, 1980)}$$

where m_C^r and m_C^s are the Malthusian fitness of chloramphenicol-resistant and chloramphenicol-sensitive cells measured in non-selective conditions, respectively (Crow and Kimura, 1970).

Results

Chloramphenicol has a toxic effect in *Scenedesmus intermedius*. Growth rate and photosynthesis performance were severely reduced by 2.2 mg L^{-1} of chloramphenicol, and at 5.8 mg L^{-1} concentration growth rate and photosynthesis performance were totally inhibited (Table 1).

When analysis of fluctuation was carried out exposing wild-type cells to inhibitory concentration of chloramphenicol (5.8 μg ml^{-1}) of the growth and the photosynthesis performance, the cell density was reduced in each culture of sets 1 and 2 due to the effect of chloramphenicol. However, after further incubations for 60 days, the majority of cultures were able to grow again suggesting that rare chloramphenicol-resistant cells occur. A high fluctuation in the set 1 was found (from 0 to more than 10^7 resistant-cells per culture flask) (Table 2). By contrast, in Set 2 all the cultures contained chloramphenicol-resistant cells showed low fluctuation (all cultures have resistant cell in the order of magnitude of 10^7) (Table 2). Besides, the fact that $CV_{set1}/CV_{set2} = 13.26$ can be inferred as chloramphenicol-resistant cells arose by rare spontaneous mutation that occurs randomly prior to chloramphenicol exposure (since set 2 samples the parental variance).

The mutation rate (μ) estimated from sensitivity to resistance for chloramphenicol in *S. intermedius* was $1.01x10^{-5}$ mutants per cell division (Table 2). It was estimated by P_0 estimator.

Chloramphenicol-resistant mutants growing in absence of chloramphenicol showed a coefficient of selection of s = 0.33 in relation to chloramphenicol-sensitive cells. The frequency (q) of chloramphenicol-resistant alleles in non-polluted environment was estimated in 5.5 chloramphenicol-resistant mutants per 10^3 sensitive-cells by using the values of μ and s, due to the balance between mutation and selection.

Discussion

We exposed a population of *S. intermedius* to increasing doses of chloramphenicol. As it was expected, *S. intermedius* showed sensibility to chloramphenicol. In our experiment, fitness and photosynthesis performance of a wild-type strain of *S. intermedius* decreased with increasing concentrations of chloramphenicol. Concentration of 5.8 mg L^{-1} caused totally growth and photosynthesis performance inhibition.

When microalgal cultures were exposed to 5.8 mg L^{-1} of chloramphenicol, they became totally inhibited in growth and photosynthesis performance. After some days of incubation, the cell concentration decreased due to the effect of chloramphenicol. However, when they were further incubated, some cultures became green again due to the growth of a cell-variant resistant to

309 chloramphenicol. This suggests that phytoplankton populations could adapt to
310 chloramphenicol.

311 Some stressful environments sustain populations of algal species at the
312 extreme limits of their physiological tolerance (Fogg, 2001). Algae survive in
313 such hostile environments as a result of physiological acclimation by
314 modifications of gene expression (Belfiore and Anderson, 2001). Beyond
315 physiological limits, adaptive evolution depends on the occurrence of new
316 mutations that confer resistance (Belfiore and Anderson, 2001, Sniegowski &
317 Lenski, 1995).

318 Under a classic neo-Darwinist point of view, genetic variability maintained
319 in natural populations is considered as the pacemaker of adaptation in changing
320 environments. By contrast, evolutionary studies of bacterial populations suggest a
321 new hypothesis including adaptive mutation, which resembles Lamarckism
322 (Cairns et al., 1988; Foster, 2000 and reviewed by Roth et al. 2006). The
323 fluctuation analysis is an appropriate procedure to discriminate between resistant
324 cells arisen by spontaneous mutation that occur prior the exposure to
325 chloramphenicol and resistant cells arisen by direct and specific adaptation (Luria
326 and Delbrük, 1943; Cairns et al., 1988; Baos et al, 2002; García-Villada et al.
327 2002 and García-Villada et al., 2004). Our results suggest that chloramphenicol
328 resistance occurs due to rare spontaneous mutation prior exposure to 5.8 mg L^{-1} of
329 chloramphenicol.

330 The rare mutation from chloramphenicol-sensitivity to chloramphenicol-
331 resistance in *Scenedesmus intermedius* ($1.01x10^{-5}$ mutation per cell division) was
332 found one order of magnitude higher than mutation rates as we have previously

described for resistance to several biocides in microalgae such as formaldehyde (López-Rodas *et al*., 2007), erythromycin (López-Rodas *et al*., 2001), herbicide DCMU (Costas *et al*., 2001) or the explosive TNT (García-Villada *et al.*, 2002). We also described mutation rates for adaptation of microalgae to heavy metals. In this case, the estimated mutation rates range from 2.12×10^{-5} in the adaptation to heavy metals mixture from the Aznalcóllar mine spill (Baos *et al*., 2002) and 2.7×10^{-7} mutants per cell division in the resistance to resistance to cooper (García-Villada *et al*., 2004). The relative high mutation rate for resistance to chloramphenicol may be consequence of the existence of several mechanisms of resistance to chloramphenicol described: reduced membrane permeability (Burns et al., 1985; Nikaido, 1989), DNA ribosomal mutation (Blanc et al., 1981) and elaboration of chloramphenicol acetyltransferase (Cohen et al., 1980), and thus several mutations may conferrer resistance.

Cloramphenicol-resistant cells to 5.8 mg L^{-1}, exhibit diminished fitness in absence of chloramphenicol compared to *S. intermedius* wild-type in absence of chloramphenicol. In this sense, resistant cells are expected to be eliminated by natural selection (Crow & Kimura, 1970; Ayala & Kiger, 1984; Spiess, 1989). However, in each generation new mutants arise allowing a balance between mutation rate and selective elimination since mutation is recurrent. At the same time, there will be a certain number of mutant cells which will have not been eliminated yet. Thus, rare spontaneous mutation from chloramphenicol-sensitivity to chloramphenicol-resistance seems to be enough to assure survival of microalgae populations in chloramphenicol contaminated environments until 5.8 mg L^{-1} of chloramphenicol.

Acknowledgement

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499 **Tables and Figures**

500

501 **Table 1.** Inhibition of growth and photosynthetic performance (effective quantum
502 yield) of *Scenedesmus intermedius* wild-type by increasing doses of
503 chloramphenicol, calculated as percentage of untreated controls.

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Chloramphenicol concentration (mg L^{-1})	Growth rate inhibition (%) (mean ± SD)	Effective quantum yield inhibition (%) (mean ± SD)
0	0 ± 0	0 ± 0
1.3	42 ± 7	80 ± 3
2.2	89 ± 5	93 ± 4
3.6	100 ± 0	100 ± 0
5.8	100 ± 0	100 ± 0

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514 **Table 2.** Fluctuation analysis of wild-type *Scenedesmus intermedius* exposed to
515 growth and photosynthesis chloramphenicol inhibitory dose (5.8 mg L^{-1}).
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	set 1	set 2
N_o. of culture replicates	100	25
N_0 (N_o cells)	100	
N_t	3.1×10^5	
N_o of cultures containing the following no. of chloramphenicol resistant cells:		
0	4	0
10^5 - 10^6	3	0
10^6 - 10^7	16	0
10^7-10^8	77	25
CV_{set1}/CV_{set2}	13.26	
Mutation rate	1.01×10^{-5}	

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525 **Figure1**. Schematic diagram of modified Luria and Delbrück fluctuation analysis.
526 Full points represent chloramphenicol-sensitive cells and empty points represent
527 chloramphenicol-resistant cells. In the set 1, 100 cultures (each one inoculated
528 small inoculum, $N_0 \approx 10^2$ cells) were propagated in BG-11 medium until a high
529 cell density ($N_t \approx 10^5$ cells) was reached. Then, inhibition dose of chloramphenicol
530 was added. If resistant cells arose by physiological acclimation or post-adaptive
531 mutations the number of resistant cells in all the cultures must be similar (Set 1A).
532 If adaptation is by rare mutations occurring in the period of cultures propagation,
533 the difference amount the number of resistant cells in each culture must be
534 different (Set 1B). Set 2 samples the variance of parental populations as an
535 experimental control. In this case, the number of resistant cells in all the cultures
536 must be similar.
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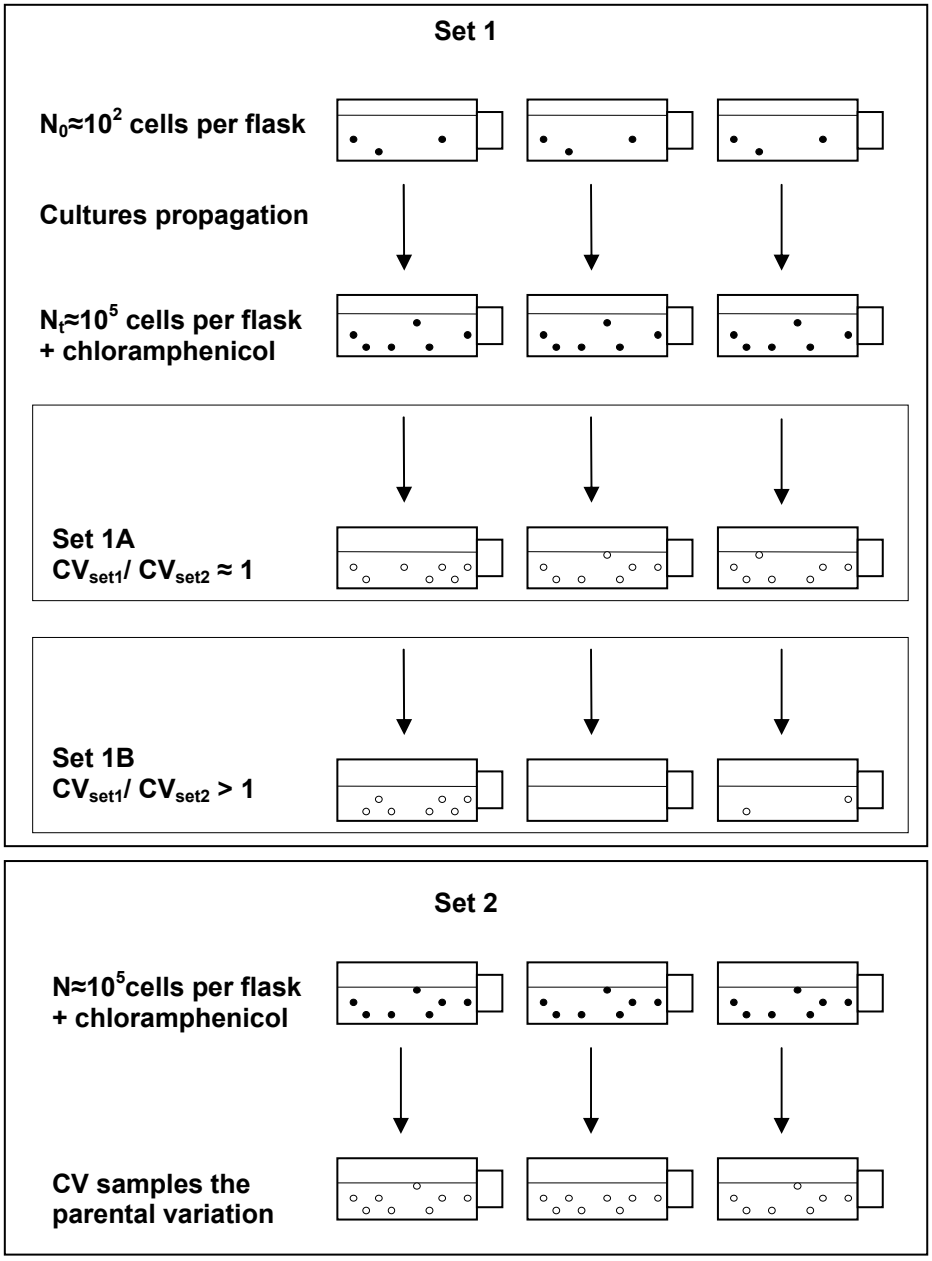

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545 **Figure 2**. Adaptation strategies of phytoplankton in herbicide-polluted waters.

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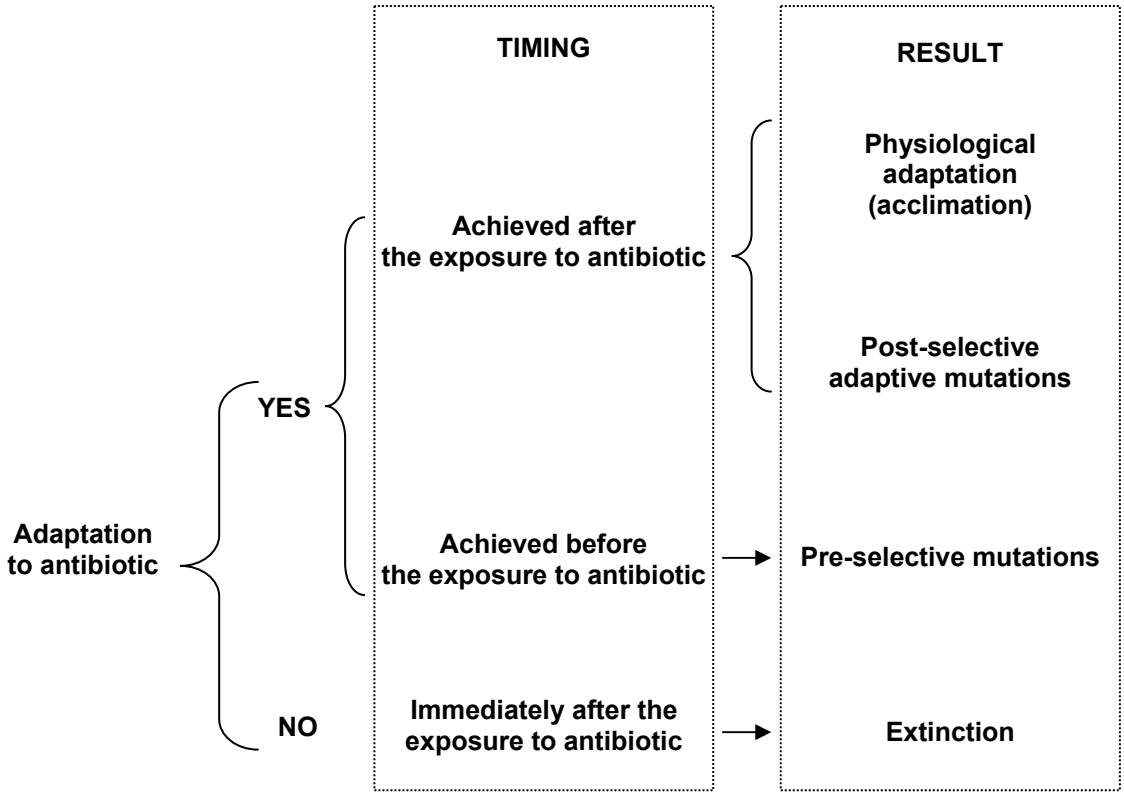

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CAPÍTULO II

¿QUÉ SUCEDE TRAS LA PRIMERA MUTACIÓN QUE PERMITE LA ADAPTACIÓN?

Introducción

Los trabajos experimentales presentados en el anterior capítulo demuestran que la mutación espontánea es una fuente variabilidad genética que permite que los microorganismos fotosintéticos del plancton se adapten a determinados cambios ambientales. Las mutaciones espontáneas son fuente de variabilidad genética y esta variabilidad es seleccionada por el ambiente siempre cambiante.

Sin embargo, para la escuela neodarwinista panadaptacionista todas las mutaciones con repercusión fenotípica están bajo la acción de la selección natural y tienen valor adaptativo (Ridley 1993; Gould 2002). Por otro lado, para la corriente neutralista el azar juega un papel muy importante en la evolución (Kimura 1983). En este caso por motivos estocásticos pueden establecerse en las poblaciones, a través de mutación, alelos que no confieren una ventaja adptativa, es decir, que sean neutros. Aunque estas teorías están basadas en poblaciones de individuos diploides de reproducción sexual, en los últimos años se ha comenzado a realizar estudios evolutivos con poblaciones haploides de reproducción asexual (Sniegowski and Lenski, 1995).

En este capítulo estudiamos los mecanismos evolutivos que tienen lugar tras la primera mutación que permite la adaptación de la clorofícea *Scenedesmus intermedius* al antibiótico cloranfenicol. Aplicamos el análisis de fluctuación y el diseño experimental realizado por Travisano y colaboradores en 1995 (Travisano et al., 1995) sobre un genotipo de *S. intermedius* resistente, para estudiar el papel que juegan las mutaciones en la evolución y determinar el peso que tiene la selección natural y la deriva genética (el azar) en la evolución de determinados caracteres.

Los resultados del análisis de fluctuación indican que *S. intermedius* es capaz de adaptarse a dosis letales de cloranfenicol gracias a mutaciones espontáneas que tuvieron lugar antes del contacto con el antibiótico (mutaciones preadaptativas). Estas células resistentes muestran una tasa de división reducida en presencia de la dosis letal de cloranfenicol. Además, mayores concentraciones de cloranfenicol consiguen inhibir por completo la capacidad de división y el rendimiento fotosintético de los mutantes resistentes. Un nuevo análisis de fluctuación sugirió la incapacidad de las células resistentes para adaptarse a la nueva concentración limitante de cloranfenicol.

En este punto desarrollamos dos experimentos independientes de evolución con células resistentes en medio suplementado con cloranfenicol. Por un lado se desarrolla el diseño de Travisano y colaboradores incubando durante aproximadamente un año bajo idénticas condiciones nueve réplicas de células resistentes al cloranfenicol procedentes de un único genotipo resistente. Periódicamente se determinó el valor medio y la varianza entre las réplicas de los caracteres tasa de crecimiento y rendimiento cuántico de la fotosíntesis. La comparación entre el valor medio de los parámetros, al inicio y al final del experimento, indica que las células resistentes evolucionan en presencia de cloranfenicol, aumentando significativamente la tasas de crecimiento y el rendimiento fotosintético. Las diferencias entre la varianza inicial y final sugieren que la evolución de rasgos ligados a la eficacia biológica, como es el caso de la tasa de crecimiento, están bajo acción de la selección natural, mientras que en la evolución de la eficiencia fotosintética el azar también juega un papel importante.

El segundo experimento de evolución consistió en aumentar gradualmente la concentración de cloranfenicol a la que se expusieron cultivos de microalgas resistentes, durante aproximadamente un año, hasta alcanzar la nueva dosis letal. Al finalizar ambos experimentos de evolución, las células resistentes fueron capaces de adaptarse a la nueva concentración letal, indicando que durante el periodo de evolución, el fenómeno de mutación fue responsable del aumento de la variabilidad genética, y la presión ambiental del cloranfenicol de seleccionar los genotipos más eficientes en este medio.

4.2.1. Experimental models to analyze adaptation of algae to anthropogenic contamination: 2. Adaptive change by mutation selection increase fitness in antibiotic-resistant green microalgae.

Experimental models to analyze adaptation of algae to anthropogenic contamination: 2. Adaptive change by mutation-selection increase fitness in antibiotic-resistant green microalgae.

Fernando Marvá[1] et al.

[1]*Genética (Producción Animal), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain and*

Keywords: Chloramphenicol, *Scenedesmus intermedius*, adaptation, natural selection, chance.

Abstract

Since phytoplankton are the principal primary producers of aquatic ecosystems their tolerance to water pollution by anthropogenic substances is relevant from an ecological point of view. We have investigated the effects of long-term selection pressure on fitness and photosynthesis performance in chloramphenicol-resistant cells, under i) constant chloramphenicol concentration and ii) increased chloramphenicol concentrations for 360 days. In the derivate population of chloramphenicol-resistant cells the mean value for fitness showed a significant increase subjected to strong selection whereas photosynthesis performance also evolved under chance influence. A fluctuation analysis demonstrated that chloramphenicol-resistant cells to 5.8 mg L^{-1} could not adapt to 40 mg L^{-1} through one-step adaptation. However the adaptation to 40 mg L^{-1} was possible after both long-term evolution experiments due to mutation-selection under chloramphenicol selective pressure.

Introduction

Water wastes of anthropogenic contaminants are producing environmental catastrophes in inland ecosystems (Ehrlich, 2001). Since phytoplankton are the principal primary producers of aquatic ecosystems the ability of adaptation to water pollutants a relevant fact from an ecological point of view (Falkowski and Raven, 1997). Recent researches suggest that phytoplankton are able to adapt to lethal doses of common water pollutants though pre-selective mutations (Costas et al., 2001; Garcia-Villada et al., 2004; Lopez-Rodas et al, 2007) but it is unknown how phytoplankton resistant-cells evolved in polluted environments and how it resistant cells face to high pollution concentrations.

Experiments were conducted in order to study the evolution of cells of the chlorophiceae *Scenedesmus intermedius* resistant to the broad-spectrum antibiotic chloramphenicol in medium supplemented with this antibiotic. The chloramphenicol-resistant cells were isolated from a previous fluctuation analysis carried out to determinate if resistant cell arise by spontaneous pre-adaptive mutation or by specific adaptation (prost-adaptive mutation or physiological acclimation) (Luria and Delbrück, 1943; López-Rodas et al. 2001)

When chloramphenicol-resistant cells were isolated from the fluctuation analysis (resistant to 5.8 mg L^{-1}) and treated with 40 mg L^{-1} of chloramphenicol fitness and photosynthesis performance were totally inhibited. In order to determine the ability of adaptation of chloramphenicol-resistant cells to this new inhibitory dose a fluctuation analysis was carried out exposing these chloramphenicol-resistant cells to 40 mg L^{-1} of chloramphenicol. This procedure

showed the impossibility of one-step adaptation neither by physiological acclimation nor by mutation. However, the same chloramphenicol-resistant cells population was able to adapt to the inhibitory chloramphenicol concentration through two different ways: i) keeping chloramphenicol-resistant cells in presence of 5.8 mg L^{-1} chloramphenicol for 360 days and ii) through multi-step adaptation increasing the concentration of chloramphenicol every 120 days for 360 days.

The Neo-Darwinist evolutionary hypothesis based in the natural selection of best adapted organisms in populations is not enough to explain all the kinds of evolution changes (Kimura, 1983). Genetic drift events and random mutations were proposed as the usually effects of chance, factor that also contribute to evolution (Kimura, 1983; Suzuki et al. 1989). Gould (1989) proposed a theoretical experiment called "Replaying life tape" to discriminate the effects of the adaptation, chance and history in evolutionary change. This theoretical experiment was carried out by Travisano through a robust empirically long-term experiment of evolution (Travisano et al., 1995). The experiment consisted in the simultaneously propagation of independent replicated of bacterial populations from a single ancestral genotype under a novel environment. The initial mean value of a specific trait was measured in each replicate. At the beginning of the experiment grand mean (M_0) and grand variance (V_0) were calculated. Values were expected to be identical within statistical limits of measurement error. After a period of time (t) the mean value was measured again for the trait in each replicate, and the grand mean (M_t) and the grand variance (V_t) were calculated again. Differences between the initial and final values are explained as a result of the effect of adaptation, chance, or both of them.

95 In this study we have adapted the model of Travisano el al. (1995) to study
96 the evolution of fitness and photosynthesis performance in chloramphenicol-
97 resistant cells after pre-adaptive mutation that allows adaptation to polluted
98 environment. Replicates form a single chloramphenicol-resistant cell genotype
99 randomly isolated from the fluctuation analysis was propagated under identical
100 new conditions (BG-11 medium supplemented with 5.8 mg L^{-1} of
101 chloramphenicol).

102 This study is also a complement to other works carried out in a framework
103 focused on understanding phytoplankton adaptation to grow in a contaminated
104 environment with anthropogenic water pollutants (López-rodas et al. 2001; Costas
105 et al. 2001; García-Villada et al. 2002; Garcia-Villada et al. 2004; López-Rodas et
106 al. 2006; Lopez-Rodas et al. 2007).

107 The main aims of this work were i) study the adaptation ability of
108 chloramphnicol-resistant to cells 5.8mg L^{-1} to higher inhibitory doses of
109 chloramphenicol. ii) Evaluate the roles of adaptation and chance in evolution of
110 fitness and photosynthetic performance in chloramphenicol polluted environment
111 and iii) study the evolution of the same traits of chloramphenicol-resistant cells
112 under increasing doses of chloramphenicol.

113

114 **Materials and methods**

115

116 *Experimental organism*

117

The experiments where conducted using a chloramphenicol-resistant *Scenedesmus intermedius* (chloroficeae) to 5.8 mg L^{-1} of chloramphenicol. It was isolated from a fluctuation analysis. Prior the experiments the cultures were re-cloned by isolating a single cell in order to decrease genetic variability.

Culture conditions, *Measure of chloramphenicol toxicity* and *Fluctuation analysis* are described in the article *Experimental models to analyze adaptation of algae to anthropogenic contamination: 1. Pre-selective mutation confers antibiotic resistance in green microalgae.*

Long-term evolution experiment of chloramphenicol-resistant cells in constant concentration of chloramphenicol

An adaptation of the Travisano et al. (1995) evolution experiment was done. Nine identical populations (replicates) propagated from one single chloramphenicol-resistant cell were grown under identical environments in presence of 5.8 mg L^{-1} of chloramphenicol for 360 days. Cultures were maintained in mid-log exponential growth by serial transfers of a cell inoculum to fresh medium (Cooper, 1991). The chloramphenicol-resistant genotype was isolated from the set 1 of the analysis of fluctuation (described in the article *Experimental models to analyze adaptation of algae to anthropogenic contamination: 1. Pre-selective mutation confers antibiotic resistance in green microalgae*) All the replicates were grown in the same culture conditions described in *Culture conditions,* except for the growth medium. It was supplemented with of 5.8 mg L^{-1} of

chloramphenicol. We reported the dynamics of two traits of the evolving algae populations: fitness and photosynthesis performance. Grand mean (*M*) and grand variance (*V*) of the two traits measured for the nine replicates were calculated at the starting point ($t = 0$) and every 120 days until the end ($t = 360$ days) of the experiment.

The effects of adaptation were defined by testing the significance change in grand mean value for a trait between ancestral (M_0) and the derived population (M_t) using Mann-Whitney nonparametric *U*-test (95% confidence limits were calculated). *F*-test was applied to demonstrate chance in evolved populations by testing the significance change in grand variance between ancestral population (V_0) and the derived one (V_t) (see Figure 1).

At the end of the experiment the evolved population was exposed to 40 mg L^{-1} of chloramphenicol and after a period of acclimation of 7 days, fitness and photosynthesis performance were measured and the mean value for each trait was calculated.

Long-term evolution experiment of chloramphenicol-resistant cells in increasing concentration of chloramphenicol. Multi-step adaptation.

Three replicates chloramphenicol-resistant *S. intermedius* were exposed to increasing doses of chloramphenicol in a multi-step adaptation experiment. The doses were 5.8 mg L^{-1}, 15.2 mg L^{-1}, 24.5 mg L^{-1} and 40 mg L^{-1}. Chloramphenicol resistant cells were exposed progressively to each concentration for 120 days, until the end of the experiment ($t = 360$ days). The mean value for fitness and

166 photosynthesis performance were estimated at the beginning and at the end of
167 each concentration exposure. The chloramphenicol-resistant cells were from the
168 same parental population than long-term evolution experiment in constant
169 concentration.
170
171 **Results**
172
173 When chloramphenicol-resistant cells (mutants resitant to 5.8 mg L^{-1}) were
174 exposed to higher doses of chloramphenicol, fitness and photosynthesis
175 performance were totally inhibited at 40 mg L^{-1} (Table 1). A fluctuation analysis
176 was carried out exposing chloramphenicol-resistant cells of *S. intermedius* to
177 fitness and photosynthesis performance inhibitory doses of chloramphenicol (40
178 mg L^{-1}). In this case, no one *S. intermedius* resistant cell was able to grow in 40
179 mg L^{-1} of chloramphenicol. It suggests the impossibility of one-step adaptation to
180 this concentration either by rare spontaneous mutation or specific adaptation in
181 response to the antibiotic exposure (Table 2). Both fluctuation analysis sets 1 and
182 2 were incubated for a year and periodically observed in order to confirm the
183 impossibility of one-step adaptation.
184 However, chloramphenicol-resistant cells were able to grow in presence of
185 40 mg L^{-1} through both long-term evolution experiments in constant and
186 increasing concentration of antibiotic.
187 In the multi-step adaptation experiment (that consist in increasing the
188 concentration of chloramphenicol every 120 days for 360 days), both fitness and
189 photosynthesis performance were increasing in each step of chloramphenicol

concentration (Figure 4 and Figure 5, respectively). Finally, chloramphenicol-resistant cells were able to growth in 40 mg L^{-1} with a Malthusian value of fitness of 0.611 ± 0.075 doublings per day (mean ± SD) (Figure 4) and a percentage of Φ_{PSII} of inhibition of 47 ± 2.3 (Figure 5).

At the same time long-term evolution experiment in constant concentration was conducted. Nine replicates of chloramphenicol-resistant cells were maintained in presence of 5.8 mg L^{-1} of chloramphenicol for 360 days. Ancestral (time t = 0) and derived (time t = 360) values for the mean of both fitness and percentage of Φ_{PSII} inhibition of the nine replicates are summarized in Table 3. The evolution of the fitness showed a statistically significant increase in the grand mean ($M_0 < M_t$) (Table 3). *F*-test indicates that the grand variance did not significantly increase between days 0 and 360 (Table 3). As far as the evolution of Φ_{PSII} inhibition is concerned, grand mean significantly decreased (Table 3); that is, the grand mean of derived populations decreased significantly from the value of the intercessor ($M_0 > M_t$). *F*-test for the evolution of percentage of Φ_{PSII} inhibition indicated that the grand variance did significantly increase between ancestral and derived values. At the end of this long-term evolution experiment (t = 360 days), every replicate were also able to adapt to 40 μg ml^{-1} of chloramphenicol with a grand mean of Malthusian value of fitness of 0.297 ± 0.069 doublings per day and a percentage of Φ_{PSII} of inhibition of 70.9 ± 9.9. Both traits were measured in presence of 40 mg L^{-1} of chloramphenicol.

Discussion

When we exposed a population of chloramphenicol-resistant cells of *S. intermedius* to increasing doses of chloramphenicol fitness and photosynthesis performance were severely diminished. 40 mg L^{-1} of chloramphenicol caused totally inhibition of the growth and the photosynthesis performance, and further incubations showed destruction of microalgal cultures.

The fluctuation analysis proposed by Luria and Delbrück (Luria and Delbrük, 1943) is an appropriate procedure to discriminate between resistant cells arisen by direct and specific adaptation and resistant cells arisen by spontaneous mutation that occur prior the exposure to chloramphenicol (Cairns et al., 1988; Baos et al, 2002; García-Villada et al., 2004). No one chloramphenicol-resistant cell was able to grow in the fluctuation analysis carried out exposing chloramphenicol-resistant cells to 40 μg ml^{-1} of chloramphenicol. This result suggests that neither physiological acclimation nor mutation allows direct one-step adaptation to 40 mg L^{-1} of chloramphenicol.

However, chloramphenicol-resistant cells were able to adapt to 40 μg ml^{-1} at the end of two different experiments of evolution carried out for 360 days: i) through exposing chloramphenicol-resistant cells to progressive increasing of chloramphenicol concentration (multi-step adaptation), showing a final growth rate of 0.611 ± 0.075 doublings per day (mean ± SD) and ii) through keeping chloramphenicol-resistant cells populations under 5.8 mg L^{-1} of chloramphenicol selective pressure for 360 days. In this case the final growth rate was 0.297 ± 0.069 doublings per day (mean ± SD). In these two evolution experiments the increasing of genetic variability and selection of positive mutations for fitness under chloramphenicol selective pressure must have happened, since adaptive

evolution depends on new mutations (Belfiore and Anderson, 2001, Sniegowski & Lenski, 1995). The fact that non-possibility of direct adaptation of chloramphenicol-resistant cells (to 5.8 ml L^{-1}) to high fitness and photosynthesis inhibitory dose though one-step adaptation (tested by the second fluctuation analysis done) confirms the increasing of genetic variability by mutation in the long-term evolution experiments. Fitness of chloramphenicol-resistant cells exposed to 40 mg L^{-1} of chloramphenicol at the end of multi-step experiment was higher than fitness of resistant cells from constant chloramphenicol exposure experiment (also measured exposing chloramphenicol-resistant cells to 40 ml L^{-1} of chloramphenicol). Thus, increasing the doses of chloramphenicol the evolutionary pressure was higher than keeping the cells in the same concentration, and the most efficient genotypes to grow in chloramphenicol were positive selected.

According to the model developed by Travisano *et al*. (1995), we propagated nine replicates of chloramphenicol-resistant cells (resistant to 5.8 mg L^{-1}) under identical culture condition (5.8 mg L^{-1} of chloramphenicol) for 360 days in order to estimate the roles of adaptation and chance in evolution of two different traits. Considering that *S. intermedius* has not sexual recombination, the only two sources of genetic variability are the exploitation of pre-existing genetic variability (it is expected to be low due to cultures were obtain for a single resistant-cell) and genetic mutation. After 360 days of serial propagation cultures under 5.8 mg L^{-1} of chloramphenicol, the fitness of the derived population was significantly greater than the one exhibited by the ancestral ones. On the other hand, *F*-test shows that variance did not significantly change between ancestral

and derived populations. Keeping chloramphenicol-resistant cells under 5.8 mg L^{-1} of chloramphenicol for 360 days, positive selection for increased fitness was induced, since fitness scores of the nine replicates converged at the end of the experiment, indicating the almost null contribution of chance. Previous studies using bacteria (travisano et al. 1995; Korona, 1996) and viruses (Cuevas et al. 2002) have also reported fitness convergence in propagation of replicates under identical novel environmental conditions.

By contrast, the decreased of the percentage of Φ_{PSII} inhibition in the derived population was due to both adaptation and chance. *U*-test showed a significantly decrease of the mean value. *F*-test suggests that the derivate variance was significantly higher than the ancestral one. Thus, the photosynthesis performance is not a trait subject to strong selection, as fitness is.

Acknowledgements

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381 **Tables and Figures**

382

383 **Table 1.** Inhibition of growth and photosynthetic performance (effective quantum
384 yield) of chloramphenicol-resistant *Scenedesmus intermedius* to 5.8 mg L^{-1} by
385 increasing doses of chloramphenicol, calculated as percentage of untreated
386 controls.

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Chloramphenicol concentration (mg L^{-1})	Growth rate inhibition (%) (mean ± SD)	Effective quantum yield inhibition (%) (mean ± SD)
5.8	0 ± 0	72 ± 2
9.8	6 ± 7	82 ± 4
15.2	47 ± 4	91 ± 2
24.5	63 ± 4	100 ± 0
40	100 ± 0	100 ± 0

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397 **Table 2.** Fluctuation analysis of chloramphenicol-resistant cells of *Scenedesmus*
398 *intermedius* exposed to growth and photosynthesis inhibitory dose (40 mg L^{-1}).
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	set 1	set 2
N_o. of culture replicates	100	25
N_0 (N_o cells)	100	
N_t	$3{,}1 \times 10^5$	
N_o. of cultures containing the following no. of chloramphenicol resistant cells:		
0	100	25

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414 **Table 3**. Mann-Whitney *U*-test and *F*-test for statistical differences in mean and
415 variance respectively, for fitness and % Φ_{PSII} inhibition estimated in 9 replicates
416 of chloramphenicol-resistant of *S. intermedius* in 5.8 mg L^{-1} of chloramphenicol
417 for t = 360 days.
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	Mean (SD)		*U*-test		*F*-test	
Traits	Time 0	Time t	*U*	*P*	*F*	*P*
Fitness	0.271 (0.027)	0.739 (0.063)	0.000	<0.0001	5.27	0,0302
% Φ_{PSII} inhibition	72.175 (1.862)	25.75 (20.599)	0.000	<0.0001	122.3	<0.0001

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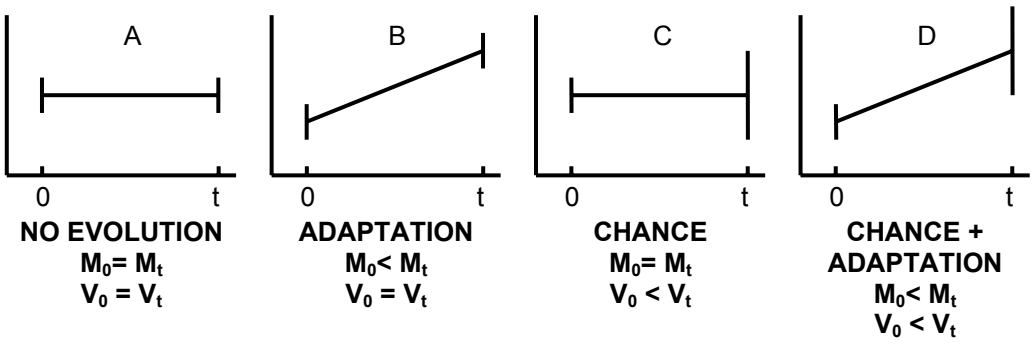
Figure 1. Schematic representation of effects due to adaptation, chance and chance plus adaptation. The difference between the mean (M) and the variance (V) of a specific trait measured at the beginning (0) and at the end (t) of the long-term evolution experiment are interfered as a consequence of the influence of adaptation, chance or both of them in the evolution the trait.



448 **Figure 2.** Grand mean and SD of fitness measured in 9 replicates of
449 chloramphenicol-resistant cells evolving in presence of 5.8 mg L^{-1} of
450 chloramphenicol for 360 days.
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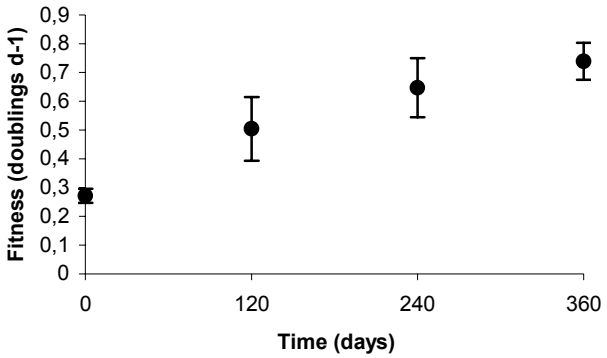

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464 **Figure 3.** Grand mean and SD of $\Phi_{(PSII)}$ inhibition measured in 9 replicates of
465 chloramphenicol-resistant cells evolving in presence of 5.8 mg L^{-1} of
466 chloramphenicol for 360 days.
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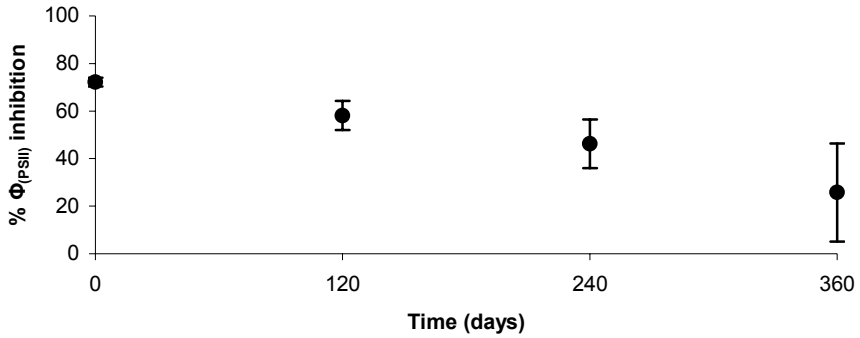

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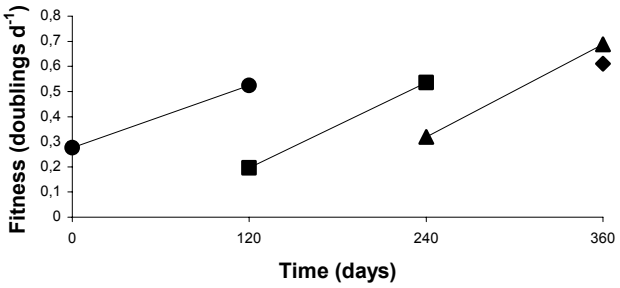
Figure 4. Grand mean of fitness measured in three replicates of chloramphenicol-resistant cell exposed to increasing doses of chloramphenicol in a multi-step adaptation experiment. Cultures were exposed to 5.8 mg L^{-1} (●), 15.2 mg L^{-1} (■), 24.5 mg L^{-1} (▲) and 40 mg L^{-1} (♦). Mean fitness was calculated at the beginning and the end of each dose exposition.



501 **Figure 5.** Grand mean of percentage of $\Phi_{(PSII)}$ inhibition measured in three
502 replicates of chloramphenicol-resistant cultures exposed to increasing doses of
503 chloramphenicol in a multi-step adaptation experiment. Cultures were exposed to
504 5.8 mg L^{-1} (●), 15.2 mg L^{-1} (■), 24.5 mg L^{-1} (▲) and 40 mg L^{-1} (♦). Mean was
505 calculated at the beginning and the end of each dose exposition.
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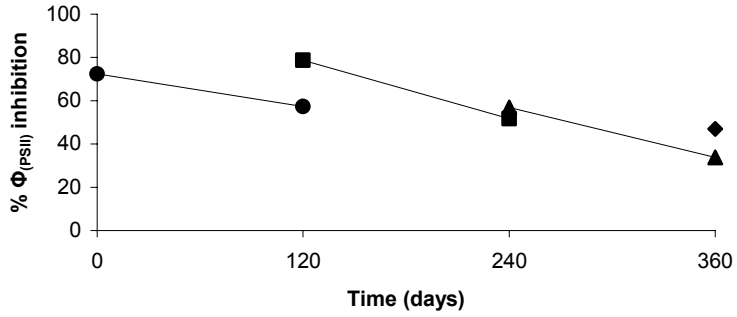

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CAPITULO III

ADAPTACIÓN DE LOS MICROORGANISMOS FOTOSINTÉTICOS DEL PLANCTON A AMBIENTES NATURALES EXTREMOS

Introducción

La capacidad de supervivencia y proliferación de microalgas en medios naturales extremos (hábitats caracterizados por valores extremos de salinidad, temperatura, pH, o deficiencias o excesos de minerales) supone un interesante campo de investigación tanto desde un punto de vista bioquímico como fisiológico. Dado que estos microorganismos son los principales productores primarios en medios acuáticos, su tolerancia a situaciones ambientales de estrés es también muy importante desde el punto de vista ecológico. Curiosamente la mayoría de los taxones fitoplanctónicos descritos en medios naturales extremos pertenecen a géneros habitualmente mesófilos (Amaral Zettler 2002). Este hecho hace de los medios naturales extremos un buen laboratorio natural para el estudio de la adaptación.

En el primer trabajo abordamos la posibilidad de adaptación de clorofíceas (eucariotas) y cianobacterias (procariotas) a aguas ácidas y sulfurosas procedentes de una charca de la isla de Vulcano (Islas Eolias, Italia), en la que identificamos *in situ* cuatro especies de algas eucariotas perfectamente adaptadas. Entre 1888 y 1890, tuvo lugar la última erupción volcánica en la isla que depositó 5 metros de material piroclástico eliminando todas las charcas de la isla y consecuentemente el fitoplancton (Cortese et al. 1986). Por tanto, dada la baja probabilidad de que estas cuatro especies fueran introducidas en la isla por algún vector procedente de condiciones naturales extremas parecidas, parece razonable pensar que la adaptación de microorganismos mesófilos a estas aguas ácidas tuvo lugar a lo largo de los últimos 100 años. Los resultados obtenidos exponiendo clorofíceas y cianobacterias mesófilas al agua ácida y sulfurosa de Vulcano parecen indicar que ciertas mutaciones preadaptativas son capaces de conferir resistencia en

la clorofícea eucariota *Dictyosphaerium chlorelloides*. Sin embargo, la cianobacteria procariota *M. aeruginosa* no se pudo adaptar a este medio hostil.

En el segundo y tercer trabajo de este capítulo investigamos la capacidad de adaptación de clorofíceas y cianobacterias a medios ácidos con elevadas concentraciones de metales pesados. Los lugares escogidos fueron el arroyo de Aguas Agrias (Tharsis, Huelva, España), y una antigua explotación minera en Mynyyd Parys (Norte de Gales, Gran Bretaña). En ambos casos, al igual que en los trabajos de adaptación realizados en el Río Tinto (Costas et al. 2007) y en las aguas sulfurosas de La Hedionda (Flores-Moya et al. 2005) los resultados indican que la adaptación tuvo lugar gracias a mutaciones espontáneas preadaptativas. Este último trabajo se ha considerado una aportación relevante a la biología evolutiva, ya que constituye una prueba empírica de evolución adaptativa (Sniegowski, 2005). Al igual que en las aguas de Vulcano, las cianobacterias no fueron capaces de adaptarse a estos medios ácidos.

4.3.1. Living in vulcan's forge: Algal adaptation to stressful geothermal ponds on Vulcano Island as result of preselective mutations.

Living in Vulcan's forge: algal adaptation to stressful geothermal ponds on Vulcano Island (S Italy) as result of pre-selective mutations

Victoria López-Rodas[1], Eduardo Costas[1], Emilia Maneiro[1], Fernando Marvá[1], Mónica Rouco[1], Antonio Delgado[2] and Antonio Flores-Moya[3*]

[1]Departamento de Producción Animal (Genética), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain, [2]Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, C/ Profesor Albareda 1, E-18008 Granada, Spain and [3]Departamento de Biología Vegetal (Botánica), Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain

**To whom correspondence should be addressed.*

Email: floresa@uma.es

SHORT RUNNING TITLE: Algal adaptation to Vulcano ponds

SUMMARY

Four species of eukaryotic algae proliferate in the sulphureous, acidic (pH 3.1) water of the largest geothermal pond on Vulcano Island (S Italy). Consequently, this pond constitutes a 'natural laboratory' for analysis of adaptation by phytoplankters to extremely stressful conditions. To distinguish between the pre-selective or post-selective origin of adaptation processes allowing the existence of phytoplankters in the pond, a Luria-Delbrück fluctuation test was performed with the chlorophycean *Dictyosphaerium chlorelloides* and the cyanobacterium *Microcystis aeruginosa*, both isolated from non-extreme waters; natural water from the Vulcano Island pond was used as selective factor. Preselective, resistant *D. chlorelloides* cells appeared with a frequency of 4.7×10^{-7} per cell per generation. We propose that the microalgae inhabiting this stressful pond could be the descendents of chance mutants that arrived in the past or are even arriving at the present. The genetic adaptation of *D. chlorelloides* to Vulcano waters could help to explain the survival of photosynthesizers in very stressful geothermal waters during the Neoproterozoic 'snowball Earth', a period when primary production collapsed in the biosphere. On the other hand, adaptation to these conditions was not observed in *M. aeruginosa*, suggesting that cyanobacteria may not be to develop any kind of adaptation to Vulcano pond water.

Key words: adaptive evolution, cyanobacteria, fluctuation analysis, microalgae, 'snowball Earth', Vulcano Island.

INTRODUCTION

Different aspects of the interactions of extreme environmental conditions from geothermal waters and the microorganisms that inhabits these environments have been previously addressed (Brock 1978; Setter 1996; Madigan & Narrs 1997; Horikoshi & Grant 1998; Elster *et al.* 2001; Rothschild & Mancinelli 2001; Newman & Banfield 2002; Seckbach 2007). However, in the case of mesophilic microalgae and cyanobacteria inhabiting geothermal ponds, a question yet remains open: how is achieved the adaptation to survive and proliferate under stressful conditions?

If the stressful conditions do not exceed the limits of the physiological tolerance of algae, the survival is the result of physiological adaptation (i.e. acclimation) supported by modifications of gene expression (Bradshaw & Hardwick 1989). On the other hand, in extreme environments characterized by values of ecological factors exceeding the physiological limits of algae, survival depends exclusively on adaptive evolution, which results from the occurrence of new mutations that confer resistance (Sniegowski & Lenski 1995; Flores-Moya *et al.* 2005; Sniegowski 2005; Costas *et al.* 2007; López-Rodas *et al.* 2008). However, recent evolutionary studies of bacteria have suggested that hypothetical adaptive mutations could be a process resembling Lamarckism which, in the absence of lethal selection, produces mutations that relieve selective pressure (Cairns *et al.* 1988; Foster 2000). Examples of adaptive mutations or related phenomena have also been recognized in yeast (Heidenreich 2007) but, as far as we know, they have not been reported in other microorganisms such as cyanobacteria and microalgae. Therefore, the key to resolving this debate is to know the pre-selective or post-selective origin of new mutations. Surprisingly, there are almost no studies of

64 the origin of favoured mutants and their survival beyond physiological limits in diploid,
65 multi-celled organisms living in well-defined populations. In contrast, cyanobacteria
66 and many lineages of microalgae are haploid, single-celled and asexual, and their
67 populations are composed of countless cells (Margulis & Schwartz 1982).
68 Consequently, these organisms are adequate to study the process of adaptation based on
69 favoured mutations.

70 Vulcano Island (Aeolian Islands, S Italy) provides an excellent model to study
71 adaptation of phytoplankters to stressful geothermal ponds that are lethal for
72 mesophilic lineages. It contains a main volcano (named Vulcano) and several
73 overlapping minor volcanic centres (Keller 1980). Vulcano is in a sense the most
74 famous volcano in the world: Vulcan, the Roman god of fire and metalworking,
75 responsible for making the weapons of the gods, made his home inside the volcano. The
76 main cone originated sometime after 11,000-8,500 years ago (Frazzeta *et al.* 1984). At
77 least four volcanic cycles contributed to the formation of Vulcano Island (Cortese *et al.*
78 1986). In addition, the last eruption of Vulcano (from 1888 to 1890) deposited 5 m of
79 pyroclastic material (Cortese *et al.* 1986) and indubitably killed all the phytoplankton of
80 the ponds on the island.

81 The aim of this work was to evaluate, from an evolutionary point of view,
82 adaptation of cyanobacteria and microalgae, to growth and survival in the stressful
83 environment of a pond on Vulcano Island. For this purpose, we first performed a survey
84 of phytoplankton inhabiting Vulcano pond water (VPW; the largest pond in the island
85 was selected). Because we found few algal species closely related to mesophilic ones,
86 we then performed a fluctuation analysis (Luria & Delbrück 1943) on a mesophilic
87 chlorophycean using VPW as selective agent; we also tested the possible adaptation to

VPW by a cyanobacterium. The fluctuation analysis is a statistical and experimental procedure which allows us to distinguish between cells that become resistant from acquired specific adaptation (including both physiological adaptation or acclimation, and possible adaptive mutations; the first case is not an evolutionary event) and resistant cells arising from spontaneous mutations that occur randomly during propagation prior to exposure to the selective agent. Here we show that cells from the mesophilic chlorophycean *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman rapidly adapt to VPW via the action of natural selection on resistant mutants that appear spontaneously in wild-type populations. Thus, the presence of microalgae in the stressful environment of the largest pond on Vulcano Island could be explained in accordance with the neo-Darwinian paradigm: natural selection selects resistant mutants that are previously present in natural populations inhabiting non-stressful environments. In contrast, the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann was unable to adapt to VPW, which is in agreement with the absence of cyanobacteria in acidic environments.

MATERIALS AND METHODS

Environmental conditions and phytoplankton community in the Isthmus pond of Vulcano Island

Sampling of water and phytoplankton was carried out in July 2006; for this purpose, the largest pond on the island (around 70 m^2 surface $\times$ 0.15 m deep) located in the periphery of Vulcano Island (named Isthmus pond; 08º25’N, 014º57’E) was selected. The values

of pH and temperature in the pond were determined by using a YSI 6820-C-M probe (Yellow Springs, OH, USA). In order to determine sulphide levels, four drops of $Zn(CH_3CO_2)_2$ 2 N were added to a 100 mL water sample and the sample bottle was immediately sealed, excluding any air. The sample was stored at 4 ºC in darkness until sulphide determination by titration with $Na_2S_2O_3$ 0.1 N, in accordance with APHA-AWWA-WPCF (1992). In addition, five 1 L VPW samples were collected and homogeneously mixed. The resulting integrated water sample was filtered (0.22 μm, Stericup, Millipore Co., Billerica, MA, USA) and kept in a closed bottle excluding any air, stored at 4 ºC in darkness until the laboratory experiments (toxicity tests and fluctuation analysis) were performed.

Phytoplankton was identified in fresh samples (directly after collection) using a McArthur portable microscope (Kirk Technology, England), and counted on fixed samples (4% formalin) in settling chambers using an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany).

Experimental organisms and culture conditions

A wild-type strain of the chlorophycean *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman was isolated from a pristine, cool-temperate (5-10 ºC), non-sulphureous, slightly alkaline (pH 7.8-8.0) high mountain lake from Sierra Nevada (S Spain); a strain of the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann was isolated from a pristine pond of non-acidic waters (pH 8.1) in Doñana National Park (SW Spain). Both strains were grown in 100 mL cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL BG-11 medium (Sigma-Aldrich Chemie,

Taufkirchen, Germany), at 22 °C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400-700 nm. Although *D. chlorelloides* forms 2- or 4-celled, rarely 16-celled colonies, and is capable of sexual reproduction in the nature (John & Tsarenko 2002), the strain was exclusively propagated by asexual reproduction, and it was represented by single-celled individuals. Cultures were axenically maintained in mid-log exponential growth (Cooper 1991) by serial transfers of subcultures to fresh medium, and only cultures without detectable bacteria were used in the experiments. Prior to the experiments, the cultures were cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the culture.

Toxicity test: effect of VPW on Malthusian fitness

In order to test the toxic effect of the VPW, we measured the changes in Malthusian fitness (*m*) when the wild-type strains of *D. chlorelloides* and *M. aeruginosa* were cultured in this selective agent. Culture samples (5.0×10^5 cells) from a mid-log exponentially growing culture of both species were placed in experimental tubes containing 1.5 mL of VPW at 30 ºC. Controls in BG-11 medium at 22 ºC were also prepared. The value of *m* was calculated in three replicates of each species in VPW as well as in three BG-11 medium controls, using the equation from Crow & Kimura (1970):

$m = \mathrm{Log_e}\,(N_t/N_0)/\,t,$ (Eqn. 1)

where t = 3 d, and N_0 and N_t are the cell numbers at the start and at the end of the experiment, respectively. Experiments and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer. The number of samples in each case was determined using the progressive mean procedure (Williams 1977), which assured a counting error <5%.

Fluctuation analysis of VPW-sensitivity →VPW-resistance transformation

A fluctuation analysis (Luria & Delbrück 1943) was carried out to discriminate between acquired adaptation (acclimation, and possible adaptive mutations) and pre-selective adaptation to VPW in *D. chlorelloides* and *M. aeruginosa* (see Fig. 1). In short, two different sets of experimental cultures were prepared. In the set 1 experiment, 70 culture flasks were inoculated with $N_0 = 10^2$ cells of *D. chlorelloides* (a number small enough to reasonably ensure the absence of pre-existing mutants in the strain); 96 culture flasks were also inoculated with $N_0 = 10^2$ cells of *M. aeruginosa*. Cultures were grown in 20 mL of BG-11 medium at 22 ºC until $N_t = 9.5 \times 10^4$ cells in *D. chlorelloides* or $N_t = 2 \times 10^5$ cells in *M. aeruginosa*, and afterwards exposed to VPW at 30 ºC. For this purpose, the cultures were centrifuged to form a pellet of cells, the medium was decanted, and 20 mL of VPW was added to the flasks. For the set 2 control, 20 aliquots of 9.5×10^4 cells of *D. chlorelloides*, and 25 aliquots 2×10^5 cells in *M. aeruginosa*, of from the same parental populations growing in 20 mL BG-11 medium at 22 ºC were separately

transferred to culture flasks containing VPW at 30 ºC. Cultures were observed for 60 d (thus insuring that one mutant cell could generate enough progeny to be detected), and the resistant cells in each culture were counted by at least three independent observers.

Two different results can be found in the set 1 experiment when conducting a fluctuation analysis, each of them being interpreted as the independent consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1A) the variance in the number of cells per culture would be low if resistant cells arose during the exposure to the selective agent (i.e. by acclimation or adaptive mutations). Because every cell is likely to have the same chance of developing resistance, inter-culture (flask-to-flask) variation would be consistent with the Poisson model (i.e. variance/mean ≈1). By contrast, if high variation in the inter-culture number of resistant cells is found (i.e. variance/mean >1), it means that resistant cells appeared by selection of rare spontaneous mutations occurring before selection, and the flask-to-flask variation would not be consistent with the Poisson model (that is to say, they occurred during the time in which the cultures grew to N_t from N_0 cells, before the exposure to VPW, Fig. 1, set 1B). Obviously, another result (0 resistant cells in each culture) could also be found, indicating that neither selection on spontaneous mutations that occur prior to VPW exposure, nor specific adaptation during the exposure to VPW, took place.

The set 2 cultures is the experimental control of the fluctuation analysis (Fig. 1). Variance is expected to be low, because set 2 samples the variance of the parental population. Thus, if a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells arose during the exposure to the VPW (by acclimation or adaptive mutations). In contrast, if the variance/mean ratio of set 1 is significantly

greater than the variance/mean ratio of set 2 (fluctuation), this confirms that resistant cells arose by rare mutations that occurred before exposure to the VPW.

The fluctuation analysis also allows estimation of the rate of appearance of resistant cells. Because of the methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of cultures of set 1 showing non-resistant cells after VPW (i.e. the first term of the Poisson distribution, named the P_0 estimator; Luria & Delbrück 1943) was the parameter used to calculate the mutation rate (μ) as:

$$\mu = -\mathrm{Log}_e P_0 / (N_t - N_0) \qquad \text{(Eqn. 2)}$$

RESULTS

The shallow pond on Vulcano Island selected for our study is characterized by high sulphide levels (1.84 ± 0.10 mg L^{-1}), and acidic (pH 3.1 ± 0.1) and relatively warm (30.3 ± 0.5 ºC) conditions.

The phytoplankton flora in the pond is very poor: only four species of eukaryotic microalgae were found. The dominant species were the chlorophyceans *Chlamydomonas variabilis* P. A. Dangeard and *Dictyosphaerium ehrenbergianum* Nägeli, with densities of 822 ± 193 and 773 ± 99 cells mL^{-1} (n = 7), respectively. The other two species were a centric diatom from the genus *Stephanodiscus* (cell density at 72 ± 26 per mL, n = 7), and the euglenophyte *Euglena* sp. (cell density at 62 ± 18 per mL, n = 7). Cyanobacteria were not detected in the pond.

The exposure of the wild-type strains of *M. aeruginosa* and *D. chlorelloides* to the filtered VPW was almost lethal in laboratory experiments performed at 30 ºC: $m = 0$ in both species. In contrast, controls cultured in BG-11 medium at 22 ºC proliferated (m = 0.68 ± 0.04 doublings d^{-1} for *D. chlorelloides*, and 0.53 ± 0.03 doublings d^{-1} for *M. aeruginosa*; $n = 3$ in both species).

When conducting the fluctuation analysis, the cell density of *D. chlorelloides* was drastically reduced in each experimental culture due to destruction of wild-type cells, sensitive to the VPW. However, after further incubation for two months, some *D. chlorelloides* cultures increased in density again, apparently due to growth of a VPW-resistant variant. In the case of set 1, three cultures recovered after 60 d under VPW exposure (Table 1). By contrast, every set 2 culture recovered, indicating the presence of VPW-resistant cells in all cultures (Table 1). A high fluctuation in the set 1 experiment (from 0 to 753,960 resistant cells per culture flask) was found (Table 1), exceeding significantly the variance of the number of resistant cells to the mean ($P<0.001$, using χ^2 as a test of goodness of fit). The fluctuation observed was not a consequence of experimental error in sampling VPW-resistant cells because the analyses of set 2 showed low fluctuation in the number of VPW-resistant cells per flask (22,258 ± 1,335). In fact, the ratio variance/mean of the no. of resistant cells per replicate in set 2 (see Table 1) was consistent with Poisson variability (i.e. variance/mean *c.* 1; $P<0.05$, using χ^2 as a test of goodness of fit). Consequently, we infer that VPW-resistant cells arose by rare, pre-selective spontaneous mutations prior to VPW exposure. The estimated μ of VPW-sensitive to VPW-resistant in *D. chlorelloides* was 4.7×10^{-7} mutants per cell per generation.

249 The cyanobacterium *M. aeruginosa* seemed to be unable to adapt to VPW (Table
250 1). Neither selection on spontaneous mutations that occur prior to VPW exposure, nor
251 specific adaptation during the exposure to VPW, was enough to allow adaptation of *M.*
252 *aeruginosa* to VPW.
253
254 DISCUSSION
255
256 Only 4 species of eukaryotic algae were found in the extreme environment of the
257 Vulcano Island pond, suggesting that they might well have undergone adaptation to
258 these hostile conditions. Moreover, the catastrophic effect of VPW on *D. chlorelloides*
259 isolated from a more moderate habitat suggests that the survival of microalgae in VPW
260 could only be achieved by some kind of adaptation.
261 When *D. chlorelloides* was cultured in VPW, cultures became clear after a few
262 days due to the massive destruction of the sensitive cells by the toxic effect of VPW.
263 However, after further incubation for two months, some cultures became coloured
264 again, due to the growth of cells that were resistant to the toxic effect of VPW. The key
265 to understanding adaptation of *D. chlorelloides* to the extremely adverse conditions of
266 the VPW is to analyse the rare variants that proliferate after the massive destruction of
267 the sensitive cells by this selective agent.
268 The large fluctuation in number of VPW-resistant cells observed in the set 1
269 experiment, in contrast to the insignificant fluctuation in set 2 controls, unequivocally
270 demonstrates that *D. chlorelloides* resistant cells arose by rare spontaneous mutations
271 and not through direct and specific adaptation in response to VPW. However, it should
272 be noted that it would be difficult to observe post-selective mutations (i.e. adaptive

mutations) using fluctuation analysis if the rate of these kinds of mutations for VPW-resistance were $<10^{-8}$. Despite this, the rapid lethal effect of VPW seems unlikely to allow the appearance of adaptive mutations, because these kinds of mutations occur in non-proliferating microbial populations after being incubated on non-lethal selective medium (Foster 2000).

The mutation rate of VPW-sensitivity→VPW-resistance transformation in *D. chlorelloides* (4.7×10^{-7} mutants per cell per generation) was comparable to that from sensitivity to resistance to the sulphureous water from La Hedionda spa (S Spain) in the chlorophycean *Spirogyra insignis* (Hassall) Kützing (2.7×10^{-7} mutants per cell per generation; Flores-Moya *et al.* 2005). These mutation rates were one or two orders of magnitude lower than those we have described for resistance to other biocides in cyanobacteria and microalgae (Costas *et al.* 2001; López-Rodas *et al.* 2001; Baos *et al.* 2002; García-Villada *et al.* 2002, 2004; Costas *et al.* 2007; López-Rodas *et al.* 2007, 2008). Taking into account the countless cells comprising algal populations and the magnitude of the mutation rate allowing adaptation to VPW found in *D. chlorelloides*, it could be hypothesized that algae colonised the sulphureous, acid, warm, shallow pond from Vulcano Island rapidly after the last eruption (1888 to 1890). Our results suggest that the eukaryotic algae living in the hostile ecosystem of the Vulcano Island pond could be the descendents of chance resistant mutants that fortuitously arrived from 1890 onwards. Moreover, this colonization could be happening continuously since the last eruption and possibly occurred also in the past, in the ponds that disappeared with the last eruption. However, in view of the fact that few lucky resistant mutants of freshwater algae can arrive at Vulcano Island because of its isolation from other freshwater

296 habitats, the phytoplankton species richness of this sulphureous, acid, warm, shallow
297 pond of Vulcano Island remains limited.

298 The rapid adaptation of *D. chlorelloides* to the geothermal waters from Vulcano
299 Island could be a model for understanding the survival of photosynthesizers during one
300 of the most critical periods in the history of life: the Proterozoic 'snowball Earth'. The
301 'snowball Earth' hypothesis proposes that a series of global glaciations, reaching the
302 Equator, occurred 850-750 million years ago (Kirschvink 1992). Negative carbon
303 isotope anomalies in carbonate rocks from this period have been interpreted as the
304 consequence of the collapse of primary production in the surface ocean for million of
305 years (Hoffman *et al.* 1998; Rothman *et al.* 2003), although some photosynthesizers
306 could have survived in refugia associated with volcanic areas, such as hot springs or
307 geothermal ponds (Schrag & Hoffman 2001). But some of such refugia, which may
308 have lethal concentrations of dissolved substances such as sulphur compounds and
309 heavy metals, and acidic or very acidic pH, are often lethal for cyanobacteria and algae,
310 which were the only photosynthesizers present in the Proterozoic biosphere (Kaufman
311 *et al.* 1997). In spite of this, the selection of rare, spontaneous mutants could be enough
312 to assure the adaptation of algae to stressful, hostile geothermal waters.

313 Cyanobacteria were not detected in the Vulcano Island pond. In fact, these
314 organisms have been reported to be absent from moderately or extremely acidic
315 environments (Brock 1973; Knoll & Bauld 1989; Albertano 1995; reviewed by
316 Gimmler 2001), and a pH limit of 4.8 was postulated for proliferation of cyanobacteria
317 (Brock 1973). However, the presence of filamentous cyanobacteria in acid lakes (pH
318 2.9) in Germany (Steinberg *et al.* 1998) renders doubtful this postulated lower pH limit.
319 The presence of cyanobacteria in acidic environments may be very unusual, and our

results are in agreement with this notion. In previous fluctuation experiments with cyanobacteria, using as selective agent acid waters from Spain's Rio Tinto (SW Spain) (Costas *et al.* 2007), adaptation was not detected. Thus, neither selection on spontaneous mutations that occur before exposure to acidic water, nor specific adaptation during the exposure to acidic water, was enough to allow adaptation of the cyanobacterium *M. aeruginosa*.

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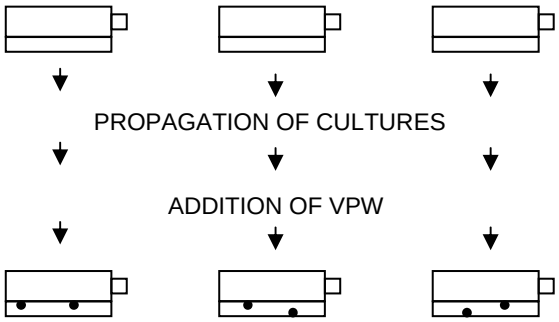
Table 1. Fluctuation analysis of VPW-resistant variants in wild-type strains of the chlorophycean *Dictyosphaerium chlorelloides* and the cyanobacterium *Microcystis aeruginosa*

		Set 1	Set 2
Dictyosphaerium chlorelloides (Chlorophyceae)			
No. of cultures		70	20
No. of cultures containing the following no. of VPW-resistant cells:			
	0	67	0
	$<10^3$	1	0
	$10^3 - 10^4$	0	0
	$10^4 - 10^5$	1	20
	$>10^5$	1	0
Variance/mean (of the no. of VPW-resistant cells per replicate)	>100*	1.3	
μ (mutants per cell per generation)	4.7×10^{-7}		
Microcystis aeruginosa (Cyanobacteria)			
No. of cultures		96	25
No. of cultures containing VPW-resistant cells:		0	0

*Variance/mean >1; $P<0.001$, using χ^2 as a test of goodness of fit

Fig. 1. Schematic diagram of possible results obtained in the experiment (modified from the classic Luria and Delbrück fluctuation analysis). In the set 1 experiment, different cultures (each started from a small inoculum, $N_0 = 10^2$ cells in both the chlorophycean *Dictyosphaerium chlorelloides* and the cyanobacterium *Microcystis aeruginosa*) were propagated under non-selective conditions (i.e. BG-11 medium, 22 ºC) until a very high cell density ($N_t = 9.5 \times 10^4$ cells in *D. chlorelloides*, and 5.0×10^5 in *M. aeruginosa*) was reached, and then transferred to VPW at 30 ºC. Set 1A: resistant cells arose during the exposure to the selective agent (physiological acclimation or adaptive mutations). In this case, the number of resistant cells in all the cultures must be similar. Set 1B: adaptation by rare mutations occurring in the period of the propagation of cultures, i.e. before exposure to the selective agent. One mutational event occurred late in the propagation of culture 1 (therefore, the density of VPW-resistant cells found is low) and early in the propagation of culture 3 (thus, density of VPW-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. In this case, the number of resistant cells in all the cultures must be different. Set 2 samples the variance of parental populations as an experimental control. In this case, the number of resistant cells in all the cultures must be similar.

SET 1A



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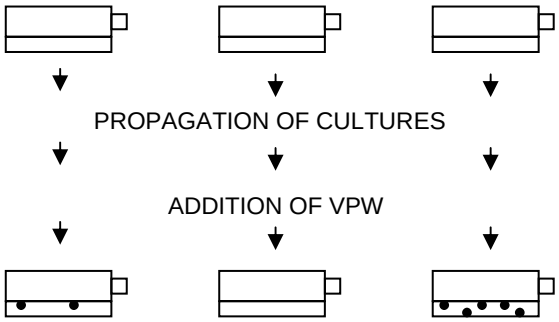
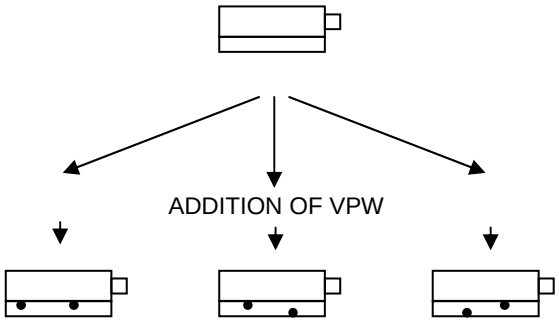


SET 2



4.3.2. Microalgal adaptation to a stressful environment (acidic, metal-rich mine waters) could be due to selection of pre-selective mutants originating in non-extreme environments.

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Microalgal adaptation to a stressful environment (acidic, metal-rich mine waters) could be due to selection of pre-selective mutants originating in non-extreme environments

Victoria López-Rodas [a], Fernando Marvá [a], Eduardo Costas [a], Antonio Flores-Moya [b,*]

[a] *Departamento de Producción Animal (Genética), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain*
[b] *Departamento de Biología Vegetal (Botánica), Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain*

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Abstract

At least six species of eukaryotic microalgae inhabit the acidic (pH 2.4–2.7), metal-rich mine waters from ponds in the copper mine district of Mynydd Parys (N Wales, UK). Consequently, these ponds constitute interesting natural laboratories for analysis of adaptation by microalgae to extremely stressful conditions. To distinguish between the pre-selective and post-selective origin of adaptation processes that allow the existence of microalgae in these ponds, a Luria-Delbrück fluctuation analysis was performed with the chlorophycean *Dictyosphaerium chlorelloides* isolated from non-acidic waters. In this analysis, natural Mynydd Parys pond water (MPW) was used as selective factor. Pre-selective, resistant *D. chlorelloides* cells appeared with a frequency of 1.6×10^{-6} per cell per generation. MPW-resistant mutants, with a diminished Malthusian fitness, are maintained in non-extreme waters as the result of a balance between new MPW-resistant cells arising by mutation and MPW-resistant mutants eliminated by natural selection (equilibrium at *ca*. 19 MPW-resistant per 10^7 wild-type cells). We propose that the microalgae inhabiting these stressful ponds could be the descendents of chance mutants that arrived in the past or are even arriving at the present.

Keywords: Acidic environments; Adaptation; *Dictyosphaerium*; Natural selection; Pre-selective mutations

1. Introduction

Some stressful environments sustain populations of organisms living at the extreme limits of their physiological tolerance as a result of physiological adaptation (i.e. acclimation) supported by modifications of gene expression (Bradshaw and Hardwick, 1989; Fogg, 2001). Beyond physiological limits, adaptive evolution depends on the occurrence of new mutations that confer resistance (Sniegowski and Lenski, 1995; Flores-Moya et al., 2005; Costas et al., 2007). The neo-Darwinian view that adaptive evolution occurs by selection of pre-existing genetic variation was early accepted for multi-celled organisms (Lewis, 1934; Huxley, 1942; reviewed by Sniegowski, 2005). However, recent evolutionary studies of bacteria have suggested that hypothetical adaptive mutations could be a process resembling Lamarckism which, in the absence of lethal selection, produces mutations that relieve selective pressure (Cairns et al., 1988; Foster, 2000). Examples of adaptive mutations or related phenomena have also been reported in yeast (Heidenreich, 2007) but, as far as we know, they have not been recognized in other microorganisms such as cyanobacteria and microalgae. The key to resolving this debate is to know the pre-adaptive or post-adaptive origin of new mutations. However, there are almost no studies that have made a direct connection between the rates of origin of favored mutants in diploid, multi-celled organisms living in well-defined populations. In contrast, many lineages of microalgae are haploid, single-celled, asexual organisms, and their populations are composed of countless cells (Margulis and Schwartz, 1982). Therefore, the study of genetic adaptation of microalgae to extreme environments (i.e. characterized by extreme values of pH, toxic substances, temperature, salinity, and mineral excess or deficiency) is an adequate approximation to the problem of the origin of favored mutants and the process of adaptation. Moreover, because of its epochal scope, evolutionary biology is often caricatured as a strictly descriptive science. However, evolutionary change can be studied on short

* Corresponding author.
E-mail address: floresa@uma.es (A. Flores-Moya).

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Nomenclature

m	Malthusian fitness parameter
m^{r}_{MPW}	Malthusian fitness parameter of MPW-resistant cells
m^{s}_{MPW}	Malthusian fitness parameter of MPW-sensitive cells
MPW	Mynydd Parys water
N_0	no. of cells at the start of the experiment
N_t	no. of cells at the end of the experiment
P_0	proportion of cultures without MPW-resistant cells in the set 1 fluctuation analysis experiment
q	frequency of MPW-resistant alleles in non-MPW-exposed populations
s	coefficient of selection
μ	mutation rate

time scales with a robust experimental approach by modifying classic evolutionary experiments such as the Luria-Delbrück fluctuation analysis (Costas et al., 2001; López-Rodas et al., 2001). In this framework, we recently proposed that algal colonization of acidic (pH 4.1–4.5), sulphureous water from La Hedionda (S Spain) spa (Flores-Moya et al., 2005) or very acid (pH 1.7–2.5) and metal-rich water from Spain's Rio Tinto (Costas et al., 2007), could be explained by natural selection of pre-selective resistant mutants of mesophilic taxa occurring in non-extreme environments.

In order to add more knowledge about mechanisms involved in the presence of microalgae in extreme environments, the aim of this work was to study the adaptive processes allowing the development of eukaryotic algae populations in the acidic, metal-rich water from ponds in the copper mine district of Mynydd Parys (N Wales, UK). Mynydd Parys water (MPW) show extreme conditions characterized by low pH values (2.4–2.7), and huge concentrations of iron, manganese, copper and zinc, where archaeal clones, as well as iron- and sulphur-oxidizer bacteria proliferate (Coupland and Johnson, 2004; Hallberg et al., 2006). To accomplish the objectives of the work, we first performed a screening of microalgae inhabiting MPW. Secondly, we analyzed whether algae that inhabit MWP could be the result of acquired adaptation (including both physiological adaptation or acclimation, and possible mutations following MPW exposure; the first case is not an evolutionary event), or could be explained as adaptive evolution. For this purpose, the statistical and experimental procedure called fluctuation analysis (Luria and Delbrück, 1943) was performed. A strain of the mesophilic, cosmopolitan chlorophycean *Dictyosphaerium chlorelloides* (Naumann) Komarek and Perman was used as experimental organism, while MPW was used as selective agent. We found that this species rapidly adapts and grows in MPW via the action of natural selection on resistant mutants that appear spontaneously in wild-type populations. Thus, the presence of microalgae in the stressful environment of the MPW could be explained as a rapid evolutionary change, in accordance with neo-Darwinian postulates.

2. Materials and methods

2.1. Phytoplankton community in the pond of Mynydd Parys

Sampling of water and phytoplankton was carried out in July 2006; for this purpose, the largest pond close to the mine area was selected. Three 1 l MPW samples were collected and homogeneously mixed. The resulting integrated water sample was filtered (0.22 μm, Stericup, Millipore Co., Billerica, MA, USA) and kept in a closed bottle excluding any air, and stored at 4 °C in darkness until the laboratory experiments (toxicity tests and fluctuation analysis) were performed.

Phytoplankton was identified in fresh samples (directly after collection) using a McArthur portable microscope (Kirk Technology, England), and counted on fixed samples (4% PBS-buffered formalin) in settling chambers using an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany). Identification of algae was carried out in accordance with Wolowski (2002) for Euglenophyta, and John and Tsarenko (2002), Pentecost (2002) and Johnson (2002) for Chlorophyta.

2.2. Experimental organism and culture conditions

A wild-type strain of the chlorophycean *D. chlorelloides* (Naumann) Komárek and Perman was isolated from a pristine, cool-temperate (5–10 °C), non-sulphureous, slightly alkaline (pH 7.8–8.0) high mountain lake from Sierra Nevada (S Spain). The strain was grown in 100 ml cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml BG-11 medium (Sigma–Aldrich Chemie, Taufkirchen, Germany), at 22 °C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Cultures were axenically maintained in mid-log exponential growth (Cooper, 1991) by serial transfers of subcultures to fresh medium, and only cultures without detectable bacteria were used in the experiments. The absence of bacteria in the cultures was tested periodically by epifluorescence microscopy after staining with acridine orange. Although *D. chlorelloides* forms 2- or 4-celled, rarely 16-celled colonies, and is capable of sexual reproduction in nature (John and Tsarenko, 2002), the strain was exclusively propagated by asexual reproduction, and it was represented by single-celled individuals. Prior to the experiments, the cultures were cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the culture.

2.3. Toxicity test: effect of MPW on Malthusian fitness

In order to test the toxic effect of the MPW, we measured the changes in Malthusian fitness (m) when the wild-type strain of *D. chlorelloides* was cultured in this selective agent. Culture samples (5.0×10^5 cells) from a mid-log exponentially growing culture were placed in experimental tubes containing 1.5 ml of MPW; controls in BG-11 medium were also prepared. The value of m was calculated in three replicates containing MPW as well as in three BG-11 medium controls, using the equation from Crow and Kimura (1970):

$$N_t = N_0 \, e^{mt}, \quad (1)$$

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where N_t and N_0 are the cell number at the end (after $t = 3$ d) and at the start of the experiment, respectively. Therefore, m was calculated as:

$$m = \frac{\log_e(N_t/N_0)}{t} \quad (2)$$

Experiments and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer. The number of samples in each case was determined using the progressive mean procedure (Williams, 1977), which assured a counting error <5%.

2.4. Fluctuation analysis of MPW-sensitivity → MPW-resistant transformation

A modified fluctuation analysis (Luria and Delbrück, 1943) was carried out (as described in Costas et al., 2001, and López-Rodas et al., 2001) to discriminate between acquired adaptation or pre-selective adaptation to MPW in *D. chlorelloides* (see Fig. 1). The modification of the analysis involves the use of liquid medium containing the selective agent rather than plating on a solid medium, as was done by Luria and Delbrück (1943) with bacterial cultures. In short, two different sets of experimental cultures were prepared. In the set 1 experiment, 50 culture flasks were inoculated with $N_0 = 10^2$ cells of *D. chlorelloides* (a number small enough to reasonably ensure the absence of pre-existing mutants in the inoculum). Cultures were grown in BG-11 medium at 22 °C until $N_t = 11 \times 10^4$ cells, and afterwards exposed to MPW. For the set 2 control, 20 aliquots of 11×10^4 cells of *D. chlorelloides* from the same parental populations growing in BG-11 medium at 22 °C were separately transferred to culture flasks containing MPW. Cultures were observed for 60 d (thus, insuring that one mutant cell could generate enough progeny to be detected), and the resistant cells in each culture were counted by at least three independent observers.

Two different results can be found in the set 1 experiment when conducting a fluctuation analysis, each of them being interpreted as the independent consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1A), the variance in the number of cells per culture would be low if resistant cells arose during the exposure to the selective agent (i.e. acclimation or adaptive mutations). Because every cell is likely to have the same chance of developing resistance, inter-culture (flask-to-flask) variation would be consistent with the Poisson model (i.e. variance/mean ≈1). By contrast, if high variation in the inter-culture number of resistant cells is found (i.e. variance/mean >1), it means that resistant cells appeared by selection of rare spontaneous mutations occurring before selection, and the flask-to-flask variation would not be consistent with the Poisson model (that is to say, they occurred during the time in which the cultures grew to N_t from N_0 cells, before the exposure to MPW, Fig. 1, set 1B). Obviously, another result (zero resistant cells in each culture) could also be found, indicating that neither selection on spontaneous mutations that occur prior to MPW exposure, nor specific adaptation during the exposure to MPW, took place.

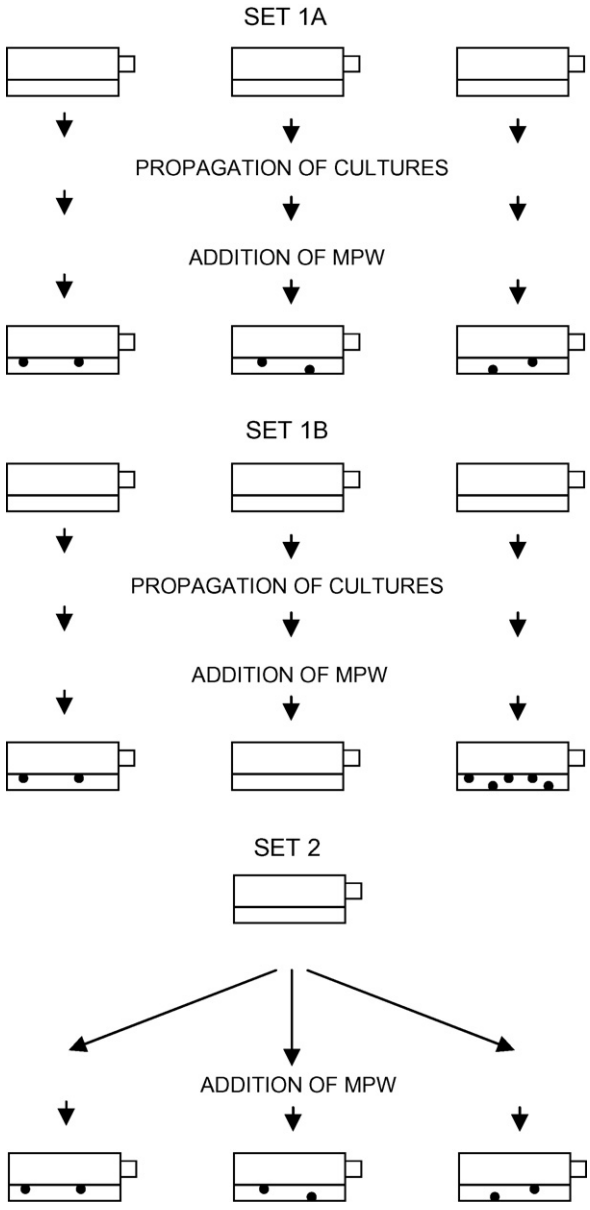

Fig. 1. Schematic diagram of possible results obtained in the experiment, modified from the classic Luria and Delbrück fluctuation analysis. In the set 1 experiment, different cultures of *Dictyosphaerium chlorelloides* (each started from a small inoculum, $N_0 = 10^2$ cells) were propagated under non-selective conditions (i.e. BG-11 medium) until a very high cell density ($N_t = 11 \times 10^4$ cells) was reached, and then transferred to the selective agent (i.e. MPW). Set 1A: Resistant cells arose during the exposure to MPW (physiological acclimation or post-adaptive mutations). In this case, the number of resistant cells in all the cultures must be similar. Set 1B: Adaptation by rare mutations occurring in the period of the propagation of cultures, i.e. before exposure to MPW. One mutational event occurred late in the propagation of culture 1 (therefore, the density of MPW-resistant cells found is low) and early in the propagation of culture 3 (thus, density of MPW-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. In this case, the number of resistant cells in all the cultures must be different. Set 2: Samples the variance of parental populations as an experimental control. In this case, the number of resistant cells in all the cultures must be similar.

The set 2 cultures serve as the experimental control of the fluctuation analysis (Fig. 1). Variance is expected to be low, because set 2 samples the variance of the parental population. Thus, if a similar variance/mean ratio between set 1 and set 2 is found,

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it confirms that resistant cells arose during the exposure to the MPW (by acclimation or adaptive mutations). In contrast, if the variance/mean ratio of set 1 is significantly greater than the variance/mean ratio of set 2 (fluctuation), this confirms that resistant cells arose by rare mutations that occurred before exposure to the MPW.

The fluctuation analysis also allows estimation of the rate of appearance of resistant cells. There are different approaches for accomplishing this estimation (Rosche and Foster, 2000). Due to methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of set 1 cultures showing non-resistant cells after MPW exposure (i.e. the first term of the Poisson distribution, termed P_0 estimator; Luria and Delbrück, 1943) was the parameter used to calculate the mutation rate (μ). The P_0 estimator is defined as follows:

$$P_0 = e^{-\mu(N_t - N_0)}, \quad (3)$$

where N_0 and N_t are the initial and the final cell population sizes (10^2 and 11×10^4 cells, respectively). Therefore, μ was calculated as:

$$\mu = \frac{-\log_e P_0}{(N_t - N_0)} \quad (4)$$

2.5. Mutation-selection equilibrium

If the mutation from a normal wild-type MPW-sensitive allele to a MPW-resistant allele is recurrent, and the MPW-resistant allele is detrimental to fitness in the absence of MPW, then new mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance (Crow and Kimura, 1970; Spiess, 1989). At any one time there will be a certain number of resistant cells that are not yet eliminated. According to Kimura and Maruyama (1966), the average number of such mutants will be determined by the balance between μ and the rate of selective elimination (s):

$$q = \frac{\mu}{(\mu + s)}, \quad (5)$$

where q is the frequency of the MPW-resistant allele and s is the coefficient of selection calculated as follows:

$$s = 1 - \left(\frac{m^{r}_{MPW}}{m^{s}_{MPW}} \right), \quad (6)$$

where m^{r}_{MPW} and m^{s}_{MPW} are the Malthusian fitness of MPW-resistant and MPW-sensitive cells measured in non-selective conditions (i.e. BG-11 medium), respectively.

3. Results

The phytoplankton community supported by MPW in July 2006 had moderate microalgal species richness, chlorophyceans and euglenophyceans being the most abundant (Table 1). Cyanobacteria were not detected.

The exposure of the wild-type strains of *D. chlorelloides* to the filtered MPW was lethal in laboratory experiments: the Malthusian fitness was totally inhibited ($n = 3$). In contrast, controls cultured in BG-11 medium proliferated ($m = 0.600 \pm 0.010$ doublings d^{-1}, $n = 3$).

Table 1
Most abundant microalgal species (cells ml^{-1}, or filaments ml^{-1} in the case of *Spirogyra communis*, mean ± S.D., $n = 10$) detected in a pond in Mynydd Parys in July 2006

Division	Species	Abundance
Chlorophyta	*Spirogyra communis* (Hassall) Kützing	29,600 ± 780
	Chlorella sp.	6,740 ± 790
	Chlamydomonas variabilis P.A. Dangeard	3,450 ± 910
	Chloroccocum infusionum (Schrank) Meneghini	1,970 ± 534
Euglenophyta	*Euglena geniculata* (F. Schmitz) Dujardin	1,980 ± 180
	Phacus longicauda (Ehrenberg) Dujardin	390 ± 60

When conducting the fluctuation analysis, the cell density of *D. chlorelloides* was drastically reduced in each experimental culture due to destruction of wild-type cells, sensitive to the MPW. However, after further incubation for two months, some *D. chlorelloides* cultures increased in density again, apparently due to growth of a MPW-resistant variant. In the case of set 1, some cultures recovered after 60 d under MPW exposure (Table 2). By contrast, every set 2 culture recovered, indicating the presence of MPW-resistant cells in all cultures (Table 2). A high fluctuation in the set 1 experiment (from 0 to 11,050 resistant cells per culture flask) was found (Table 2), exceeding significantly the variance of the number of resistant cells to the mean ($P < 0.001$, using χ^2 as a test of goodness of fit). The fluctuation observed was not a consequence of experimental error in sampling MPW-resistant cells because the analyses of set 2 showed low fluctuation in the number of MPW-resistant cells per flask (from 29,850 to 30,250). In fact, the ratio variance/mean of the no. of resistant cells per replicate in set 2 (see Table 2) was consistent with Poisson variability (i.e. variance/mean *ca.* 1; $P < 0.05$, using χ^2 as a test of goodness of fit). Consequently, it could be inferred that MPW-resistant cells arose by rare, pre-selective spontaneous mutations prior to MPW exposure. The estimated mutation rate (μ) of MPW-sensitive to MPW-resistant in *D. chlorelloides* was 1.6×10^{-6} mutants per cell per generation.

Isolated *D. chlorelloides* MPW-resistant mutants growing in the absence of the selective agent (i.e. in BG-11 medium)

Table 2
Fluctuation analysis of $MPW^s \rightarrow MPW^r$ transformation in *Dictyosphaerium chlorelloides*

	Set 1	Set 2
No. of replicate cultures	50	20
No. of cultures containing the following no. of MPW-resistant cells:		
0	42	0
$1–10^3$	3	0
$10^3–10^4$	2	0
$>10^4$	3	20
Variance/mean (of the no. of resistant cells per replicate)	27.69	0.72
μ (Mutants per cell per generation)	1.6×10^{-6}	

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showed a Malthusian fitness of 0.085 ± 0.009 doublings d^{-1} ($n = 6$). The overall mean m^{r}_{RTW} and m^{s}_{RTW} values (0.085 and 0.600 doublings d^{-1}, respectively) were used to compute the coefficient of selection of MPW-resistant mutants ($s = 0.86$). By using the values of μ and s, the frequency (q) of resistant alleles was calculated: *ca.*19 *D. chlorelloides* MPW-resistant mutants per 10^7 cells as the consequence of the balance between mutation and selection.

4. Discussion

Evolution by natural selection is driven by the continuous generation of genetic variability based on mutations. Although the pre-selective origin of mutations is the consensus (Sniegowski and Lenski, 1995), the occurrence of adaptive mutations in bacteria and yeast (Foster, 2000; Heidenreich, 2007) add a new view on evolutionary biology. In particular, there is great interest in understanding whether adaptive mutations play any evolutionary role in adaptative processes (Foster, 2000; Perfeito et al., 2007). However, it is supposed that they can provide a valuable supplement to the conventional process of mutation and selection only when the selective stress is non-lethal (Wintersberger, 1991). Moreover, the occurrence of adaptive mutations in phytoplankters is unknown. In the present work we examined the process of adaptation in a microalgal species resulting from a sudden exposure to a lethal stress, in order to explain the presence of mesophilic algae (at least 4 species of chlorophyceans and 2 species of euglenophycenas; see Table 1) in the extreme environment of MPW. The experimental and statistical approach that we employed allowed us to discriminate between pre-selective and post-selective origins of adaptation.

The catastrophic effect of MPW on Malthusian fitness of *D. chlorelloides* (isolated from pristine waters) suggests that the survival of eukaryotic algae in the MPW could only be achieved by some kind of adaptation to these adverse conditions. A massive destruction of the sensitive cells by the toxic effect of MPW was observed when *D. chlorelloides* cultures were treated with MPW. However, after further incubation for several weeks, some cultures showed growth of cells that were resistant to the effect of MPW. The key to understanding adaptation of *D. chlorelloides* to the extremely adverse conditions of the MPW is to analyze the origin of the rare algal variants that occur after the massive destruction of the sensitive cells by MPW. Fluctuation analysis is the suitable method to discriminate between MPW-resistant cells arising through specifically acquired adaptation in response to MPW environmental selection (i.e. acclimation or possible adaptive mutations), and MPW-resistant cells arising by rare spontaneous mutations occurring randomly during replication of organisms under non-selective conditions (i.e. prior to exposure to MPW). The large fluctuation in number of MPW-resistant cells observed in the set 1 experiment, in contrast to the minor variation in set 2 controls, unequivocally demonstrates that *D. chlorelloides* resistant cells arose by rare spontaneous mutations and not through direct and specific adaptation in response to MPW (i.e. MPW did not stimulate the appearance of resistant cells). It should be noted that using fluctuation analysis, it would be difficult to observe post-selective mutations occurring at very low rates ($<10^9$). Despite this, it seems very unlikely that adaptive mutations occurred in our cultures, because the rapid lethal effect of MPW seems unlikely to allow the appearance of adaptive mutations. Adaptive mutations are only observed in non-proliferating microbial populations after being incubated on non-lethal selective medium plates (Cairns et al., 1988; Foster, 2000). Consequently, the occurrence pre-selective mutations prior to exposure to MPW seems to be the sole mechanism that allows adaptation of algae to MPW.

The rate of mutation from MPW-sensitivity to MPW-resistance in *D. chlorelloides* (1.6×10^{-6} mutants per cell division) was in the middle of the range of the mutation rates (from 2.1×10^{-5} to 2.7×10^{-7} mutants per cell division) we have described for resistance to several biocides and severe environments in other cyanobacteria and microalgal species (Costas et al., 2001, 2007; López-Rodas et al., 2001, 2007; Baos et al., 2002; García-Villada et al., 2002, 2004; Flores-Moya et al., 2005). Mutation from MPW-sensitivity to MPW-resistance occurs prior to exposure to MPW. However, MPW-resistant cells have very diminished fitness compared to wild-type MPW-sensitive cells. Consequently, the balance between the recurrent appearance of mutants by rare pre-selective mutation and their elimination by natural selection controls the presence of MPW-resistant cells in the populations of *D. chlorelloides* in a non-extreme environment. As a result, an equilibrium frequency of around 19 MPW-resistant mutants per 10^7 wild-type cells should arise in non-extreme waters. Taking into account both the relatively high number of resistance-mutants and the countless cells comprising algal populations, it could be hypothesized that algal colonization of Mynydd Parys ponds was almost instantaneous when they first formed, because MPW-resistant cells were already present in non-extreme waters. The results reported here indicate that eukaryotic algae living in the hostile ecosystem of the Mynydd Parys mine district could be the descendents of mutants that fortuitously arrived at the ponds in the past; moreover, this event could be happening continuously.

Cyanobacteria were not detected in the ponds from Mynyyd Parys. In fact, these organisms have been reported to be absent from moderately or extremely acidic environments (Brock, 1973; Knoll and Bauld, 1989; Albertano, 1995; reviewed by Gimmler, 2001), and a pH limit of 4.8 was postulated for proliferation of cyanobacteria (Brock, 1973). It has been proposed that the lack of specific ion-transporter H^+-ATPase may be the molecular basis for the lack of successful adaptation by cyanobacteria to acidic environments (Amaral Zettler et al., 2003). Moreover, in previous fluctuation experiments with the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann, using as selective agent acid waters from Spain's Rio Tinto (Costas et al., 2007), adaptation was not detected. Thus, neither selection on spontaneous mutations that occur before exposure to acidic water, nor specific adaptation during the exposure to acidic water, was enough to allow adaptation of the cyanobacterium. For this reason, we did not carry out any fluctuation analysis with cyanobacteria using MPW as selective agent. However, the presence of filamentous cyanobacteria in acid lakes (pH 2.9) in Germany (Steinberg et al., 1998) renders doubtful this postulated lower pH limit.

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4.3.3. Adaptation of the chlorophycean *Dictyosphaerium chlorelloides* to stressful acidic, mine metal-rich waters as result of pre-selective mutations.

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Adaptation of the chlorophycean *Dictyosphaerium chlorelloides* to stressful acidic, mine metal-rich waters as result of pre-selective mutations

Victoria López-Rodas [a], Fernando Marvá [a], Mónica Rouco [a], Eduardo Costas [a], Antonio Flores-Moya [b,*]

[a] *Producción Animal (Genética), Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain*
[b] *Biología Vegetal (Botánica), Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain*

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ABSTRACT

Several species of microalgae, closely related to mesophilic lineages, inhabit the extreme environment (pH 2.5, high levels of metals) of the Spain's Aguas Agrias Stream water (AASW). Consequently, AASW constitutes an interesting natural laboratory for analysis of adaptation by microalgae to extremely stressful conditions. To distinguish between the pre-selective or post-selective origin of adaptation processes allowing the existence of microalgae in AASW, a Luria–Delbrück fluctuation analysis was performed with the chlorophycean *Dictyosphaerium chlorelloides* isolated from non-acidic waters. In the analysis, AASW was used as selective factor. Preselective, resistant *D. chlorelloides* cells appeared with a frequency of 1.1×10^{-6} per cell per generation. AASW-resistant mutants, with a diminished Malthusian fitness, are maintained in non-extreme waters as the result of a balance between new AASW-resistant cells arising by mutation and AASW-resistant mutants eliminated by natural selection (equilibrium at *c.* 12 AASW-resistants per 10^7 wild-type cells). We propose that the microalgae inhabiting this stressful environment could be the descendents of chance mutants that arrived in the past or are even arriving at the present.

1. Introduction

In extreme environments characterized by values of ecological factors exceeding the physiological limits of organisms, survival depends exclusively on adaptive evolution which occurs by selection on pre-existing genetic variation (Sniegowski and Lenski, 1995; Hughes, 1999; Sniegowski, 2005). Surprisingly, there are almost no studies that have made a direct connection between the rates of origin of favored mutants and the process of adaptation in diploid, multi-celled organisms living in well-defined populations. In contrast, many lineages of microalgae are haploid, single-celled and asexual, and their populations are composed of countless cells (Margulis and Schwartz, 1982). Consequently, these organisms are adequate to study the process of adaptation based on favored mutants. Moreover, there are few circumstances under which a specific mutation rate that is of relevance to adaptation can be measured.

The study of genetic adaptation of microalgae to extreme environments (i.e. characterized by extreme values of pH, toxics, and mineral excess) is an adequate approximation to the problem of the origin of favored mutants and the process of adaptation. Recently we demonstrated that the green alga *Spirogyra insignis* (Hassall) Kützing (Streptophyta) inhabiting the acidic (pH 4.1–4.5), sulphureous water from La Hedionda spa (S Spain) could be explained by selection of pre-selective mutants of mesophilic algal lineages inhabiting in non-acidic waters (Flores-Moya et al., 2005). Similarly, we suggested that eukaryotic microalgae, resistant to acidic (pH 1.7–2.5) and metal-rich waters from the Spain's Rio Tinto, arose randomly by rare spontaneous mutations and, as a result, algal populations could able to instantaneously adapt to Rio Tinto water by means of selection of resistant-mutants growing in non-extreme populations (Costas et al., 2007). An interesting question is to elucidate if adaptation to these stressful environments always take place rapidly through selection of resistant mutants or, in some cases, it could be the consequence of physiological adaptation (i.e. acclimation).

The aim of this work was to analyze adaptation of microalgae in the fascinating example of the extreme environment of Aguas Agrias Stream water (AASW) (meaning sour waters in English), located near the village of Tharsis (north of the city of Huelva,

Abbreviations: AASW, Aguas Agrias Stream water; F'_m, maximum fluorescence of light-adapted cells; F_t, steady-state fluorescence of light-adapted cells; m^r_{AASW}, Malthusian fitness parameter from AASW-resistant cells; m^s_{AASW}, Malthusian fitness parameter from AASW-sensitive cells; N_0, no. of cells at the start of the experiment; N_t, no. of cells at the end of the experiment; P_0, proportion of cultures without AASW-resistant cells in the set 1; q, frequency of AASW-resistant alleles in non-AASW-exposed populations; s, coefficient of selection; Φ_{PSII}, effective quantum yield; μ, mutation rate.

* Corresponding author. Tel.: +34 952 131951; fax: +34 952 131944.
E-mail address: floresa@uma.es (A. Flores-Moya).

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SW Spain). AASW is very acid (pH 2.5), and contains high concentrations of metals (Fe at 1435 mg l^{-1}, Al at 551 mg l^{-1}, Zn at 324 mg l^{-1}, Mn at 143 mg l^{-1}, Cu at 64 mg l^{-1}, As(V) at 657 μg l^{-1} and Pb at 650 μg l^{-1}) (Sánchez-Rodas et al., 2006). Even in such extreme conditions, green patches of algae can be observed. To accomplish the objectives of the work, we initially performed a screening of microalgae species inhabiting AASW. Secondly, we studied if algal community inhabiting AASW could be explained as the result of selection of resistant mutants, or as result of other different adaptive process. For this purpose, a fluctuation analysis (Luria and Delbrück, 1943) was performed by using a wild-type strain of the mesophilic, cosmopolitan chlorophycean *Dictyosphaerium chlorelloides* (Naumann) Komarek and Perman isolated from a pristine, slightly alkaline (pH 8.0) high mountain lake from Sierra Nevada (S Spain) as experimental organism, and AASW as selective agent. This species rapidly adapt to grow in AASW via the action of natural selection on resistant mutants that appear spontaneously in wild-type populations. Thus, the presence of microalgae in the stressful environment of the AASW could be explained in accordance with the neo-Darwinian adaptive evolution hypothesis.

2. Materials and methods

2.1. Phytoplankton community in the AASW

Sampling of water and phytoplankton was carried out in May 2006, nearby Tharsis mine (37°35′28 N, 007°03′31 W), an area that has been exploited in ancient times and during the last two centuries until the 1990s. The sample point was located within the so-called Iberian Pyrite Belt, a volcanogenic massive sulfide province (Sáez et al., 1999). AASW is heavily loaded in mine drainage due to the lixiviates from the Tharsis mine (Checkland, 1967). Three AASW samples of 5 l were collected and homogeneously mixed. The resulting integrated water sample was filtered (0.22 μm, Stericup, Millipore Co., Billerica, MA, USA).

Phytoplankton was identified in fresh samples (directly after collection) using a McArthur portable microscope (Kirk Technology, England), and counted on fixed samples (4% PBS-buffered formalin) in settling chambers using an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany). Identification of algae was carried out in accordance with Cox (1996) for diatoms, Wolowski (2002) for euglenophytes, and John and Tsarenko (2002), Johnson (2002) and Pentecost (2002) for green algae.

2.2. Experimental organism

A wild-type strain of *D. chlorelloides* from the Algal Culture Collection of the Faculty of Veterinary, Complutense University (Madrid, Spain) was grown in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, at 20 °C. The strain was isolated from a pristine, slightly alkaline (pH 7.8–8.0) high mountain lake from Sierra Nevada (S Spain). Cultures were maintained axenically in mid-log exponential growth (Cooper, 1991) by serial transfers of subcultures to fresh medium. Only cultures without detectable bacteria were used in the experiments. Although *D. chlorelloides* forms 2- or 4-celled, rarely 16-celled colonies, and is capable of sexual reproduction in the nature (John and Tsarenko, 2002), the strain tested here exclusively propagated by asexual reproduction, and it was represented by single-celled individuals. Prior to the experiments, the culture was cloned (by isolating a single cell) to avoid including any previous spontaneous mutants that accumulated previously.

2.3. Toxicity test: effect of AASW on Malthusian fitness and effective quantum yield

With the purpose to test the toxic effect of the AASW, we measured the changes in Malthusian fitness (m) and effective quantum yield from photosystem II (Φ_{PSII}) when the wild-type strains of *D. chlorelloides* were cultured in AASW.

Culture samples (5×10^5 cells) from mid-log exponentially growing cultures of *D. chlorelloides* wild-type strain were placed in experimental tubes containing 1.5 ml of AASW. Controls in BG-11 medium were also prepared. Malthusian fitness values were calculated in three replicates in AASW as well as in three controls, using the equation from Crow and Kimura (1970):

$$m = \mathrm{Log_e}(N_t/N_0)/t \quad (1)$$

where t = 5 d, $N_0 = 5 \times 10^5$ cells, and N_t is the cell number at the end of the experiment. Experiments and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer.

The effective quantum yield (Φ_{PSII}) was also measured in triplicates of experiments and controls using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) at five different time points (1, 12, 24, 48, and 72 h). Effective quantum yield was calculated as follows:

$$\Phi_{PSII} = (F'_m - F_t)/F'_m \quad (2)$$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al., 1986; Maxwell and Johnson, 2000).

2.4. Fluctuation analysis of AASW-sensitive → AASW-resistant transformation

A Luria and Delbrück (1943) fluctuation analysis was carried out as described in Fig. 1. Two different sets of experimental cultures were prepared. In the set 1 experiment, 95 test tubes were inoculated with $N_0 = 10^2$ cells of *D. chlorelloides* wild-type strain (a number small enough to reasonably ensure the absence of pre-existing mutants in the strain). Cultures were grown in 10 ml BG-11 medium until $N_t = 1.04 \times 10^5$ cells and afterwards exposed to AASW. For this purpose, the cultures were centrifuged to form a pellet of cells in the tube, the medium was decanted, and 10 ml of AASW was added to the tubes. In the set 2 controls, 20 aliquots of 1.04×10^5 cells from the same parental population (growing in 10 ml BG-11 medium) were separately transferred to test tubes containing AASW. Cultures were observed for 80 d (thereby insuring that one mutant cell could generate enough progeny to be detected), and the resistant cells in each culture (both in set 1 and set 2) were counted. The cell count was performed by at least two independent observers.

Two different results can be found in the set 1 experiment, each of them being interpreted as the consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1), the variance in the number of cells per culture would be low if resistant cells arose during the exposure to the selective agent (i.e. by physiological adaptation). Because every cell is likely to have the same chance of developing resistance, inter-culture (test tube-to-test tube) variation would be consistent with the Poisson model (i.e. variance/mean = 1). On the contrary, if high variation in the inter-culture number of resistant cells is found (i.e. variance/mean > 1), it means that resistant cells appeared by rare spontaneous mutations occurring before AASW exposure (that is to say, they occurred during the time in which the cultures grew to N_t from N_0 cells, before the exposure to AASW, Fig. 1, set 1B). Therefore, the test tube-to-test tube variation would not be consistent with the Poisson model.

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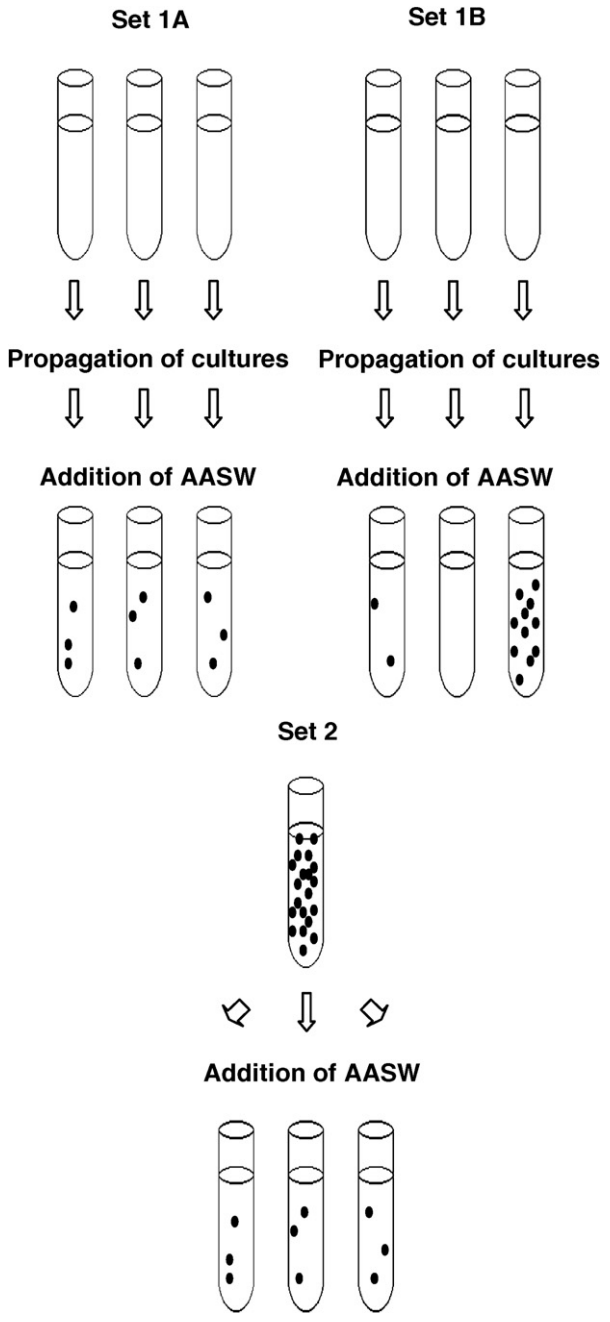

Fig. 1. Schematic diagram of possible results obtained in the experiment (modified from the classic Luria and Delbrück fluctuation analysis). Set 1: different cultures of *Dictyosphaerium chlorelloides* (each started from a small inoculum, $N_0 = 10^2$ cells) were propagated under non-selective conditions (i.e. BG-11 medium) until a very high cell density was reached ($N_t = 1.04 \times 10^5$ cells), and then transferred to the selective agent (i.e. AASW). If resistant cells arose during the exposure to AASW (physiological adaptation or post-adaptive mutations), the number of resistant cells in all the cultures must be similar (set 1A). If resistant cells arose by rare mutations occurring in the period of the propagation of cultures (i.e. before exposure to AASW) the number of resistant cells in all the cultures must be different (set 1B). In the figure, one mutational event occurred late in the propagation of culture 1 (therefore, the density of AASW-resistant cells found is low) and early in the propagation of culture 3 (thus, density of AASW-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. Set 2: Different replicates from the same parental culture sampling the variance of the parental population are used as an experimental control. In this case, the number of resistant cells in all the cultures must be similar.

Set 2 is the experimental control of the fluctuation analysis (Fig. 1). Either way resistance appears, variance is expected to be low, because set 2 samples the variance of the parental population. If a different variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells appeared by rare spontaneous mutations that occurred before exposure to the selective agent. If a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells appeared after exposure to the selective agent (i.e. by acclimation).

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. Due to the methodological limitations imposed by a fluctuation analysis using liquid cultures of algae, the proportion of cultures of set 1 showing no resistant mutants (P_0 estimator; Luria and Delbrück, 1943) after AASW exposure was the parameter used to calculate the mutation rate (μ) as follows:

$$\mu = -\mathrm{Log_e} P_0/(N_t - N_0) \quad (3)$$

2.5. Mutation–selection equilibrium

If the AASW-sensitive → AASW-resistance mutation is recurrent, and the mutant allele is detrimental to fitness in the absence of AASW, then new resistant alleles arise in each generation by mutation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance. As a result, at any one time there will be a certain number of resistant cells that are not yet eliminated. The average number of such mutants will be determined by the balance between μ and the rate of selective elimination (s), in accordance with Kimura and Maruyama (1966):

$$q = \mu/(\mu + s) \quad (4)$$

where q is the frequency of the AASW-resistant allele and s is the coefficient of selection against this resistant allele, calculated as follows:

$$s = 1 - (m^r_{AASW}/m^s_{AASW}) \quad (5)$$

where m^r_{AASW} and m^s_{AASW} are the fitness of AASW-resistant and AASW-sensitive cells measured in non-selective conditions (i.e. in BG-11 culture medium), respectively.

3. Results

The phytoplankton community of AASW in May 2006 was composed by diatoms, chlorophyceans and euglenophyceans (Table 1); cyanobacteria were not detected.

The toxic effect of AASW on Φ_{PSII} and m from *D. chlorelloides* wild-type strain cells was very dramatic: Φ_{PSII} was completely inhibited (100% inhibition with respect to unexposed controls) at all the time points tested (from 1 to 72 h). Similarly, a value of $m = 0$ was found in AASW exposed cultures; in contrast, the Malthusian fitness value in the controls (m^s_{AASW}) was 0.600 ± 0.011 doublings d^{-1} ($n = 3$).

Table 1
Most abundant microalgal species (cells ml^{-1} in single-celled species or filaments ml^{-1} in filamentous species, mean ± SD, $n = 5$) detected in AASW in May 2006

Phylum	Species	Abundance
Bacillariophyta	*Nitzschia acicularis* (Kützing) W. Smith	438 ± 41
	Navicula exigua Gregory	92 ± 38
	Fragilaria sp.	78 ± 34
Streptophyta	*Spirogyra* sp.	415 ± 65
Chlorophyta	*Chlorella* sp.	310 ± 56
	Oocystis borgei J. Snow	40 ± 3
	Chlamydomonas sp.	17 ± 11
	Scenedesmus arcuatus Lemmermann	8 ± 4
Euglenophyta	*Euglena* sp.	19 ± 11
	Trachelomonas granulosa Playfair	7 ± 3

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Table 2
Fluctuation analysis of AASW-sensitive → AASW-resistance transformation in *Dictyosphaerium chlorelloides*

	Set 1	Set 2
No. of replicate cultures	95	20
No. of cultures containing the following no. of AASW resistant cells:		
0	85	0
$1–10^3$	4	0
$10^3–10^4$	3	0
$>10^4$	3	20
Variance/mean (of the no. of resistant cells per replicate)	153.1	0.9
μ (mutants per cell per generation)	1.1×10^{-6}	

When conducting the fluctuation analysis, first of all the cell density was drastically reduced in each experimental culture of sets 1 and 2 due to massive destruction of sensitive cells. However, after further incubation for several weeks, some cultures increased in density again, apparently due to growth of a AASW-resistant variant. Ten of the initial 95 cultures in the set 1 recovered after 80 d under AASW exposure (Table 2). By contrast, every set 2 culture recovered, and AASW-resistant cells were detected in all cultures (Table 2). A high fluctuation was detected in set 1 cultures; the variance significantly exceeded the mean (variance/mean $\gg 1$; $P < 0.001$, using χ^2 as a test of goodness of fit). In contrast, almost null fluctuation was observed in set 2 (variance/mean ≈ 1, consistent with Poisson variability; $P < 0.05$, using χ^2 as a test of goodness of fit) (Table 2). Consequently, the high fluctuation found in set 1 cultures should be due to processes other than sampling error, and it could be inferred that AASW-resistant cells arose prior to AASW exposure by rare, spontaneous mutations rather than by specific adaptation (i.e. acclimation) during AASW exposure. The estimated μ value for AASW-sensitive → AASW-resistance transformation in *D. chlorelloides* was of 1.1×10^{-6} mutants per cell per generation (Table 2).

AASW-resistant mutants growing in BG-11 medium (i.e. in absence of the selective agent) showed a Malthusian fitness value (m^r_{AASW}) of 0.048 ± 0.003 doublings d^{-1}. The overall mean values of m^s_{AASW} and m^r_{AASW} measured in non-selective conditions (0.600 and 0.048 doublings d^{-1}, respectively) were used to estimate the coefficient of selection of AASW-resistant mutants ($s = 0.92$). By using the values of μ and s, the frequency of AASW-resistant allele was calculated: *c.* 12 *D. chlorelloides* AASW-resistant mutants per 10^7 cells as the consequence of the balance between mutation and selection.

4. Discussion

An astonishing diversity of eukaryotic microalgae, closely related to neutrophilic lineages, inhabit the acidic and metal-rich waters from Aguas Agrias Stream (see Table 1). This result is comparable to algal sampling carried out in the similar environment from Spain's Rio Tinto (also located in the Iberian Pyrite Belt), where green algae and euglenophytes closely related to their corresponding neutrophilic lineages have been detected both by molecular, cultivation-independent techniques (Amaral Zettler et al., 2002) and direct isolation (Costas et al., 2007) samplings. However, the catastrophic effect of AASW on *m* and Φ_{PSII} of *D. chlorelloides* isolated from non-extreme waters suggests that the survival of this species in AASW could only be achieved by some kind of adaptation. The key to understanding adaptation of this species to the extreme environment of the AASW is to examine the rare algal variants that occur after the massive destruction of the sensitive cells by AASW.

The fluctuation analysis allows us to distinguish between cells that became resistant to AASW from acquired specific resistance (i.e. physiological adaptation) and cells resistant due to spontaneous mutations that occur randomly during propagation of organisms prior to exposure to AASW (Luria and Delbrück, 1943). The large fluctuation in number of AASW-resistant cells observed in set 1 experiments, in contrast with the no fluctuation in set 2, unequivocally demonstrates that resistant cells arose by rare spontaneous mutation (AASW did not stimulate the appearance of resistant cells at all) and not through direct and specific adaptation in response to AASW. It should be noted that it is impossible to detect post-selective mutations (adaptive mutations) if the rate of these kinds of mutations for AASW-resistance were smaller than 10^{-8} using fluctuation analysis. Despite this, the rapid lethal effect of AASW seems unlikely to allow the appearance of adaptive mutations, which are observed in non-proliferating microbial populations after being incubated on non-lethal selective medium plates (Cairns et al., 1988; Foster, 2000).

Evolutionary biology is often caricatured as a strictly descriptive science. However, here we show that evolutionary change in microalgae can be studied on short time scales with a robust experimental approach such as the Luria–Delbrück fluctuation analysis. Moreover, we followed a similar approach to study adaptation to stressful lethal conditions (originated by anthropogenic contamination, or extreme natural environments) in cyanobacteria and microalgae; in all the cases, the adaptation was achieved as the result of a rare event: the spontaneous mutation from sensitivity to resistance (with frequencies of 1 resistant mutant cell per 10^5–10^7 wild-type cells) that occurs randomly prior to the cells coming into contact with the selective agent (Costas et al., 2001; López-Rodas et al., 2001; Baos et al., 2002; García-Villada et al., 2002, 2004; Flores-Moya et al., 2005; Costas et al., 2007; López-Rodas et al., 2007, 2008). If it is assumed that this is true under any stressful lethal conditions, it could be hypothesized that future changes in the biosphere, as consequence of Global Change, are not a compromise for survival of cyanobacteria and microalgae because they can develop genetic adaptation and, simultaneously, their populations are composed by countless cells. Thus, the probability to survive to sudden stressful changes could be enough high to avoid any future environmental crisis, but with lower values of productivity (Costas et al., 2007; López-Rodas et al., 2007), and the arising of morphological novelties (López-Rodas et al., 2006, 2007). The only vital limit that could not be avoided is acidification of waters in the case of cyanobacteria. These organisms have been reported to be absent from moderately or extremely acidic environments (Brock, 1973; Knoll and Bauld, 1989; Albertano, 1995; Gimmler, 2001), and a pH limit of 4.8 was postulated for proliferation of cyanobacteria (Brock, 1973). In fact, we did not detect cyanobacteria in the phytoplankton sampling of AASW. Moreover, in previous fluctuation experiments with the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann, using as selective agent acid waters from Spain's Rio Tinto (Costas et al., 2007), adaptation was not detected.

Summarizing, eukaryotes rarely flourish in extreme environments; however, we propose that these environments could be colonized by mesophilic organisms inhabiting non-extreme environments by neo-Darwinian adaptive evolution.

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CAPITULO IV

APLICACIÓN: BIOSENSORES MICROALGALES SENSIBLES Y ESPECÍFICOS

Introducción

En este último capítulo se exponen los trabajos referentes a la aplicación de microorganismos fotosintéticos sensibles y resistentes en la construcción de biosensores para la detección de contaminantes ambientales. La base de los biosensores basados en estos microorganismos es la sensibilidad que presentan a contaminantes disueltos en agua. Cuando se exponen a contaminantes ambientales, determinados parámetros biológicos relacionados con la fotosíntesis disminuyen. En general los parámetros más ampliamente utilizados son la producción de oxigeno y la fluorescencia del fotosistema II.

Las medidas simultáneas de la actividad fotosintética en una cepa sensible y una específicamente resistente al tóxico que queremos detectar da especificidad al biosensor microalgal. Mientras que en las microalgas sensibles los parámetros fotosintéticos se ven rápidamente afectados por la presencia de bajas concentraciones de cualquier tóxico, en las específicamente resistentes son necesarias concentraciones muy altas y tiempos continuados de exposición al tóxico (al que son resistentes) para disminuir su rendimiento fotosintético (Altamirano et al., 2001). Sin embargo con la presencia de cualquier otro tóxico los parámetros fotosintéticos disminuyen prácticamente por igual en los genotipos sensibles y resistentes.

Este capítulo lo ilustramos con la utilización de dos cloroficeas eucariotas como biosensores frente a un amonio cuaternario y a cromo hexavalente.

El primer trabajo de este capítulo es un ejemplo de la gran sensibilidad que el fitoplancton eucariota tiene frente a contaminantes ambientales. Estudiamos la respuesta de diferentes parámetros fotosintéticos de dos

clorofíceas (*Scenedesmus intermedius* y *Dictyosphaerium chlorelloides*) a la exposición a un amonio cuaternario ampliamente utilizado en la industria textil y como biocida. Los resultados indican que las dos familias de clorofíceas tienen distinta sensibilidad frente a este compuesto, pero que en ambas la producción de oxígeno y el rendimiento cuántico de la fotosíntesis se ven afectados a bajas concentraciones del tóxico.

El segundo trabajo demuestra cómo las células resistentes pueden proporcionar especificidad al biosensor microalgal. Los resultados indican que las microalgas resistentes al Cromo (VI) aisladas a través de un análisis de fluctuación, mantienen mejores parámetros fotosintéticos en presencia de Cr(VI) que células sensibles de la misma especie. Las diferencias en la respuesta al Cr(VI) entre microalgas sensibles y resistentes, tanto en rendimiento cuántico de la fotosíntesis como en producción de oxígeno, pueden utilizarse para la construcción de un biosensor sensible y específico a este metal pesado.

4.4.1 Inhibition of growth and photosynthesis of selected green microalgae as tools to evaluate toxicity of dodecylethyldimethyl-ammonium bromide.

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Inhibition of growth and photosynthesis of selected green microalgae as tools to evaluate toxicity of dodecylethyldimethyl-ammonium bromide

S. Sánchez-Fortún · F. Marvá · A. D'ors · E. Costas

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Abstract The effect of dodecylethyldimethyl-ammonium bromide (DEAB), a quaternary ammonium, compound widely used as disinfectant, on phytoplankton of inland water systems was analysed by using an experimental model. A toxicity test was based on inhibition of photosynthesis performances (effective quantum yield from photosystem II, Φ_{PSII} and O_2 production) of the phytoplanktonic species *Scenedesmus intermedius* and *Dictiosphaerium chlorelloides* (Chlorophyceae) under growing doses of DEAB. A concentration-dependent toxic response was obtained in both parameters analysed. In addition, this response was almost immediate. Consequently, the measurement of both parameters (Φ_{PSII} and O_2 production) allows to assess DEAB toxicity with higher standards of precision and repeatability. We propose that this procedure could be used to detect presence of quaternary ammonium pollutants in freshwater.

Keywords Toxicity · *Scenedesmus intermedius* · *Dictyosphaerium chlorelloides* · Dodecylethyldimethyl-ammonium bromide · Photosynthesis performance · Disinfectants

S. Sánchez-Fortún (✉) · A. D'ors
Department of Toxicology and Pharmacology, School of Veterinary Sciences, Complutense University, Avda. Puerta de Hierro, s/n, Madrid 28040, Spain
e-mail: fortun@vet.ucm.es

F. Marvá · E. Costas
Department of Genetics, School of Veterinary Sciences, Complutense University, Avda. Puerta de Hierro, s/n, Madrid 28040, Spain

Introduction

Algae are the major contributors to primary production in aquatic ecosystems (Falkowski and Raven 1997). For this reason, they are used in environmental studies for assessing the relative toxicity of various chemicals and/or waste discharges (ASTM 1994; ISO 1987; USEPA 1996; OECD 1984). Currently, the batch technique is adopted by most protocols of standard algal test for regulatory purposes. The conventional response endpoints applied in algal toxicity test include final yield (estimated as biomass or cell densities), growth rate, chlorophyll concentration and total biovolume. Analysis of experimental results from International Standards Organization's ring tests (Hanstveit and Oldersma 1981; Hanstveit 1982) shows that EC_{50} values based on final yield (based on biomass measurements) were generally lower and could differ by a factor of 2 compared with those based on growth rate. On the other hand, algal growth rate has been considered as a more meaningful and consistent parameter than total cell number or biomass as expressed, for instance, by cell volume. Moreover, toxicity data based on growth rate were found to provide greater reproducibility and, therefore, it has been proposed to use this approach in order to compare test results from different laboratories (Nyholm 1985; OECD 2000). However, growth rate measurements using classic procedures (haemocytometers, settling chambers) are very hard to obtain when compared to automatic measures of photosynthesis performances. Thus, inhibition of photosynthetic performance could also be used as a tool to evaluate the presence of pollutants in aquatic ecosystems.

Dodecylethyldimethyl-ammonium bromide (DEAB) is a quaternary ammonium compound (QAT) with special commercial significance due to its relevant properties: surface activity, adsorption onto negatively charged solids

and biocide activity (Garcia et al. 2001). Because of these qualities QATs have a widespread use as detergents, antistatic, wetting and softening agents, biocides, germicides, deodorizers and emulsifiers. In quantitative terms, this utilization translates into mostly fabric softeners (66%), coated clays (16%) and biocides (8%) (Cross 1994). Their application in Europe and the United States industry exceeds 32,000 tons each (Giolando et al. 1995). After using, the active ingredients of fabric softeners are discharged predominantly into sewers as part of household wastewaters. This sewage is processed at municipal wastewater treatment plants, where QATs may affect the activated sludge. The treated effluents are finally discharged into surface waters, being eliminated in sewage by forming a neutral ion pair with anionic surfactants and other anionic compounds. The biodegradation of most QATs is poor and their presence may reduce the biodegradation of linear alkylbenzene sulfonates (Kümmurer et al. 1997). Most disinfectants containing QATs are used on surfaces and it is unclear the percentage of the active compounds that persists in the effluent (Kümmurer et al. 1997).

The goals of this study are to determine the acute toxicity of DEAB on the green microalgae species *Scenedesmus intermedius* Chodat and *Dictyosphaerium chlorelloides* (Naum.) Kom. and Perm., by determining the values of mean effective concentration (EC_{50}) for the growth based on algal cell number as well as on the photosynthetic quantum yield from photosystem II (Genty et al. 1989). Growth and phothosynthesis performance of these algal species are widely studied previously (Boyle 1984; OECD 1984; Girling et al. 2000; Altamirano et al. 2004).

Material and methods

Test chemical

Dodecylethyldimethyl-ammonium bromide was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The purity of this QAT was >97%. It was used dissolved in distilled water.

Experimental organisms

Haploid vegetative cells of *S. intermedius* and *D. chlorelloides* (algal culture collection of Genetics, Faculty of Veterinary, Complutense University, Madrid, Spain) were grown axenically in cell-culture flasks with 20 ml of BG-11 medium (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), at 20°C and a photon irradiance of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, in a 16:8 h light-dark photoperiod. Cells were maintained in mid-log exponential growth by serial cell transfers to fresh medium. Prior to the experiments, the culture cells were re-cloned (by isolating a single cell) to assure genetic homogeneity in all the cultures.

Inoculations were taken from pre-cultures set-up three days before the experiment and replicated under the same conditions. The initial cell densities were adjusted to 10^4 cells ml^{-1}.

Toxicity tests

To determine the inhibition of algal growth and the decrease of Φ_{PSII}, the toxicity tests were performed in 13 ml polystyrene sterile tubes (Sarstedt Co., Nümbrecht, Germany) filled with BG-11 medium. Previous studies determined the suitability in the use of polystyrene sterile tubes for these toxicity assays, assuring that neither chemicals nor microalgal cells adhered to the tube walls (Costas et al. 2001; García-Villada et al. 2004). The water used for media preparation was of ultrapure quality, distilled by means of Milli-Q device (Millipore, Bedford, MA, USA). DEAB was added to culture medium in the appropriate amount to achieve final concentration, determined in preliminary experiments for each tested organism. Four DEAB concentrations, as well as four controls, were established and tested. In addition, as an internal quality control, the bioassays were also performed on the reference chemical potassium dichromate ($K_2Cr_2O_7$), and each assay was repeated eight times ($n = 8$). Both control and test tubes were inoculated with 10^4 cells ml^{-1} as initial concentration.

All the cultures (control and treatments) were incubated for 72 h at 20°C in a thermostatically controlled chamber (Velp Scientifica, Usmate, Italy) at 60 μmol m^{-2} s^{-1} to ensure exponential algal growth. Every 24 h the algal density was quantified under the light microscope with a Neubauer's chamber.

The concentration causing 50% growth inhibition of algae was determined by using light microscope and Neubauer's chamber, while the quantity causing 50% inhibition of photosynthetic yield was obtained by means of the dual-channel pulse amplitude modulation (PAM) chlorophyll fluorometer (ToxY-PAM, Heinz Walz GmbH, Germany). These parameters were used as toxic endpoints and expressed as EC_{50}.

Toxic variability in time-dependent assays

It has been studied the toxic effect dependent on time induced by DEAB on exposed algal cultures. For this, it

has been used eight replicates of controls and eight replicates treated with the EC_{50} concentration, performing measurements during 72 h. Under these conditions, photosynthetic O_2 production was analysed.

Dissolved O_2 was measured in a 1 ml reaction chamber from an Oxytherm system (Hansatech, Norfolk, UK) which employs a Clark-type oxygen electrode. A small stir bar maintains the cells in suspension, and a Peltier heating block maintains the temperature at 21°C.

Data analysis

The 72 h EC_{50} values were calculated according to the 'area under the curve' method prescribed by the ISO (1989). EC_{50}-values were determined by nonlinear regression analysis, and all the results are presented as mean ± SD. The photosynthetic response was monitored on the software package ToxyWin v1.14 (Heinz Walz GmbH, Germany), and the results are presented as mean ± SD of inhibition percentage respect to control. The data from dissolved O_2 were exported to a computerized chart recorder (Oxigraph v1.01, Hansatech, Norfolk, UK). Statistical analysis was performed using the computer software package GraphPad Prism v4.0 (Graph-Pad Software Inc., USA). The experimental data were analysed by the one-way analysis of variance and the differences were considered significant at $P < 0.05$.

Results

Algal growth and acute toxicity

Dodecylethyldimethyl-ammonium bromide induced acute toxicity in the green algae *S. intermedius* and *D. chlorelloides* (Table 1), being EC_{50} values for growth and photosynthetic inhibition higher in *S. intermedius* (2.63 and 2.58 mg l^{-1}, respectively) than *D. chlorelloides* (0.96 and 1.57 mg l^{-1}, respectively) ($P < 0.05$). The dose–response curves reflected a correlation between DEAB concentration and the inhibition of growth and photosynthesis in *S. intermedius* (Fig. 1) and *D. chlorelloides* (Fig. 2).

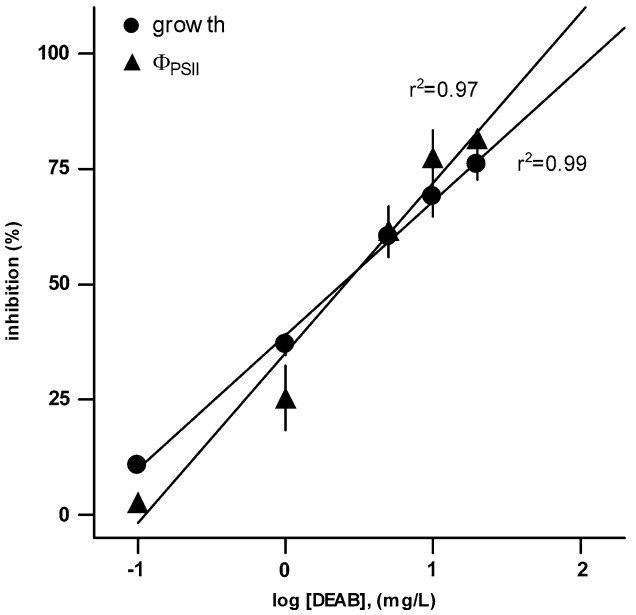

Fig. 1 Dose–response relationship of DEAB to *S. intermedius* in growth rate (●) and Φ_{PSII} (▲). Points represent means with vertical lines showing SE mean ($n = 8$)

Effect of DEAB on in situ measurements of Φ_{PSII} photochemical efficiency

The assays with *S. intermedius* using the calculated EC_{50} (2.63 and 2.58) have shown that, as whole, a reduction of effective Φ_{PSII} quantum yield induced by DEAB was time-dependent effect and proportional to the growth inhibition (Fig. 3). However, analysing the behaviour of this cells at different stages of the experiment, it has been found a significant inhibition of the photosynthetic capacity at the beginning of the exposure, having observed an inhibition of about 24% after the first 5 min, while the growth inhibition has not suffered any modification. In the following 12 h after exposure, it has been achieving gradually a balance between growth and photosynthesis inhibition.

When the photochemical efficiency of Φ_{PSII} in *D. chlorelloides* was analysed, the results have indicated that the inhibition of Φ_{PSII} was also proportional to those obtained in growth inhibition analysis (Fig. 4). However, *D. chlorelloides* has shown a significant increment of the photosynthetic capacity, in the order of 40% at 30 min

Table 1 Comparison of 72 h EC_{50} values and associated 95% confidence limits (CL), expressed in mg l^{-1}, of DEAB to *S. intermedius* and *D. chlorelloides*

Species	*n*	Growth inhibition		Photosynthesis inhibition	
		$EC_{50(72)}$	CL (95%)	$EC_{50(72)}$	CL (95%)
Scenedesmus intermedius	8	2.63	(2.17–3.33)	2.58	(1.44–8.33)
Dictiosphaerium chlorelloides	8	0.96	(0.85–1.09)	1.57	(1.23–2.02)

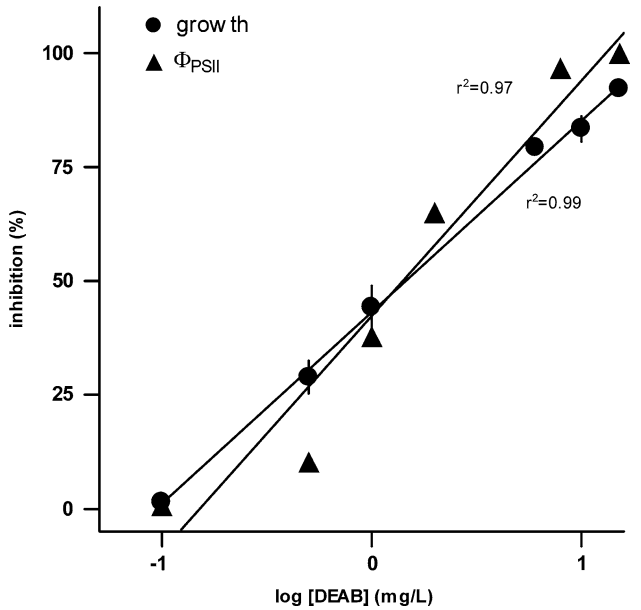

Fig. 2 Dose–response relationship of DEAB to *D. chlorelloides* in growth rate (●) and Φ_{PSII} (▲). Points represent means with vertical lines showing SE mean ($n = 8$)

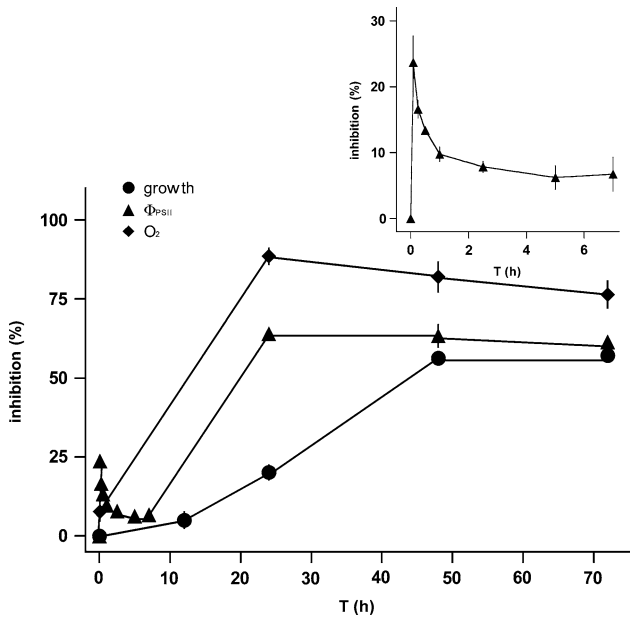

Fig. 3 The inhibitory time-dependent effects on the Φ_{PSII} (▲), photosynthetic O_2 production (◆) and growth rate (●) of *S. intermedius* exposed to $EC_{50}(72)$ concentration of DEAB. The inset represents the effect of Φ_{PSII} in the time window from 0 to 7 h. Points represent means with vertical lines showing SE mean ($n = 8$)

after initial exposure compared to *S. intermedius*, while the growth inhibition has not suffered any modification. After, the inhibition values of Φ_{PSII} were parallel to those obtained in the growth inhibition assays.

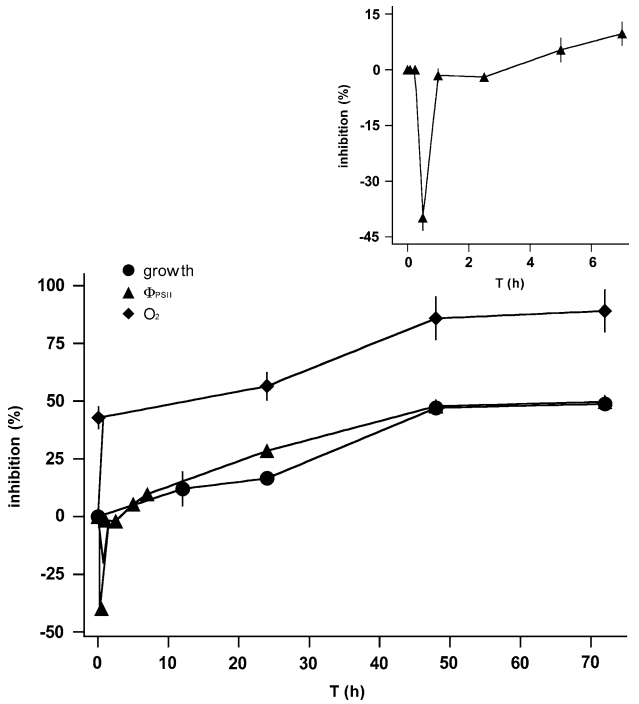

Fig. 4 The inhibitory time-dependent effects on the Φ_{PSII} (▲), photosynthetic O_2 production (◆) and growth rate (●) osf *D. chlorelloides* exposed to $EC_{50}(72)$ concentration of DEAB. The inset represents the effect of Φ_{PSII} in the time window from 0 to 7 h. Points represent means with vertical lines showing SE mean ($n = 8$)

Effect of DEAB on dissolved oxygen

In both kinetic assays of microalgae there has been a significant inhibition of the dissolved oxygen from the start of the experiments. However, the initial inhibitory response to exposure (15 min) has been higher in *D. chlorelloides* (42.82%) than in *S. intermedius* (7.73%) ($P < 0.05$). At the end of the kinetic assays, in both cases there has been a significant decrease of the dissolved oxygen, showing levels of 80 and 90% for *S. intermedius* and *D. chlorelloides*, respectively (Figs. 3 and 4).

Discussion

In conformity with classification UE Directive 92/32/EEC (UE 1992), DEAB appeared to be highly toxic for algae. We have demonstrated that DEAB is toxic agent for *S. intermedius* and *D. chlorelloides*. Active substances are easily absorbed by the cationic surface of the algae cells (Waters 1982) and it has been supposed that absorption and reaction of algae cells was the reason for the high-toxic effect of these pollutants on them. As it has been pointed in literature, algae are sensitive to the presence of detergents (Issa and Ismail 1995) and they could be good indicators of surface water pollution by surfactants.

The data, describing the effects of QUATs on algae, found in literature, are few and quite old; however, all of them are in agreement with the idea that the cationic and amphoteric surfactants are good inhibitors of growth, just like some anionic and non-ionic compounds (Nyberg 1988). Similar results were obtained by Singh et al. (2002) when tested seven surfactants for toxicity on six freshwater microbes; likewise, other studies have demonstrated the high-toxicity level induced by QUATS on aquatic organisms (Utsunomiya el al. 1997; Verge et al. 2000; Temart et al. 2001).

Our results have clearly shown that DEAB significantly inhibited the Φ_{PSII} photochemical activity in *S. intermedius* and *D. chlorelloides*. These results are in agreement with those obtained by other authors, and have demonstrated that chlorophyll fluorescence quenching analysis has been proven as a rapid, precise, non-invasive and reliable method to assess photosynthetic performance under changing environmental conditions (Krause and Weis 1991; Schreiber et al. 1994). PAM fluorometer may be an useful tool in the field of toxicology when the assays include microalgae, since it allow us to locate the target sites of cells induced by agents in stressed environments.

With a Clark-type oxygen electrode, we have obtained the O_2-production activity of the algae in presence of DBA by recording the oxygen produced in medium. When the solutions containing DBA were tested, a continuous and reproducible decrease in the signal was observed. The inhibitory effect induced by this compound on photosynthetic activity was similar to those obtained by other authors dealing in both inorganic (Takamura et al. 1990; Pandard et al. 1993) and organic pollutants (Peterson et al. 1994; Carrasco and Sabater 1997).

In conclusion, the results of this study have shown that DEAB acts as a sensitive and rapid agent inhibiting the relative quantum yield of chlorophyll fluorescence of Φ_{PSII} and has diminished the oxygen production in both *S. intermedius* and *D. chlorelloides*. Our results have demonstrated that physiological tools, such as analysis of the relative quantum yield of chlorophyll fluorescence by PAM fluorimeter, can be very fast and precise useful in detecting biocides toxicity, such as that caused by DEAB, at an early stage.

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4.4.2. Resistance of phytoplankton to chromium contamination: physiological acclimation *versus* genetic adaptation

Resistance of Phytoplankton to Chromium Contamination: Physiological acclimation *versus* Genetic Adaptation.

S. Sánchez-Fortún[1], F. Marvá[2], M. Rouco[2], D. Haigh-Florez[3] , V. López-Rodas[2] and E. Costas[2]*

[1]Dpto. Toxicología y Farmacología and [2]Genética. Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain.

[3]Dpto. Química Organica. Facultad de Quimicas, Universidad Complutense, E-28040 Madrid, Spain.

*Corresponding author:

E. Costas

Tel./Fax: +34 913 94 37 69

Email: ecostas@vet.ucm.es

Abstract

Adaptation of microalgae to resist the hexavalent chromium (a serious pollutant of inland water systems) was analysed by using an experimental model with *Dictyosphaerium chlorelloides* (Chlorophyceae). Growth and photosynthetic performance of algal cells were inhibited at 25 mg/L hexavalent chromium. However, after further incubation in chromium during several moths, occasionally some rare cells resistant to the heavy metal were found. A fluctuation analysis was carried out to distinguish between resistant cells arising from rare spontaneous mutations and resistant cells arising from physiological acclimation and other mechanisms of adaptation. Resistant cells arose only by spontaneous mutations prior to the addition of chromium, with rate $5.3x\ 10^{-5}$ mutants per cell division. Chromium-resistant mutants have diminished growth rate and photosynthesis performances. However, they are maintained in uncontaminated waters as the result of the balance between new resistant cells arising from spontaneous mutation and cells eliminated by selection at a frequency of the Cr^r allele was in $4.2x10^{-4}$. Under a practical point of view, the use of both chromium sensitive and chromium resistant genotypes could allow a specific algal biosensor for chromium.

Key words: Adaptation; Chromium; microalgae; mutation; toxicity.

Abreviations and notations

Cr^r	Cr(VI)-resistant cells
Cr^s	Cr(VI)-sensitive cells
Cr(VI)	Hexavalent chromium
F'_m	Maximum fluorescence of light-adapted cells
F_t	Steady-state fluorescence of light-adapted cells
m	Malthusian fitness parameter
$m_S{}^r$	Malthusian fitness parameter from Cr(VI)-resistant cells
$m_S{}^s$	Malthusian fitness parameter from Cr(VI)-sensitive cells
N_0	No. of cells at the start of the experiment
N_t	No. of cells at the end of the experiment
P_0	Proportion of cultures without Cr(VI)-resistant cells in the set 1
q	Frequency of Cr(VI)-resistant alleles in non Cr(VI)-exposed populations
s	Coefficient of selection
Φ_{PSII}	Effective quantum yield
μ	Mutation rate

Introduction

Modern materials of water pollution is altering aquatic biosphere-level processes and causing biodiversity crisis, Woodruff (2001). For example, heavy metals are often spilled out from industrial sources into the environment. The most famous example about water supply contaminated with chromium and its repercussions on public health was reflected in the film titled “Erin Brockovich”, based on the lawsuit filed by the town of Hinkley (California, USA) against Pacific Gas and Electric Company (PG&E). The residents of Hinkley attributed many of their illnesses including cancer to chromium contamination produced by PG&E, and finally this company settled the dispute with $333 million as compensation for damages.

Chromium (Cr) is extensively used in industry and it is a serious pollutant of water, Khasim et al. (1989), Armienta-Hernández and Rodríguez-Castillo (1995). Their natural concentration in fresh waters varies from 0.1 to 0.5 ppm but this can be altered as a consequence of industrial waste, reaching 80 ppm becoming a grave threat to biota and environment, De Filippis (1994). The biological effects of chromium, which exists in hexavalent (VI) and trivalent (III) form, depend on its oxidation state. The majority of Cr (VI) found in nature comes from industrial emissions and it is known that it is the most toxic form to plant, animal and microorganism species that inhabit aquatic environments, Wong and Trevors (1988), Katz and Salem (1993).

Because algae and cyanobacteria are the principal primary producers of aquatic ecosystems, Kirk (1994), Falkowski and Raven (1997), the tolerance of these organisms to contaminated environments is very relevant from an ecological point of view. Usually, the phytoplankton populations are tolerant to the presence of heavy metals when previously they have contacted them, Corradi et al. (1995) Gorbi et al. (1996),

101 Abd-El-Momem et al. (1998). In this sense, we have analysed the possible implications
102 of the use of copper as algaecide in a cyanobacterial population (*Microcystis*
103 *aeruginosa*), Garcia-Villada et al. (2004), Costas and Lopez-Rodas (2006) and the effect
104 of a serious toxic spill of acid wastes rich in heavy metals (Aznalcóllar mine) in an
105 eukaryotic population (*Scenedesmus intermedius*), Baos et al. (2002). We have reported
106 also the negative consequences of their adaptation, appearance of resistant genotypes
107 and reduction of primary production and biomass.

108 Although little is known about the mechanisms to propitiate tolerance of
109 microalgae to Cr contamination, they may survive as a result of two different processes:
110 i) physiological adaptation (acclimation or tolerance), usually resulting from
111 modifications of gene expression and ii) adaptation by natural selection if mutations
112 provide the appropriate genetic variability revealing the existence of resistant genotypes
113 (neo-Darwinist point of view), Sniegowski and Lenski (1995), Sniegowski (2005).
114 Fluctuation analysis, Luria and Delbrück (1943), is an experimental model to
115 discriminate between adaptation by spontaneous mutation (pre-adaptive mechanisms)
116 and short-term phenotypic consequences of environment changes by directed mutation
117 (post-adaptive mechanisms).

118 The aim of this work was: (i) to assess the effect of Cr(VI) on the growth rate
119 and photosynthetic performance of microalgae, (ii) to determine if some kind of Cr(VI)-
120 resistant cells could arise when microalgae are exposed to a lethal concentration of
121 Cr(VI), (iii) to discriminate between resistan cells arising by physiological acclimation
122 in response to Cr(VI) and Cr(VI)-resistant cells arising by spontaneous mutations
123 occurring randomly before Cr(VI) exposure, and (iv) to estimate the mutation rate from
124 Cr(VI) sensitivity to Cr(VI) resistance. For this purpose, we have performed a
125 fluctuation analysis, modified by López-Rodas et al. (2001) and Costas et al. (2001),

126 using Cr(VI) as selective agent. In this way, we have investigated the mechanisms that
127 allow *D. chlorelloides* to withstand the increasing exposure to Cr(VI), demonstrating
128 that the resistant cells arise from rare spontaneous mutations that occur randomly during
129 propagation of *D. chlorelloides* prior to the Cr(VI) exposure. We show the existence of
130 very rapid adaptation of *D. chlorelloides* as a result of pre-selective mutations from
131 sensitive (Cr^s) to resistant chromium (Cr^r) cells.
132
133 **Material and methods**
134
135 ***Experimental organism and culture conditions***
136
137 *Dyctiophaerium chlorelloides*, strain Dc, from the algae culture collection of the
138 Genetics laboratory, Faculty of Veterinary, Complutense University, Madrid, Spain.
139 Cell cultures were grown axenically in culture flasks (Greiner, Bio-One Inc.,
140 Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie,
141 Taufkirchen, Germany), at 20 ºC under continuous light of 60 μmol m^{-2} s^{-1} over the 400-
142 700 nm waveband. Cells were maintained in mid-log exponential growth by serial
143 transfers of a cell inoculum to fresh medium once every two weeks. Prior to the
144 experiments, the cultures were re-cloned (by isolating a single cell) to avoid including
145 any previous spontaneous mutants accumulated in the cultures.
146
147
148 ***Effect of Cr(VI) on the growth rate and photosynthetic performance***
149

Hexavalent chromium oxide "Cr(VI)" (98% purity from Sigma-Aldrich Chemical Co. St. Louis, MO, USA) was used dissolved in distilled water. First of all, the toxic effect of Cr(VI) on the growth rate of *D. chlorelloides* was tested using 13 mL polystyrene sterile tubes (Sarstedt Co., Nümbrecht, Germany). No adherence neither chemicals nor microalgae to the tube walls was previously checked. Serial dilutions of Cr(VI) in BG-11 medium were prepared to obtain of 0, 0.1, 0.5, 1, 5 and 10 mg L^{-1}. Eight replicates were inoculated with 10^4 cells from mid-log exponentially growing cell cultures and were used for each dose.

The effect of Cr(VI) was estimated after 72 hours by calculating the acclimated maximal growth rate (m) in mid-log exponentially growing cells, that derives from the equation: $N_t = N_0\, e^{mt}$, where t = 3 d, and N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively. Therefore, m was calculated as: $m = \log_e (N_t / N_0)/t$. Acclimated maximal growth rate (m) is the Malthusian parameter of fitness under conditions of r selection, Crow and Kimura (1970), Spiess (1989). Experiments and controls were counted blind (i.e. the person counting the test did not know the identity of the tested samples) each 24 hours, using a haemocytometer in an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany). The number of samples in each case was determined by using the progressive mean procedure, Williams (1977), which assures a counting error lower than 5%.

The photosynthetic response was measured as effective fluorescence quantum yield (Φ_{PSII}) in triplicates of experiments and controls, using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) at 72 h. Effective quantum yields were calculated as follows: $\Phi_{PSII} = (F'_m - F_t)/ F'_m$, where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively, Schreiber *et al*. (1986).

The concentration causing 50% of growth inhibition in algae was evaluated according to the 'area under the curve' method prescribed by the ISO (ISO, 1982). IC_{50}-values were determined by nonlinear regression analysis, and all the results are expressed as mean ± SD. Data were presented as inhibition percentage of growth rate and Φ_{PSII} with regard to control (unexposed to metal cells).

Statistical analysis was performed using the computer software package GraphPad Prism v 4.0 (Graph-Pad Software Inc., USA).

Ability of sensitive cells to adapt to Cr (VI)

In addition, the ability of sensitive cells from *D. chlorelloides* to acclimate or adapt to Cr (VI) exposure were studied by longer-term cultures (3 months). Massive cultures of *D. chlorelloides* were exposed to a high Cr(VI) concentration (25 mg L^{-1}), equivalent approximately to 2.5 times the 100% inhibitory concentration ($IC_{100(72)}$) obtained in the toxicity assays. Growth rate and photosynthetic yield of resistan cells (surviving cells to lethal concentration of metal) were determined, as previously described.

Fluctuation analysis: from chromium-sensitive to chromium-resistant cells

A modified Luria-Delbrück analysis (1943) was performed as previously described by López-Rodas *et al*. (2001) and Costas *et al*. (2001) to distinguish between resistant cells arising as result of rare spontaneous mutations (occurred prior to Cr(VI)

199 exposure) and cells arising through acquired physiological acclimation (during the
200 exposure to Cr(VI)). In short, two different sets (experimental and control) were
201 prepared. In the set 1 experiment, 105 culture flasks were inoculated with $N_0 = 10^2$ cells
202 (a number small enough to ensure reasonably the absence of pre-existing mutants in the
203 strain). Cultures were allowed to grow until $N_t = 1.13x10^5$ cells and then were
204 supplemented with 25 mg L^{-1} of Cr(VI). In the set 2 control, 30 aliquots of $1.13x10^5$
205 cells from the same parental population were separately transferred to culture flasks
206 containing fresh liquid medium with Cr(VI) at the same concentration as set 1. Cultures
207 were observed for 60 days (to ensure resistant mutant cell could generate enough
208 progeny to be detected). The resistant cells from each culture (both in set 1 and set 2)
209 were counted. The cell count was performed by at least two independent observers.

210 According to Luria and Delbrück (1943), two different results can be found in
211 the set 1 experiment when performing a fluctuation analysis, each of them being
212 interpreted as an independent consequence of two different phenomena of adaptation.
213 The variance in the number of cells per culture would be low if resistant cells arose by
214 physiological acclimation because every cell is likely to have the same chance of
215 developing resistance (flask-to-flask variation would be consistent with the Poisson
216 model). In contrast, if resistant cells have appeared by random preselective mutations
217 that occurs prior to Cr(VI) exposure, variation in the number of resistant cells among
218 cultures is found (i.e. variance/mean >1). The flask-to-flask variation would not be
219 consistent with the Poisson model. Resistant cells arose during the time in which the
220 cultures reached N_t from N_0 cells, before the exposure to Cr(VI).

221 In the set 2, if resistant cells arose by preselective mutations, variance is
222 expected to be low, due to set 2 samples the variance of the parental population. Thus,
223 despite the way of resistant cells appear, the variance among cultures of resistant cells in

set 2 should be similar to the average of resistant cells in set 2 cultures. Since this set constitutes the experimental control for the fluctuation analysis, if a similar variance/mean ratio between set 1 and set 2 is found, it would confirm that resistant cells appear by physiological acclimation, rather than by preselective mutations.

In addition, the fluctuation analysis allows estimating the mutation rate. Due to the methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of set 1 cultures showing no mutant cells after Cr(VI) exposure (P_0 estimator) was the parameter used to calculate the mutation rate (μ). The P_0 estimator, Luria and Delbrück (1943), is defined as follows: $P_0 = e^{-\mu(Nt-N0)}$, where P_0 is the proportion of cultures showing no resistant cells. Therefore, μ was calculated as: $\mu = -\log P_0/(N_t - N_0)$.

Mutation-selection equilibrium

If the $Cr^s \rightarrow Cr^r$ mutation from a normal wild-type, Cr(VI)-sensitive allele to a Cr(VI)-resistant allele is recurrent, and the Cr(VI)-resistant allele is detrimental to fitness in absence of the Cr(VI), then new resistant mutants would arise in each generation, but most of these mutants would be eliminated sooner or later by natural selection, if not by chance, Spiess (1989). At any time there will be a certain number of cells that have not been eliminated yet. The average number of such mutants will be determined by the balance between new resistant mutants and the rate of selective elimination, according to the equation: $(1-q)\mu = qs$ where q is the frequency of the Cr(VI)-resistant allele and s is the coefficient of selection of Cr(VI)-resistant calculated as follows: $s = 1 - (m_S{}^r/m_S{}^s)$, where $m_S{}^r$ and $m_S{}^s$ are the Malthusian fitness of Cr^r and Cr^s cells measured in non-selective conditions, respectively, Crow and Kimura (1970).

Results

Ability of sensitive cells to adapt to Cr (VI)

When *D. chlorelloides* cultures were exposed to a 25 mg L^{-1} Cr(VI), they became clear after some days due to the destruction of the sensitive cells by the toxic effect of Cr(VI). However, after further incubation for three months some cultures became colored again, due to the growth of an algal-variant that was resistant to the effect of Cr(VI). The key to understand adaptation of microalgae to survive in a chromium contaminated environment seems likely to characterize the algal-variant that appears after the massive destruction of the sensitive cells.

Effect of Cr(VI) on growth rate and photosynthetic performance

Cr(VI) showed acute toxicity on sensitive microalgae inhibiting both cell growth and photosynthetic performance (Table 1). The $IC_{50(72)}$ values obtained for growth inhibition were similar than those of $\Phi_{(PSII)}$ quantum yield assays. However, resistant cells (obtained from long-term exposure cultures at high Cr(VI) levels) are in order of 12 times more resistant than those sensitive cells obtained from Cr(VI)-free cultures. The concentration–response relationships to Cr(VI) to growth rate and $\Phi_{(PSII)}$ quantum yield confirm that the resistant algae (obtained from Cr(VI) long-term exposure cultures) have developed a higher tolerance to this heavy metal (Figures 1 and 2)

274

275 ***Fluctuation analysis from chromium-sensitive to chromium-resistant cells***

276

277 The fluctuation analysis has demonstrated that cell density was drastically
278 reduced in each experimental culture of sets 1 and 2 due to destruction of sensitive cells.
279 However, after further incubation for several weeks, some cultures of *D. chlorelloides*
280 have increased in density again, apparently due to the growth of a Cr(VI)-resistant
281 variant. In the case of set 1, a high fluctuation (from 0 to more than 10^5 resistant cells
282 per culture flask) was observed after 60 d of Cr(VI) exposure (Table 2). By contrast,
283 around $1.5x10^5$ Cr(VI)-resistant cells were detected in all cultures from set 2 (Table 2).
284 In addition, a low Cr(VI)-resistant cell fluctuation was observed in set 2 (variance/mean
285 $\approx$ 0.2), indicating that the high fluctuation found in set 1 cultures should be due to
286 processes other than sampling error (Table 2). Like in set 1 cultures, the variance has
287 significantly exceeded the mean (variance/mean $\approx$ 2.5), so it could be inferred that
288 Cr(VI)-resistant cells have arisen by rare, pre-selective spontaneous mutations rather
289 than by physiological acclimation or post-selective mutations appearing in response to
290 Cr(VI). The estimated μ of Cr(VI)-sensitive to Cr(VI)-resistant in *D. chlorelloides* was
291 calculated in $5.3x10^{-5}$ mutants per cell division (Table 2).

292

293

294 ***Mutation-selection equilibrium***

295

296 Isolated Cr^r mutants growing in absence of Cr(VI) have shown a small
297 diminution of growth rates with respect to those found in Cr^s cells. The coefficient of

298 selection of Cr^r mutants was s = 0.125. By using μ and s values, the frequency of
299 Cr(VI)-resistant alleles was estimated in $4.2x10^{-4}$.
300
301 **Discussion**
302
303 The $IC_{50(72)}$ value obtained with Cr(VI) exposures in *Dyctiophaerium*
304 *chlorelloides* cultures demonstrates that phytoplankton is very sensitive to this metal.
305 These results are in agreement with those obtained for other authors in toxicity test
306 assays with marine, Kusk and Nyholm (1992) and freshwater algal species, Rojickova
307 and Marsalek (1999). However, there is a wide information in the literature that
308 demonstrate the high variability in sensitivity of different algal species to the presence
309 of Cr(VI) in aquatic medium, Stauber (1995), Peterson and Stauber (1996). These
310 sensitive differences can be explained by the morphology, cytology, physiology and
311 genetics of the organisms. For example, the difference in sensitivity between
312 *Selenastrum capricornutum* and *Chlorella vulgaris* (more tolerant) to different organic
313 compounds was due to some characteristics such as thick cell wall and the presence of
314 more active enzymes in *Chlorella vulgaris,* Kasai and Hatakeyama (1993).
315 It is difficult to explain why these algae have been able to be less sensitive to
316 chromium exposures. It is known that there are mechanisms involved in the reduction of
317 the metal to Cr(III) and/or formation of bonds between Cr(VI) and excreted organic
318 molecules. Different authors demonstrated that the algal effectiveness in reducing
319 chromium toxicity does not correspond with those exhibited by other aquatic organisms,
320 Corradi et al. (1998), because if they were the only mechanisms involved, we would
321 expect a similar detoxifying effect in all cases. Further experiments with algae
322 suggested another type of interaction. In these experiments, the only cells able to grow

were those which had previously undergone a short-time stress by chromium and were subsequently maintained in the filtrate containing the exudates, before the long-term treatment with the metal. Therefore it seems that the reduction of chromium effects on algae is not only a consequence of an interaction between chromium and exudates, it is also due to an interaction between exudates and algal cells, which may be an algae-specific mechanism to counteract Cr poisoning. This mechanism could be similar to the one suggested by Campbell et al. (1997) for the interaction among chromium, dissolved organic matter, and cell surface. According to this author, the presence of accumulated dissolved organic matter at the cell surface tends to retard metal diffusion, thus affecting its bioavailability. The peculiarity of the interaction of chromium-algae and their own exudates is that it takes place only after the algae have been stressed by a previous contact with the metal.

The gene expression in microorganisms can vary as a consequence of changes in the environment. This shift of gene expression (tolerance) within the genetically defined limits for this microorganism is known as physiological acclimation. *D. chlorelloides* has shown a range of tolerance to Cr(VI) from 0.1 to 0.65 mg L^{-1} in sensitive cells. However, these values increased in 3.16-17.38 mg L^{-1} in resistant cells. The key to understand the adaptation of *D. chlorelloides* to Cr(VI) exposure is analyse the rare algal variants that occur after the massive destruction of the sensitive cells by Cr(VI). The large fluctuation observed in number of Cr(VI)-resistant cells in the set 1 experiment, in contrast to the scant variation in set 2 controls, demonstrates that *D. chlorelloides* resistant cells arose by rare spontaneous mutations and not through physiological acclimation in response to Cr(VI). Hexavalent chromium did not stimulate the appearance of resistant cells since using fluctuation analysis would be difficult to observe post-selective mutations, because these kinds of mutations is usually

348 observed in non-proliferating microbial populations after being incubated on non-lethal
349 selective medium plates, Foster (2000). The rapid lethal effect of Cr(VI) seems unlikely
350 to allow the appearance of adaptive mutations.

351 The rate of mutation from Cr(VI)-sensitivity to Cr(VI)-resistance in *D.*
352 *chlorelloides* (5.3×10^{-5} mutants per cell division) was highest found among our
353 estimated mutation rates for adaptation to heavy metals mixture from the Aznalcóllar
354 mine spill (2.1×10^{-5} mutants per cell division), Baos et al. (2002), La Hedionda Spa
355 waters (2.7×10^{-7} mutants per cell division), Flores-Moya et al. (2005), Rio Tinto
356 (1.4×10^{-6} mutants per cell division), Costas et al. (2007) or Mynydd Parys (1.6×10^{-6})
357 Lopez-Rodas et al (2008). Resistance to other biocides as glyphosate herbicide
358 (3.6×10^{-7} mutants per cell division) Lopez-Rodas et al. (2007) or formaline (3.6×10^{-6}
359 mutants per cell division) Lopez-Rodas et 2008, also occurs at lower rate. Only
360 mutation rate from TNT sensitivity to TNT resistance occurs at higher rate (1.4×10^{-5}
361 mutants per cell division) Garcia-Villada et al. (2002), Altamirano et al. (2004).
362

363 The presence of Cr(VI)-resistant cells in populations of *D. chlorelloides* is due
364 to rare spontaneous mutation that occur prior Cr(VI) exposure. New resistant mutants
365 arise in each generation, but most of these mutants are eliminated because Cr(VI)-
366 resistant mutants are some detrimental in fitness in absence of chromium contamination.
367 At any one time there will be around 4 resistant mutants per each 10,000 sensitive wild
368 type cells as the result of balance between new resistant cells arising from spontaneous
369 mutation and resistant cells eliminated by natural selection. Taking into account both
370 the relatively high number of Cr(VI)-resistant mutants in unexposed populations and the

371 countless cells comprising algal populations, it could be hypothesized that algal
372 adaptation to chromium has been almost instantaneous.

373 Finally, we tentatively propose that the different response respect to
374 photosynthetic activity observed between sensitive and resistant cells of *D.chlorelloides*
375 in the presence of Cr(VI) could be used to obtain an chromium-specific microalgal
376 biosensors. Hexavalent chromiun and other heavy metals in water are usually analyzed
377 with time-consuming techniques that require laboratory hardware, so that they are not
378 appropriate for in situ, continuous monitoring of those pollutants, Gomez and Callao
379 (2006). Because of this many efforts have done to develop biosensors for continuous
380 monitoring of hexavalent chromium Zlatev et al. (2006). Unfortunately, microalgal
381 biosensors are not specific. However, Altamirano et al (2004) propose a new genetic
382 approach for increasing specificity of microalgal biosensors based on the use of a
383 sensitive genotype (sensitivity) and a resistant mutant (specificity). In this sense, it
384 should be possible to use the differential response of the photosynthetic activity of
385 sensitive and resistant cells of *D. chlorelloides* in the presence of Cr(VI) species for
386 continuous monitoring of such pollutant in water. The biosensor could be based on the
387 use of two different genotypes jointly to detect chromium: i) the sensitive Cr^s to obtain
388 sensitivity, and ii) the resistant Cr^r mutant to obtain specificity.
389
390
391 **Conclusions**
392
393 1. Cr(VI) showed acute toxicity on sensitive microalgae inhibiting both cell growth and
394 photosynthetic performance. However, Cr(VI)-resistant cells arise when microalgae are
395 exposed to a lethal concentration of Cr(VI) during 3 moths. These resistant cells are in

order of 12 times more resistant than those sensitive cells. The different response respect to photosynthetic activity observed in sensitive and resistant cells of *D.chlorelloides* in the presence of Cr(VI) could be used as a microalgal biosensor to detect chromium-contaminated waters.

2. Physiological acclimation is unable to allow resistance of microalgae to high doses of Cr(VI). Additionally, Cr(VI) does not facilitate the occurrence of Cr(VI)-resistant cells; rather it was found that Cr(VI)-resistant cells appears spontaneously by rare mutation under nonselective conditions ($5.3x10^{-5}$ mutants per cell division) prior the incorporation of Cr(VI).

3. Spontaneous pre-selective mutants are enough to ensure the survival of microalgal populations to Cr(VI)-contaminated habitats. At any one time, in a population unexposed to Cr(VI) there will be $2.1x10^{-2}$ resistant mutants as the result of balance between new resistant cells arising from spontaneous mutation and resistant cells eliminated by natural selection. Taking into account both the relatively high number of Cr(VI)-resistant mutants in unexposed populations and the countless cells comprising algal populations, it could be hypothesized that algal adaptation to chromium has been almost instantaneous.

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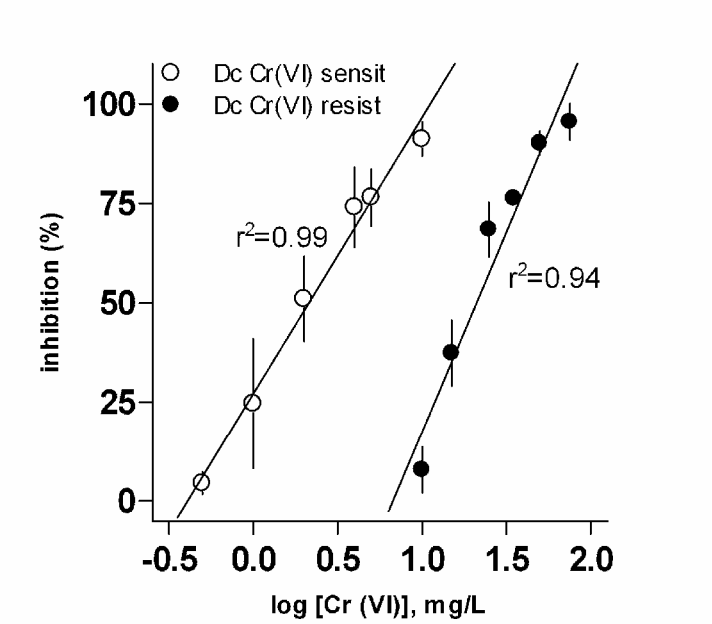
540

Figure 1. Growth inhibition response (a) and $\Phi_{(PSII)}$ inhibition response (b) induced by Cr(VI) exposure in sensitive (○) and resistant (●) *Dictyosphaerium chlorelloides* populations. Points represent means with vertical lines showing SD (n = 8).

A



B

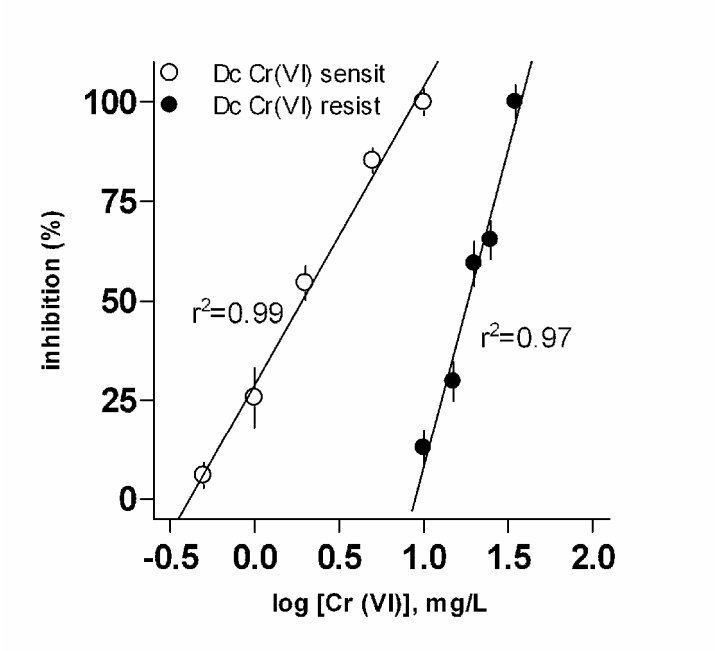


Table 1. Comparison of 72-h IC_{50} values and associated 95% confidence limits (CL), expressed in mg L^{-1}, correspondent to sensitive and resistant algal populations of *Dyctiosphaerium chlorelloides* to hexavalent chromium oxide.

D. chlorelloides	**n**	**Growth inhibition**		**$\Phi_{(PSII)}$ inhibition**	
		$IC_{50(72)}$	**CL (95%)**	**$IC_{50(72)}$**	**CL (95%)**
Sensitive Populations	8	1.64	(1.43-1.98)	1.54	(1.29-1.89)
Resistant Populations	8	20.61*	(19.57-21.66)	17.26*	(15.04-19.48)

* Significant differences (P<0.05) with respect to values obtained for sensitive populations.

Table 2. Fluctuation analysis of *D. chlorelloides* Cr(VI)-resistant cells exposed to 25 mg L^{-1} concentrations of hexavalent chromium in culture medium.

	Set 1	**Set 2**
Nº of replicate cultures	105	30
Nº of cultures containing the following nº of Cr(VI) resistant cells mL^{-1}		
0	86	0
$< 1.5 \times 10^5$	7	0
$1.5 \times 10^5 - 2 \times 10^5$	9	6
$> 2 \times 10^5$	3	24
Variance/Mean representative of nº of resistant cells per replicate	2.48	0.19
Mutants per cell division (μ)	5.30×10^{-5}	

5. DISCUSIÓN

Adaptación a contaminantes de origen antropogénico

En el primer capítulo de esta memoria estudiamos la capacidad de las poblaciones de protistas fotosintéticos y cianobacterias para adaptarse a contaminantes de origen antropogénico. En primer lugar determinamos los límites de adaptación fisiológica de algunas de las especies de microalgas y cianobacterias más comunes a determinados contaminantes de origen antropogénico, y los mecanismos genéticos que permiten la adaptación a concentraciones superiores a estos límites.

Todas la sustancias contaminantes de origen antropogénico aplicadas en los trabajos experimentales (glifosate, simazina, diquat, formol, cloranfenicol, DEAB y Cr(VI)), demostraron ejercer una fuerte presión selectiva sobre las poblaciones de fitoplancton a bajas concentraciones. Cuando aplicamos dosis letales de los contaminantes sobre cultivos de fitoplancton, la densidad celular se redujo rápidamente debido al efecto tóxico de las sustancias. Sin embargo tras incubaciones en condiciones adecuadas observamos que algunos cultivos fueron capaces de proliferar de nuevo. Este fenómeno desveló la existencia de variantes celulares resistentes a los diferentes tóxicos ensayados. La clave para entender la adaptación de estos organismos a medios contaminados está en averiguar la naturaleza de las células resistentes que proliferan tras la destrucción masiva de las células sensibles.

El análisis de fluctuación desarrollado por Luria y Delbrück en 1943 es el procedimiento experimental adecuado para determinar la naturaleza de la adaptación. Este método es capaz de discernir si la adaptación a una sustancia tóxica se debe a mutaciones que tienen lugar al azar, antes de que las células entren en contacto con la sustancia tóxica, o si por el contrario la adaptación es una respuesta directa y específica al medio selectivo (Luria y Delbrück, 1943). La primera hipótesis de adaptación corrobora la hipótesis evolucionista neodarwinista (mayoritariamente aceptada por la comunidad científica para

organismos pluricelulares) basada en la idea de que la evolución tiene lugar por la selección de la variabilidad genética preexistente en una población (Lewis 1934; Huxley 1942). La segunda hipótesis propone la adaptación por respuesta directa al medio, ya sea por una aclimatación fisiológica (Fogg, 2001) o a través de mutaciones desencadenadas por la presencia de la sustancia tóxica. Recientes estudios evolutivos en bacterias sugieren la existencia de ciertas mutaciones, llamadas “adaptive mutation”, que podrían formar parte de procesos evolutivos similares a los propuestos por el lamarckismo (Cairns et al. 1988; Foster 2001).

En los experimentos descritos en los artículos aplicamos una modificación experimental del clásico análisis de fluctuación (López-Rodas et al. 2001) que permite aplicar este método en cultivos en medio líquido. El análisis de fluctuación original se diseñó para determinar el mecanismo de resistencia de la bacteria *E. coli* al bacteriófago T1, y por tanto los medios de cultivos empleados fueron sólidos.

Todos los los análisis de fluctuación realizados con microorganismos fotosintéticos del plancton con diferentes sustancias contaminantes, indican que estos microorganismos son capaces de adaptarse a concentraciones letales de los diferentes contaminantes a través de mutaciones preadaptativas, apoyando experimentalmente la teoría evolutiva neodarwinista. En ningún caso los resultados sugirieren que la adaptación a dosis letales de contaminantes ambientales se deba a mutaciones postadaptativas. Debido a que las “adaptive mutation” solamente se han descrito en cultivos que se encuentran en fase estacionaria y sometidos a una presión selectiva no letal (Foster, 2001), no podemos descartar que tras la adaptación tengan lugar fenómenos de esta clase.

El análisis de fluctuación nos permite determinar la frecuencia con la que tiene lugar la mutación que permite la adaptación específica a una sustancia. Esta aproximación se realiza a través del estimador P_0 desarrollado por Luria y Delbrük y basado en la proporción de cultivos del set 1 del análisis de

fluctuación en los que no encontramos células resistentes (Luria y delbrük, 1943).

Si sólo los mutantes preselectivos son capaces de asegurar la supervivencia de la especie, ¿Cómo se mantienen los mutantes resistentes en las poblaciones antes de la aparición del contaminante? La mutación que permite la adaptación lleva asociados determinados costes que impiden que las células resistentes proliferen con la misma eficiencia que las células sensibles en condiciones no selectivas (Coustau et al., 2000). En todos los casos el fitoplancton resistente a los diferentes contaminantes de origen antropogénico presentan una tasa de crecimiento significativamente menor que las células sensibles en condiciones no selectivas. Este fenómeno tiene como consecuencia que las células resistentes serán eliminadas de la población de fitoplancton por selección natural (Spiess, 1980). Mientras que en cada generación aparecen nuevos mutantes resistentes a la sustancia tóxica por mutación espontánea (ya que es un fenómeno recurrente), simultáneamente desaparecen por selección natural. Existe un equilibrio entre los microorganismos resistentes que aparecen por mutación y los que desaparecen por selección (Kimura y Muruyama, 1966). En todos los casos estudiados, la proporción de genotipos resistentes que se mantiene en equilibrio en la población supone una cantidad suficiente para asegurar la supervivencia de la especie en medios contaminados con concentraciones superiores al límite de adaptación fisiológico.

En este sentido, a la hora de entender la adaptación de estos organismos hay que tener en cuenta una de sus mayores peculiaridades biológicas: su tamaño de población. Presentan tamaños de población verdaderamente grandes. *Microcystis sp.* puede alcanzar densidades celulares de 500.000 células por mililitro en manchas que abarcan centenares de Km^2 de superficie. Por ejemplo, el seguimiento por satélite ha desvelado manchas de la cianobacteria *Trichodesmium* que abarcan millones de Km^2 cuadrados de superficie a altísimas densidades celulares. Tales tamaños poblacionales aseguran que, incluso habiendo un solo mutante resistente por cada millón de individuos, el número de resistentes en un momento dado, (fruto de los que,

apareciendo por mutación espontánea, caminan hacia su eliminación por selección) sería elevadísimo en términos absolutos.

En el primer trabajo del primer capítulo el análisis de fluctuación sugiere que la cianobacteria *Microcystis aeruginosa* es capaz de adaptarse al herbicida glifosate a través de mutaciones preadaptativas, es decir, mutaciones que tienen lugar al azar y espontáneamente antes de que las células entren en contacto con el herbicida. Los procedimientos experimentales de este trabajo fueron aplicados sobre dos cepas de la cianobacteria *M. aeruginosa* aisladas del Parque Nacional de Doñana, una hiperproductora y una no productora de microcistina (cepas Ma7D y Ma3D respectivamente). Las frecuencias con las que tiene lugar la mutación espontánea que permite la adaptación al glifosate se estimaron en $3{,}6x10^{-7}$ y $3{,}1x10^{-7}$ para las cepas Ma3D y Ma7D respectivamente. Estas tasas de mutación son uno o dos ordenes menores que las descritas en otras cianobacterias y en microalgas para la resistencia a diferentes biocidas (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al.; 2002, 2004), pero en el mismo orden de magnitud que para la resistencia a aguas sulfurosas en la cianobacteria *Spyrogira insignis* ($2.7 \cdot 10^{-7}$ mutantes por división celular) (Flores-Moya et al. 2005).

Respecto a las fluctuaciones llevadas a cabo para estudiar la adaptación de las microalgas clorofíceas al herbicida simazina, los resultados sugieren que las clorofíceas son capaces de adaptarse al herbicida triacínico simazina, al igual que la cianobacteria *M. aeruginosa* al glifosate, a través de mutaciones espontáneas que suceden al azar antes del contacto con la sustancia tóxica. Las frecuencias con la que tienen lugar las mutaciones espontáneas que permite la adaptación de las diferentes cepas a la simazina ($9{,}2x10^{-6}$, $3{,}0x10^{-6}$ y $3{,}0x10^{-6}$ mutaciones por división celular para las cepas Dc1, SiD y SiM respectivamente) se encuentran en mitad del rango de tasas de mutación (de $2{,}1x10^{-5}$ a $2{,}7x10^{-7}$ mutantes por división celular) descritas en cianobacterias y protistas fotosintéticos para la resistencia a otros biocidas y a ambientes naturales extremos (Costas et al. 2001, 2007; López-Rodas et al. 2001, 2007, 2008a, 2008b; Baos et al. 2002; García-Villada et al. 2002, 2004; Flores-Moya et al. 2005). Cabe destacar las similares tasas de mutación determinadas en

las dos cepas de *Scenedesmus sp.*, aunque una fue aislada del Parque Nacional de Doñana (habitualmente expuesta a herbicidas) y la otra del desierto del Sahel (nunca expuestas a herbicidas). Sin embargo, la constante presencia de herbicida en un determinado hábitat podría causar la aparición de cepas con un mayor coeficiente de selección, y consecuentemente aumentar la frecuencia de alelos resistentes como consecuencia del equilibrio entre la mutación y la selección. En el caso de *Scenedesmus sp*. en ausencia de simazina, la frecuencia del alelo resistente es 2,5 veces mayor en la cepa procedente del Parque Nacional de Doñana que en la procedente del Sahel. Este fenómeno se debe a la diferencia en el coeficiente de selección de las cepas SiM y SiD.

Por otro lado el trabajo realizado para estudiar la adaptación de la clorofícea *Dictyosphaerium chlorelloides* al formaldehído indica que el fenómeno de adaptación a dosis letales fue también posible gracias a mutaciones preadaptativas. En este caso la tasa de mutación que permite la adaptación fue de 3.61×10^{-6} mutaciones por división celular.

La variabilidad genética está directamente relacionada con la capacidad que tienen las poblaciones de adaptarse a los cambios ambientales bruscos (Falconen y Mackay, 1996, Bürger, 1999). Dado que los microorganismos experimentales usados carecen de reproducción sexual (y por tanto de recombinación cromosómica en la meiosis) la única fuente de variabilidad genética es la mutación. Los resultados de adaptación descritos en esta memoria indican que la mutación preadaptativa confiere a las poblaciones de fitoplancton variabilidad genética suficiente para asegurar la supervivencia de la especie en condiciones de contaminación.

Aunque las poblaciones de procariotas y protistas fotosintéticos tienen una elevada capacidad para sobrevivir a episodios de contaminación aguda con contaminantes de origen antropogénico, estos episodios tienen un efecto negativo sobre estos productores primarios, y por tanto sobre los ecosistemas acuáticos. En estos episodios se seleccionan genotipos con una capacidad de división baja, por lo que se reduce la cantidad de materia orgánica que estos

microorganismos son capaces de introducir en la cadena trófica. El uso de de pesticidas afecta de forma selectiva a los productores primarios, de modo que su acción repercute sobre los niveles superiores de la red trófica (Fleeger *et al.*, 2003).

Algunos ambientes contaminados mantienen las poblaciones de fitoplancton al límite de su capacidad de adaptación fisiológica (Fogg, 2001). En los trabajos del primer capítulo determinamos el mecanismo genético de adaptación a contaminantes de origen antropogénico a concentraciones superiores a los límites de aclimatación fisiológica. Pero, ¿Qué sucede a concentraciones muy superiores a estos límites de aclimatación fisiológica? ¿Es el fitoplancton capaz de adaptarse a estas concentraciones? La incapacidad de adaptación del fitoplancton a nuevas condiciones ambientales se traduciría en el colapso de los ecosistemas acuáticos (Woodruff, 2001).

¿Qué sucede tras la primera mutación que permite la adaptación?: evolución de poblaciones resistentes en medios contaminados

Los resultados de los trabajos expuestos en esta memoria sugieren que la resistencia a determinados contaminantes tiene un coste asociado, las células resistentes muestran una clara disminución en la capacidad de división en comparación con las células sensibles en condiciones no selectivas. Sin embargo, las poblaciones naturales de microalgas que habitan en lugares altamente contaminados (y que son portadoras de mutaciones que les permiten adaptarse a las condiciones de su hábitat) no manifiestan el coste que suele observarse en las poblaciones resistentes aisladas experimentalmente (Sajjaphan *et al*., 2002). Este resultado parece indicar que, bajo una presión selectiva constante, se induce el establecimiento de una nueva población de individuos con mutaciones compensadoras que reducen el efecto negativo de las mutaciones que confieren resistencia.

En una aproximación experimental a este complejo problema, estudiamos los cambios adaptativos por mutación-selección en poblaciones de

Scenedesmus intermedius resistentes a cloranfenicol bajo la presión selectiva del antibiótico.

Cuando exponemos las microalgas resistentes al cloranfenicol (a 5,8 ppm) a dosis superiores de antibiótico, detectamos que parámetros como la tasa de crecimiento y el rendimiento fotosintético se ven afectados. A concentraciones iguales o superiores a 40 ppm las células resistentes no son capaces de dividirse ni de realizar la fotosíntesis. Todos los cultivos de células resistentes colapsaron tras pocas horas de exposición a esta concentración. Un análisis de fluctuación indicó que las células resistentes a 5,8 ppm de cloranfenicol son incapaces de adaptarse directamente a esta nueva concentración letal de 40 ppm. Los 130 cultivos del análisis de fluctuación fundado con células resistentes y expuestas a 40 ppm, fueron incubados durante un año y periódicamente revisados en busca de células capaces de adaptarse a esta nueva concentración. Durante este periodo de tiempo no se encontró ninguna célula resistente, todos los cultivos colapsaron hasta la extinción.

Sin embargo, las células resistentes a 5,8 ppm de cloranfenicol son capaces de adaptarse a la dosis letal de 40 ppm tras un periodo de evolución bajo la presión selectiva del cloranfenicol. En un experimento se mantuvieron los cultivos, en crecimiento exponencial, a una concentración de 5,8 ppm de cloranfenicol durante un año. Paralelamente, en otro experimento, fuimos aumentando progresivamente la dosis de cloranfenicol durante un año hasta alcanzar los 40 ppm. En este último, observamos una adaptación más eficaz a 40 ppm, ya que las microalgas resistentes en esta concentración presentaban una tasa de crecimiento de aproximadamente el doble que en el caso de las células resistentes expuestas a una concentración constante de 5,8 ppm de cloranfenicol.

El hecho de que las células resistentes a 40 ppm tuvieran una tasa de división menor que las células resistentes a 5,8 ppm en ausencia de cloranfenicol indica que tuvo lugar una adaptación por cambios genéticos. Durante los experimentos de evolución las mutaciones favorables para la tasa

de crecimiento y para el rendimiento fotosintético fueron seleccionadas positivamente por la constante exposición a cloranfenicol. Estudios llevados a cabo con bacterias y virus, ponen de manifiesto que tras un proceso de selección las poblaciones resistentes aumentan su eficacia, ya que el coste asociado a las mutaciones, inicialmente adaptativas, se diluye debido al establecimiento de mutaciones que compensan su efecto (revisado por Levin *et al*., 2000). La selección de estas mutaciones que favorecen el crecimiento en medios contaminados con cloranfenicol fue más efectiva en el caso del experimento de aumento progresivo de la concentración.

Debido la importancia del fitoplancton en el mantenimiento de la estructura y dinámica de los ecosistemas, los resultados de este trabajo indican que, al menos en el caso de contaminación de ecosistemas acuáticos por cloranfenicol es mejor que la concentración de antibiótico en el medio aumente paulatinamente, en vez de aumentar bruscamente. De esta manera aumenta la probabilidad de supervivencia de las poblaciones de fitoplancton gracias a que hay tiempo suficiente, bajo presión ambiental a concentraciones no letales de antibiótico, para que las mutaciones que permiten el cambio adaptativo se fijen en la población.

El dogma central de la teoría evolutiva neodarwinista (uno de los actuales paradigmas en la biología) consiste en que la evolución de las poblaciones es fruto de la adaptación, que se produce por selección natural de los caracteres por el ambiente. Para la corriente panadaptacionista, muy extendida en la comunidad científica, todas las diferencias fenotípicas tienen un valor adaptativo (Ridley 1993; Gould 2002).

Sin embargo, existen corrientes dentro de la teoría Neodarwinista que proponen que el azar y la contingencia histórica también juegan un papel muy importante en la evolución. Según Kimura, padre de la teoría neutralista, por motivos estocásticos se pueden establecer en las poblaciones mutaciones que no confieren una ventaja adaptativa, es decir, mutaciones neutras debidas simplemente al azar (Kimura, 1983).

La contingencia histórica postula que no todos los cambios evolutivos son posibles, sino que determinados cambios que en el pasado tuvieron valor adaptativo podría actuar como lastre histórico (Gould and Lewontin, 1979).

Para determinar la influencia del azar y la adaptación, en la evolución de la tasa de crecimiento y el rendimiento fotosintético de mutantes de *Scenedesmus intermedius* resistentes al cloranfenicol en presencia del antibiótico, desarrollamos el experimento planteado por Travisano y colaboradores en 1995 (Travisano et al. 1995). Los caracteres cuantitativos que determinamos fueron la tasa de crecimiento y el rendimiento fotosintético. Los resultados sugieren que la evolución de la tasa de crecimiento está muy influida por la adaptación, ya que hay una convergencia entre los valores finales estimados en las diferentes réplicas. Este fenómeno de convergencia en la tasa de crecimiento también ha sido descrito en experimentos similares llevados a cabo en cultivos de virus (Cuevas et al. 2002) y bacterias (Travisano et al. 1995; Korona, 1996), lo que podría dar fuerza a la hipótesis de que los rasgos ligados a la eficacia biológica (como es la tasa de crecimiento) están fuertemente unidos a la evolución adaptativa, mientras que el azar influye muy poco. Sin embargo, en la evolución del rendimiento fotosintético, el azar juega un papel importante. Este carácter no está tan fuertemente ligado a la eficacia biológica como la tasa de crecimiento.

Adaptación a ambientes naturales extremos

Las cianobacterias pueblan la tierra desde hace aproximadamente 3.500 millones de años y forman parte del tapete bacteriano fosilizado que constituye el fósil más antiguo encontrado (Schopf, 1996). Actualmente, podemos encontrar cianobacterias y microalgas en casi todos los ecosistemas. Han logrado colonizar prácticamente todos los medios acuáticos presentes en la Tierra: desde aguas continentales hasta aguas marinas, pasando por fuentes termales, neutras, alcalinas y ácidas. Además han conseguido adaptarse a sistemas edáficos como suelos tropicales y desérticos (Stanier et al., 1984; Rai and Gaur, 2001). No es extraño identificar estos microorganismos en ambientes extremos como las aguas alcalinas y termales del Parque Nacional

de Yellowstone en Estados Unidos, las aguas hipersalinas de la bahía de Shark en Australia (Fogg, 2001) o aguas sulfurosas de La Hedionda en Málaga (Flores-Moya et al. 2005). Aunque se han descrito cianobacterias filamentosas en lagos ácidos (pH 2,9) en Alemania (Steinberg et al. 1998), este fenómeno no es frecuente. La excepción a esta ubicuidad es la ausencia de cianobacterias en la inmensa mayoría de medios extremadamente ácidos (Brock, 1973; Albertano, 1995, revisado por Gimmler, 2001).

Indiscutiblemente, los microorganismos fotosintéticos del plancton han logrado colonizar una gran variedad de hábitats. En el caso de la adaptación a ambientes naturales extremos como las aguas ácidas de las minas de cobre de Mynyyd Parys, Gales, con unas condiciones similares a las del Río Tinto (pH cercano a 2 y una elevada concentración de metales pesados) cabe esperar que la adaptación de estos microorganismos hubiera sido un proceso lento y gradual. Sin embargo, el hecho de que los grupos taxonómicos que encontramos en estos ambientes corresponden a grupos habitualmente mesófilos (Amaral Zettler et al. 2003), nos hace pensar que la adaptación a estos hábitats puede ser un fenómeno rápido.

La presencia del agua tóxica no facilita la adaptación inicial de las microalgas al medio hostil, sino que determinadas mutaciones al azar proporcionan resistencia a estos ambientes extremos antes de la colonización. Esta hipótesis explica la elevada diversidad de eucariotas fotosintéticos que encontramos en estos ambientes extremos y como la colonización puede ser casi instantánea. Se han descrito mecanismos similares de adaptación de microalgas para ambientes extremos como la mezcla de metales pesados del vertido tóxico de las minas de Aznalcollar (Baos et al., 2002), adaptación al Río Tinto (Costas et al., 2007) y a aguas sulfurosas de origen natural (Flores-Moya et al., 2005).

Sin embargo, en los tres casos estudiados no se produjo la adaptación de la cianobacteria *M. aeruginosa*. De hecho, está ampliamente descrita la ausencia de estos microorganismos en ambientes moderada y extremadamente ácidos (revisado por Gimmler, 2001; Nixdorf et al., 2001).

Además un pH de 4,8 fue postulado como límite para la proliferación de cianobacterias (Brock, 1973). El hecho de que las cianobacterias, al contrario que las células eucariotas, carezcan de bombas ATPasa específicas transportadoras de iones, puede ser la base molecular del fracaso adaptativo de las cianobacterias a medios ácidos (Amaral Zettler et al., 2003). Sin embargo, se ha descrito la presencia de cianobacterias filamentosas en lagos ácidos (pH 2.9) en Alemania (Steimberg et al., 1998).

La rápida adaptación de *D. chlorelloides* a las aguas tóxicas de la isla de Vulcano puede ser un modelo para entender la supervivencia de los microorganismos fotosintéticos del plancton durante uno de los periodos más críticos en la historia de la vida: el periodo propuesto por la hipótesis de “la bola de nieve” en el Proterozoico. Esta hipótesis sugiere que hace 850-750 millones de años una serie de glaciaciones globales (que llegaron hasta el ecuador) convirtieron la tierra en una gran bola de hielo (Kirschvink, 1992). Las discontinuidades de determinadas proporciones de isótopos de carbono procedentes de rocas calizas del periodo proterozoico se han interpretado como la consecuencia del colapso de la producción primaria en la superficie de los océanos durante aproximadamente un millón de años (Hoffman et al. 1998; Rothman et al. 2003). Algunos microorganismos fotosintéticos pudieron sobrevivir a este periodo en las zonas asociadas a volcanes activos, únicas aguas superficiales en estado líquido (Schrag and Hoffman 2001). Sin embargo, estos refugios para los microorganismos fotosintéticos tienen concentraciones letales de sustancias como compuestos sulfurosos, metales pesados, pH ácido o extremadamente ácido, habitualmente letales para los microbios fotosintéticos, los cuales eran los únicos fotosintetizadotes en la biosfera proterozoica (Kaufman et al.1997). A pesar de esta toxicidad, la selección de genotipos con mutaciones aleatorias y espontáneas pudo ser suficiente para asegurar la adaptación de determinados eucariotas fotosintéticos a las hostiles condiciones que suponen las aguas de origen volcánico.

Bajo esta hipótesis, una mayor capacidad de adaptación a medios contaminados con sustancias de origen volcánico debió proporcionar una

ventaja evolutiva en este periodo de grandes glaciaciones. Los resultados sugieren que los microorganismos fotosintéticos del plancton eucariotas tienen una mayor probabilidad de adaptación a medios naturales extremos que los procariotas, en especial a los medios ácidos (revisado por Gimmler, 2001).

Sorprendentemente no hay demasiados estudios que demuestren experimentalmente la relación entre la aparición de mutaciones espontáneas y su implicación en los procesos de adaptación a ambientes naturales. Existen dos posibles razones que explican esta situación. En primer lugar la imposibilidad experimental para medir tasas específicas de mutación en organismos pluricelulares y la dificultad para realizar estas mismas medidas en organismos unicelulares. En segundo lugar, hay pocas circunstancias bajo las cuales una determinada tasa de mutación específica, que juegue un papel relevante para la adaptación, puede ser medida en condiciones naturales (Lenski, 2005).

Aplicación: microalgas resistentes para la construcción de biosensores sensibles y específicos

Los resultados del primer trabajo del último capítulo revelan la sensibilidad que tienen alunas de las clorofíceas estudiadas a bajas concentraciones de amonio cuaternario DEAB. Determinadas sustancias (como es el caso del DEAB) son fácilmente adsorbidas por la superficie cationica de las clorofíceas (Waters, 1982). Está descrito en la literatura la sensibilidad de las clorofíceas a la presencia de detergentes, de manera que se convierten en buenos bioindicadores en caso de contaminación con surfactantes (Issa and Ismael, 1995).

Los resultados indican que el DEAB inhibe significativamente el rendimiento cuántico de la fotosíntesis (medido con un fluorímetro de pulso de amplitud modulada) de las clorofíceas *S. intermedius* y *D. chlorelloides.* Los resultados coinciden con los de otros autores en apuntar que estas medidas son un método rápido, preciso y no invasivo, para determinar la capacidad

fotosintética de las clorofíceas en ambientes cambiantes (Krause and Weis, 1991; Schreiber et al., 1994).

El efecto del DEAB sobre el rendimiento de fotosíntesis también fue determinado mediante la producción de oxígeno a través de medidas con un electrodo de Clarck. La presencia del tóxico induce una disminución en la cantidad de oxígeno producido por las clorofíceas. El efecto inhibitorio producido por este compuesto sobre la actividad fotosintética es similar a otros descritos para contaminantes orgánicos (Carrasco and Sabater, 1997) e inorgánicos (Pandard et al., 1993).

Los resultados indican que a clorofícea *D. chlorelloides* es más sensible al DEAB que *S. intermedius*, tanto para los valores de inhibición de rendimiento cuántico fotosintético como para las medidas de producción de oxígeno. Por este motivo, después de los experimentos realizados el microorganismo escogido como especie sensible para la construcción de un biosensor para detectar la presencia de DEAB, sería *D. chlorelloides*

En el segundo trabajo proponemos las diferencias en la respuesta fotosintética entre células de *D. chlorelloides* resistentes y sensibles al cromo hexavalente como mecanismo biosensor. El trabajo experimental muestra que las células resistentes al cromo hexavalente son doce veces más resistentes que las células sensibles. Es decir, para obtener igual inhibición fotosintética hay que exponer las células resistentes a una concentración de Cr(VI) doce veces mayor que a las células sensibles. Actualmente la detección de cromo hexavalente y otros metales pesados en embalses de abastecimiento se realiza mediante cuantificaciones por métodos analíticos químicos como el espectrómetro de absorción atómica (Sperling et al., 1999), que requieren tiempo en la preparación y procesado de las muestras en laboratorio, pero como contrapartida tienen una elevada especificidad y sensibilidad.

El modelo de biosensor presentado para detección de cromo hexavalente tiene un límite de detección de 0,3 mg L^{-1}, muy superior al método analítico clásico basado en un espectrómetro de absorción atómica de $0{,}9x10^{-3}$

mg L^{-1} (Sperling et al., 1999). Sin embargo el biosensor microalgal tiene ventajas sobre los métodos analíticos clásicos como la facilidad de manejo, el bajo coste y la sencillez en el mantenimiento del dispositivo (D'Souza, 1997).

La obtención de células resistentes es relativamente sencilla y económica. Además, las muestras no requieren ningún tratamiento previo antes del análisis. Simplemente midiendo la fluorescencia con un fluorímetro de pulso de amplitud modulada simultáneamente en células resistentes y sensibles expuestas a la muestra podemos determinar la presencia de cromo (VI), al igual que en el modelo descrito por Altamirano y colaboradores (Altamirano et al. 2004). Otra ventaja es el poco tiempo que transcurre entre que recogemos la muestra y obtenemos los resultados. Además, el biosensor permite hacer medidas de campo *in situ*, ya que hay fluorímetros de pulso de amplitud modulada portátiles.

La toxicidad de los metales pesados depende de factores como la concentración iónica (dependiente del pH del medio), la accesibilidad a las células (Collard y Matagne, 1990; Bruins et al., 2000) y la presencia de otros metales pesados en el medio (Ince et al. 1999), ya que en muchos casos parece existir antagonismos en el efecto sobre las microalgas (Pawlik-Skowronska, 2001). Los mecanismos descritos implicados en la resistencia a metales pesados por las microalgas son: un incremento en la producción de fitoquelatinas, la excreción de materia orgánica para quelar iones, el secuestro intracelular y la alteración en la composición de la membrana (revisado por Collard y Matagne, 1990). En este modelo desarrollado para la detección de cromo hexavalente, por tanto, cabe la posibilidad de que exista resistencia cruzada con otros metales pesados. Este aspecto aún está en vías de investigación.

El biosensor propuesto, es una aproximación experimental a un método barato y rápido, que nos permite distinguir entre muestras que tienen Cr(VI) de las que no. Este método de detección se presenta como un buen mecanismo de alerta temprana para la detección desustancias toxicas dado su reducido tiempo de respuesta y la facilidad que presenta para realizar medidas *in situ*.

Para concluir esta discusión citaré una conclusión a la que llegó, en 1994, el paleontólogo W. Schopf: “En contraste con la evolución normal de animales y plantas (convertirse en especialistas, que se especializan y se extinguen tras pocos millones de años), las reglas de la evolución de procariotas y protistas parecen haber sido las contrarias: convertirse en generalistas con una excepcional supervivencia a largo plazo cifrada en eones” (Schopf, 1994). En conclusión, los procariotas y protistas evolucionaron siguiendo la navaja de Occam: la máxima simplicidad. Solo unas pocas mutaciones espontáneas preselectivas permiten asegurar la supervivencia de cianobacterias y protistas, incluso al cambio ambiental derivado de la contaminación de origen antropogénico. Para sobrevivir a los cambios ambientales con esta estrategia solamente hace falta tener un elevado tamaño poblacional. Así, las cianobacterias han conseguido sobrevivir más de 3.500 millones de años, sin necesidad de complicaciones como el sexo y la recombinación. Pasaron, aparentemente sin problemas, a través de las cinco grandes extinciones y, previsiblemente, pasarán a través de la sexta y los problemas de contaminación antropogénica.

6. CONCLUSIONES

1. El fitoplancton es capaz de adaptarse a dosis letales de contaminantes de origen antropogénico como los herbicidas glifosate, simazina y diquat, el formaldehído, el antibiótico cloranfenicol, el amonio cuaternario DEAB y el metal pesado cromo (VI), a través de mutaciones preadaptativas, espontáneas y que tienen lugar al azar antes del contacto con el agente selectivo.

2. Las tasas mutación determinadas para los diferentes contaminantes de origen antropogénico fueron, para *Microcystis aeruginosa* glifosate sensible a glifosate resistente de $3{,}1 \times 10^{-7}$ mutaciones por división celular (mut./div.cel); para *Scenedesmus intermedius* simazina sensible a simazina resistente de 3×10^{-6} mut./div.cel.; para *Dictyosphaerium chlorelloides* simazina sensible a simazina resistente de $9{,}2 \times 10^{-6}$ mut./div.cel.; para *D. chlorelloides* formaldehído sensible a formaldehído resistente de $3{,}6 \times 10^{-6}$ mut./div.cel.; para *S. intermedius* diquat sensible a diquat resistente de $17{,}9 \times 10^{-6}$ mut./div.cel.; para *D. chlorelloides* cloranfenicol sensible a cloranfenicol resistente de 1×10^{-5} mut./div.cel. y para *D. chlorelloides* cromo hexavalente sensible a cromo hexavalente resistente de $5{,}3 \times 10^{-5}$ mut./div.cel.

3. La adaptación tiene un coste elevado: los mutantes resistentes tienen una tasa de división menor que las células sensibles en condiciones no selectivas. Estimamos el coeficiente de selección para células resistentes de *M. aeruginosa* a glifosate en 0,83; para *S. intermedius* resistente a simazina en 0,26; para *D. chlorelloides* resistente a simazina en 0,3; para *S.intermedius* resistente a diquat en 0,21; para *D. chlorelloides* resistente a formaldehído en 0,06; para *D. chlorelloides* resistente a cloranfenicol en 0,33 y para *D. chlorelloides* resistente a cromo hexavalente en 0,12.

4. Tras la primera mutación los microorganismos fotosintéticos resistentes son capaces de adaptarse progresivamente a la presencia de la sustancia a la que son resistentes, mediante nuevas mutaciones y acción de la selección natural. En concreto, los mutantes de *Scenedesmus intermedius* resistentes al cloranfenicol, cultivados en presencia de este antibiótico durante un largo periodo de tiempo, aumentan significativamente su tasa de crecimiento gracias a que la selección natural juega un papel muy importante su evolución, mientras que el rendimiento fotosintético está más influenciado por el azar.

5. Los microorganismos fotosintéticos del plancton eucariotas son capaces de adaptarse a determinados ambientes naturales extremos. La clorofícea *Dictyosphaerium chlorelloides* es capaz de adaptarse a aguas ácidas y con elevadas concentraciones de metales pesados de de Tharsis (Huelva) y Mynyyd Parys (Gales), y a ambientes con sustancias tóxicas de origen volcánico de la isla de Vulcano (Italia). La adaptación es rápida y tiene lugar gracias a mutaciones preadaptativas que suceden, en el caso de las Aguas Agrias de Tharsis, con una frecuencia de $1{,}1 \times 10^{-6}$ mut./div.cel., para las aguas ácidas de Mynyyd Parys, la frecuencia es de $1{,}6 \times 10^{-6}$ mut./div.cel. y para la adaptación a aguas tóxicas de Vulcano la frecuencia de mutación es de $4{,}7 \times 10^{-7}$ mut./div.cel.

6. En último lugar, proponemos que las microalgas resistentes aisladas a través de análisis de fluctuación pueden usarse en la construcción de biosensores sensibles y específicos. La especificidad se logra combinando simultáneamente un genotipo sensible y uno resistente a la sustancia que deseamos detectar.

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