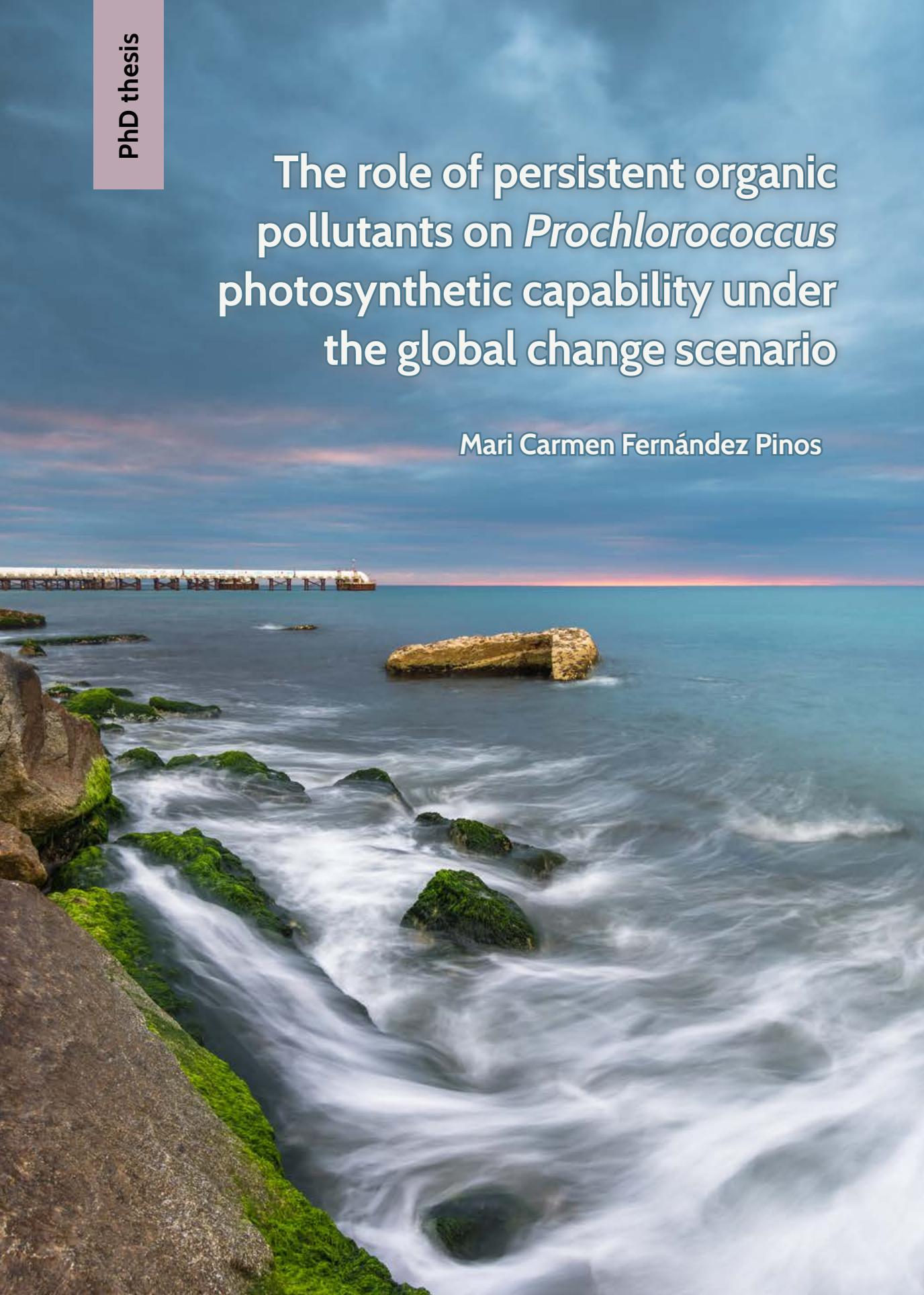
PhD thesis

The role of persistent organic pollutants on *Prochlorococcus* photosynthetic capability under the global change scenario

Mari Carmen Fernández Pinos



Cover and back cover photographies:
Juan Carlos Fajardo Juan

A mis padres, Fernando y Mari Carmen,
los pilares de mi persona.

A Juan Carlos, mi otra mitad.

INSTITUTE OF ENVIRONMENTAL ASSESMENT AND WATER RESEARCH

SPANISH NATIONAL RESEARCH COUNCIL

POLYTECHNIC UNIVERSITY OF CATALONIA

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And by Dr. Agustín Sánchez-Arcilla Conejo as UPC advisor.

Barcelona, June 2016

‹‹In nature nothing exists alone››
Rachel Carson

This thesis was founded by the JAE Predoctoral Program of the Spanish National Research Council (CSIC).

AGRADECIMIENTOS

Hace no mucho se cumplieron cinco años del comienzo de esta aventura del doctorado; recuerdo como si fuera ayer la inesperada llamada de Jordi que la inició. A él he de agradecerle su confianza ciega en una niña que miraba pájaros, su constante aliento, que me ha llevado incluso a aprender algo de cromatografía, y su genialidad. ¡Jordi, has sido un excelente padre científico!

A mi otro padre científico, por su parte, he de agradecerle su inquebrantable fe en las causas perdidas (contagiosa hasta el extremo de hacerme creer que llegaría hasta aquí...), su eterna disposición y su paciencia. ¡Benjamí, trabajar a tu lado ha sido extraordinariamente estimulante!

Desde aquella llamada hasta conseguir este libro de título raro el camino ha sido largo y, en ocasiones, abrupto, pero en él no sólo me he cruzado con contaminantes, *Prochlorococcus*, y "real times", he encontrado personas que, sin duda alguna, son la esencia de esta aventura. Personas que me han llevado de la mano en los tramos en que me encontraba perdida. Personas que han derramado su sudor recogiendo mis "maricarmen de noche" y "maricarmen de día" (y otras tantas...). Personas con las que he vivido una de las mejores experiencias de mi vida en ese barco rojo chillón. Friendly people that made me feel like home during my American experience. Personas que tan amablemente han invertido su tiempo en responder mis infinitas preguntas. Personas que me cedieron sus datos para que los míos no se sintieran solos. Personas con las que compartir deliciosos desayunos a las 11, mojitos a las 20, comidas de navidad, de semana santa, de verano... ¡cualquier excusa es buena! Personas que me han escuchado con paciencia y me han dado ánimos cuando más lo necesitaba. Incluso personas que se han convertido en mi familia de Barcelona, y a las que les agradezco los mejores momentos de este viaje.

¡GRACIAS A TODOS LOS QUE HABÉIS HECHO ESTO POSIBLE!

María Vila Marta Casado Eva Oliveira Belén González
Javi Zuñiga MariJose Ojeda Sofía Mesquita Rita Jordão
Naiara Berrojalbiz Sergi Pelayo Anna Navarro Alba García
Mariana Pizarro Paulo Casal Elena Cerro Cristina Pizzaro
Denise Fenandes Eli Perez María Blanco Laura Morales
Elena Jurado Javi Castro Linda Gioia Ana Cabrerizo
Cristobal Galbán Gemma Caballero Laia Navarro Melissa
Faria Bruno Campos Claudia Rivetti Carlos Barata Eva
Prats Cinta Porte José María Ripol Pep Gasol Clara
Cardelús Vanessa Balagué Ángel López Maribel Cerezo
Eneko Aierbe Sara-Jeanne-Royer Rafel Simó Luis Lubián
Fran Rodríguez Raquel Gutiérrez Paqui García Zuriñe
Baña Luis Resines Irene Forn Max Galindo Teresa
Serrano Axa Molina Igor Fernández Bieito Fernández
Eli Fernández María Huete Daffne López José Luis
Acuña Eva Mayol Txetxu Arrieta Elena Tel Camilo
Gómez Carlos Duarte Susana Agustí Paloma Carrillo
Fidel Echevarría Nacho González Cristina Sobrino
Mario Fernández Pepelu Roscales Begoña Jiménez Erik
Zinser Lanying Ma Marty Szul Jeremy Chandler Ashley
Frank Jackson Gainer Morgan Steffen Nana Ankrah
Mohammad Moniruzzaman Tiana Pimentel Alise Ponsero
Courtney Apperson Taylor Presley Amanda Snider
Roser Chaler Dori Fanjul Inma Fernández Elvira Méndez
Genoveva Comas Zlaten del Castillo Paola de la Higuera
Noelia Fernández JuanCarlos Fajardo Pumba Bartumeu

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ABSTRACT

Global Change is altering the Earth System so fast that a new era has been proposed, the Anthropocene. Among the nine planetary boundaries established for the transition from the Holocene to the Anthropocene, only two of them, chemical pollution and atmospheric aerosol loading, remain still unexplored at a global level. As a part of both global change drivers, persistent organic pollutants (POPs) are of special concern by virtue of their persistence, ubiquity, bioaccumulation tendency and high toxicity to both ecosystems and living beings. They are prone to long-range transport by atmospheric or oceanic circulation and are ubiquitous in fresh and marine waters, soils, organisms and the atmosphere, including remote regions such as open oceans. Oceans play an important role in controlling the environmental transport, fate and sinks, and acting as a critical reservoir of POPs, but there was limited knowledge on the influence of POPs on the marine carbon cycle. POPs bioconcentrate in phytoplankton and bacteria, and they bioaccumulate in higher trophic levels. Previous works have demonstrated the toxicity of some POPs on marine photosynthetic organisms, which could explain in part the global phytoplankton decline and the global changes in chlorophyll concentrations and primary production rate that occurred during the past century. However, most published studies focused on the effects of single compounds, whereas organisms are exposed in their natural environment to complex mixtures of chemicals. In addition, most reported toxic effects have been measured in cellular terms without considering molecular effects that could also influence phytoplankton ecosystem functions, like photosynthesis.

Oceanic photosynthesis accounts for about a half of the global photosynthesis on Earth. Between 5% and 10% of the global marine primary production is carried out by the cyanobacterial genus *Prochlorococcus*. This is the smallest known free-living oxygenic phototroph and the most abundant photosynthetic organism in the world. *Prochlorococcus* is numerically dominant in the oligotrophic waters of tropical and subtropical world's oceans between 40° N and 40° S latitude, where it is responsible

for 50–80% of primary production. *Prochlorococcus* genus has been broadly divided into two major clades, high-light (HL) and low-light (LL) adapted strains. HL strains are usually found in surface waters, whereas LL strains usually have their abundance maximum in the base of the euphotic zone. In previous studies, *Prochlorococcus* was considered as the most sensitive phytoplanktonic organism to several organic pollutants, due to its tiny size. The same studies demonstrated that complex mixture of organic pollutants were more toxic than single pollutants separately.

This thesis took a step forward aiming to assess the effects of the complex mixture of organic pollutants on the oceanic photosynthesis. The study was performed in field conditions, at sublethal toxicity levels, and in a global change context where multitude of interlinked drivers act at the same time. The choice of *Prochlorococcus* as sentinel organism allowed us to follow a truly global approach. We designed a feasible and simple sample collection methodology that reduced by minimum degradation or changes on mRNA levels, and kept the integrity of nucleic acid until these were quantified. To study the effects of global change drivers on *Prochlorococcus* photosynthetic capability, we designed a high sensitivity and high selectively molecular tool based on quantify the expression of *rbc*L and *psb*A genes by qRT-PCR, as proxies of carbon fixation and photosystem II (PSII) functionality, respectively. Gene expression levels were separately quantified for HL and LL *Prochlorococcus* clades. This technique was applied to laboratory experiments under controlled conditions in which axenic cultures of HL-MED4 or LL-MIT9313 *Prochlorococcus* strains were challenged with either polycyclic aromatic hydrocarbons (PAHs) or organoclorine pesticides (OClPs) commercial mixtures, which consisted of few representative compounds of the corresponding family. Although no cellular effects were detected, we observed a decrease of 20% of the *rbc*L relative expression, which may trigger a reduction of CO_2 fixation by *Prochlorococcus*. Both strains showed differences in the timing and amplitude of response to pollutants. The *rbc*L/*psb*A ratio of both strains decreased as well when exposed to pollutants, possibly meaning a limitation of electron transport.

We performed similar experiments using wild populations from the deep chlorophyll maximum (DCM) during the Malaspina circumnavigation. In these experiments, natural communities were challenged with PAH and OClP mixtures, as

well as with a complex mixture (CM) of organic pollutants obtained by concentrating seawater that contained both known and unknown organic compounds. The pollutants concentration increase in relation to environmental levels was much more higher in PAH (70 - 150 times) and OClP (260 - 470 times) experiments than in CM (1.3 - 1.8 times) experiments. None of the treatments induced cellular death, but *rbc*L expression of both HL and LL decreased after 2 h of exposure to the CM, suggesting that this mixture contained not only the known and quantified organic pollutants, but also uncharacterized organic pollutants that could either contribute to the observed toxicity or have synergistic effects. We also found a decrease of LL-*psb*A expression following a chronic toxicity pattern during the experiments performed with the PAH mixture. No significant effects were observed with the addition of the OClP mixture.

Finally, we sampled 62 stations at 3 m, DCM and DCM+40 m depth during the Malaspina circumnavigation. Relative gene expression levels of *Prochlorococcus rbc*L and *psb*A were determined for a total of 183 samples and the results were compared to as many environmental variables as possible (159), including the concentrations of a number of tracers for three families of organic pollutants. We found clear correlations between our molecular measurements and different independent variables related to photosynthesis. We also corroborated the different physiological adaptations of both clades, especially significant at DCM, where both clades overlap. Light conditions resulted determinant on *Prochlorococcus* photosynthetic capability, especially on HL strains, and on LL strains sampled at the deepest depths. However, the most important finding from these analyses was the relevant role of organic pollutants on the modulation of *Prochlorococcus* photosynthetic capability. Organic pollutants were estimated to decrease the HL-*rbc*L expression up to 20% at 3 m depth. In addition, *Prochlorococcus* abundance was negatively correlated to the concentrations of several bioavailable PAHs, and, even more dramatically, the maximum yield of PSII from the entire photosynthetic community and the chlorophyll *a* concentration from picophytoplankton were also negatively correlated with the organic pollutant concentrations. Hence, this is the first evidence of that a small increase in the concentration of organic pollutants present in seawater can reduce the *Prochlorococcus* photosynthetic capability in field, and, even more alarming, it is the first sign of that the organic pollutant mixture present in oceans already has a strong influence on the oceanic primary production at a global scale.

RESUMEN

El Cambio Global está alterando tan rápidamente el Sistema Tierra que algunos autores lo consideran como el comienzo de una nueva era, el Antropoceno. De entre los nueve límites planetarios que se han propuesto para establecer el paso de Holoceno a Antropoceno, solo dos, la contaminación química y la carga atmosférica de aerosoles, permanecen sin estudiar a nivel global. Los contaminantes orgánicos persistentes (POPs) son parte de estos dos vectores del cambio global, y especialmente preocupantes debido a su persistencia, ubicuidad, tendencia a bioacumularse y su alta toxicidad tanto para el medio ambiente como para los seres vivos. Los POPs son propensos a ser transportados a larga distancia mediante procesos naturales de las aguas continentales y marinas, los suelos, los organismos y, en mayor medida, de la atmósfera, siendo capaces de alcanzar regiones remotas como el océano abierto. Los océanos cumplen una labor significativa en el control del transporte, destino y almacenamiento de los POPs, y además actúan como un importante depósito de estos compuestos. Una vez que los POPs se encuentran en la columna de agua, se pueden bioconcentrar en el fitoplancton y las bacterias, y, consecuentemente, bioacumular en niveles tróficos superiores, acoplándose de esta manera al ciclo del carbono. En trabajos previos se ha demostrado que algunos POPs son tóxicos para los organismos fotosintéticos marinos, lo que podría explicar parcialmente la reducción global de fitoplancton y los cambios globales en las concentraciones de clorofila y producción primaria que han tenido lugar durante el último siglo. Sin embargo, la mayoría de estos trabajos se centraron en analizar los efectos de compuestos individuales, mientras que en el ambiente los organismos están expuestos a complejas mezclas de compuestos químicos. Además, la mayoría de los efectos se midieron a nivel celular, sin considerar los posibles efectos moleculares que también podrían influenciar las funciones ecosistémicas del fitoplancton, por ejemplo la fotosíntesis.

La fotosíntesis oceánica representa aproximadamente la mitad de la fotosíntesis de la Tierra, y un único género, la cianobacteria *Prochlorococcus*, es el responsable de

entre el 5% y el 10% de esta producción primaria oceánica global. *Prochlorococcus* es el organismo fotosintético de vida libre más pequeño que se conoce y, a la vez, el más abundante del mundo. Esta cianobacteria domina numéricamente las aguas oligotróficas de los océanos tropicales y subtropicales de todo el mundo entre los 40° N y 40° S, donde es responsable del 50 – 80% de la producción primaria. El género *Prochlorococcus* está dividido en dos clados principales, high-light (HL) y low-light (LL), según la adaptación de los respectivos clados a altas o bajas intensidades de luz, respectivamente. Las cepas HL se encuentran normalmente en aguas superficiales, mientras que las cepas LL presentan su mayor abundancia en la base de la zona eufótica. En estudios previos se observó que, entre un grupo de organismos fitoplanctónicos, *Prochlorococcus* era el más sensible a diversos contaminantes orgánicos debido a su diminuto tamaño. Además, se descubrió que las mezclas complejas de contaminantes orgánicos resultaban más tóxicas que los compuestos individuales. Sin embargo, estos estudios se realizaron bajo condiciones experimentales controladas, y los efectos de los contaminantes se midieron únicamente a nivel celular.

La presente tesis va un paso más allá al establecer como principal objetivo analizar los efectos de la compleja mezcla de contaminantes orgánicos que se encuentra en el océano sobre la fotosíntesis oceánica en condiciones naturales, a nivel subletal y en el contexto del cambio global, en el que multitud de vectores interrelacionados entre sí actúan a la vez. Usar *Prochlorococcus* como organismo centinela nos permitió un enfoque global de este tema. Diseñamos una metodología útil y sencilla de recolección de muestras para reducir al mínimo la degradación y los cambios del ARNm, manteniendo la integridad de los ácidos nucleicos durante varios meses. Para estudiar los efectos de los vectores del cambio global sobre la capacidad fotosintética de *Prochlorococcus*, diseñamos una herramienta molecular de gran sensibilidad y altamente específica, basada en la cuantificación de la expresión de los genes *rbc*L y *psb*A mediante qRT-PCR, en representación de la fijación de carbono y la funcionalidad del fotosistema II (FSII), respectivamente. La expresión de estos genes fue normalizada por el gen de referencia *rnp*B. Debido a las diferencias fisiológicas, cuantificamos de forma independiente la expresión génica de los clados HL y LL. Esta técnica fue aplicada a experimentos de laboratorio realizados bajo condiciones controladas, donde exponíamos cultivos axénicos de la cepa HL MED4 o la cepa

LL MIT9313 a una de estas dos mezclas comerciales compuestas de unos pocos representantes de la familia correspondiente: hidrocarburos aromáticos policíclicos (PAHs) o pesticidas organoclorados (OClPs). Aunque no detectamos efectos a nivel celular, observamos una reducción del 20% de la expresión relativa del gen *rbc*L, lo que podría desencadenar una reducción de la fijación de CO_2 llevada a cabo por *Prochlorococcus*. Respecto a esta reducción, ambas cepas mostraron diferencias en el tiempo y la amplitud de su respuesta. Además, la ratio *rbc*L/*psb*A de ambas cepas también decreció, lo que podría significar una limitación en el transporte de electrones.

También realizamos experimentos similares usando comunidades naturales del máximo de clorofila profundo (DCM) durante la circunnavegación Malaspina. En esta ocasión, además de las mezclas de PAHs y OClPs, también expusimos a la comunidad natural a una mezcla compleja (CM) de contaminantes orgánicos que provenía de la concentración de agua marina, y contenía tanto contaminantes orgánicos conocidos como posiblemente aquellos que aún no han sido caracterizados. El incremento de la concentración de contaminantes sobre los niveles ambientales fue mucho mayor en los experimentos realizados con las mezclas de PAHs (70 - 150 veces) y OClPs (260 - 470 veces) que los realizados con la CM (1.3 - 1.8 veces). Ninguno de los tratamientos indujo muerte celular, sin embargo la expresión de *rbc*L de ambos clados, HL y LL, disminuyó tras 2 horas de exposición a la CM. Este resultado sugiere que esta mezcla contenía, además de los contaminantes orgánicos conocidos y cuantificados, otros contaminantes orgánicos no caracterizados que también podrían contribuir a la toxicidad observada o ejercer efectos sinérgicos. También encontramos una reducción de la expresión de LL-*psb*A, que seguía un patrón crónico de toxicidad, durante los experimentos realizados con la mezcla de PAHs. Por el contrario, no encontramos ningún efecto significativo cuando añadimos la mezcla de OClPs.

Finalmente, durante la circunnavegación Malaspina muestreamos 62 estaciones, cada una de ellas a tres profundidades: 3 m, DCM y DCM+40 m. Para cada muestra analizamos la expresión génica relativa de *rbc*L y *psb*A en *Prochlorococcus* en relación a todas la variables ambientales que nos fue posible medir, incluyendo las concentraciones de numerosos indicadores para tres familias de contaminantes

orgánicos. La correspondencia ente nuestros parámetros moleculares y diversas variables relacionadas con la fotosíntesis oceánica fue obvia. También corroboramos las diferentes adaptaciones fisiológicas entre los dos clados de *Prochlorococcus*, que fueron especialmente evidentes en el DCM, profundidad a la cual se solapan ambos clados. Las condiciones de luz resultaron ser determinantes para la capacidad fotosintética de *Prochlorococcus*, especialmente para las cepas HL, y para las cepas LL cuando se encontraban a las mayores profundidades analizadas. No obstante, el hallazgo más importante de este trabajo fue que, entre las numerosas variables analizadas, los contaminantes orgánicos tenían un importante papel en la modulación de la capacidad fotosintética de *Prochlorococcus*. Estimamos que los contaminantes orgánicos fueron responsables de una reducción del 20% en la expresión de HL-*rbc*L a 3 m de profundidad. Además, la abundancia de *Prochlorococcus* estaba negativamente correlacionada con varios PAHs biodisponibles. Incluso más preocupante fue que el rendimiento máximo del FSII de la comunidad fotosintética completa y la concentración de clorofila *a* de todo el picofitoplancton también estaban negativamente correlacionadas con la concentración de los contaminantes orgánicos. Por tanto, en esta tesis no solo se evidencia por primera vez que un pequeño incremento de la concentración de los contaminantes orgánicos presentes en el océano pueden reducir la capacidad fotosintética de *Prochlorococcus*, sino también que la compleja mezcla de contaminantes orgánicos que se encuentra presente en el océano ya está teniendo una fuerte influencia sobre la producción primaria oceánica a escala global.

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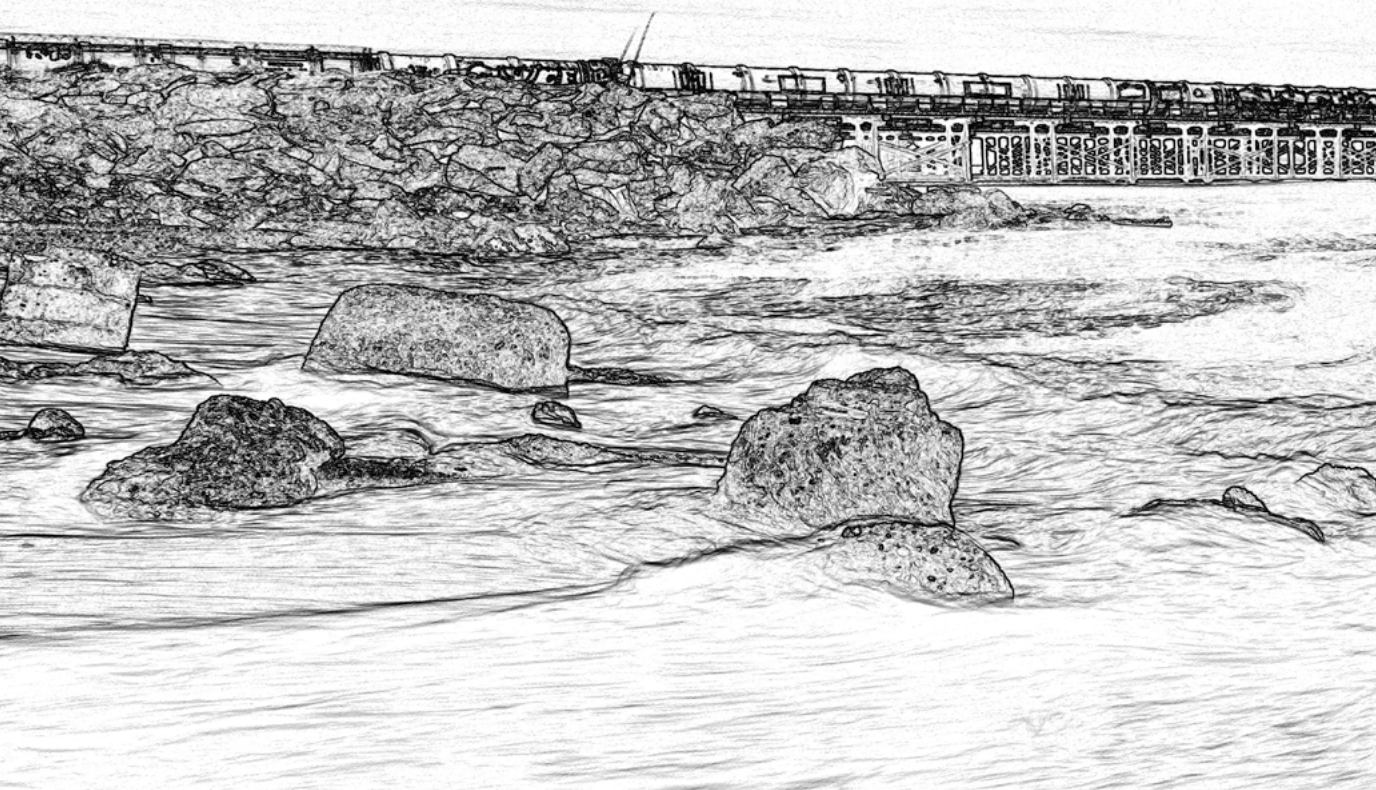
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
CM	Complex mixture of organic pollutants from seawater
Cp	Crossing point in qPCR
CTD	Conductivity temperature and depth sensor
DCM	Deep chlorophyll maximum
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EPA	United States Environmental Protection Agency
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HL	High-ligh adapted *Prochlorococcus* clade
HPLC	High-performance liquid chromatography
IMO	International marine organization
ITS	Internal transcribed spacer
LC10	Lethal concentration to 10% of the population
LL	Low-ligh adapted *Prochlorococcus* clade
mRNA	Messenger RNA
OClP	Organoclorine pesticides
PAH	Polycyclic aromatic hydrocarbon
PAR	Photosynthetically active radiation

PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PLSR	Partial least squares regression
POP	Persistent organic pollutant
PSII	Photosystem II
qPCR	Quantitative real time PCR
qRT-PCR	Quantitative real-time reverse transcription PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
Tm	Melting temperature in qPCR
UNECE	United Nations Economic Commission for Europe
UNEP	United Nations Environment Programme



Chapter I

General introduction

I.1. THE CHALLENGE OF GLOBAL CHANGE

The first concerns about environmental degradation were raised during the 1960s by publications such as "Implications of Rising Carbon Dioxide Content of the Atmosphere" (1), "Silent Spring" (2), or "The Tragedy of the Commons" (3). During the 1970s a global awareness about the rapid environmental modifications started within the scientific community. In 1975 the term "global warming" was used for the first time to refer to the surface temperature increases (4). Later, in the first decisive National Academy of Science study of carbon dioxide's impact on climate, Jule Charney used "climate change" when discussing the many other changes that would be induced by increasing carbon dioxide besides the global warming. But it was one decade later that the term "global change" entered the lexicon during the late 1980s to encompass not only "climate change" but also other critical drivers of environmental change that may alter the Earth System (5-7).

The Earth System is the sum of interacting physical, chemical, biological and ecological processes that support our planet. It consists of land, oceans, atmosphere, every natural cycle (carbon, water, nitrogen, phosphorus, sulphur, etc.) and energy flux. Life is also an integral part of the Earth System that interacts with the rest of the components and includes human society and our activities. Variability and change are realities of the Earth System in a wide range of space and time scales, and the equilibrium conditions are unlikely (8). Nevertheless, this variability remained within domains characterized by well-defined limits and periodic patterns. In this sense, global change refers to the planetary-scale biophysical and socioeconomics changes occurring during the past few decades that are altering the patterns of the internal dynamics of the Earth System. Two main characteristics of global change are the rapidity of these changes, and being humans as dominant force of them (9, 10). It comprises alterations of many spheres: climate, carbon, nutrients and hydrological cycles, food chains, ecosystems, biodiversity, land uses, natural resources, transports, urbanization, population dynamics, and economy, among others. The principal cause of this phenomenon is the fast growth of human population accompanied by the technological development and the increase of natural resources consumption.

Human population has kept an exponential growth for a million of years (11) but the industrial revolution caused a tremendous acceleration of the growth rate, with a population increase from 1 billion people in 1800 to more than seven billion people at the present (12). In addition, life expectancy has also increased globally by almost 20 years since 1960s by about a 40% (13). So many people inhabit now the planet, with so much impact on Earth System, that it is considered that we have entered in a new era, the Anthropocene (see Box I.1). This era is characterized by global changes of several environmental factors, which already have broken the limits of Holocene as consequence of the increasing demands on global resources. Human activities supporting these demands are the immediate drivers of global change. For example, land uses have played an important role in changing environmental processes. Clearing by deforestation and burning for cropland and urbanization affects the global carbon cycle, and probably the global climate: during the Anthropocene, approximately 35% of anthropogenic CO_2 emissions have resulted directly from land use changes (14). Regional climates are affected by land-cover changes through alterations of surface energy and water balance (15, 16). An extreme example of how land use modifies regional climate are urban "heat islands", resulting from reduced vegetation cover, the impervious surface and the morphology of buildings, together with the emission of greenhouse gases and pollutants (17, 18). Agricultural practices such as tillage and irrigation transform the hydrologic cycle and contribute to soil erosion (19-21); chemical fertilizers and pesticides represent an anthropogenic input of nutrients and contaminants that has changed the natural nutrient cycles and has affected widespread air and water quality, terrestrial, freshwater and oceanic ecosystems, and also human health (22-25).

Industry, vehicles and other activities related with the burning of fossil energy sources are also important drivers of global change, since they are responsible of most of the greenhouse-gas, atmospheric pollutant and aerosol emissions (26-29). Roughly three-quarters of the anthropogenic CO_2 emitted between 1980 and 2000 proceeded from fossil fuel burning (30). The atmosphere has been also affected by the significant increase of methane, sulfur dioxide and nitrous oxide concentrations, and the emission of new synthetic chemicals such as halocarbon gases that are both ozone-depleting and greenhouse gases, or the perfluorocarbons that are also greenhouse gasses (30). These perturbations of atmospheric composition are

intimately related to climate change because these greenhouse gases have long (decades to centuries) atmospheric lifetimes and they tend to accumulate in the atmosphere. The result is that over the 20th century global surface temperature increased about 0.6°C, the global average sea level rose between 0.1 and 0.2 meters and the snow cover decreased by 10% (30). Moreover, atmospheric circulation plays an important role in global change because it is responsible of the large-scale transport and redistribution of gaseous and particulate materials found in the atmosphere. Currently we are using more than 100,000 synthetic compounds (31) and about 1,000 of them have been identified in the atmosphere. However, the total number of anthropogenic substances that have already reached the environment is still unknown (32). Regional emissions of long-lifetime compounds can affect remote planetary places thorough atmospheric circulation, and subsequent depositions into soils and oceans. This is the case of persistent organic pollutants (POPs), chemical substances that persist in the environment, bioaccumulate and biomagnify through food webs and pose a risk of causing adverse effects to human health and ecosystems (33, 34). Volatile and semivolatile POPs have a long-range transportation potential mainly by atmospheric circulation (35, 36) and have been found worldwide (37-39).

BOX I.1 - THE ANTHROPOCENE

The Holocene is the postglacial geological epoch that began at approximately 11,700 years BP and is characterized by the accommodating environment for the humankind development (106). It allowed permanent settlements, agriculture and the flourish of more complex societies.

In 2000 Paul Crutzen and Eugene Stoermer coined the term Anthropocene to describe the current geological era in which human activities have become the major geological and morphological force of Earth's natural system (107). The resilience of the planet is associated with the natural variability of the Holocene. This variability has been surpassed during the past three centuries because of the growing pressure of human population that has leaded to a new state marked by global environmental changes (108). The Anthropocene started concurrently with industrialization around 1800, and its central feature is the fast spread of fossil fuel-based energy systems (108). The transition to this new epoch has especially accelerated after the Second World War. Since then, population doubled in 50 years and the environment changed more rapidly and extensively than in any other period in human history (109). Will Steffen and coworkers (8) showed several examples of human activities that experienced an

exponential increase during Anthropocene, such as the water use, the damming of rivers, the fertilizer consumption or the number of motor vehicles (Fig. I.1). The resulting global changes in the Earth System can be used as indicators to track the progression of the Anthropocene. In summary, the global indicators are:

1. Atmospheric composition has been perturbed by the dramatic increase of greenhouse gases: concentrations of CO_2, N_2O and CH_4 have risen by 40%, 18% and 147%, respectively, during Anthropocene (110-113). The ozone content in the total atmospheric column began to decrease in the late 1970's, dropping below 125 D.U. in 1987 versus the 300 to 320 D.U. of the preindustrial era (114).

2. Climate change comprises, among others factors, an increase of land surface temperature at a rate of 0.09- 0.10 °C decade^{-1} since 1901 and 0.23- 0.28 °C decade^{-1} since 1971, resulting in a rise of the mean surface temperature of about 0.6 °C (115). This increase is assume to be responsible for an increment of the frequency of great floods (116).

3. Ocean environmental damage is driven by diverse factors: overfishing has collapsed large-fish populations. Of the fish stocks assessed and monitored by FAO since 1971, 57.4% were fully exploited, 29.9% overexploited and only 12.7% non-fully exploited in 2009 (117). Steffen and coworkers (8) used the annual shrimp farming production as a proxy for coastal zone anthropogenic alteration, which is not only caused by the aquaculture but also by the tremendous growth rates of coastal cities that presents a population density of 80 persons per square kilometre (118), and the subsequent anthropogenic activities on the shoreline including constructions, dredgings and so on. Human activities have also increased dramatically the nutrients loadings into the ocean. Global fluxes in rivers to coastal oceans are some twofold greater for nitrogen (119, 120) and two-to three-fold greater for phosphorus (121) than before the Industrial and Agricultural Revolutions. Moreover, it should be added the entrance of nutrients through atmospheric deposition (122).

4. Changes in land-use are one of the most evident global changes. By the late twentieth century a half of the amount of forest in 1,850 was converted to other land covers (123) and this conversion has been accelerating until reaching the current average annual net loss of forest of about 5.2 million hectares (124). This forest loss is consistent with the increase of cropland and pasture areas more than six-fold during Anthropocene (125).

5. Biodiversity loss rates are higher than would be expected from the fossil record, so a sixth mass extinction may be under way (126). Human activity has forced 869 species to extinction in the last 500 years, and currently 18,788 species are known to be threatened with extinction. This may be a gross underestimate because only 52,017 species out of 1.9 million described species on the world have been assessed (less than 3%) (127).

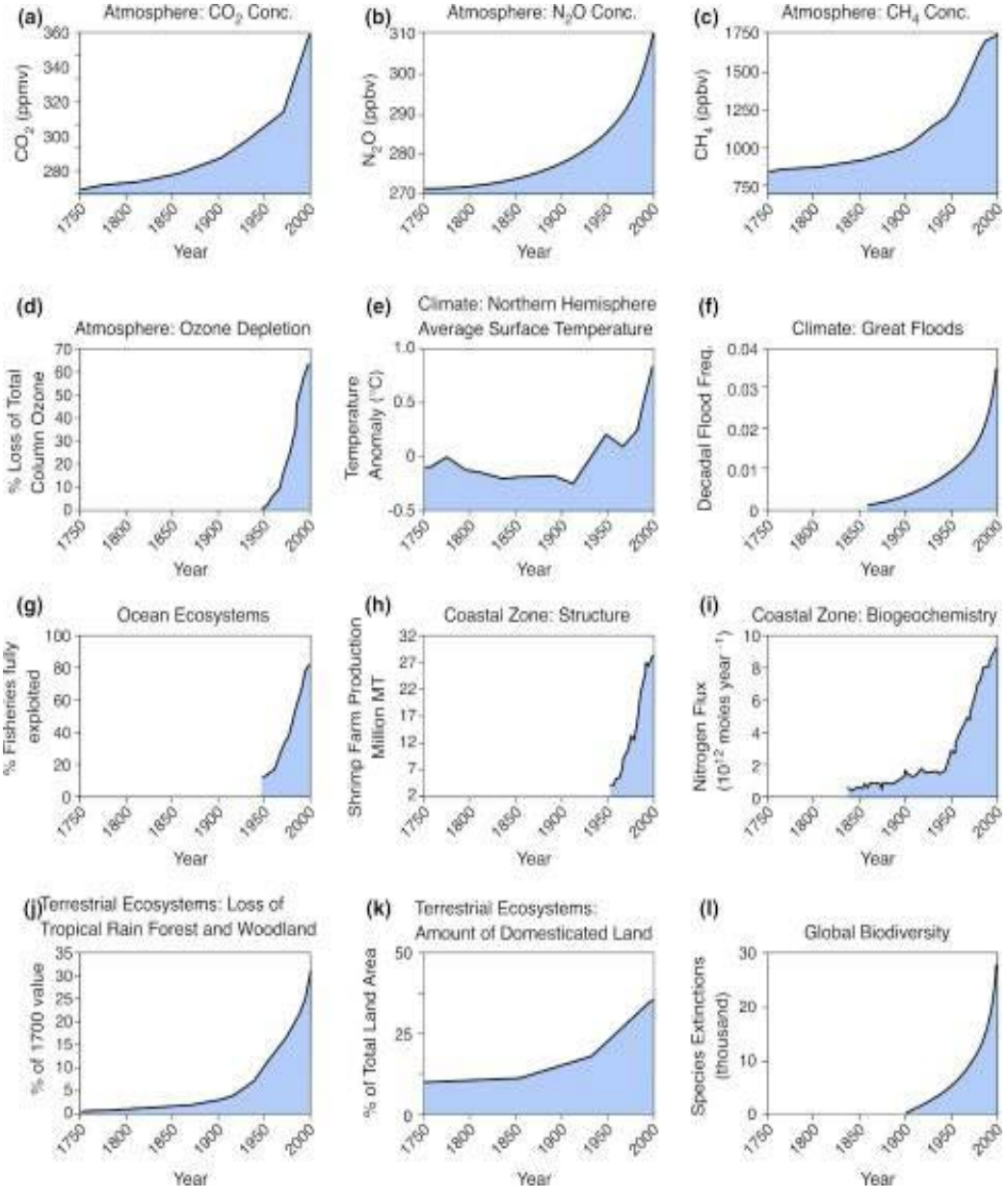


FIGURE I.1 - THE ANTHROPOCENE

Planetary-scale changes in the Earth System that support the transition to a new epoch: The Anthropocene. Figure: Steffen et al., 2004 (8).

Furthermore, climate change is contributing to a stronger dispersion of POPs, since increasing temperature enhances volatilization and therefore leads to increased emissions into air (40, 41). In this sense, the intensification of global warming would likely increase the exposure of the environment and ecosystems to POPs (40, 42, 43).

Environmental research focused mainly in the study of changes that occur on land (e.g., land use), or in the atmosphere (e.g., climate change), rather than in the ocean. However, ocean is equally, if not arguably more important than land or atmosphere in the functioning of the Earth System. Ocean covers 71% of the Earth' s surface, comprises 97% of the water, and holds a larger amount of heat than the land surfaces, regulating global climate (44), and stores much more carbon than the land and atmosphere combined (10). Most marine organisms are small, can readily distribute through oceanic currents, and present short turnover times, therefore they react rapidly to environmental changes (45).

Oceanic phytoplankton accounts for about 46% of the global primary production (46, 47). Around 35% of new organic carbon sinks below the upper sunlit layer (48). The CO_2 sequestration by phytoplankton is named the biological pump and it is a key process in regulating oceanic carbon cycle and the CO_2 exchange between atmosphere and ocean (49, 50). Given that oceanic and atmospheric gas concentrations tend towards equilibrium, any CO_2 increase in atmosphere will drive more CO_2 into the ocean. Over the past 200 years the ocean has been a net sink of anthropogenic CO_2 (51) and its uptake has increase acutely since the 1950s. Therefore, ocean has taken up approximately one-third of the anthropogenic CO_2 released into the atmosphere during Anthropocene (51), buffering the effects of increasing levels of CO_2 in the atmosphere and limiting the rise of global temperatures. When CO_2 dissolves in seawater it forms carbonic acid (H_2CO_3), decreasing the oceanic pH. As direct consequence of the atmospheric CO_2 increase, the ocean pH has dropped by 0.1 during the last two centuries (52). This process is known as ocean acidification (52, 53) and is responsible for reducing calcification in corals, coralline macroalgae, and planktonic organisms (54-56), ultimately slowing down the CO_2 sequestration by the biological pump.

Despite the buffering effects of oceans, the greenhouse effect of rising atmospheric CO_2 has increased the mean sea-surface temperatures by 0.13 °C per decade since 1979 (57). Even though this rate is about a half as that for land (57), the impacts on marine systems are evident (58): temperature increase influences water and ice volumes resulting in a sea level rise (59); oceanic primary productivity is undergoing a seasonal shift in some areas (60); and many organisms are expanding towards the poles (61, 62). In addition, CO_2 solubility reduces with higher temperatures contributing to the biological pump drawdown (51). The same occurs with oxygen, which availability has been declining during the last decades as the ocean warmed, generating hypoxia in diverse areas (63-66). Hence, these changes caused by ocean warming are affecting the trophic webs with implications for ocean-climate interactions and living marine resources (67). This situation grows worse with the eutrophication originated by increased riverine and atmospheric inputs of nitrogen and phosphorous (68, 69) that enhances the microbial activity and therefore increases the consumption of dissolved oxygen.

Besides the increase in carbon and nutrients entry into the ocean, hundreds of anthropogenic organic pollutants have been detected in marine environments (70, 71). These contaminants are coupled to carbon cycle (72-74) and their fate, occurrence and toxicity depend on nutrient loads (75, 76, 77). Most of them are lipophilic and tend to bioaccumulate and biomagnify through food webs (78-81). Moreover, semivolatile organic pollutants are prone to long-range atmospheric transport to remote open sea areas and even to the poles (36, 82, 83). Given the variety of organic pollutants, their effects on marine organisms and on their habitats are diverse. In general, they reduce the species richness in the vast majority of studied habitats, being the most sensitive species the first one to disappear (84). In phytoplankton, an inverse relationship between sensitivity and cell size has been observed (85). Organic substances used as herbicides, like diuron, reduce the growth rate and photosynthetic activity of microalgae, seagrass and coral symbiotic dinoflagellates (86-88), decreasing the primary production and hence affecting the entire ecosystem. Insecticides can also affect the photosynthesis; for instance, lindane decreases biomass production and photosynthetic rate of cyanobacteria (89, 90). Some others organic pollutants are endocrine disruptors, like the antifouling agent tributyltin that causes irreversible sexual disruption of female molluscs and

has led to local extinctions because the affected individuals cannot reproduce (91). The adverse effects of polycyclic aromatic hydrocarbons (PAH) on oceanic ecosystem have been thoroughly studied since they are also directly released to the ocean from oil drillings, accidental spillages or intentional discharges by vessels. PAHs toxicity has been demonstrated in bacteria (92, 93), phytoplankton (85, 94, 95), algae (93, 96), invertebrates (97-99) , fishes (100, 101), marine mammals, and seabirds in a cascade of direct and indirect impacts of PAHs, which can change an entire ecosystem (102).

Synergistic effects of all these drivers of global change could be responsible of the global phytoplankton decline and global changes in chlorophyll concentrations and primary production rates over the past century (103, 104). Although there is still considerable uncertainty about the influence of each driver and even more about their synergistic effects (104), it is clear that global change is being increasingly manifested in important and tangible ways. Nine planetary boundaries for the transition from the Holocene to the Anthropocene have been established: climate change, ocean acidification, stratospheric ozone depletion, changes to the biogeochemical nitrogen and phosphorus cycles, global freshwater use, land system change, biodiversity loss rate, chemical pollution, and atmospheric aerosol loading (105, Fig. I.2). Analysis of the first seven drivers based on the values recorded during the Holocene proposed that three of these safe boundaries have already been crossed: climate change, biodiversity loss rate, and changes to the global nitrogen cycle. Unfortunately, there is insufficient data on chemical pollution and atmospheric aerosol loading quantifications to determine the safe boundaries for Earth (105, Fig. I.2).

Planetary boundaries and their underlying processes are tightly coupled, and the transgression of one of them can drive the transgression of the rest. That is the reason why Global Change must be studied comprehensively and holistically towards the goal of understanding the dynamics of the planetary life support system as a whole. Rapid changes of Earth System have forced a fast generation of scientific knowledge and the integration of different research disciplines. These efforts must be sustained and increased in the future to support the societal action to achieve the sustainability and face this challenge of Global Change.

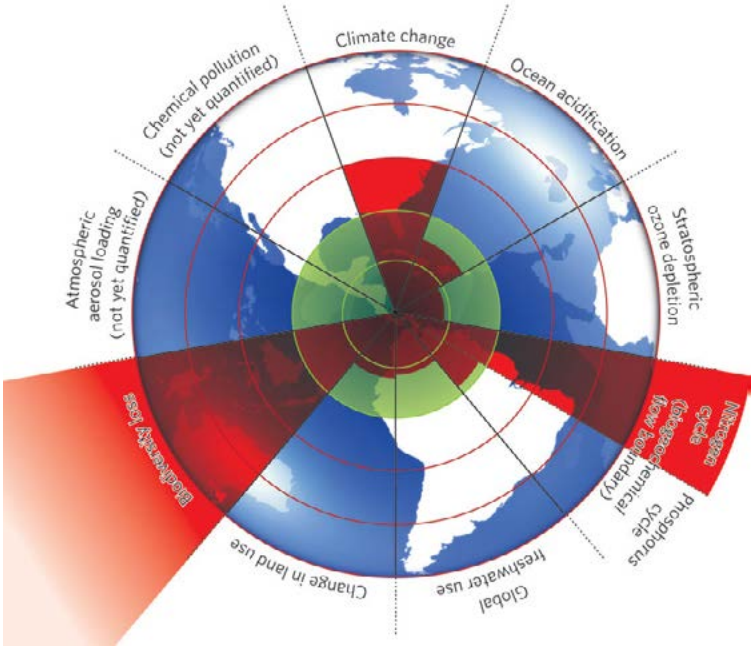


FIGURE I.2 - PLANETARY BOUNDARIES

Planetary boundaries of nine Earth System processes that must not be transgressed to avoid a global environmental change. Green shading represents the safe operating space and red wedges represent the current position for each variable. Three boundaries (rate of biodiversity loss, climate change and anthropogenic changes of nitrogen cycle), have already been crossed. Figure: Rockström et al., 2009 (105).

I.2. PERSISTENT ORGANIC POLLUTANTS AS A VECTOR OF GLOBAL CHANGE

More than 100 million chemicals are commercially available, but only 340,000 of them are reported to be inventoried/regulated according to the Chemical Abstracts Service (CAS) web site (128). Out of these, about three million synthetic substances are organic (129), being thousands or tens of thousands of them released into the environment (32, 130). Some of these chemicals, the persistent organic pollutants (POPs), have become an issue of global concern due to their impact on the environment and human health (40). POPs constitute a diverse group of organic compounds, principally of anthropogenic origin, that possess an intrinsic

combination of physical and chemical properties that determine their behaviour once released into the environment (35, 131, 132):

- As the name indicates, POPs are persistent, i.e., they remain intact for exceptionally long periods of time (many years), since they are highly resistant to photolytic, chemical and biological degradation (133, 134).
- POPs are ubiquitous throughout the globe as result of their propensity to long-range transportation by natural processes involving fresh and marine waters, soils, organisms and, most notably, the atmosphere (36, 135, 136). Consequently, they reach regions where they have never been produced or used (37, 137-139).
- POPs are hydrophobic and bioaccumulative in lipid-rich tissues of biota. They biomagnify through food webs (34), being found at higher concentrations at higher levels in the food chain, where concentrations can become magnified by up to 70,000 times the background levels (140). Thus, POPs have been reported in humans tissues throughout the world (141, 142).
- POPs are highly toxic to humans and wildlife, and they can result in both acute and chronic effects. Numerous studies have demonstrated that POPs can cause reduction of reproductive success (143), behavioural changes (144), endocrine and immune systems disruption (145, 146), damage to the nervous system (144) and the liver (147), and cancer (148).

Since World War II, thousands of synthetic chemicals, including POPs, were introduced into commercial use with the aims of increasing the crop production, controlling diseases and facilitating industrial development (149, 150). However, despite their effectiveness in many applications, they had unforeseen effects on human health and on the environment. The first public warning about their hazard came with the publication of Silent Spring in 1962 (2). Since then, a raising awareness on POPs impact prompted the emergence of a new scientific field during the 1970s (151-153) dealing with environmental risks associated to the occurrence and fate of POPs, and it has had a decisive impact on regional and international legislation. Beginning in the 1970s, the use of several organochlorine pesticides (OClPs) were banned or restricted in North America, Western Europe, and Japan. In 1976

United States passed the Toxic Substances Control Act (TSCA) with the purpose of regulating new commercial chemicals before they enter the market and the existing chemicals that pose a risk to health or to the environment. Over the following years similar legislation on chemicals was introduced in Canada, Japan and Europe among others regions, but it was in 1987 when the first international treaty on chemicals including POPs was adopted (Box I.2). Since then, international concern on POPs regularization has been evidenced in diverse agreements that included it among their objectives (Box I.2). Efforts to eliminate or restrict the production and use of POPs reached their highest international landmark with the Stockholm Convention

BOX I.2 - POP INTERNATIONAL REGULARIZATION

1987 - Montreal Protocol on Substances that Deplete the Ozone Layer of the Vienna Convention for the Protection of the Ozone Layer (UNEP). Objective: phasing out the production of numerous substances that are responsible for ozone depletion

1989 - Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal (UNEP). Objective: protecting human health and the environment against the adverse effects of hazardous wastes.

1998 - Aarhus Protocol on Persistent Organic Pollutants (POPs) of the Convention on Long-range Transboundary Air Pollution (CLRTAP, UNECE). Objective: eliminating any discharges, emissions and losses of POPs.

1998 - Rotterdam Convention on the Prior Informed Consent (PIC) Procedure for Certain Hazardous Chemicals and Pesticides in International Trade (UNEP). Objective: promoting shared responsibility and cooperative efforts among Parties in the international trade of certain hazardous chemicals in order to protect human health and the environment

2001 - Stockholm Convention on Persistent Organic Pollutants (POPs, UNEP). Objective: protecting human health and the environment from persistent organic pollutants.

2001 - International Convention on the Control of Harmful Anti-fouling Systems on Ships (IMO). Objective: prohibiting the use of harmful organotins in anti-fouling paints used on ships and prevent the future use of other harmful substances in anti-fouling systems.

2002 - Globally Harmonized System of Classification and Labelling of Chemicals (GHS, UNECE). Objective: ensuring that information on physical hazards and toxicity from chemicals is available in order to enhance the protection of human health and the environment during their handling, transport and use.

on Persistent Organic Pollutants. This global treaty was adopted in 2001, entered into force in 2004 (140) and currently has been ratified by 179 parties that have agreed to reduce or eliminate the production, use, and/or release of the POPs listed in the Convention. At first, it included 12 POPs of global concern, the "dirty dozen", but new chemical have been added to the agreement by a scientific review procedure until reaching a total of 23 listed POPs nowadays (Box I.3), and a few others are proposed as potential POPs. Additional substances and substance groups are recognized as POPs by the Aarhus Protocol on Persistent Organic Pollutants adopted under the auspices of the Convention on Long-range Transboundary Air Pollution (CLRTAP) (154), such as the polycyclic aromatic hydrocarbons (PAHs).

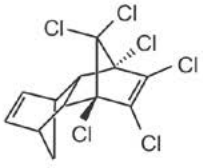
Despite of the great progress in research and regularization on POPs during the two last decades, the listed substances in these agreements represent only a small fraction of the chemicals widely used. Some chemicals have shown POP-like properties but they have not yet been measured in the environment (32). Emerging POPs present some differences with respect to legacy POPs, for example, some of them are polar and their sampling and analysis must be adapted taking into account their distinct behaviour in water (35). Obtaining relevant and reliable information on production and use of POPs is still difficult, and the few existing inventories include only the intentionally produced chemicals but not the by-products (35). In addition, ecotoxicological tests are usually performed for a single chemical or a limited group of them whereas organisms are exposed to very complex mixtures of contaminants, which may have biological effects different from those caused by their isolated components (155-157). Therefore, we have just begun to accumulate some experience on POPs occurrence, fate, and effects, but there is still a lot of research to be done.

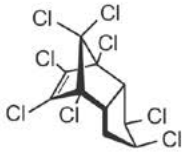
I.2.1. TYPES OF PERSISTENT ORGANIC POLLUTANTS

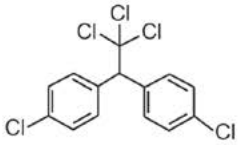
POPs constitute a diverse group of organic substances (Box I.3) that share some particular characteristics when they are released into the environment. In this context, "substance" means: a) a single chemical species or b) a set of chemical

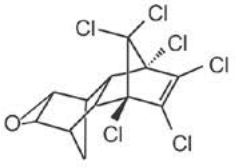
POPs listed in Stockholm Convention

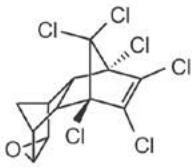
Dirty dozen (2001)

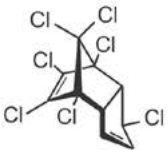

Aldrin

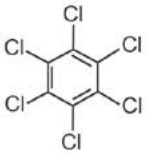

Chlordane

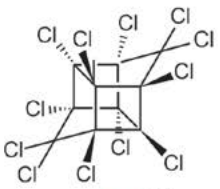

DDT

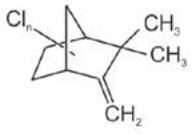

Dieldrin

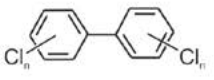

Endrin

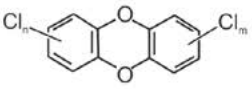

Heptachlor

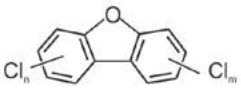

Hexachlorobenzene


Mirex

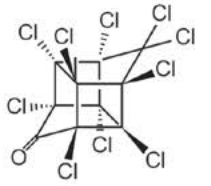

Toxaphene

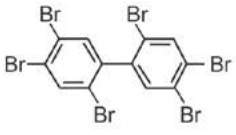

Polychlorinated biphenyl (PCB)


Polychlorinated dibenzo-p-dioxins (PCDD)

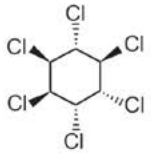

Polychlorinated dibenzofurans (PCDF)

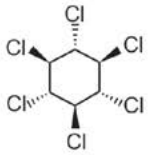
Added in 2009

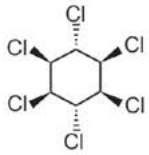

Chlordecone

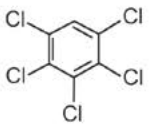

Hexabromo-biphenyl


Hexabromobiphenyl ether (Hexa-BDE) & Hepta-BDE

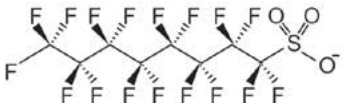

α-hexachlorocyclo-hexane (α-HCH)


β-HCH


Lindane (γ-HCH)


Pentachlorobenzene (PeCB)


Tetra-BDE & Penta-BDE


Perfluorooctane sulfonic acid (PFOS),its salts & perfluorooctane sulfonyl floride (PFOS-F)

BOX I.3 - PERSISTENT ORGANIC POLLUTANTS

POPs listed in the Aarhus Protocol and the Stockholm Convention on Persistent Organic Pollutants. The Aarhus Protocol was adopted in 1998 and focused on 16 substances singled out because of their potential risk to human health and to the environment. Years later, in 2001, the Stockholm Convention was built on the Aarhus Protocol raising the POPs issue to the global level. It started including 12 substances ("the dirty dozen") and new substances have been added on three occasions (2009, 2011 and 2013) to reach the current 23 listed POPs. The Convention includes all substances listed under the Protocol, except hexachlorobutadien, the rest of hexachlorocyclohexane isomers, polychlorinated naphthalenes, polycyclic aromatic hydrocarbon and short-chain chlorinated paraffins. Both agreements classify POPs under three different groups: "for elimination" (red circle), "for restriction" (yellow circle) and "for reduction" (blue circle). POPs are also arranged according to their use in three categories: "Industrial chemical" (industry icon), pesticide (sprayer icon) and unintentional production (flame icon).

species that have similar properties and are emitted together or form a mixture normally marketed as a single product. The most commonly used criteria for classifying POPs is based on their production and application (158, 159). We can distinguish intentionally produced chemicals from unintentionally produced ones, which are formed as accidental by-products of various combustion processes.

A) **Intentionally produced POPs:** according to their applications, intentionally produced POPs comprise two majors groups among others: organochlorine pesticides (OClPs) and industrial chemicals.

- Organochlorine Pesticides (OClPs): pesticides are chemicals used to control organisms considered pest. They comprise mainly insecticides, fungicides and herbicides, but also rodenticides, nematicides, molluscicides, and acaricides. Since the 1950s they were widely used throughout the world in agriculture and disease and pest control. For example, Dichlorodiphenyltrichloroethane (DDT) was used extensively during World War II to protect soldiers and civilians from insect-borne diseases such as malaria and typhus. After the war, it continued being used on agricultural crops, particularly cotton (160). In 1970–1980 DDT was one of the most widely used pesticides in the world, together with hexachlorobenzene (HCB), used against fungi that affect food crops such as the wheat bunt, and hexachlorocyclohexane (HCH), a broad-spectrum insecticide used to treat seeds, trees, wood, soils and against ectoparasites in both domestic animals and humans (140). Today, the Stockholm Convention regulates a number of these pesticides: aldrin, chlordane, DDT, dieldrin, endrin, heptochlor, HCB, mirex, toxaphene, chlordecone, HCH, pentachlorobenzene and endosufan (Box I.3).

- Industrial chemicals: this group contains diverse substances (Box I.3) useful in a variety of industrial applications. Common examples are the polychlorinated biphenyls (PCBs), extensively used until the late 1970s in electrical transformers and large capacitors, as hydraulic and heat exchange fluids, and as additives in paints, lubricants, carbonless copy paper, and plastics (140, 160). HCB, besides its use as a fungicide, was used to make fireworks and ammunition, as a peptizing agent in the production of synthetic

rubber, as a porosity controller in the manufacture of graphite electrodes, and as a chemical intermediate in dye manufacturing (161). Other industrial chemicals listed in the Stockholm Convention are hexabromobiphenyl (HBB), hexabromocyclododecane (HBCD), pentachlorobenzene (PeCB) and four polybrominated diphenyl ethers (PBDEs), which have been used as flame retardants in a wide variety of products, including plastics, furniture, electrical equipment, electronic devices, textiles, etc. The Stockholm Convention also regulates the perfluorooctane sulfonic acid (PFOS), its salts, and the perfluorooctane sulfonyl fluoride (PFOSF), widely used in products such as in electric and electronic parts, firefighting foam, photo imaging, hydraulic fluids, and textiles (140). On the other hand, the Aarhus Protocol regulates hexachlorobutadiene (HCBD), which most important use is as an intermediate in the production of rubber (160), and polychlorinated naphthalenes (PCNs) that are mainly used in cable insulation, wood preservation and engine oil additives among others (162).

B) **Unintentionally produced chemicals:** some of the intentionally produced substances previously mentioned may also be accidentally produced and released into the environment (Box I.3). For instance, HCB is also produced unintentionally during the manufacture of several chlorinated compounds (pesticides, solvents, and so on) (161). Likewise, PeCB occurs as a byproduct in combustions, thermal and industrial processes and is found at trace levels in solvents and pesticides (140). Other unintentional way of releasing POPs into nature is through an inappropriate waste management. This is the case for PCBs that in spite of being banned they are still released from waste sites, electrical transformers or by the incineration of garbage containing PCBs (160). However, the three main groups of unintentionally produced POPs are polychlorinated dibenzo-p-dioxins (dioxins), polychlorinated dibenzofurans (furans) and polycyclic aromatic hydrocarbons (PAHs) (Fig. I.3). Unlike the rest of the substances listed in the Convention and the Protocol, these chemicals do not have any use and are not intentionally manufactured for any purpose (140):

- Dioxins and furans: these two groups comprise chlorinated aromatic compounds that generally are considered together by virtue of their similar structure, chemical properties, toxic effects, and sources. Dioxins are formed as by-products of incomplete combustion involving chlorine-containing compounds (burning of hospital waste, municipal waste, automobile emissions, etc.) and in the production of chlorinated substances (e.g., pesticides). Furans are produce from the same processes that dioxins, and also during the manufacture of PCBs. There are 75 different dioxin and 135 furan congeners, and their toxicities vary, but a number of them are considered to be of concern (140).

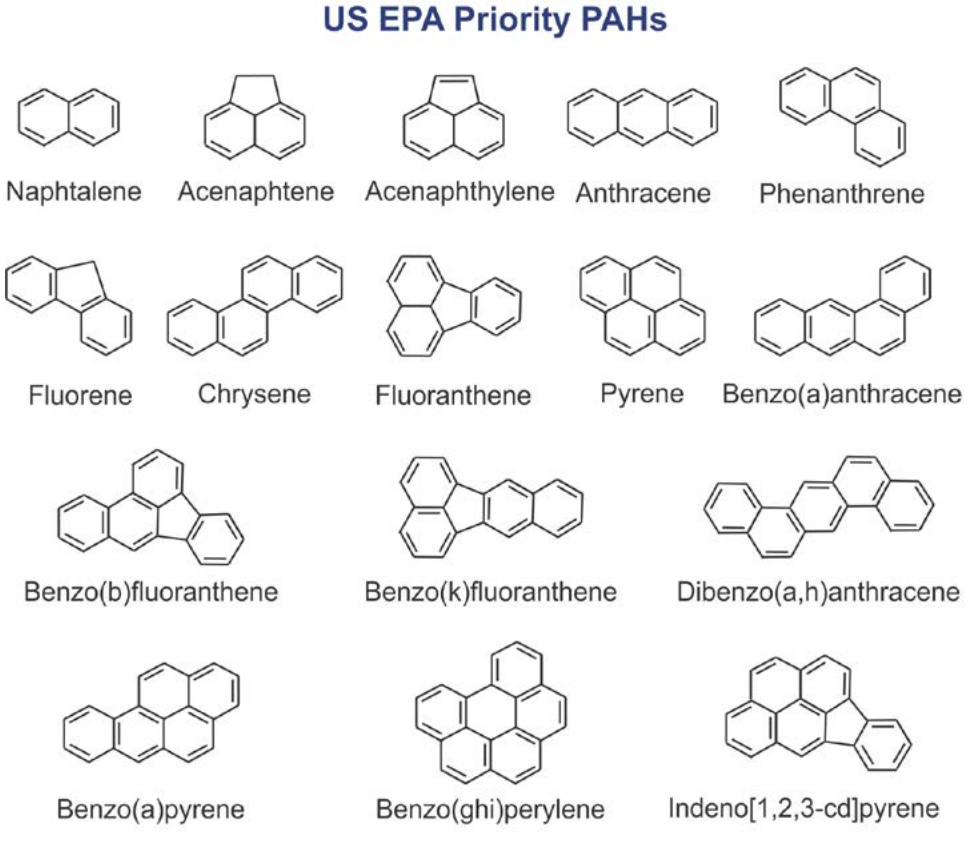


FIGURE I.3 - POLYCYCLIC AROMATIC HYDROCARBONS

16 Polycyclic aromatic hydrocarbons (PAHs) on the United States Environmental Protection Agency (EPA) priority pollutant list.

- PAHs: these compounds contain only carbon and hydrogen that form two or more fused benzene rings in linear, angular or cluster arrangements. They are always found together in groups of several PAHs (160). PAHs occur as result of incomplete combustions of material containing carbon and hydrogen like coal, oil, gas, petrol or wood (160). They can have natural sources such as forest and prairie fires, volcanoes (163, 164) or biosynthesis by plants, algae, phytoplankton and microorganisms (165). However, the greatest amounts of PAHs released into the environment come from anthropogenic fossil fuel combustion (166). Their main differences in relation to the legacy POPs are a shorter atmospheric half- lives (167), soot carbon affinity (168, 169) and a distribution influenced by the ongoing emissions (166, 170). These dissimilarities make PAHs unfit to be listed in the Stockholm Convention and hence they are only included in the Aarhus Protocol (154). Regardless, PAHs are semivolatile and stable enough in the atmosphere to enable them to travel long distances before deposition and thus reach remote areas (171, 172). They have been detected in soil, air, sediments, seawater, rivers and consumable products worldwide (173-175), and in addition they are bioaccumulative and highly toxic (166). Indeed, 16 PAHs were included on the United States Environmental Protection Agency (EPA) list of priority pollutants as possible human carcinogens (160, Fig I.3).

I.2.2. OCCURRENCE AND FATE OF PERSISTENT ORGANIC POLLUTANTS

The behaviour and fate of POPs in the environment are determined by a combination of three set of factors: a) their physicochemical properties, determined by the nature of the atoms present in the molecule and its structure, which control persistence, reactivity and partitioning in the environmental matrices; b) environmental conditions such as temperatures, wind speed, or organic matter content; and c) their primary and secondary sources, including patterns of use, the compartment in which they are stored and released and frequency of introduction (167). Hence, although substances with similar properties tend to move together,

they can be differently fractionated during transport, owing to cold trapping (36) or selective sequestration by the ocean (176).

Most POPs are volatile enough to cycle at the environmental temperature range through volatilization to the atmosphere and subsequent deposition to proximate or remote soils and water (36, Fig. I.4). Three natural processes are responsible of POPs distribution: convection by water and/or atmospheric circulation, and biotransport by organism migration (Fig. I.4). Transport by organism migration happens when POPs are bioaccumulated in organisms that move periodically from place to place, acting as biovectors (177) on both local (e.g., amphipods that move from surface waters to seafloor in the ocean (178) and long-range scales (e.g., gulls that breeding in remote areas far away from their overwintering areas (178). Transport by water can occur in ocean currents and rivers, and it may be significant for polar emerging POPs, such as perfluorooctane sulfonic acid (PFOS) (35). Once in the water, POPs can dissolve or remain sorbed to particles. In any case, they evaporate or become degraded or sequestered by sinking particles or in sediments. Nonetheless, atmospheric transport has been identified as the key global dispersal mechanism for most

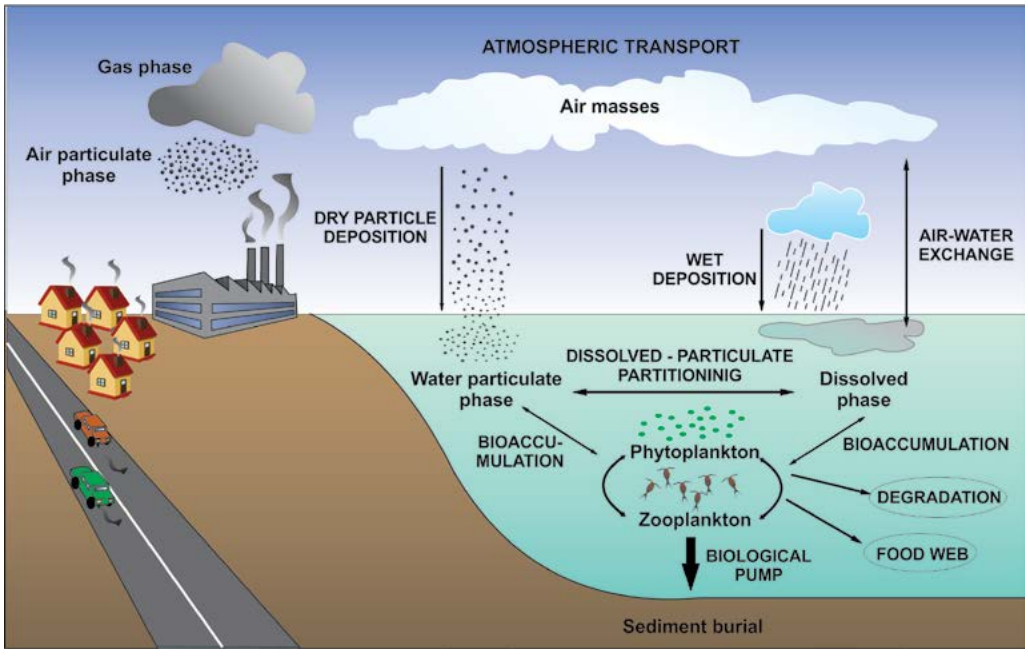


FIGURE I.4 - GLOBAL CYCLE OF PERSISTENT ORGANIC POLLUTANTS (POPS)

Main processes affecting fate and transport of POPs at their different phases. Figure: Adapted from Berrojalbiz, 2011 (215)

legacy POPs (35). Semivolatile POPs are partitioned in the atmosphere between the gas and aerosol phases (135). Highly volatile substances tend to volatilize and to remain in the atmosphere until they are degraded (flyer compounds). However most POPs are semivolatile, hydrophobic and have high affinity for organic matter. These characteristics allow them to move around the globe by repeated cycles of volatilization, long-range air transport and deposition. This cycle is often called the "grasshopper effect" and the implicated POPs nicknamed as "grasshopper" (36). There are three mechanisms for atmospheric deposition of POPs, and the relative importance of one process over the other depends on the POP's physicochemical properties:

1. Atmospheric dry deposition is the process by which aerosol particles fall to soil or water surface driven by gravity (coarse aerosols) or turbulence (fine aerosols). This is the dominating depositional process for POPs tending to associate with atmospheric aerosol particles (e.g., high molecular weight PAHs). Dry deposition velocity depends on many factors related with particle nature (size, density), surface properties (humidity, organic matter) and meteorology (wind speed, relative humidity) (179, 180).

2. Atmospheric wet deposition refers to scavenging of gas and aerosol phase POPs by rainwater or snow (181-183). The magnitude of this process is intimately related to the intensity and spatial and seasonal variations of precipitation (181).

3. Diffusive air-water vapour exchange is the transfer of POPs molecules between the atmospheric gas phase and the water dissolved phase driven by a deviation of the chemical equilibrium between the air and water phase (184). Chemical equilibrium means that the escaping tendency, or fugacity, is equal in both phases, but it does not imply equal concentrations (185). Diffusive fluxes may be significant for POPs with a large fraction in the atmospheric gas phase, accounting for 50-85% of the atmospheric deposition of organochlorine compounds to the sea (186). For 2-4 ring PAHs, these fluxes can be several order of magnitude higher than the dry deposition rates (187).

POPs cycling in the environment can be strongly retarded or prevented by their accumulation in organic matter (188) (74, 189). This coupling with organic

carbon is highly relevant for the fate of POPs, for example, being a key vector for their transport and partitioning in oceans and deep lakes (72, 73, 76, 136, 190), or influencing the spatial, horizontal and vertical POPs distribution in soils (191). POPs can remain stored in these reservoirs for long periods of time. If disturbed, however, POPs can remobilize again into the atmosphere/water. The emissions from these reservoirs are the secondary sources of POPs, and can be more important than primary sources for those that are no longer produced (136, 192) (193).

High temperatures and low surface organic matter enhance volatilization and therefore leads to increased emissions into the air (40, 41, 194). In the scenario of global change, POPs remissions from reservoirs will accelerate due to global warming (40, 42, 43), increasing their concentrations in environmental compartments (193, 195). Global warming also will increase the frequency of extreme events such as melting ice, storms, floods, and forest fires (57), which will modify POPs transport, deposition and remobilization. Global changes in land use and nutrient cycle will also affect ecological processes such as photosynthesis and respiration (196-198), disturbing the fluxes and sinks of carbon and, consequently, of POPs (199).

I.2.3. PERSISTENT ORGANIC POLLUTANTS IN THE OCEAN

Oceans play an important role in controlling the environmental transport, fate and sinks of POPs at both regional and global scales (36, 74). Since oceans cover 70% of the planet's surface, they can act as a critical reservoir accumulating a large inventory of POPs (74, 200). Riverine inputs and run-offs may dominate the POPs entry at coastal sites, rather than atmospheric loadings (201, 202). However, atmospheric deposition is the main source of POPs in the open sea (181, 203) and explains the presence of the major POP families in remote marine areas worldwide (170, 204, 205). Water-column processes remove POPs from the mixed surface water layer, reducing the volatilization rates (206, 207). POPs can dissolve in water or couple to organic matter, mainly by bioconcentration (Box. I.4) in phytoplankton (208) and bacteria (209). This uptake occurs in two steps, where the first compartment for POPs is the cellular surface and the second compartment is the cytoplasm (78).

BOX I.4 - GLOSSARY OF TERMS

- **Bioconcentration**: process by which the concentration of a chemical in an aquatic organism exceeds that in water as a result of the passive uptake (partitioning) of a chemical by an aquatic organism from water. It can occur through cell wall, skin or respiratory surfaces.
- **Bioaccumulation**: process by which the concentration of a chemical in an aquatic organism exceeds that in water as a result of uptake through all possible routes of chemical exposure, including actives mechanisms (diet, respiration, dermal absorption, etc.)
- **Biomagnification**: process by which the concentration of a chemical in an aquatic organism exceeds that in organism's diet as a result of dietary absorption. It is due to the slowly elimination of POPs from organisms because of their hydrophobicity. As a consequence of this persistence they become more concentrated the further they move up through food chains.

POPs accumulated in primary producers may become part of the biological pump and be sequestered in the deep ocean (74), which together with atmospheric degradation are the main sinks of POPs (74, 180, 210). This is a highly relevant process, since biological pump influences the transport, occurrence, and distribution of POPs in aquatic environments (74, 168, 211), affecting the air– water diffusive exchange (76, 206) and atmospheric deposition (212) of POPs, and even modulate their atmospheric transport (83, 212). The biological pump transports POPs to deep waters and sediments that constitute their main reservoir in the open ocean. Sediments ordinarily present higher concentrations of POPs than seawater (136, 213).

Despite sinking is the dominant flux for the more hydrophobic substances, especially in poorly stratified waters, sediments can also be a secondary source of POPs when resuspended (136). This source strongly influence levels of POPs in deep water and in some environments make them available for mixing throughout the water column and for bioconcentration in organisms (136, 192). Multicompartimental diffusion between air, water, sediments and organisms drives POPs towards chemical equilibrium (205, 214, 215). Non-equilibrium conditions appear under the influence

of convection, the biological and degradative pumps, and other non-fugacity driven processes (Fig. I.4).

POPs bioconcentration in phytoplankton, as well as bioaccumulation in higher trophic levels (81, 216), accounts for their entry and biomagnification in the food web (Box. I.4) (78, 80, 217). Some organisms, especially bacteria, are able to degrade different POPs (218, 219). However, effects of pollutants have been demonstrated worldwide throughout the marine food web, on phytoplankton (85, 94, 220), herbivores (221, 222) and predators (223, 224).

Regarding the coupling between carbon and POPs cycles, effects on photosynthesis are particularly important. Toxicity of diverse individual compounds and of their mixtures of them on marine photosynthetic organisms has been demonstrated in some previous works (85, 86, 225-227). On the contrary, effects of the complex mixture of POPs present in the ocean have been less explored. Most toxicity tests and risk assessments of chemicals mainly focus on the effects of single compounds (228), whereas in their natural environment, organisms are exposed to complex mixtures of chemicals (229-233). Echeveste and coworkers (94) showed that complex mixtures of organic pollutants from concentrated seawater were about 103 times more toxic than expected for a single compound, affecting abundance, viability and Chlorophyll *a* concentration of phytoplankton. Some other approaches on effects of real pollutant mixtures in the ocean have been done with different objects of study, such as the crustacean *Daphnia magna* (156), the bacterium *Vibrio fischeri* (157) or the human cell line AREc32 (155). All these studies showed that non-toxic chemicals can become toxic in a mixture, which may be attributed to additive effects of the pollutants. These results point out the need for a systematic investigation on the consequences of the cocktail of POPs that already is accumulating in oceans. It must take into account not only well-known and/or regulated chemicals, but also unknown pollutants, including those chemicals below the analytical detection limits or for which no chemical analytical method is available, with the ultimate goal of establishing the pressure of the anthropogenic chemosphere on marine environments.

I.3. *PROCHLOROCOCCUS* AS MODEL SENTINEL SPECIES

The first reference to *Prochlorococcus* was just 36 years ago when an uncommon cyanobacterium was identified in the ocean by transmission electron microscopy (234). It presented an ultrastructure typical of chroococcoid but with the photosynthetic thylakoid membranes more closely spaced than its congeners. Years later, an unusual derivative of chlorophyll was detected in the ocean by high-performance liquid chromatography (HPLC) analysis (235). But not until 1988, after the development of shipboard flow cytometry, *Prochlorococcus* was described as a novel free-living prochlorocophyte (236). This technology enabled the detection, quantification and isolation of the tiny *Prochlorococcus* cells (237, 238). Subsequent analysis verified that the unusual photosynthetic pigments, divinyl-chlorophyll *a* and *b* belonged to *Prochlorococcus* (238, 239), which is the only marine phytoplanktonic organism that uses these two pigments to harvest light energy (239). This pigments cause the faintly red fluorescence under blue light excitation that distinguished *Prochlorococcus* from the cyanobacterium *Synechococcus* (240, 241). Since then, flow cytometry has been applied worldwide to study *Prochlorococcus* and in time it revealed that this is the most abundant photosynthetic organism known on Earth and that it contributes significantly to global primary production (242).

Prochlorococcus satisfies the requirements to elucidate reciprocal links between gene expression and environmental processes and nowadays is considered a model organism that is providing valuable knowledge of biological systems through cross-scale biology assessments (243).

I.3.1. THE ECOLOGICAL RELEVANCE OF *PROCHLOROCOCCUS*

Prochlorococcus is a unicellular cyanobacterium only found in marine environments. Its cell diameter, between 0.5 to 0.8 µm (Fig. I.5), makes it the smallest known free-living oxygenic phototroph (238, 242-244). Its genome is the smallest one found in any photosynthetic organism on the planet, about 1.65 Mbp, with only around 1,700 genes (245). Its growth rate depends on radiation and temperature

(241, 246, 247), but as a general rule it is a slow growing bacterium doubling every 1–2 days (241, 248, 249). *Prochlorococcus* abundance is typically 10^4–10^5 cells per mL throughout the euphotic zone of the oceans (242, 250, 251). This high density and its broad horizontal and vertical distribution make it the most abundant photosynthetic organism on the world (242, 250, 251), with a global estimated population of more than 10^{27} cells (252, 253).

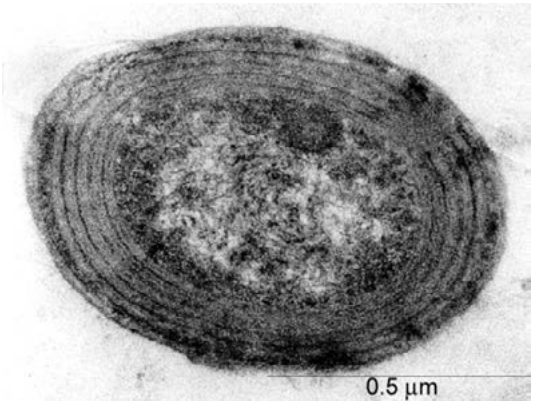


FIGURE I.5 - THE *PROCHLOROCOCCUS* CELL

Transmission electron micrograph of an ultradian section of *Prochlorococcus* SS120 strain.
Figure: Bryant, 2003 (254).

Prochlorococcus is numerically dominant in the oligotrophic waters of tropical and subtropical world's oceans between 40 °N and 40 °S latitude (236, 242, 255), although it can be found further north in smaller numbers (256). It thrives better in warm, stratified nutrient-poor waters, so temperature limits its dispersion to this latitudinal band (242, 250, 251, 256-258). *Prochlorococcus* depth distribution depends on the clarity of the water column. While in oligotrophic, stably stratified waters it is found at depths of 150-200 m, in less clear and well-mixed waters its distribution is substantially shallower (242). Given the vertical gradients of light, temperature and nutrients throughout the euphotic zone (Box I.5), *Prochlorococcus* supports light intensities spanning four orders of magnitude (241, 259, 260), temperatures ranging from 11 °C to 31 °C (241, 247, 256) and a wide range of nutrient sources (256, 261). Its abundance also varies seasonally in waters where temperatures change considerably over the yearly cycle. Highest abundances are in summer, whereas in winter the mixing of the column water causes a decrease of *Prochlorococcus* density and a concurrent increase of *Synechococcus* and eukaryotic phytoplankton (251, 255, 262).

Prochlorococcus is an important global primary producer. Beyond its abundance, it is highly efficient in transforming light energy into compounds usable by the cell.

BOX I.5 - LIVING IN AN OLIGOTROPHIC ENVIRONMENT

Prochlorococcus habitat is an extremely dilute environment in terms of both chemistry and biology. It thrives in the entire euphotic zone, throughout gradients of light, temperature and nutrients. The water column consists of an upper mixed layer, where the distributions of nutrients and cells are homogeneous due to the forces of wind and heat-driven turbulences, and deeper stratified waters with gradients of nutrients (Fig. I.6). Solar radiation and temperature are higher at the surface and decrease with depth, whereas nutrient concentration is usually low at the surface and increases with depth. Metals also show a concentration gradient in the water column, being generally greater at the surface than in deeper waters (296). According to the concentrations measures in the North Pacific Ocean it is estimated that each *Prochlorococcus* individual is hundreds of cell diameters away from other individuals of its genera, a few cell diameters away from essential nutrients and tens of cell lengths away from the closest phage (299) (Fig. I.6). This dilute habitat exerts a selective pressure on its inhabitants and hence *Prochlorococcus* shows diverse adaptations to oligotrophy. Compared to other bacteria, *Prochlorococcus* has a reduced level of nitrogen in its proteome (300) and a low GC composition in its genome (301), reducing its need of nitrogen. Its phosphorus requirement is also unusually low compared to other phytoplankton, with a cellular P:N ratio of 1:16-24 (302-304), partially due to the replacements of phospholipids with sulfolipids in the cell membrane (305) and to its small genome size (302). *Prochlorococcus* spherical shape and small size result in a high surface-to-volume ratio that enhances nutrient acquisition and light absorption. This feature together with its unique pigmentation makes it the most efficient light absorber among the photosynthetic organisms (264). In fact, Prochlorococcus is the only phytoplankton known to absorb more light than it scatters (240), and the phototroph found deepest in the water column (257), defining the lower limit of photosynthesis in the ocean.

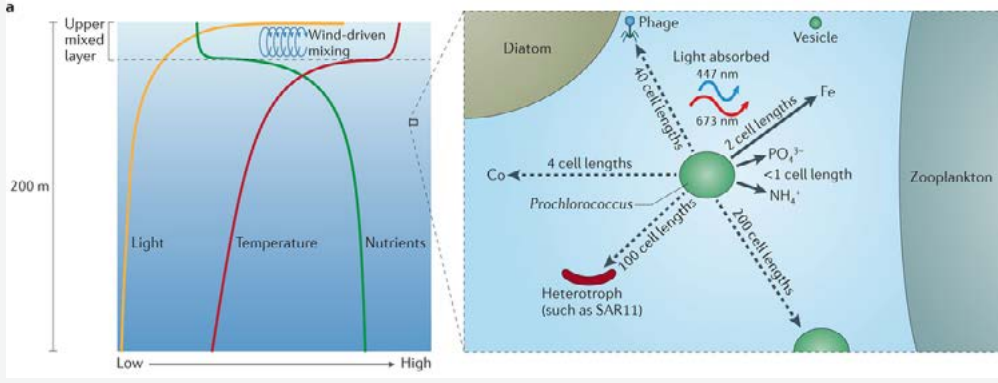


FIGURE I. 6 - THE *PROCHLOROCOCCUS* HABITAT

Characteristics of the oceanic water column in oligotrophic regions and distances between a *Prochlorococcus* cell and diverse biological and chemical drivers of its habitat. Distances that are to scale are marked with a solid line, whereas those that are not to scale are represented by dashed lines. Figure: Biller *et al.*, 2015 (299).

The carbon dioxide-concentrating mechanism is an essential part of this process, which improves the effectiveness of photosynthesis (263). Indeed, CO_2 fixation rates of *Prochlorococcus* are 1.5–2.0 times higher than those of *Synechococcus* and plastidic protists (264). It contributes up to 50–80% of primary production in tropical and subtropical oceanic regions (242, 249), which means that it is responsible for 5–10% of the global marine primary production (252, 265, 266). Its primary productivity is estimated to be 4 gigatons of fixed carbon each year (253), this is threefold the carbon uptake by global tropical forests (267). Carbon dioxide is taken up only passively but bicarbonate (the predominant source of inorganic carbon in the oceans) may be transported actively into the cell (245, 268). Then it is likely transformed in carbon dioxide by carbonic anhydrase for subsequent fixation through the Calvin cycle, and stored as glycogen (269). *Prochlorococcus* releases diverse organic molecules into the water (270) by different mechanisms such as direct secretion (271, 272) or cell lysis by either phages or grazers (273). Heterotrophic bacteria in the euphotic zone will take up a part of the fixed carbon by *Prochlorococcus* and it will be incorporated to the food web (273). In contrast, other part of *Prochlorococcus*-derived carbon will be exported to deep waters by aggregation and sinking, being part of the biological pump (274, 275).

The photosynthetic apparatus of this genus is unique. It is the only organism to use divinyl-chlorophyll *a* and *b* as the major light-harvesting pigments (245). The light-harvesting antenna is made up of divinyl-chlorophyll *a/b* binding proteins (Pcbs) instead of phycobilisomes used by most cyanobacteria, including its close relative, marine *Synechococcus* (268, 276, 277). Likewise, ribulose-1,5-bisphosphate carboxylase (RuBisCO), the enzyme involved in the first major step of carbon fixation, and the carboxysome shell proteins, are structurally different from those found in many other cyanobacteria (268). The daily light–dark cycle synchronizes cellular processes and gene expression in *Prochlorococcus* (246, 248, 269, 278, 279), including the photosynthetic activity that exhibits reproducible diel fluctuations. Carbon fixation occurs exclusively during the day and is maximal at midday (260, 269). Transcript concentration of genes related to photosynthesis and cell cycle undergo fluctuations along the day (260, 269, 278-280). However, these diel variations in gene expression and metabolic activity can be regulated not only by direct responses to changes in light intensity, but also by an endogenous circadian

rhythm. For example, the expression of photosystem core reaction centre genes is maximal in the middle of the day, which suggests they follow the light intensity (269, 280). In contrast, transcript levels of Calvin cycle genes are maximal at dawn anticipating the carbon fixation optimum by a diel timing mechanism (260, 269).

A major challenge for *Prochlorococcus* cells is to deal with the scarcity of nutrients in the oligotrophic oceans where it thrives. Nutrient deprivation causes a significant decline in transcript levels of 2–10 % of the genome, including a decrease in the expression of many photosynthetic genes (277, 281-284). For example, nitrogen deprivation reduces severely the efficiency of photosystem II (282, 284, 285). As a consequence, *Prochlorococcus* has developed different mechanisms to face productivity limitation by nutrient availability (see Box 1.5). It is a non-nitrogen fixing cyanobacterium (245), but it can use ammonium, nitrite, nitrate, cyanate, and even organic compounds such as urea and amino acids as sources of nitrogen (243, 286-291). Phosphorus assimilation can be in form of phosphite, phosphate and, as organic sources, phosphonate, b-glycerophosphate, glucose-6-phosphate, ATP, and cAMP (268, 292-294). Trace metals are also required for many cell processes (268). Iron has a key role in photophysiology and is required in large amounts, but it is found at very low bioavailable concentrations in the open ocean, therefore limiting primary production (295). Conversely, higher concentrations of these metals can be toxic (283, 296).

Although *Prochlorococcus* is certainly photosynthetic, its mixotrophic capacity has been demonstrated. Besides the import of organic compounds as sources or nitrogen and phosphorus, glucose uptake has been identified, which could serves as source of carbon and energy to supplement photoautotrophic growth (297, 298).

I.3.2. THE DIVERSITY OF *PROCHLOROCOCCUS*

The numerical abundance and the broad distribution of *Prochlorococcus* throughout the euphotic zone of the world's tropical and subtropical oceans can be largely explained by its microdiversity. Phylogenetic analyses using a number of genes (16S rRNA, *rpo*C1, *ntc*A) or intergenic regions (ITS, petB-D) show that the *Prochlorococcus* genus is made up of a number of discrete lineages that form a

of the basic housekeeping functions and constitute the core genome (310). Of these, only 13 genes are absent from marine *Synechococcus* (310). The rest of the genome is composed by the so-called "flexible genes", about 150 different genes of each sequenced genome, which presumably contribute to the relative fitness of each distinct lineage within its local environment (248, 310, 320). The sum of core genome and flexible genes from all members of *Prochlorococcus* genus make up the *Prochlorococcus* pangenome, which for now includes about 6,000 genes (310) but continues to grow as more strains are sequenced. It is estimated that *Prochlorococcus* pangenome contains more than 57,000 genes (323), which is almost three times the size of human genome.

Horizontal gene transfer mediated by phages is not only responsible of the flexible gene gains from other microorganisms (320, 324) but also of intragenic recombination between core photosynthesis genes that are shared by both *Prochlorococcus* and their phages (325, 326). This mechanism, combined with the selective pressures of each niche where *Prochlorococcus* can thrive, are likely the evolutive forces that shaped *Prochlorococcus* genetic microdiversity (327).

I.3.3. *PROCHLOROCOCCUS* IN A CHANGING WORLD

Because of the ecological significance of *Prochlorococcus*, effects of global change on its abundance, distribution or functions may have impacts on ocean ecosystems and biogeochemical cycles. The oceanic temperature increase is thought to cause a displacement of the subtropical linages by their tropical counterparts, although for the moment there are no evidences for this change (328). Models predict that at an oceanic temperature corresponding to 650 ppm atmospheric CO_2, the global abundance of *Prochlorococcus* may increase around 25% and expand towards the poles (253). However, this predicted increase of abundance worldwide may not result in a parallel increase of the *Prochlorococcus*-derived organic matter that enters the ecosystem, since *Prochlorococcus* synchronizes its cell production with its mortality rate (329).

In addition, the increase of UV radiation as a consequence of stratospheric ozone depletion may induce high levels of oxidative stress that have deleterious effects on

monophyletic clade with marine *Synechococcus* lineages (268, 306), well separated from all other cyanobacteria (237, 307-309). *Prochlorococcus* and *Synechococcus* are the only marine picocyanobacteria and are thought to descend from a common ancestor (259). Indeed, on basis of their 16S rRNA sequences most members of both groups could be considered to be the same species but key physiological and ecological features and whole-genome phylogenies distinguish them (261, 308-310). *Prochlorococcus* genus consists of distinct ecotypes or clades adapted to different light intensities, temperatures and nutrient concentrations, and no single ecotype can grow throughout the entire range of ecological conditions supported by the genus as a whole (247, 256, 259, 311). *Prochlorococcus* ecotypes have been broadly divided into two major groups or clades, adapted to either high-light (HL) or low-light (LL) conditions (259). HL-adapted cells grow optimally at higher light intensities than LL-adapted cells, and hence they distribute differently in the water column (247, 261, 312). HL-adapted cells are orders of magnitude more abundant in surface waters, whereas LL-adapted cells usually have their abundance maximum in the base of the euphotic zone (256, 313, 314). These discrepancies in light adaptation are due to different pigment ratios (241, 259, 315) and are further supporter by significant differences at the genetic level (308, 309, 311, 313). HL-adapted *Prochlorococcus* strains form a more recently diverged monophyletic group divided into at least six ecotypes (HLI-HLVI) (241, 311, 313). Conversely, LL-adapted strains are polyphyletic and form at least seven distinct ecotypes (LLI-LLVII) (316-318) (Fig. I.7). These ecotypes are genetically and physiologically distinct populations that display different requirements and tolerances of light, temperature and nutrients (Table I.1).

Around 20 *Prochlorococcus* strains have been described, and the genomes of 12 of them have been completely sequenced (245, 310, 319, 320). These genomes are among the smallest for any free-living organism (1716–2201 protein-coding genes and 1.6– 1.9 Mbp) (268, 321), likely as a result of a rapid loss of genes after their divergence from a common ancestor with *Synechococcus* (268). This decrease in genome size seems to be related to the stable environmental conditions of subtropical and tropical oceans (245, 319) or to their capacity of taking advantage of metabolic functions provided by other organism (322). Approximately 1,250 genes are shared among all 12 sequenced *Prochlorococcus* genomes. They are responsible

Prochlorococcus growth and productivity (330), and even cause cell mortality (331), being *Prochlorococcus* more sensitive than *Synechococcus* (330, 331). Atmospheric aerosol deposition is an important source of beneficial nutrients and trace metals to the open ocean (332) but also of pollutants (203). Its effect on *Prochlorococcus* depends on specific components in aerosols; essential micronutrients input enhance productivity and growth (333), but some components can have toxic effects and reduce the metabolic activity of *Prochlorococcus* (334). The increment of organic pollutant concentrations in surface waters may affect phytoplankton growth, abundance and cell viability (335, 336). *Prochlorococcus* resulted to be the most sensitive among a set of phytoplanktonic organism to polycyclic aromatic hydrocarbons (PAHs) due to its higher surface to volume ratio (85). This PAHs toxicity is enhanced by the increasing UV radiation (227). In addition, complex

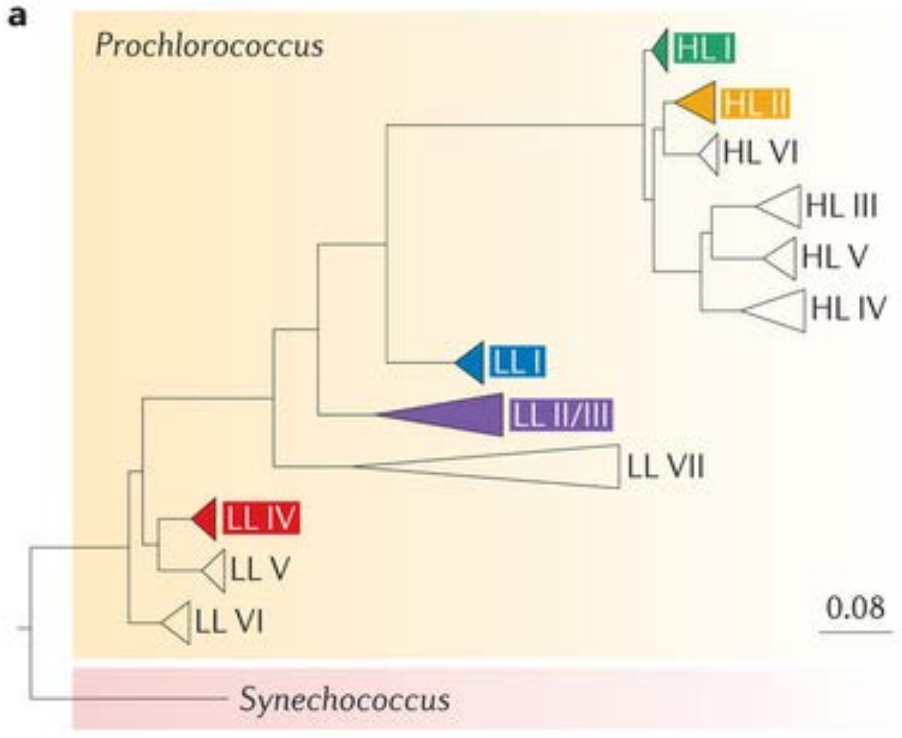


FIGURE I.7 - *PROCHLOROCOCCUS* PHYLOGENY

Phylogenetic distribution of the 12 known major clades of *Prochlorococcus* determined by rRNA internal transcribed spacer (ITS) sequence diversity. Coloured clades comprise cultured strains, the rest have been identified using environmental sequence data only.
Figure: Biller *et al.* 2015 (299)

TABLE I.1 - THE MAIN CLADES AND STRAINS OF *PROCHLOROCOCCUS*

Clade	Alternative name for the clade	Representative cultured strains	Ecology	References
HLI	low B/A clade I, eMED4	MED4, MIT9515	• Subtropical oceans (35° -48°N and 35°-45°S) • Cool weakly stratified surface water • Lower optimum growth temperature than HLII. They can grow at temperatures as low as 11-15 °C, setting the high- latitude cold-water limits of the *Prochlorococcus* range.	247, 256, 258, 268, 308, 312
HLII	low B/A clade II, eMIT9312	AS9601, MIT9301, MIT9215, MIT9312, SB	• Tropical and subtropical oceans (30°N-30°S) • Warm strongly stratified surface waters • Throughout the euphotic zone • Higher optimum growth temperature than HLI. They still grow at 30° C. • They seem to be the most abundant *Prochlorococcus* cells on a global scale.	256, 258, 268, 308, 312, 314, 337
HLIII	HNLC1, HNLC2	None	• Waters with high nitrogen and phosphorus, but low iron availability. • Adapted to iron-limited environments by decreasing cellular iron requirements • Physiological differences between these three ecotypes are still unknown.	317, 318, 338, 339
HLIV	HNLC1, HNLC2	None		
HLV	NA	None		
HLVI	NA	None	• Middle to lower euphotic zone of the South China Sea. • Data on this ecotype are limited, but is thought that they might be adapted to lower light intensities than HLI and HLII ecotypes.	317
LLI	high B/A clade I, eNATL2A	NATL1A, NATL2A, PAC1	• High latitude (above 40°N/30°S) • Throughout the euphotic zone up to the surface • LLI cells have intermediate characteristics between HL-adapted and the rest of LL-adapted ecotypes. • They tolerate light intensity fluctuations better than others LL- adapted cells.	247, 256, 268, 308, 312, 337, 340
LLII	high B/A clade II, eSS120	SS120	• Deep waters of the euphotic zone at low concentrations. • Their abundance decreases during deep mixing events.	256, 268, 308, 312
LLIII	high B/A clade III, eMIT9211	MIT9211		
LLIV	high B/A clade IV, eMIT9313	MIT9313, MIT9303, MIT0701	• Widely distributed between 40°N and 35°S. • Found at the base of the euphotic zone. The most tolerant to extremely low radiation. • LLIV cells are the most similar to *Synechococcus* among the cultured *Prochlorococcus* lineages. • They display the largest and most diverse genomes in *Prochlorococcus*.	247, 256, 257, 268, 272, 308, 312-314, 337
LLV	NA	None	• Only detected in oxygen-poor zones.	317, 431
LLVI	NA	None	• Little is known about these ecotypes.	317, 341
LLVII	NC1	None	• Subtropical oceans. • Lower euphotic zone. • Data on this ecotype are lacking.	341

mixtures of organic pollutants present in seawater are know to be much more toxic for *Prochlorococcus* than single pollutants separately (94).

Although the effects of distinct global change drivers on *Prochlorococcus* have been assessed, the complex relationships between the cells, its community and the changing environment are still unpredictable. Hence, multivariate studies, considering as many drivers as possible, are needed to advance our understanding of the effects of global change on *Prochlorococcus*, and consequently on oceanic ecosystems and biogeochemical cycles.

I.4. OUTLINE AND GOALS OF THE THESIS

Chemical pollution is one of the most unknown drivers of global change (105). As a part of it, persistent organic pollutants (POPs) are of special concern by virtue of their persistence, ubiquity, bioaccumulation tendency and high toxicity to both environment and human health (140, 154). The influence of carbon cycle on occurrence, fate and transport of POPs is well-known (72, 73, 76, 136, 190) whereas the inverse relationship, i.e. the effect of POPs on carbon cycle remains unexplored.

Global change could be responsible for global perturbations on oceanic primary production (104) and for the global decline of phytoplankton (103) occurred during the past century. Since oceanic phytoplankton accounts for about 46% of the global primary production (46, 47), these changes may affect the global carbon cycle.

Oceans also have an important role in POPs cycle (36, 74) and represent one of the major reservoirs for them (74, 200, 342) . POPs have been shown to be toxic for a number of marine organisms (221-224), including phytoplankton (85, 94, 220). Among them, the cyanobacterium *Prochlorococcus* stands out because of being a relevant contributor for oceanic photosynthesis (252, 253, 265, 266, 286), and one of the most sensitive phytoplankton to POPs (85, 94, 227).

While our knowledge of the connection between POPs and disturbances of marine photosynthesis is expanding, most ecotoxicological works focus on the effects of single compounds (228) whereas in their natural environment organisms

are exposed to complex mixtures of chemicals (229-233). In addition, toxicity is typically measured as mortality (85, 94, 227) but effects of POPs on photosynthetic metabolism at sublethal levels are yet to be discovered. With the final goal of moving forward in our understanding on the interrelationships between POPs and carbon global cycles, this thesis pretends to elucidate the effects of the complex mixture of POPs present in the oceans on the photosynthesis capability of *Prochlorococcus*.

The specific objectives are as follows:

1. Development of a molecular method to quantify the expression of genes related to photosynthesis in *Prochlorococcus* that allows detection of effects at sublethal levels.
2. Verification of the specificity of the method on *Prochlorococcus* and its ability to discern *Synechococcus* by testing with laboratory cultures of both genera.
3. Method validation by its application to wild populations of *Prochlorococcus*.
4. Assessment of the possible effects of POPs on photosynthetic capability of laboratory cultured *Prochlorococcus*.
5. Assessment of the possible effects of POPs on photosynthetic capability of wild populations of *Prochlorococcus*.
6. Evaluation of the hypothesis "non-toxic chemicals can become toxic in a mixture" by exposing wild populations of *Prochlorococcus* to both simple commercial mixtures of POPs and a complex mixture of POPs accumulated in seawater.
7. Study of photosynthesis capability of wild populations of *Prochlorococcus* worldwide in a comprehensive approach integrating as many environmental stressors, including POPs, as possible.

I.5. HIGH THROUGHPUT GENE EXPRESSION ANALYSIS OF WILD *PROCHLOROCOCCUS*

I.5.1. QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

The quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is a molecular technique for the detection and quantification of messenger ribonucleic acid (mRNA) (343, 344). It is based on the revolutionary method of polymerase chain reaction (PCR), developed by Kary Mullis in the 1980s (345), which allows to copy and amplify specific pieces of DNA more than a billion-fold (345-348). The PCR relies on the *in vitro* use of DNA polymerase enzymes to amplify fragments of DNA using short, sequence-specific oligonucleotides added to the reaction to act as primers (Box I.6), and by repeating cycles of heating and cooling the polymerization reaction solution. After each round of DNA copying, the resulting double-stranded DNA (dsDNA) is denatured, or melted, by heating the reaction tube

BOX I.6 - GLOSSARY OF TERMS RELATED TO qRT-PCR

- **DNA polymerase** is a type of enzyme that functions in the replication and repair of DNA by catalysing the linking of nucleotides in a specific order, using single-stranded DNA as a template. The most popular DNA polymerase used in PCR is the Taq DNA polymerase, from *Thermus aquaticus*, because of its resistance to the high temperatures needed for PCR.
- **Oligonucleotide** is a short nucleic-acid chain, generally below 100 nt (ssDNA).
- **Primer** is a short, single-stranded DNA sequence, usually of approximately 20 nucleotides, used in the PCR technique to hybridize with the sample DNA and to define the region of the DNA that will be amplified.
- **Amplicon** or PCR product is the DNA fragment that has been synthesized using PCR.
- **Housekeeping gene** is a gene involved in basic functions needed for the sustenance of the cell. It is constitutively expressed at a relatively constant level across all studied conditions.

to 95 °C. Then, the reaction is cooled to allow the primers to anneal to the now single-stranded DNA (ssDNA), which is used as template, and direct the DNA polymerase to initiate elongation by adding single complementary nucleotides to create a new complete strand of dsDNA. This new dsDNA will then be melted for the next cycle of copying, setting in motion a chain reaction in which DNA is exponentially amplified (Fig. I.8). However, PCR loses efficiency after a given number of cycles because of consumptions of reactants, until it reaches a plateau (Fig. I.9) (349).

The novelty of the real-time PCR is the possibility to monitor the ongoing DNA amplification process by adding to the reaction of a fluorescent dye (350, 351). The intensity of the fluorescent signal correlates with the amount of DNA, thereby enabling quantification. How quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence. This lineal correlation occurs only during the exponential phase (Fig. I.9), when the amplification efficiency is at its maximum. The key advantage of qRT-PCR is the conversion of the changes in the fluorescent signal into a numerical value is named quantitative real-time PCR (qPCR). In addition, a DNA melting curve is usually performed on the final amplification product, as its melting point is a unique property only dependent on its length and nucleotide composition (Fig. I.9, B). Therefore, the melting curve characterizes the uniqueness and nature of the final product.

A major limitation of DNA polymerase amplification reaction and, consequently, of PCR, is that it requires DNA as template, not RNA. When RNA abundance is to be determined (for example, for transcript abundance studies), it should be retro-transcribed to DNA using a reverse transcriptase (352). Reverse transcriptases are used in nature by retroviruses to generate complementary DNA (cDNA) from a viral RNA template in a process named reverse transcription (RT). Therefore, the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) consist of the reverse transcription of the target RNA into cDNA *in vitro*, and its subsequent amplification and quantification by qPCR. Under the appropriate reaction conditions, the relative amount of a given cDNA generated by reverse transcription is proportional to the relative amount of its mRNA template. For this reason, and because of its precision and sensitivity, qRT-PCR has become one of the most popular methods of quantitating steady-state mRNA levels (352).

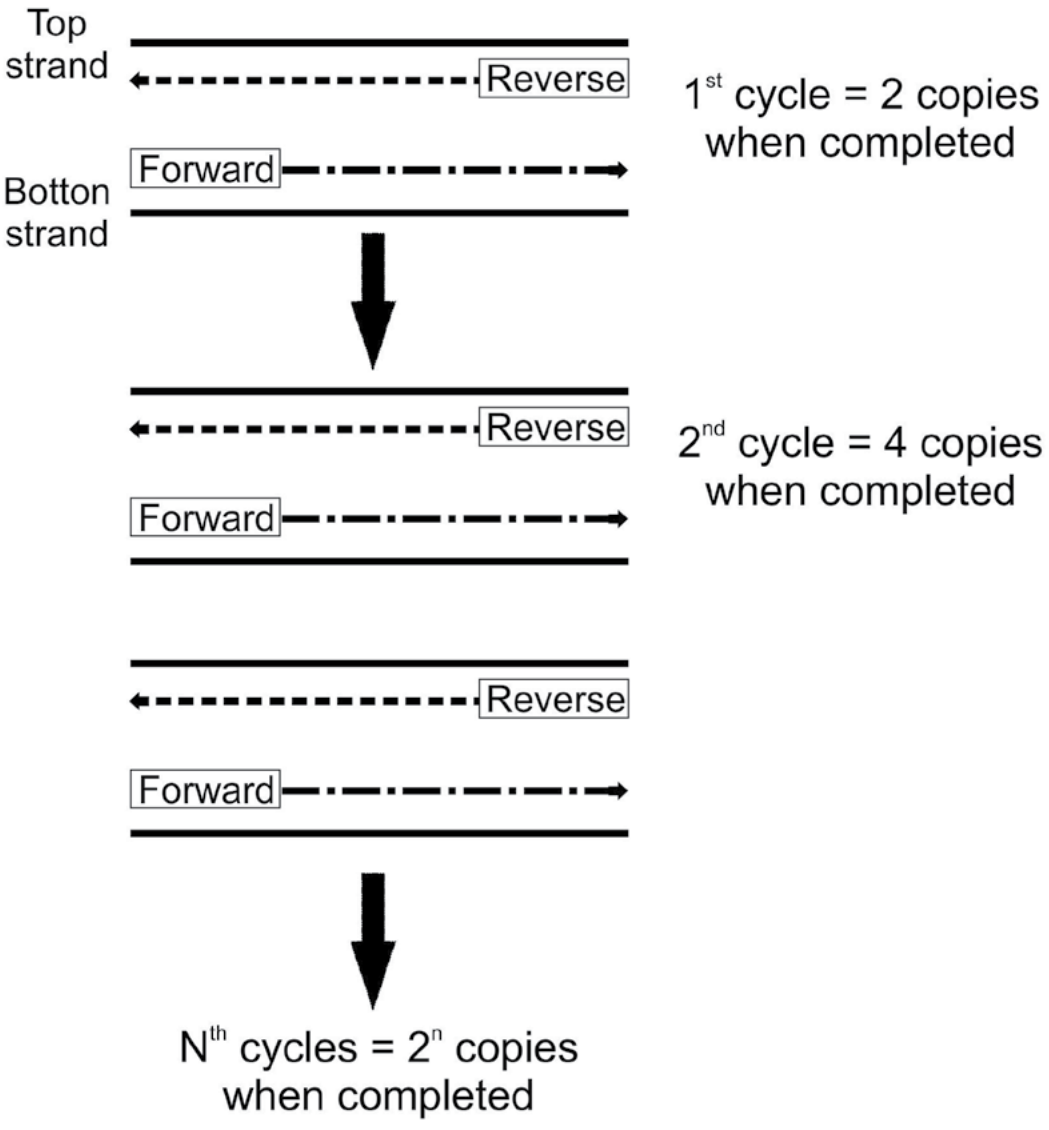


FIGURE I.8 - THE POLYMERASE CHAIN REACTION

PCR at its exponential phase is depicted. High temperatures are used to melt dsDNA into two strands. This mixture is cooled for the annealing between the DNA target and its sequence-specific primers, which both forward (Fw), i.e. complementary to the sense strand (5' to 3' direction), and reverse (Rw), i.e. complementary to the sense strand (3' to 5' direction). Then, DNA elongation occurs at the optimal reaction temperature for the thermostable DNA polymerase (around 72 °C). This cycle is repeated a number of times, generally from 15 to 40, amplifying exponentially the amount of amplicon produced.

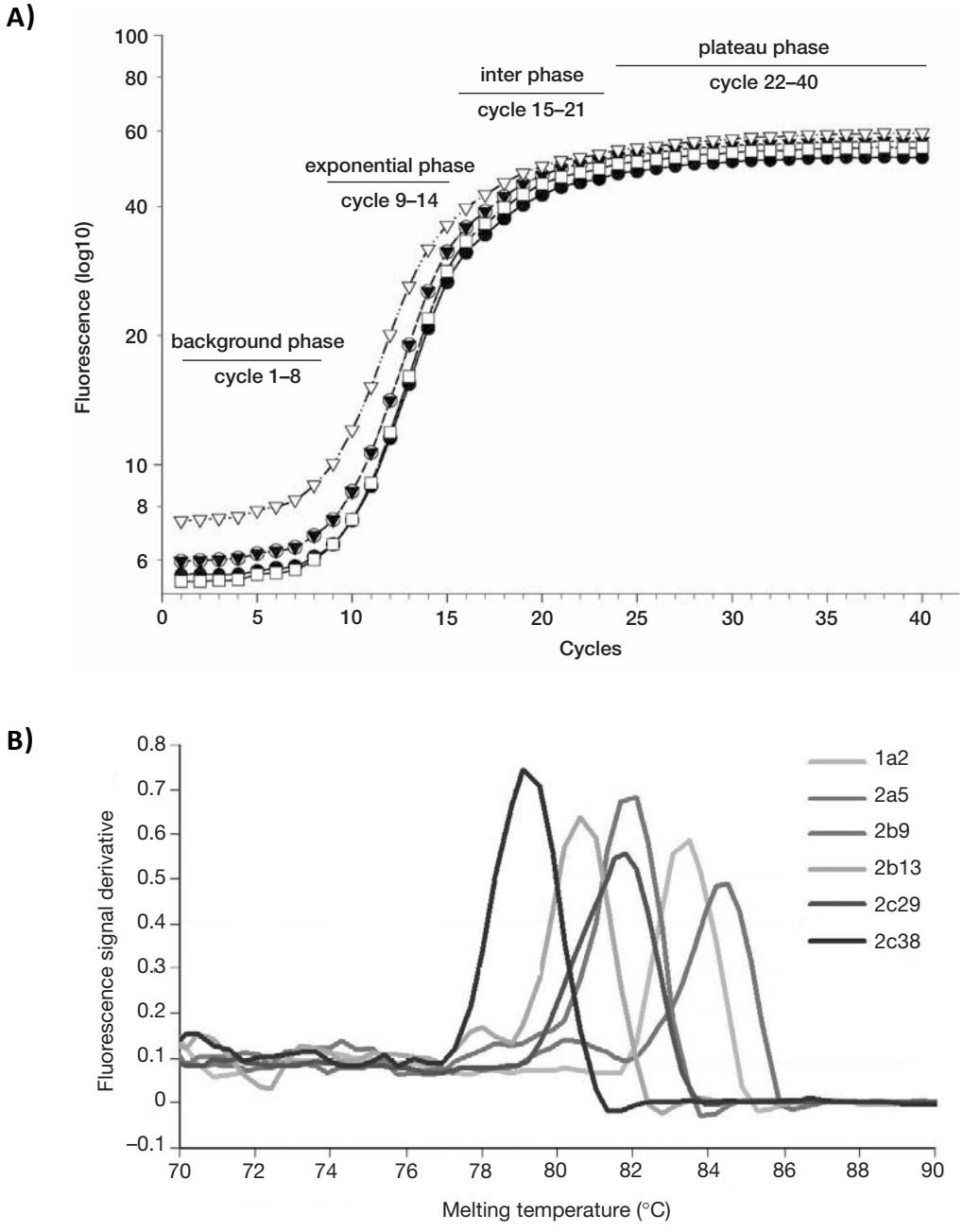


FIGURE I.9 - RESULTS FROM REAL-TIME PCR

A) amplification plot illustrating the increase in fluorescent reported signal (y-axis, note the log scale) with each PCR cycle (x-axis). Four phases can be distinguished along the process: background, exponential, inter and plateau. **B)** melting curve of PCR product, showing a single peak for each of the 6 products tested. This suggests that only a specific PCR amplicon was generated during the reaction for each amplicon. Figures: Dorak, 2006 (358)

Several types of qPCR hardwares exist, but the basis of all of them is a thermocycler, which controls the temperatures of each PCR cycle, an energy source to excite the fluorescent molecules, and a photodetector to measure the produced fluorescence. In this work, the LightCycler 480 (Roche Diagnostics, Indianapolis, IN) was used. It employs a high-intensity Xenon lamp that emits light over a broad wavelength range (430–630 nm) and allows the use of a variety of fluorescent dyes and detection formats. The selected dye here was SYBR Green I (SYBR Green Master Mix, Takara Bio Inc., Siga, Japan) that binds specifically to the minor groove of dsDNA (353). It is the most economical, easiest to use and widely used among the qRT-PCR product detection methods when an absolute sequence identity between the amplicon and the target genes is not required (353-356). If such a high specificity is indeed needed, a third, fluorescently labelled probe (e.g., Taqman probes) should be designed and added to the reaction. SYBR Green I absorbs light of 480-nm wavelength and emits light of 520-nm wavelength, which is a 1,000-fold greater fluorescence than when it is free in solution (357). Given that in a PCR the input cDNA is minimal, the only dsDNA present in sufficient amounts to be detected is the PCR product itself. During the initial PCR cycles, the fluorescence signal emitted by SYBR Green bound to the PCR product is usually too weak to register above background. It will be detectable only after significant amplification (> 15 cycles for most cDNA) (358). During the exponential phase of the PCR, the fluorescence doubles at each cycle. After 30-35 cycles, the intensity of the fluorescent signal usually begins to plateau, indicating that the PCR has reached saturation (Fig. I.9). Finally, the PCR product can be verified by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (359) (Fig. I.9). If there are two or more peaks, it suggests that more than one amplified sequence was obtained, and the amplification was not specific for a single DNA target.

There are several quantification strategies in qRT-PCR (360), but most of them can be classified into two types: (i) relative quantification based on the relative expression of a target gene versus a reference gene; and (ii) absolute quantification based on the use of internal or external standards and the corresponding calibration curve (353, 359). Absolute quantification requires standards with known concentrations and its accuracy depends entirely on the accuracy of the standards. On the contrary, relative quantification only requires a reference gene that can be any constantly

expressed gene (housekeeping gene, Box I.6) (361, 362), and the relative quantities of mRNA can be compared across multiple qRT-PCR experiments (363-365). Since the objective here is to quantify mRNA levels of wild populations of *Prochlorococcus*, which consist of several coexisting strains, relative quantification was selected.

To calculate the expression of a target gene in relation to an appropriate reference gene various estimation approaches are established. Calculations are based on the comparison of the distinct cycle determined by various methods, such as crossing points (Cp) and threshold values (Ct) at a constant level of fluorescence, or Cp acquisition according to a mathematical algorithm (349, 366-368). In the present work we used the "Second Derivative Maximum" analysis method, which is available from the LightCycler 480 software. This algorithm is based on the kinetics of a PCR reaction and identifies the crossing point (Cp) as the point where the fluorescence reaches the maximum of the second derivative of the amplification curve. This corresponds to the point of the maximal acceleration of the fluorescence signal and it is therefore located in the middle of the log-linear portion of the PCR amplification plot (Fig. I.9). The advantage of this analysis method is that it requires little user input and produces consistent results. The "delta-delta Ct" mathematical model, using Cp as determined cycle number, (349, 369) was applied for the relative quantification of gene expression. This model requires that PCR efficiencies (E) of both target and reference genes should be close to 100% (E=1) and not to differ by more than 10 %

$$\mathrm{E} = 10^{(-1/\mathrm{slope})}$$

one each other (370). Efficiencies were calculated on the basis of a linear regression

$$N = N_0 \times (1 + E)^n$$

slope of a dilution row,

The equation that describes the PCR amplification during the exponential phase is

$$N = N_0 \times (1 + E)^{Cp}$$

where N is the number of molecules at cycle n; N_0 is the initial number of molecules; *E* is the PCR efficiency; and n is the number of cycles. The Cp is the cycle number of the maximal second derivative of the amplification curve, thus

$$\frac{N_{target}}{N_{ref.}} = \frac{N_0^{target} \times (1+ E_{target})^{Cp^{target}}}{N_0^{ref.} \times (1+ E_{ref.})^{Cp^{ref.}}} = K$$

This equation is calculated for both target and reference genes. Dividing the number of molecules in the Cp of the target gene (N_{target}) by the number of molecules in the Cp of the reference gene ($N_{ref.}$) gives the expression

where N_0^{target} and $N_0^{ref.}$ are the initial number of molecules; E_{target} and $E_{ref.}$ are

$$R = \frac{N_0^{target}}{N_0^{ref.}} = K \times \frac{(1+E_{ref.})^{Cp^{ref.}}}{(1+E_{target})^{Cp^{target}}}$$

the efficiencies; Cp^{target} and $Cp^{ref.}$ are the crossing points of the target gene and

$$R = K \times 2^{(Cp^{ref.} - Cp^{target})} = K \times 2^{-\Delta Cp}$$

the reference gene, respectively in each case; and K is a constant. Thus, the initial number of molecules of the target gene normalized to the reference gene (R) is

When efficiencies of both target and reference genes are close to 100% (E ≈ 1),

$$\frac{R_{treatment}}{R_{control}} = \frac{K \times 2^{-\Delta Cp^{treatmebt}}}{K \times 2^{-\Delta Cp^{control}}} = 2^{-\Delta\Delta Cp}$$

where ΔCp is the difference in the crossing point for target and reference genes ($\Delta Cp = Cp^{target} - Cp^{ref.}$).

Then, possible changes in the relative expression of the same target gene between two different samples (treatment and control) are calculated as

where $\Delta\Delta Cp$ is the difference in the crossing point for the relative expressions of the treatment and the control ($\Delta\Delta Cp = \Delta Cp^{treatment} - \Delta Cp^{control}$).

In conclusion, qRT-PCR is a powerful method to amplify and detect trace amounts of mRNA (343, 344) given its sensitivity, high specificity, good reproducibility and wide dynamic quantification range (351, 352, 354, 363). It is particularly useful for the elucidation of small changes in mRNA expression levels, even with very low transcripts abundance (354, 371-374). As a consequence, it has been gaining in popularity over recent years for the study of phytoplankton from the marine environment (312, 337, 375-379), including works about expression changes of genes related to photosynthesis (379, 380).

I.5.2. SELECTED GENES TO ANALYSE *PROCHLOROCOCCUS*' PHOTOSYNTHETIC CAPABILITY

To analyse the photosynthetic capability of *Prochlorococcus* populations we focus on quantifying the expression of two functional photosynthetic protein-coding genes, *rbc*L and *psb*A.

The *rbc*L gene in *Prochlorococcus* is part of a highly conserved cluster of genes in the genome (263). It encodes the large protein subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). This enzyme is responsible of carbon dioxide fixation preceding the Calvin cycle. RbcL protein abundance is considered a good representative for the activity of the entire Calvin cycle (381). Hence, *rbc*L gene has been considered a useful target for molecular assays that quantify clade-specific RNA transcript concentrations as a proxy for the carbon fixation activity of marine phytoplankton (256, 289, 340, 382). *rbc*L expression is regulated under a circadian clock, having a peak at dawn and its minimal level toward the end of the day (269), although is known to be strongly influenced by light intensity (248, 251, 340) and, to a lesser extent, by other environmental variables (311).

The *psb*A gene encodes the D1 protein of photosystem II (PSII) core, which is the primary target of photo-inactivation and protects the cell from photo-oxidative stress (259). In contrast to *Synechococcus*, all *Prochlorococcus* strains possess a single D1 isoform, and its damage results in photo-inhibition and a decrease of the photosystem II efficiency and of the photosynthetic carbon fixation (290, 313). Levels of the PsbA protein subunit have been shown to reflect the cellular PSII content (381). We therefore consider the *psb*A transcript concentration as a proxy of the functionality of the PSII in the cell. The expression of *psb*A also experiences a daily variation but, unlike *rbc*L, it co-varied with light intensity, with maximum levels at mid-day, and minimum in the middle of the night (269, 280, 383). In addition, *psb*A expression is also influenced by environmental variables such as UV radiation, iron starvation and glucose availability (290, 337).

The *rnp*B gene was selected as reference gene of the quantification of the two target genes. It encodes the RNA component of RNase P, a ubiquitous enzyme required for tRNA 5' end maturation in prokaryotes. *Prochlorococcus rnp*B gene presents a low variance among strains (384), and its expression is highly stable at different conditions of irradiation, iron, phosphate, glucose, UV radiation, nitrogen, or light quality (248, 312, 314-317, 338, 339, 385, 386). Therefore, it has been widely used as reference gene qRT-PCR assays on *Prochlorococcus* (251, 318, 339, 387).

I.5.3. THE MALASPINA 2010 CIRCUMNAVIGATION

The Malaspina 2010 circumnavigation (www.expedicionmalaspina.es) was a multidisciplinary project led by the Spanish National Research Council (CSIC) and funded by the Spanish Ministry of Science and Technology, and supplementary private and public funding (388). Its main goals were to conduct a multidisciplinary study evaluating the impact of global change and exploring the biodiversity of the deep ocean. Furthermore, it aimed at prompting the interest for marine science by the Spanish public and to foster scientific vocations among its youth. Over 400 scientists from the CSIC, the Spanish Institute of Oceanography, 11 Spanish

universities, and 16 associated foreign institutions, including the NASA and the European Space Agency participated in this project.

The Malaspina Expedition sailed the ocean between December 2010 and July 2011, involving two research vessels (R/V). The R/V Sarmiento de Gamboa, operated by the Spanish National Research Council (CSIC) conducted a detailed

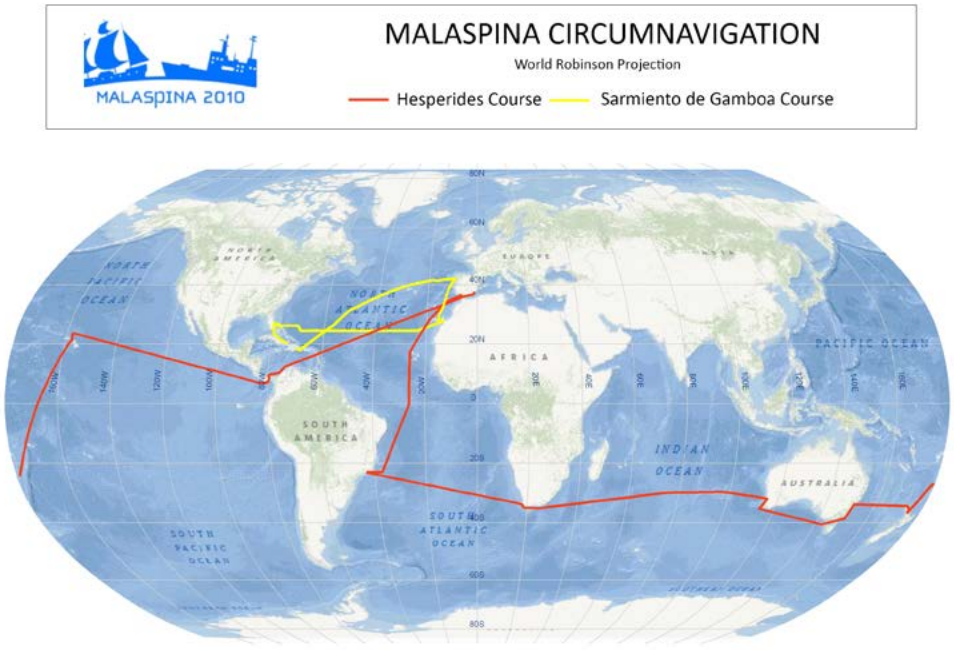


FIGURE I.10 - THE MALASPINA CIRCUMNAVIGATION

World Robinson projection of the courses of both vessels involved in the Malaspina Circumnavigation, Hesperides (red course) and Sarmiento de Gamboa (yellow course).

study of a section, along 24.5 N, of the Atlantic Ocean (Fig. I.10). It sailed from the Canary Islands (Spain) to Florida (USA) between 28 January and 9 March 2011, and from Santo Domingo (Dominican Republic) to Vigo (Spain) from 23 March to 8 April, serving in its return as a floating university. On the other hand, the R/V BioHesperides covered a total distance of 31,798 nautical miles (58,890 km) across the Atlantic, Indian, and Pacific Oceans, spanning latitudes from 34 °N to 40 °S (Fig. I.10). This cruise comprised seven legs (i.e. routes between two harbours):

- Leg 1: 14 December 2010, Cadiz (Spain) – 13 January 2011, Rio de Janeiro (Brazil)
- Leg 2: 17 January, Rio de Janeiro (Brazil) – 6 February, Cape Town (South Africa)
- Leg 3: 11 February, Cape Town (South Africa) – 13 March, Perth (Australia)
- Leg 4: 17 March, Perth (Australia) – 30 March, Sydney (Australia)
- Leg 5: 16 April, Auckland (New Zealand) – 8 May, Honolulu (Hawaii, USA)
- Leg 6: 13 May, Honolulu (Hawaii, USA) –10 June, Cartagena de Indias (Colombia)
- Leg 7: 19 June, Cartagena de Indias (Colombia) –14 July, Cartagena (Spain)

A total of 147 stations were sampled from surface to 4,000 m depth, and dozens of environmental, biological and chemical variables were measured in each of them. Therefore, the BioHesperides cruise was a great opportunity to achieve the objectives of the present work, and the samples collected during it are the base of the experimental work in this manuscript.

I.6. REFERENCES

1. Anonymous (1963) Implications of Rising Carbon Dioxide Content of the Atmosphere. (The Conservation Fundation, New York).
2. Carson R (1962) Silent spring (Houghton Mifflin, Boston) 368 p.
3. Hardin G (1968) The tragedy of the commons. Science 162(3859):1243-1248.
4. Broecker WS (1975) Climatic change: Are we on the brink of a pronounced global warming? Science 189(4201):460-463.
5. Clark WC & Holling CS (1985) Sustainable development of the biosphere: human activities and global change. Global change. Proc. ICSU symposium, Ottawa, 1984:474-490.
6. Diamante JM, Pyle TE, Carter WE, & Scherer W (1987) Global change and the measurement of absolute sea-level. Progress in Oceanography 18(1-4):1-21.
7. Fleagle RG (1988) Effects of global change. Nature 333(6176):794-794.
8. Steffen W et al. (2004) Global Change and the Earth System: a Planet under Pressure (Springer-Verlag, New York)
9. Crutzen PJ (2006) The anthropocene. Earth System Science in the Anthropocene), pp 13-18.
10. Steffen W, Crutzen PJ, & McNeill JR (2007) The anthropocene: Are humans now overwhelming the great forces of nature? Ambio 36(8):614-621.
11. Cohen JE (1995) How Many People Can the Earth Support? The Sciences 35(6):18-23.
12. Anonymous (2015) United States Census Bureau. Acces date September 28, 2015.
13. Anonymous (2014) The World Population Situation in 2014. A Concise Report. (United Nations, Department of Economic and Social Affairs, Population Division).
14. Houghton RA, JL Hackler. (2001) Carbon Flux to the Atmosphere from Land-Use Changes: 1850 to 1990. (ORNL/CDIAC-131, NDP-050/R1. Carbon Dioxide Information Analysis Center, U.S. Department of Energy, Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A).
15. Pielke Sr RA, et al. (2002) The influence of land-use change and landscape dynamics on the climate system: Relevance to climate-change policy

beyond the radiative effect of greenhouse gases. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 360(1797):1705-1719.

16. Kalnay E & Cai M (2003) Impact of urbanization and land-use change on climate. Nature 423(6939):528-531.

17. Arnfield AJ (2003) Two decades of urban climate research: A review of turbulence, exchanges of energy and water, and the urban heat island. International Journal of Climatology 23(1):1-26.

18. Landsberg HE (1982) The urban climate. The urban climate.

19. S. Wood KS, S. J. Scherr (2000) Pilot Analysis of Global Ecosystems: Agroecosystems. (International Food Policy Research Institute and World Resources Institute, Washington, DC).

20. Sahin V & Hall MJ (1996) The effects of afforestation and deforestation on water yields. Journal of Hydrology 178(1-4):293-309.

21. Costa MH, Botta A, & Cardille JA (2003) Effects of large-scale changes in land cover on the discharge of the Tocantins River, Southeastern Amazonia. Journal of Hydrology 283(1-4):206-217.

22. Matson PA, Parton WJ, Power AG, & Swift MJ (1997) Agricultural intensification and ecosystem properties. Science 277(5325):504-509.

23. Tilman D, et al. (2001) Forecasting agriculturally driven global environmental change. Science 292(5515):281-284.

24. Bennett EM, Carpenter SR, & Caraco NF (2001) Human impact on erodable phosphorus and eutrophication: A global perspective. BioScience 51(3):227-234.

25. Weisenburger DD (1993) Human health effects of agrichemical use. Human Pathology 24(6):571-576.

26. Grandesso E, Gullett B, Touati A, & Tabor D (2011) Effect of moisture, charge size, and chlorine concentration on PCDD/F emissions from simulated open burning of forest biomass. Environ Sci Technol 45(9):3887-3894.

27. Pacca S & Horvath A (2002) Greenhouse gas emissions from building and operating electric power plants in the upper Colorado river basin. Environ Sci Technol 36(14):3194-3200.

28. Van Der Werf GR, et al. (2006) Interannual variability in global biomass burning emissions from 1997 to 2004. Atmospheric Chemistry and Physics 6(11):3423-3441.

29. Charlson RJ, et al. (1992) Climate Forcing by Anthropogenic Aerosols. Science 255(5043):423-430.

30. IPCC (2001) Climate Change 2001: Synthesis Report. A Contribution of Working Groups I, II, and III to the Third Assessment Report of the Integovernmental Panel on Climate Change. (Cambridge University Press, Cambridge, United Kingdom, and New York, NY, USA), p 398.

31. Anonymous (1990) EINECS: European Inventory of Existing commercial Chemical Substances, Annex to the Official Journal C146A, 15 June 1990, page 1 - 1802. Office for Official Publications of the European Communities. L-2985. (Luxembourg).

32. Muir DCG & Howard PH (2006) Are there other persistent organic pollutants? A challenge for environmental chemists. Environ Sci Technol 40(23):7157-7166.

33. UNEP (2005) General technical guidelines for the environmentally sound management of wastes consisting of, containing or contaminated with persistent organic pollutants (POPs). Basel convention series. SBC Nr.2005/1.

34. Ma J, Hung H, & Blanchard P (2004) How Do Climate Fluctuations Affect Persistent Organic Pollutant Distribution in North America? Evidence from a Decade of Air Monitoring. Environ Sci Technol 38(9):2538-2543.

35. Lohmann R, Breivik K, Dachs J, & Muir D (2007) Global fate of POPs: current and future research directions. Environ Pollut 150(1):150-165.

36. Wania F & Mackay D (1996) Tracking the distribution of persistent organic pollutants. Environ Sci Technol 30(9):390A-397A.

37. Galbán-Malagón C, Cabrerizo A, Caballero G, & Dachs J (2013) Atmospheric occurrence and deposition of hexachlorobenzene and hexachlorocyclohexanes in the Southern Ocean and Antarctic Peninsula. Atmospheric Environment 80:41-49.

38. Cabrerizo A, Dachs J, Barcelo D, & Jones KC (2012) Influence of organic matter content and human activities on the occurrence of organic pollutants in antarctic soils, lichens, grass, and mosses. Environ Sci Technol 46(3):1396-1405.

39. Carrera G et al. (2001) Persistent organic pollutants in snow from European high mountain areas. Atmospheric Environment 35(2):245-254.

40. Lamon L, et al. (2009) Modeling the global levels and distribution of polychlorinated biphenyls in air under a climate change scenario. Environ Sci Technol 43(15):5818-5824.

41. Gioia R, et al. (2011) Evidence for major emissions of PCBs in the West African region. Environ Sci Technol 45(4):1349-1355.

42. Ma J & Cao Z (2010) Quantifying the perturbations of persistent organic pollutants induced by climate change. Environ Sci Technol 44(22):8567-8573.

43. MacLeod M, Riley WJ, & McKone TE (2005) Assessing the influence of climate variability on atmospheric concentrations of polychlorinated biphenyls using a global-scale mass balance model (BETR-Global). Environ Sci Technol 39(17):6749-6756.

44. Bigg GR, Jickells TD, Liss PS, & Osborn TJ (2003) The role of the oceans in climate. International Journal of Climatology 23(10):1127-1159.

45. Sarmiento JL, et al. (2004) Response of ocean ecosystems to climate warming. Global Biogeochemical Cycles 18(3):GB3003 3001-3023.

46. Field CB (1998) Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. Science 281(5374):237-240.

47. Jennings S, et al. (2008) Global-scale predictions of community and ecosystem properties from simple ecological theory. Proceedings of the Royal Society B: Biological Sciences 275(1641):1375-1383.

48. Reid PC, et al. (2009) Chapter 1. Impacts of the oceans on climate change. Advances in marine biology 56:1-150.

49. Milliman JD (1993) Production and accumulation of calcium carbonate in the ocean: budget of a nonsteady state. Global Biogeochemical Cycles 7(4):927-957.

50. Holligan PM & Robertson JE (1996) Significance of ocean carbonate budgets for the global carbon cycle. Global Change Biology 2(2):85-95.

51. Sabine CL, et al. (2004) The oceanic sink for anthropogenic CO2. Science 305(5682):367-371.

52. Raven J, et al. (2005) Ocean acidification due to increasing atmospheric carbon dioxide. (The Royal Society, Cardiff, UK).

53. Denman KL et al. (2007) Couplings Between Changes in the Climate System and Biogeochemistry. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, ed Solomon S, D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA).

54. Riebesell U, et al. (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407(6802):364-366.

55. Gattuso JP, et al. (1998) Effect of calcium carbonate saturation of seawater on coral calcification. Global and Planetary Change 18(1-2):37-46.

56. Langdon C, et al. (2000) Effect of calcium carbonate saturation state on the calcification rate of an experimental coral reef. Global Biogeochemical Cycles 14(2):639-654.

57. IPCC (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. p 996.

58. Halpern BS, et al. (2008) A global map of human impact on marine ecosystems. Science 319(5865):948-952.

59. Rahmstorf S (2007) A semi-empirical approach to projecting future sea-level rise. Science 315(5810):368-370.

60. Edwards M, Reid P, & Planque B (2001) Long-term and regional variability of phytoplankton biomass in the Northeast Atlantic (1960-1995). ICES Journal of Marine Science 58(1):39-49.

61. Beaugrand G, Reid PC, Ibañez F, Lindley JA, & Edwards M (2002) Reorganization of North Atlantic marine copepod biodiversity and climate. Science 296(5573):1692-1694.

62. Mackas DL, Batten S, & Trudel M (2007) Effects on zooplankton of a warmer ocean: Recent evidence from the Northeast Pacific. Progress in Oceanography 75(2):223-252.

63. Garcia HE, et al. (2005) On the variability of dissolved oxygen and apparent oxygen utilization content for the upper world ocean: 1955 to 1998. Geophysical Research Letters 32(9):1-4.

64. Ono T, Midorikawa T, Watanabe YW, Tadokoro K, & Saino T (2001) Temporal increases of phosphate and apparent oxygen utilization in the subsurface water of western subarctic Pacific from 1968 to 1998. Geophysical Research Letters 28(17):3285-3288.

65. Whitney FA, Freeland HJ, & Robert M (2007) Persistently declining oxygen levels in the interior waters of the eastern subarctic Pacific. Progress in Oceanography 75(2):179-199.

66. Stramma L, Johnson GC, Sprintall J, & Mohrholz V (2008) Expanding oxygen-minimum zones in the tropical oceans. Science 320(5876):655-658.

67. Edwards M & Richardson AJ (2004) Impact of climate change on marine pelagic phenology and trophic mismatch. Nature 430(7002):881-884.

68. Smith SV, et al. (2003) Humans, hydrology, and the distribution of inorganic nutrient loading to the ocean. BioScience 53(3):235-245.

69. Duce RA, et al. (2008) Impacts of atmospheric anthropogenic nitrogen on the open ocean. Science 320(5878):893-897.

70. Dachs J & Méjanelle L (2010) Organic Pollutants in Coastal Waters, Sediments, and Biota: A Relevant Driver for Ecosystems During the Anthropocene? Estuaries and Coasts 33(1):1-14.

71. Ma Y, et al. (2015) Persistent organic pollutants in ocean sediments from the North Pacific to the Arctic Ocean. Journal of Geophysical Research C: Oceans.

72. Persson NJ, et al. (2002) Soot-carbon influenced distribution of PCDD/Fs in the marine environment of the Grenlandsfjords, Norway. Environ Sci Technol 36(23):4968-4974.

73. Lohmann R, Macfarlane JK, & Gschwend PM (2005) Importance of black carbon to sorption of native PAHs, PCBs, and PCDDs in Boston and New York Harbor sediments. Environ Sci Technol 39(1):141-148.

74. Dachs J, et al. (2002) Oceanic biogeochemical controls on global dynamics of persistent organic pollutants. Environ Sci Technol 36(20):4229-4237.

75. Olsson M (2000) Comparison of temporal trends (1940s-1990s) of DDT and PCB in Baltic sediment and biota in relation to eutrophication. Ambio 29(4-5):195-201.

76. Dachs J, Eisenreich SJ, & Hoff RM (2000) Influence of eutrophication on air-water exchange, vertical fluxes, and phytoplankton concentrations of persistent organic pollutants. Environ Sci Technol 34(6):1095-1102.

77. Koelmans AA, Van der Heijde A, Knijff LM, & Aalderink RH (2001) Integrated modelling of eutrophication and organic contaminant fate & effects in aquatic ecosystems. A Review. Water Research 35(15):3517-3536.

78. Del Vento S & Dachs J (2002) Prediction of uptake dynamics of persistent organic pollutants by bacteria and phytoplankton. Environmental Toxicology and Chemistry 21(10):2099-2107.

79. Roche H, et al. (2009) Organochlorines in the Vaccarès Lagoon trophic web (Biosphere Reserve of Camargue, France). Environ Pollut 157(8-9):2493-2506.

80. Berrojalbiz N, et al. (2009) Accumulation and cycling of polycyclic aromatic hydrocarbons in zooplankton. Environ Sci Technol 43(7):2295-2301.

81. Johnson-Restrepo B, Kannan K, Addink R, & Adams DH (2005) Polybrominated diphenyl ethers and polychlorinated biphenyls in a marine foodweb of coastal Florida. Environ Sci Technol 39(21):8243-8250.

82. Corsolini S, et al. (2002) Polychloronaphthalenes and other dioxin-like compounds in arctic and antarctic marine food webs. Environ Sci Technol 36(16):3490-3496.

83. Galbán-Malagón C, Berrojalbiz N, Ojeda MJ, & Dachs J (2012) The oceanic biological pump modulates the atmospheric transport of persistent organic pollutants to the Arctic. Nature Communications 3:862.

84. Johnston EL & Roberts DA (2009) Contaminants reduce the richness and evenness of marine communities: A review and meta-analysis. Environ Pollut 157(6):1745-1752.

85. Echeveste P, Agusti S, & Dachs J (2010) Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations. Environ Pollut 158(1):299-307.

86. Magnusson M, Heimann K, & Negri AP (2008) Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae. Mar Pollut Bull 56(9):1545-1552.

87. Haynes D, Ralph P, Prange J, & Dennison B (2000) The impact of the herbicide diuron on photosynthesis in three species of tropical seagrass. Mar Pollut Bull 41(7-12):288-293.

88. Jones RJ & Kerswell AP (2003) Phytotoxicity of Photosystem II (PSII) herbicides to coral. Marine Ecology Progress Series 261:149-159.

89. Bueno M, et al. (2004) Effects of lindane on the photosynthetic apparatus of the cyanobacterium Anabaena: Fluorescence induction studies and immunolocalization of ferredoxin-NADP+ reductase. Environmental Science and Pollution Research 11(2):98-106.

90. Suresh Babu G, Hans RK, Singh J, Viswanathan PN, & Joshi PC (2001) Effect of lindane on the growth and metabolic activities of cyanobacteria. Ecotoxicol Environ Saf 48(2):219-221.

91. Sumpter JP (2009) Protecting aquatic organisms from chemicals: The harsh realities. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 367(1904):3877-3894.

92. Herbarth O, et al. (2001) Effects of pre-incubation period on the photoinduced toxicity of polycyclic aromatic hydrocarbons to the luminescent bacterium Vibrio fischeri. Environmental Toxicology 16(3):277-286.

93. Petersen DG, Reichenberg F, & Dahllöf I (2008) Phototoxicity of pyrene affects benthic algae and bacteria from the Arctic. Environ Sci Technol 42(4):1371-1376.

94. Echeveste P, Dachs J, Berrojalbiz N, & Agusti S (2010) Decrease in the abundance and viability of oceanic phytoplankton due to trace levels of complex mixtures of organic pollutants. Chemosphere 81(2):161-168.

95. Southerland HA & Lewitus AJ (2004) Physiological responses of estuarine phytoplankton to ultraviolet light-induced fluoranthene toxicity. Journal of Experimental Marine Biology and Ecology 298(2):303-322.

96. Simpson SL, et al. (2007) Establishing cause-effect relationships in hydrocarbon-contaminated sediments using a sublethal response of the benthic marine alga, Entomoneis cf punctulata. Environmental Toxicology and Chemistry 26(1):163-170.

97. Pelletier MC, et al. (1997) Phototoxicity of individual polycyclic aromatic hydrocarbons and petroleum to marine invertebrate larvae and juveniles. Environmental Toxicology and Chemistry 16(10):2190-2199.

98. Boese BL, Lamberson JO, Swartz RC, & Ozretich RJ (1997) Photoinduced toxicity of fluoranthene to seven marine benthic crustaceans. Archives of Environmental Contamination and Toxicology 32(4):389-393.

99. Karacik B, Okay OS, Henkelmann B, Bernhöft S, & Schramm KW (2009) Polycyclic aromatic hydrocarbons and effects on marine organisms in the Istanbul Strait. Environment International 35(3):599-606.

100. Marty GD, et al. (1997) Ascites, premature emergence, increased gonadal cell apoptosis, and cytochrome P4501A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. Canadian Journal of Zoology 75(6):989-1007.

101. Heintz RA, et al. (2000) Delayed effects on growth and marine survival of pink salmon Oncorhynchus gorbuscha after exposure to crude oil during embryonic development. Marine Ecology Progress Series 208:205-216.

102. Peterson CH, et al. (2003) Long-Term Ecosystem Response to the Exxon Valdez Oil Spill. Science 302(5653):2082-2086.

103. Boyce DG, Lewis MR, & Worm B (2010) Global phytoplankton decline over the past century. Nature 466(7306):591-596.

104. Henson SA, et al. (2010) Detection of anthropogenic climate change in satellite records of ocean chlorophyll and productivity. Biogeosciences 7(2):621-640.

105. Rockström J, et al. (2009) A safe operating space for humanity. Nature 461(7263):472-475.

106. Walker M, et al. (2009) Formal definition and dating of the GSSP (Global Stratotype Section and Point) for the base of the Holocene using the Greenland NGRIP ice core, and selected auxiliary records. Journal of Quaternary Science 24(1):3-17.

107. Crutzen PJ (2002) Geology of mankind. Nature 415(6867):23.

108. Steffen W, et al. (2011) The anthropocene: From global change to planetary stewardship. Ambio 40(7):739-761.

109. Anonymous (2005) Ecosystems and human well-being: Synthesis. (Millennium Ecosystem Assessment, Washington), p 137.

110. Etheridge DM, et al. (1996) Natural and anthropogenic changes in atmospheric CO_2 over the last 1000 years from air in Antarctic ice and firn. Journal of Geophysical Research Atmospheres 101(D2):4115-4128.

111. Machida T, Nakazawa T, Fujii Y, Aoki S, & Watanabe O (1995) Increase in the atmospheric nitrous oxide concentration during the last 250 years. Geophysical Research Letters 22(21):2921-2924.

112. Blunier T, et al. (1995) Variations in atmospheric methane concentration during the Holocene epoch. Nature 374(6517):46-49.

113. IPCC (2014) Climate Change 2014: Impacts, Adaptation, and Vulnerability. Working Group II Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. (Cambridge, United Kingdom and New York, NY, USA), p 1132.

114. F. S. Rowland JA, et al. (1988) Trends in Total Column Ozone Measurements Report of the International Ozone Trends Panel 1988).

115. AMS (2012) State of the Climate in 2011. Bulletin of the American Meteorological Society 93:S1-S264.

116. Milly PCD, Wetherald RT, Dunne KA, & Delworth TL (2002) Increasing risk of great floods in a changing climate. Nature 415(6871):514-517.

117. FAO (2011) Review of the state of world marine fishery resources. FAO Fisheries and Aquaculture Technical Paper 569:334.

118. Small C & Nicholls RJ (2003) A global analysis of human settlement in coastal zones. Journal of Coastal Research 19(3):584-599.

119. Galloway JN, et al. (2004) Nitrogen cycles: Past, present, and future. Biogeochemistry 70(2):153-226.

120. Boyer EW & Howarth RW (2008) Nitrogen Fluxes from Rivers to the Coastal Oceans. Nitrogen in the Marine Environment), pp 1565-1587.

121. Howarth RW JH, Marino R, and Postma H (1995) Transport to and processing of phosphorus in near-shore and oceanic waters. Phosphorus in the global environment: transfers, cycles, and management, ed H T (John Wiley and Sons, Chichester, UK).

122. Howarth RW, et al. (1996) Regional nitrogen budgets and riverine N & P fluxes for the drainages to the North Atlantic Ocean: Natural and human influences. Biogeochemistry 35(1):75-139.

123. Williams M (1990) Forest. The Earth as Transformed by Human Action, ed Turner BLI, Clark W.C., Kates R.W., Richards J.F., Mathews J.T., and Meyer W.B. (Cambridge Univ. Press, New York, USA).

124. FAO (2010) Global Forest Resources Assessment 2010. Main report. FAO Forestry Paper 163:340.

125. Goldewijk KK (2001) Estimating global land use change over the past 300 years: The HYDE database. Global Biogeochemical Cycles 15(2):417-433.

126. Barnosky AD, et al. (2011) Has the Earth's sixth mass extinction already arrived? Nature 471(7336):51-57.

127. IUCN (2014) 2014 IUCN Annual Report. (International Union for Conservation of Nature).

128. CAS (World's Authority for Chemical Information.

129. Grzybowski BA, Bishop KJM, Kowalczyk B, & Wilmer CE (2009) The 'wired' universe of organic chemistry. Nature Chemistry 1(1):31-36.

130. Daughton CG (2005) "Emerging" chemicals as pollutants in the environment: A 21st century perspective. Renewable Resources Journal 23(4):6-23.

131. Wania F (2003) Assessing the potential of persistent organic chemicals for long-range transport and accumulation in polar regions. Environ Sci Technol 37(7):1344-1351.

132. Wania F (2006) Potential of degradable organic chemicals for absolute and relative enrichment in the Arctic. Environ Sci Technol 40(2):569-577.

133. Buccini J (2003) The development of a global treaty on persistent organic pollutants (POPs). The Hand Book of Environmental Chemistry, Persistent Organic Pollutants, ed Fiedler H (Springer-Verlag, Berlin/Heidelberg).

134. Wong MH, et al. (2005) A review on the usage of POP pesticides in China, with emphasis on DDT loadings in human milk. Chemosphere 60(6):740-752.

135. Wania F, Axelman J, & Broman D (1998) A review of processes involved in the exchange of persistent organic pollutants across the air-sea interface. Environ Pollut 102(1):3-23.

136. Jurado E, et al. (2007) Fate of persistent organic pollutants in the water column: does turbulent mixing matter? Mar Pollut Bull 54(4):441-451.

137. de Wit CA, Herzke D, & Vorkamp K (2010) Brominated flame retardants in the Arctic environment - trends and new candidates. Science of the Total Environment 408(15):2885-2918.

138. Gamberg M, et al. (2005) Spatial and temporal trends of contaminants in terrestrial biota from the Canadian Arctic. Science of the Total Environment 351-352:148-164.

139. Evans MS, et al. (2005) Persistent organic pollutants and metals in the freshwater biota of the Canadian Subarctic and Arctic: An overview. Science of the Total Environment 351-352:94-147.

140. UNEP (The Stockholm Convention on Persistent Organic Pollutants.

141. Mes J (1987) PCBs in human populations. PCBs and the Environment, vol. III, ed Waid JS (CRC Press, Boca Raton, FL).

142. Kuhnlein HV & Chan HM (2000) Environment and contaminants in traditional food systems of northern indigenous peoples. in Annual Review of Nutrition, pp 595-626.

143. Pocar P, et al. (2012) Effects of polychlorinated biphenyls in CD-1 mice: Reproductive toxicity and intergenerational transmission. Toxicological Sciences 126(1):213-226.

144. Weinand-Härer A, Lilienthal H, Bucholski KA, & Winneke G (1997) Behavioral effects of maternal exposure to an ortho-chlorinated or a coplanar PCB congener in rats. Environmental Toxicology and Pharmacology 3(2):97-103.

145. Ellis-Hutchings RG, Cherr GN, Hanna LA, & Keen CL (2006) Polybrominated diphenyl ether (PBDE)-induced alterations in vitamin A and thyroid hormone concentrations in the rat during lactation and early postnatal development. Toxicology and Applied Pharmacology 215(2):135-145.

146. Hayashi K, et al. (2013) Immunotoxicity of the organochlorine pesticide methoxychlor in female ICR, BALB/c, and C3H/He mice. Journal of Immunotoxicology 10(2):119-124.

147. Kumar J, et al. (2014) Persistent organic pollutants and liver dysfunction biomarkers in a population-based human sample of men and women. Environmental Research 134:251-256.

148. Cole P, Trichopoulos D, Pastides H, Starr T, & Mandel JS (2003) Dioxin and cancer: A critical review. Regulatory Toxicology and Pharmacology 38(3):378-388.

149. Selin H & Eckley N (2003) Science, Politics, and Persistent Organic Pollutants: The Role of Scientific Assessments in International Environmental Co-operation. International Environmental Agreements: Politics, Law and Economics 3(1):17-42.

150. Krueger J & Selin H (2002) Governance for sound chemicals management: The need for a more comprehensive global strategy. Global Governance 8(3):323-342.

151. Jensen S, Johnels AG, Olsson M, & Otterlind G (1969) DDT and PCB in marine animals from Swedish waters. Nature 224(5216):247-250.

152. Bitman J & Cecil HC (1970) Estrogenic activity of DDT analogs and polychlorinated biphenyls. Journal of Agricultural and Food Chemistry 18(6):1108-1112.

153. Pitts Jr JN, Grosjean D, Mischke TM, Simmon VF, & Poole D (1977) Mutagenic activity of airborne particulate organic pollutants. Toxicology Letters 1(2):65-70.

154. UNECE (1998) The 1998 Aarhus Protocol on Persistent Organic Pollutants (POPs). Protocol to the 1979 Convention on Long-Range Transboundary Air Pollution on Persistent Organic Pollutants. (United Nations Economic Commission for Europe).

155. Escher BI, Van Daele C, Dutt M, Tang JYM, & Altenburger R (2013) Most oxidative stress response in water samples comes from unknown chemicals: The need for effect-based water quality trigger values. Environ Sci Technol 47(13):7002-7011.

156. Smith KEC, et al. (2013) Baseline toxic mixtures of non-toxic chemicals: "solubility addition" increases exposure for solid hydrophobic chemicals. Environ Sci Technol 47(4):2026-2033.

157. Tang JYM, et al. (2013) Mixture effects of organic micropollutants present in water: Towards the development of effect-based water quality trigger values for baseline toxicity. Water Research 47(10):3300-3314.

158. Vallack HW, et al. (1998) Controlling persistent organic pollutants-what next? Environmental Toxicology and Pharmacology 6(3):143-175.

159. El-Shahawi MS, Hamza A, Bashammakh AS, & Al-Saggaf WT (2010) An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. Talanta 80(5):1587-1597.

160. EPA (United States Environmental Protection Agency.

161. ATSDR (2015) Toxicological Profile for Hexachlorobenzene. (U.S. Department of Health and Services. Public Health Service. Agency for Toxic Substances and Disease Registry).

162. E. van de Plassche AS (2002) Polychlorinated Naphthalenes. (United Nations Economic Commission for Europe (UNECE)).

163. Baek SO, et al. (1991) A review of atmospheric polycyclic aromatic hydrocarbons: Sources, fate and behavior. Water, Air, and Soil Pollution 60(3-4):279-300.

164. Howsam M & Jones K (1998) Sources of PAHs in the Environment. PAHs and Related Compounds, The Handbook of Environmental Chemistry, ed Neilson A (Springer Berlin Heidelberg), Vol 3 / 3I, pp 137-174.

165. UNECE (1994) State of knowledge report of the UNECE task force on persistent organic pollutants. (UNECE Convention on Long-range Transboundary Air Pollution).

166. Wild SR & Jones KC (1995) Polynuclear aromatic hydrocarbons in the United Kingdom environment: A preliminary source inventory and budget. Environ Pollut 88(1):91-108.

167. Mackay DS, Y. W.; Ma, K. C (1992) Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals (Lewis Publisher, Boca Raton, FL).

168. Dachs J & Eisenreich SJ (2000) Adsorption onto aerosol soot carbon dominates gas-particle partitioning of polycyclic aromatic hydrocarbons. Environ Sci Technol 34(17):3690-3697.

169. Mader BT & Pankow JF (2002) Study of the effects of particle-phase carbon on the gas/particle partitioning of semivolatile organic compounds in the atmosphere using controlled field experiments. Environ Sci Technol 36(23):5218-5228.

170. Jaward FM, Barber JL, Booij K, & Jones KC (2004) Spatial distribution of atmospheric PAHs and PCNs along a north-south Atlantic transect. Environ Pollut 132(1):173-181.

171. Van Den Berg M, et al. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106(12):775-792.

172. Guzzella L, et al. (2005) Evaluation of the concentration of HCH, DDT, HCB, PCB and PAH in the sediments along the lower stretch of Hugli estuary, West Bengal, northeast India. Environment International 31(4):523-534.

173. Wilcke W (2000) Polycyclic aromatic hydrocarbons (PAHs) in soil - A review. Journal of Plant Nutrition and Soil Science 163(3):229-248.

174. Laflamme RE & Hites RA (1978) The global distribution of polycyclic aromatic hydrocarbons in recent sediments. Geochimica et Cosmochimica Acta 42(3):289-303.

175. González-Gaya B, Zúñiga-Rival J, Ojeda MJ, Jiménez B, & Dachs J (2014) Field measurements of the atmospheric dry deposition fluxes and velocities of polycyclic aromatic hydrocarbons to the global oceans. Environ Sci Technol 48(10):5583-5592.

176. Jurado E & Dachs J (2008) Seasonality in the "grasshopping" and atmospheric residence times of persistent organic pollutants over the oceans. Geophysical Research Letters 35(17).

177. Blais JM, et al. (2007) Biologically mediated transport of contaminants to aquatic systems. Environ Sci Technol 41(4):1075-1084.

178. Svendsen TC, et al. (2007) Polyaromatic hydrocarbons, chlorinated and brominated organic contaminants as tracers of feeding ecology in polar benthic amphipods. Marine Ecology Progress Series 337:155-164.

179. del Vento S & Dachs J (2007) Influence of the surface microlayer on atmospheric deposition of aerosols and polycyclic aromatic hydrocarbons. Atmospheric Environment 41(23):4920-4930.

180. Jurado E (2004) Latitudinal and seasonal capacity of the surface oceans as a reservoir of polychlorinated biphenyls. Environ Pollut 128(1-2):149-162.

181. Jurado E, et al. (2005) Wet deposition of persistent organic pollutants to the global oceans. Environ Sci Technol 39(8):2426-2435.

182. Franz TP & Eisenreich SJ (1998) Snow scavenging of polychlorinated biphenyls and polycyclic aromatic hydrocarbons in Minnesota. Environ Sci Technol 32(12):1771-1778.

183. Van Ry DA, et al. (2002) Wet deposition of polychlorinated biphenyls in urban and background areas of the Mid-Atlantic States. Environ Sci Technol 36(15):3201-3209.

184. Liss P & Merlivat L (1986) Air-Sea Gas Exchange Rates: Introduction and Synthesis. The Role of Air-Sea Exchange in Geochemical Cycling, NATO ASI Series, ed Buat-Ménard P (Springer Netherlands), Vol 185, pp 113-127.

185. Mackay D & Paterson S (1991) Evaluating the multimedia fate of organic chemicals: A level III fugacity model. Environ Sci Technol 25(3):427-436.

186. GESAMP (1989) The Atmospheric Input of Trace Species to the World Ocean. (WMO Reports and Studies No. 38, Joint Group of Experts on the Scientific Aspects of Marine Pollution).

187. Castro-Jiménez J, Berrojalbiz N, Wollgast J, & Dachs J (2012) Polycyclic aromatic hydrocarbons (PAHs) in the Mediterranean Sea: Atmospheric occurrence, deposition and decoupling with settling fluxes in the water column. Environ Pollut 166:40-47.

188. Karickhoff SW, Brown DS, & Scott TA (1979) Sorption of hydrophobic pollutants on natural sediments. Water Research 13(3):241-248.

189. Mackay D (2004) Finding fugacity feasible, fruitful, and fun. Environmental Toxicology and Chemistry 23(10):2282-2289.

190. Larsson P, Okla L, & Cronberg G (1998) Turnover of polychlorinated biphenyls in an oligotrophic and an eutrophic lake in relation to internal lake processes and atmospheric fallout. Canadian Journal of Fisheries and Aquatic Sciences 55(8):1926-1937.

191. Cousins IT, Mackay D, & Jones KC (1999) Measuring and modelling the vertical distribution of semi-volatile organic compounds in soils. II: Model development. Chemosphere 39(14):2519-2534.

192. Ko FC, Sanford LP, & Baker JE (2003) Internal recycling of particle reactive organic chemicals in the Chesapeake Bay water column. Marine Chemistry 81(3-4):163-176.

193. Nizzetto L, et al. (2010) Past, present, and future controls on levels of persistent organic pollutants in the global environment. Environ Sci Technol 44(17):6526-6531.

194. Cabrerizo A, et al. (2011) Factors influencing the soil-air partitioning and the strength of soils as a secondary source of polychlorinated biphenyls to the atmosphere. Environ Sci Technol 45(11):4785-4792.

195. Ma J, Hung H, Tian C, & Kallenborn R (2011) Revolatilization of persistent organic pollutants in the Arctic induced by climate change. Nature Climate Change 1(5):255-260.

196. Imhoff ML, et al. (2004) The consequences of urban land transformation on net primary productivity in the United States. Remote Sensing of Environment 89(4):434-443.

197. Korner C (2000) Biosphere responses to CO_2 enrichment. Ecological Applications 10(6):1590-1619.

198. Ross DJ, Tate KR, Scott NA, & Feltham CW (1999) Land-use change: Effects on soil carbon, nitrogen and phosphorus pools and fluxes in three

adjacent ecosystems. Soil Biology and Biochemistry 31(6):803-813.

199. Cabrerizo A, Dachs J, Barceló D, & Jones KC (2013) Climatic and biogeochemical controls on the remobilization and reservoirs of persistent organic pollutants in Antarctica. Environ Sci Technol 47(9):4299-4306.

200. Iwata H, Tanabe S, Sakai N, & Tatsukawa R (1993) Distribution of persistent organochlorines in the oceanic air and surface seawater and the role of ocean on their global transport and fate. Environ Sci Technol 27(6):1080-1098.

201. Yan S, Rodenburg LA, Dachs J, & Eisenreich SJ (2008) Seasonal air-water exchange fluxes of polychlorinated biphenyls in the Hudson River Estuary. Environ Pollut 152(2):443-451.

202. García-Flor N, et al. (2009) Surface waters are a source of polychlorinated biphenyls to the coastal atmosphere of the North-Western Mediterranean Sea. Chemosphere 75(9):1144-1152.

203. Jurado E, et al. (2004) Atmospheric dry deposition of persistent organic pollutants to the atlantic and inferences for the global oceans. Environ Sci Technol 38(21):5505-5513.

204. Lohmann R, et al. (2009) Organochlorine pesticides and PAHs in the surface water and atmosphere of the North Atlantic and Arctic Ocean. Environ Sci Technol 43(15):5633-5639.

205. Galbán-Malagón C, et al. (2013) Polychlorinated biphenyls, hexachlorocyclohexanes and hexachlorobenzene in seawater and phytoplankton from the Southern Ocean (Weddell, South Scotia, and Bellingshausen Seas). Environ Sci Technol 47(11):5578-5587.

206. Dachs J, Eisenreich SJ, Baker JE, Ko FC, & Jeremiason JD (1999) Coupling of phytoplankton uptake and air-water exchange of persistent organic pollutants. Environ Sci Technol 33(20):3653-3660.

207. Scheringer M, Stroebe M, Wania F, Wegmann F, & Hungerbühler K (2004) The Effect of Export to the Deep Sea on the Long-Range Transport Potential of Persistent Organic Pollutants. Environmental Science and Pollution Research 11(1):41-48.

208. Gerofke A, Kömp P, & McLachlan MS (2005) Bioconcentration of persistent organic pollutants in four species of marine phytoplankton. Environmental Toxicology and Chemistry 24(11):2908-2917.

209. Broman D, et al. (1996) Significance of bacteria in marine waters for the distribution of hydrophobic organic contaminants. Environ Sci Technol 30(4):1238-1241.

210. Lohmann R, Jurado E, Dachs J, Lohmann U, & Jones KC (2006) Quantifying the importance of the atmospheric sink for polychlorinated dioxins and furans relative to other global loss processes. Journal of Geophysical Research Atmospheres 111(21).

211. Nizzetto L, et al. (2012) Biological pump control of the fate and distribution of hydrophobic organic pollutants in water and plankton. Environ Sci Technol 46(6):3204-3211.

212. Galbán-Malagón CJ, Berrojalbiz N, Gioia R, & Dachs J (2013) The "degradative" and "biological" pumps controls on the atmospheric deposition and sequestration of hexachlorocyclohexanes and hexachlorobenzene in the North Atlantic and Arctic Oceans. Environ Sci Technol 47(13):7195-7203.

213. Dueri S, Dahllöf I, Hjorth M, Marinov D, & Zaldívar JM (2009) Modeling the combined effect of nutrients and pyrene on the plankton population: Validation using mesocosm experiment data and scenario analysis. Ecological Modelling 220(17):2060-2067.

214. Marinov D, et al. (2009) Integrated modelling of Polycyclic Aromatic Hydrocarbons in the marine environment: coupling of hydrodynamic, fate and transport, bioaccumulation and planktonic food-web models. Mar Pollut Bull 58(10):1554-1561.

215. Berrojalbiz N, et al. (2011) Persistent organic pollutants in Mediterranean seawater and processes affecting their accumulation in plankton. Environ Sci Technol 45(10):4315-4322.

216. Gobas FAPC, Wilcockson JB, Russell RW, & Haffner GD (1999) Mechanism of biomagnification in fish under laboratory and field conditions. Environ Sci Technol 33(1):133-141.

217. Cropp R, Kerr G, Bengtson-Nash S, & Hawker D (2011) A dynamic biophysical fugacity model of the movement of a persistent organic pollutant in Antarctic marine food webs. Environmental Chemistry 8(3):263-280.

218. Berrojalbiz N, et al. (2011) Biogeochemical and physical controls on concentrations of polycyclic aromatic hydrocarbons in water and plankton of the Mediterranean and Black Seas. Global Biogeochemical Cycles 25(4):n/a-n/a.

219. Gupta S, Pathak B, & Fulekar MH (2015) Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: a review. Reviews in Environmental Science and Biotechnology 14(2):241-269.

220. Hook SE & Osborn HL (2012) Comparison of toxicity and transcriptomic profiles in a diatom exposed to oil, dispersants, dispersed oil. Aquat Toxicol 124-125:139-151.

221. Camacho M, et al. (2013) Potential adverse health effects of persistent organic pollutants on sea turtles: Evidences from a cross-sectional study on Cape Verde loggerhead sea turtles. Science of the Total Environment 458-460:283-289.

222. Hermanussen S, Matthews V, Päpke O, Limpus CJ, & Gaus C (2008) Flame retardants (PBDEs) in marine turtles, dugongs and seafood from Queensland, Australia. Marine Pollution Bulletin 57(6-12):409-418.

223. Hall AJ, Kalantzi OI, & Thomas GO (2003) Polybrominated diphenyl ethers (PBDEs) in grey seals during their first year of life - Are they thyroid hormone endocrine disrupters? Environ Pollut 126(1):29-37.

224. Panti C, et al. (2011) Ecotoxicological diagnosis of striped dolphin (Stenella coeruleoalba) from the Mediterranean basin by skin biopsy and gene expression approach. Ecotoxicology 20(8):1791-1800.

225. Arrhenius A, Gronvall F, Scholze M, Backhaus T, & Blanck H (2004) Predictability of the mixture toxicity of 12 similarly acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities. Aquat Toxicol 68(4):351-367.

226. Othman HB et al. (2012) Toxicity of benz(a) anthracene and fluoranthene to marine phytoplankton in culture: Does cell size really matter? Journal of Hazardous Materials 243:204-211.

227. Echeveste P, Agusti S, & Dachs J (2011) Cell size dependence of additive versus synergetic effects of UV radiation and PAHs on oceanic phytoplankton. Environ Pollut 159(5):1307-1316.

228. Anonymous (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/ 45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155 EEC, 93/67/EEC. (European Union).

229. Altenburger R & Greco WR (2009) Extrapolation Concepts for dealing with multiple contamination in environmental risk assessment. Integrated Environmental Assessment and Management 5(1):62-68.

230. Syberg K, Jensen TS, Cedergreen N, & Rank J (2009) On the use of mixture toxicity assessment in reach and the water framework directive: A review. Human and Ecological Risk Assessment 15(6):1257-1272.

231. Spurgeon DJ, et al. (2010) Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. Science of the Total Environment 408(18):3725-3734.

232. Backhaus T & Faust M (2012) Predictive environmental risk assessment of chemical mixtures: A conceptual framework. Environ Sci Technol46(5):2564-2573.

233. McCarty LS & Borgert CJ (2006) Review of the toxicity of chemical mixtures containing at least one organochlorine. Regulatory Toxicology and Pharmacology 45(2):104-118.

234. Johnson PW & Sieburth JM (1979) Chroococcoid cyanobacteria in the sea: A ubiquitous and diverse phototrophic biomass. Limnol. Oceanogr. 24:928-935.

235. Gieskes WWC & Kraay GW (1983) Unknown chlorophyll a derivatives in the North Sea and the tropical Atlantic Ocean revealed by HPLC analysis. Limnol. Oceanogr. 28:757-766.

236. Chisholm SW, et al. (1988) A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334(6180):340-343.

237. Palenik B & Haselkorn R (1992) Multiple evolutionary origins of prochlorophytes, the chlorophyll b-containing prokaryotes. Nature 355(6357):265-267.

238. Chisholm SW, et al. (1992) Prochlorococcus marinus nov. gen. nov. sp.: An oxyphototrophic marine prokaryote containing divinyl chlorophyll a and b. Archives of Microbiology 157(3):297-300.

239. Goericke R & Repeta DJ (1992) The pigments of Prochlorococcus marinus: the Presence of Divinyl Chlorophyll a and Chlorophyll b in a marine prokariote. Limnol. Oceanogr. 37:425-433.

240. Morel A, et al. (1993) Prochlorococcus and Synechococcus: a comparative study of their optical properties in relation to their size and pigmentation. Journal of Marine Research 51(3):617-649.

241. Moore LR, Goericke R, & Chisholm SW (1995) Comparative physiology of Synechococcus and Prochlorococcus: Influence of light and temperature on growth, pigments, fluorescence and absorptive properties. Marine Ecology Progress Series 116(1-3):259-276.

242. Partensky F, Hess WR, & Vaulot D (1999) Prochlorococcus, a marine photosynthetic prokaryote of global significance. Microbiology and Molecular Biology Reviews 63(1):106-127.

243. Coleman ML & Chisholm SW (2007) Code and context: Prochlorococcus as a model for cross-scale biology. Trends Microbiol 15(9):398-407.

244. Partensky F & Garczarek L (2010) Prochlorococcus: Advantages and limits of minimalism. Annual Review of Marine Science 2(1):305-331.

245. Rocap G, et al. (2003) Genome divergence in two Prochlorococcus ecotypes reflects oceanic niche differentiation. Nature 424(6952):1042-1047.

246. Shalapyonok A, Olson RJ, & Shalapyonok LS (1998) Ultradian growth in Prochlorococcus spp. Appl Environ Microbiol 64(3):1066-1069.

247. Zinser E, et al. (2007) Influence of light and temperature on Prochlorococcus ecotype distributions in the Atlantic Ocean. Limnol. Oceanogr. 52(5):2205-2220.

248. Vaulot D, Marie D, Olson RJ, & Chisholm SW (1995) Growth of Prochlorococcus, a photosynthetic prokaryote, in the equatorial Pacific Ocean. Science 268(5216):1480-1482.

249. Liu H, Nolla HA, & Campbell L (1997) Prochlorococcus growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. Aquatic Microbial Ecology 12(1):39-47.

250. Campbell L, Liu H, Nolla HA, & Vaulot D (1997) Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean at Station ALOHA during the 1991-1994 ENSO event. Deep-Sea Research Part I: Oceanographic Research Papers 44(2):167-192.

251. Durand MD, Olson RJ, & Chisholm SW (2001) Phytoplankton population dynamics at the Bermuda Atlantic Time-series station in the Sargasso Sea. Deep-Sea Research Part II: Topical Studies in Oceanography 48(8-9):1983-2003.

252. Partensky F, Blanchot J, & Vaulot D (1999) Differential distribution and ecology of Prochlorococcus and Synechococcus in oceanic waters: A review. Marine Cyanobacteria 19(SPEC. ISSUE):457-475.

253. Flombaum P, et al. (2013) Present and future global distributions of the marine Cyanobacteria Prochlorococcus and Synechococcus. Proceedings of the National Academy of Sciences of the United States of America 110(24):9824-9829.

254. Bryant DA (2003) The beauty in small things revealed. Proc Natl Acad Sci U S A 100(17):9647-9649.

255. Olson RJ, Chisholm SW, Zettler ER, Altabet MA, & Dusenberry JA (1990) Spatial and temporal distributions of prochlorophyte picoplankton in the North Atlantic Ocean. Deep Sea Research Part A, Oceanographic Research Papers 37(6):1033-1051.

256. Johnson ZI, et al. (2006) Niche partitioning among Prochlorococcus ecotypes along ocean-scale environmental gradients. Science 311(5768):1737-1740.

257. Zwirglmaier K, et al. (2008) Global phylogeography of marine Synechococcus and Prochlorococcus reveals a distinct partitioning of lineages among oceanic biomes. Environ Microbiol 10(1):147-161.

258. Bouman HA, et al. (2006) Oceanographic basis of the global surface distribution of Prochlorococcus ecotypes. Science 312(5775):918-921.

259. Moore LR & Chisholm SW (1999) Photophysiology of the marine cyanobacterium Prochlorococcus: Ecotypic differences among cultured isolates. Limnol. Oceanogr. 44(3 I):628-638.

260. Bruyant F, et al. (2005) Diel variations in the photosynthetic parameters of Prochlorococcus strain PCC 9511: Combined effects of light and cell cycle. Limnol. Oceanogr. 50(3):850-863.

261. Scanlan DJ & West NJ (2002) Molecular ecology of the marine cyanobacterial genera Prochlorococcus and Synechococcus. FEMS Microbiology Ecology 40(1):1-12.

262. Lindell D & Post AF (1995) Ultraphytoplankton succession is triggered by deep winter mixing in the Gulf of Aqaba (Eilat), Red Sea. Limnol. Oceanogr. 40(6):1130-1141.

263. Ting CS, et al. (2015) The Prochlorococcus carbon dioxide-concentrating mechanism: Evidence of carboxysome-associated heterogeneity. Photosynthesis Research 123(1):45-60.

264. Hartmann M, et al. (2014) Efficient CO2 fixation by surface Prochlorococcus in the Atlantic Ocean. ISME Journal 8(11):2280-2289.

265. Campbell L, Nolla HA, & Vaulot D (1994) The importance of Prochlorococcus to community structure in the central North Pacific Ocean. Limnol. Oceanogr. 39(4):954-961.

266. Buitenhuis ET, et al. (2012) Picophytoplankton biomass distribution in the global ocean. Earth Syst. Sci. Data 4:37-46.

267. K. Trumper; M. Bertzky BD, G. van der Heijden, M. Jenkins, P. Manning (2009) The Natural Fix? The role of ecosystems in climate mitigation. A UNEP rapid response assessment. (United Nations Environment Programme, UNEP-WCMC, Cambridge, UK).

268. Scanlan DJ, et al. (2009) Ecological genomics of marine picocyanobacteria. Microbiol Mol Biol Rev 73(2):249-299.

269. Zinser ER, et al. (2009) Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, Prochlorococcus. PLoS ONE 4(4).

270. Becker JW, et al. (2014) Closely related phytoplankton species produce similar suites of dissolved organic matter. Frontiers in Microbiology 5(MAR).

271. Bertilsson S, Berglund O, Pullin MJ, & Chisholm SW (2005) Release of dissolved organic matter by Prochlorococcus. Vie et Milieu 55(3-4):225-231.

272. Biller SJ, et al. (2014) Bacterial vesicles in marine ecosystems. Science 343(6167):183-186.

273. Azam F & Malfatti F (2007) Microbial structuring of marine ecosystems. Nature Reviews Microbiology 5(10):782-791.

274. Richardson TL & Jackson GA (2007) Small phytoplankton and carbon export from the surface ocean. Science 315(5813):838-840.

275. Goericke R, Strom SL, & Bell MA (2000) Distribution and sources of cyclic pheophorbides in the marine environment. Limnol. Oceanogr. 45(1):200-211.

276. La Roche J, et al. (1996) Independent evolution of the prochlorophyte and green plant chlorophyll a/b light-harvesting proteins. Proceedings of the National Academy of Sciences of the United States of America 93(26):15244-15248.

277. Bibby TS, Mary I, Nield J, Partensky F, & Barber J (2003) Low-light-adapted Prochlorococcus species possess specific antennae for each photosystem. Nature 424(6952):1051-1054.

278. Holtzendorff J, et al. (2001) Diel expression of cell cycle-related genes in synchronized cultures of Prochlorococcus sp. strain PCC 9511. Journal of Bacteriology 183(3):915-920.

279. Holtzendorff J, et al. (2002) Synchronized expression of ftsZ in natural Prochlorococcus populations of the Red Sea. Environmental Microbiology 4(11):644-653.

280. Garczarek L, et al. (2001) Differential expression of antenna and core genes in Prochlorococcus PCC 9511 (Oxyphotobacteria) grown under a modulated light-dark cycle. Environmental Microbiology 3(3):168-175.

281. Steglich C, Futschik M, Rector T, Steen R, & Chisholm SW (2006) Genome-wide analysis of light sensing in Prochlorococcus. J Bacteriol 188(22):7796-7806.

282. Tolonen AC, et al. (2006) Global gene expression of Prochlorococcus ecotypes in response to changes in nitrogen availability. Mol Syst Biol 2:53.

283. Thompson AW, Huang K, Saito MA, & Chisholm SW (2011) Transcriptome response of high- and low-light-adapted Prochlorococcus strains to changing iron availability. ISME Journal 5(10):1580-1594.

284. Steglich C, et al. (2001) Nitrogen deprivation strongly affects Photosystem II but not phycoerythrin level in the divinyl-chlorophyll b-containing cyanobacterium Prochlorococcus marinus. Biochimica et Biophysica Acta - Bioenergetics 1503(3):341-349.

285. Lindell D, et al. (2002) Nitrogen stress response of Prochlorococcus strain PCC 9511 (Oxyphotobacteria) involves contrasting regulation of ntcA and amt1. Journal of Phycology 38(6):1113-1124.

286. Berube PM, et al. (2015) Physiology and evolution of nitrate acquisition in Prochlorococcus. ISME Journal 9(5):1195-1207.

287. Zubkov MV, et al. (2003) High rate of uptake of organic nitrogen compounds by Prochlorococcus cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Appl Environ Microbiol 69(2):1299-1304.

288. Garcia-Fernandez JM, de Marsac NT, & Diez J (2004) Streamlined regulation and gene loss as adaptive mechanisms in Prochlorococcus for optimized nitrogen utilization in oligotrophic environments. Microbiol Mol Biol Rev 68(4):630-638.

289. Martiny AC, Kathuria S, & Berube PM (2009) Widespread metabolic potential for nitrite and nitrate assimilation among Prochlorococcus ecotypes. Proc Natl Acad Sci U S A 106(26):10787-10792.

290. Moore LR, Post AF, Rocap G, & Chisholm SW (2002) Utilization of different nitrogen sources by the marine cyanobacteria Prochlorococcus and Synechococcus. Limnol. Oceanogr. 47(4):989-996.

291. Kamennaya NA & Post AF (2011) Characterization of cyanate metabolism in marine Synechococcus and Prochlorococcus spp. Appl Environ Microbiol 77(1):291-301.

292. Martinez A, Tyson GW, & Delong EF (2010) Widespread known and novel phosphonate utilization pathways in marine bacteria revealed by functional screening and metagenomic analyses. Environmental Microbiology 12(1):222-238.

293. Feingersch R, et al. (2012) Potential for phosphite and phosphonate utilization by Prochlorococcus. ISME Journal 6(4):827-834.

294. Martinez A, Osburne MS, Sharma AK, DeLong EF, & Chisholm SW (2012) Phosphite utilization by the marine picocyanobacterium Prochlorococcus MIT9301. Environ Microbiol 14(6):1363-1377.

295. Boyd PW, et al. (2007) Mesoscale iron enrichment experiments 1993-2005: Synthesis and future directions. Science 315(5812):612-617.

296. Mann EL, Ahlgren N, Moffett JW, & Chisholm SW (2002) Copper toxicity and cyanobacteria ecology in the Sargasso Sea. Limnol. Oceanogr. 47(4):976-988.

297. Gómez-Baena G, et al. (2008) Glucose uptake and its effect on gene expression in Prochlorococcus. PLoS ONE 3(10).

298. Muñoz-Marín MC, et al. (2013) Prochlorococcus can use the Pro1404 transporter to take up glucose at nanomolar concentrations in the Atlantic Ocean. Proceedings of the National Academy of Sciences of the United States of America 110(21):8597-8602.

299. Biller SJ, Berube PM, Lindell D, & Chisholm SW (2015) Prochlorococcus: The structure and function of collective diversity. Nature Reviews Microbiology 13(1):13-27.

300. Grzymski JJ & Dussaq AM (2012) The significance of nitrogen cost minimization in proteomes of marine microorganisms. ISME Journal 6(1):71-80.

301. Bragg JG & Hyder CL (2004) Nitrogen versus carbon use in prokaryotic genomes and proteomes. Proceedings of the Royal Society B: Biological Sciences 271(SUPPL. 5):S374-S377.

302. Bertilsson S, Berglund O, Karl DM, & Chisholm SW (2003) Elemental composition of marine Prochlorococcus and Synechococcus: Implications for the ecological stoichiometry of the sea. Limnol. Oceanogr. 48(5):1721-1731.

303. Heldal M, Scanlan DJ, Norland S, Thingstad F, & Mann NH (2003) Elemental composition of single cells of various strains of marine Prochlorococcus and Synechococcus using X-ray microanalysis. Limnol. Oceanogr. 48(5):1732-1743.

304. Grob C, et al. (2013) Elemental composition of natural populations of key microbial groups in Atlantic waters. Environmental Microbiology 15(11):3054-3064.

305. Van Mooy BAS, Rocap G, Fredricks HF, Evans CT, & Devol AH (2006) Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. Proceedings of the National Academy of Sciences of the United States of America 103(23):8607-8612.

306. Fuller NJ, et al. (2005) Dynamics of community structure and phosphate status of picocyanobacterial populations in the Gulf of Aqaba, Red Sea. Limnol. Oceanogr. 50(1):363-375.

307. Penno S, Lindell D, & Post AF (2006) Diversity of Synechococcus and Prochlorococcus populations determined from DNA sequences of the N-regulatory gene ntcA. Environmental Microbiology 8(7):1200-1211.

308. Rocap G, Distel DL, Waterbury JB, & Chisholm SW (2002) Resolution of Prochlorococcus and Synechococcus Ecotypes by Using 16S-23S Ribosomal DNA Internal Transcribed Spacer Sequences. Appl Environ Microbiol 68(3):1180-1191.

309. Urbach E, Scanlan DJ, Distel DL, Waterbury JB, & Chisholm SW (1998) Rapid diversification of marine picophytoplankton with dissimilar light- harvesting structures inferred from sequences of Prochlorococcus and Synechococcus (cyanobacteria). Journal of Molecular Evolution 46(2):188-201.

310. Kettler GC, et al. (2007) Patterns and implications of gene gain and loss in the evolution of Prochlorococcus. PLoS Genetics 3(12):2515-2528.

311. Moore LR, Rocap G, & Chisholm SW (1998) Physiology and molecular phylogeny of coexisting Prochlorococcus ecotypes. Nature 393(6684):464-467.

312. Ahlgren NA, Rocap G, & Chisholm SW (2006) Measurement of Prochlorococcus ecotypes using real-time polymerase chain reaction reveals different abundances of genotypes with similar light physiologies. Environ Microbiol 8(3):441-454.

313. West NJ & Scanlan DJ (1999) Niche-partitioning of Prochlorococcus populations in a stratified water column in the eastern North Atlantic Ocean? Appl Environ Microbiol 65(6):2585-2591.

314. West NJ, et al. (2001) Closely related Prochlorococcus genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides. Microbiology 147(7):1731-1744.

315. Partensky F, La Roche J, Wyman K, & Falkowski PG (1997) The divinyl-chlorophyll a/b-protein complexes of two strains of the oxyphototrophic marine prokaryote Prochlorococcus - Characterization and response to changes in growth irradiance. Photosynthesis Research 51(3):209-222.

316. Martiny AC, Tai AP, Veneziano D, Primeau F, & Chisholm SW (2009) Taxonomic resolution, ecotypes and the biogeography of Prochlorococcus. Environ Microbiol 11(4):823-832.

317. Huang S, et al. (2012) Novel lineages of Prochlorococcus and Synechococcus in the global oceans. ISME J 6(2):285-297.

318. Malmstrom RR, et al. (2013) Ecology of uncultured Prochlorococcus clades revealed through single-cell genomics and biogeographic analysis. ISME Journal 7(1):184-198.

319. Dufresne A, et al. (2003) Genome sequence of the cyanobacterium Prochlorococcus marinus SS120, a nearly minimal oxyphototrophic genome. Proc Natl Acad Sci U S A 100(17):10020-10025.

320. Coleman ML, et al. (2006) Genomic islands and the ecology and evolution of Prochlorococcus. Science 311(5768):1768-1770.

321. Hess WR (2011) Cyanobacterial genomics for ecology and biotechnology. Current Opinion in Microbiology 14(5):608-614.

322. Morris JJ, Lenski RE, & Zinser ER (2012) The black queen hypothesis: Evolution of dependencies through adaptive gene loss. mBio 3(2).

323. Baumdicker F, Hess WR, & Pfaffelhuber P (2012) The infinitely many genes model for the distributed genome of bacteria. Genome Biology and Evolution 4(4):443-456.

324. Lindell D, et al. (2007) Genome-wide expression dynamics of a marine virus and host reveal features of co-evolution. Nature 449(7158):83-86.

325. Zeidner G, et al. (2005) Potential photosynthesis gene recombination between Prochlorococcus and Synechococcus via viral intermediates. Environ Microbiol 7(10):1505-1513.

326. Sullivan MB, et al. (2006) Prevalence and evolution of core photosystem II genes in marine cyanobacterial viruses and their hosts. PLoS biology 4(8).

327. Kashtan N, et al. (2014) Single-cell genomics reveals hundreds of coexisting subpopulations in wild Prochlorococcus. Science 344(6182):416-420.

328. Mella-Flores D, et al. (2011) Is the distribution of Prochlorococcus and Synechococcus ecotypes in the Mediterranean Sea affected by global warming? Biogeosciences 8(9):2785-2804.

329. Ribalet F, et al. (2015) Light-driven synchrony of Prochlorococcus growth and mortality in the subtropical Pacific gyre. Proceedings of the National Academy of Sciences of the United States of America 112(26):8008-8012.

330. Mella-Flores D, et al. (2012) Prochlorococcus and Synechococcus have evolved different adaptive mechanisms to cope with light and uv stress. Frontiers in Microbiology 3(AUG).

331. Agustí S & Llabrés M (2007) Solar radiation-induced mortality of marine pico-phytoplankton in the oligotrophic ocean. Photochemistry and Photobiology 83(4):793-801.

332. Okin GS, et al. (2011) Impacts of atmospheric nutrient deposition on marine productivity: Roles of nitrogen, phosphorus, and iron. Global Biogeochemical Cycles 25(2).

333. Paytan A, et al. (2009) Toxicity of atmospheric aerosols on marine phytoplankton. Proc Natl Acad Sci U S A 106(12):4601-4605.

334. Hill PG, Zubkov MV, & Purdie DA (2010) Differential responses of Prochlorococcus and SAR11-dominated bacterioplankton groups to atmospheric dust inputs in the tropical Northeast Atlantic Ocean. FEMS Microbiol Lett 306(1):82-89.

335. Hjorth M, Forbes VE, & Dahllöf I (2008) Plankton stress responses from PAH exposure and nutrient enrichment. Marine Ecology Progress Series 363:121-130.

336. Gilde K & Pinckney JL (2012) Sublethal Effects of Crude Oil on the Community Structure of Estuarine Phytoplankton. Estuaries and Coasts 35(3):853-861.

337. Zinser ER, et al. (2006) Prochlorococcus ecotype abundances in the North Atlantic Ocean as revealed by an improved quantitative PCR method. Appl Environ Microbiol 72(1):723-732.

338. Rusch DB, Martiny AC, Dupont CL, Halpern AL, & Venter JC (2010) Characterization of Prochlorococcus clades from iron-depleted oceanic regions. Proceedings of the National Academy of Sciences of the United States of America 107(37):16184-16189.

339. West NJ, Lebaron P, Strutton PG, & Suzuki MT (2011) A novel clade of Prochlorococcus found in high nutrient low chlorophyll waters in the South and Equatorial Pacific Ocean. ISME J 5(6):933-944.

340. Malmstrom RR, et al. (2010) Temporal dynamics of Prochlorococcus ecotypes in the Atlantic and Pacific oceans. ISME J 4(10):1252-1264.

341. Lavin P, Gonzalez B, Santibanez JF, Scanlan DJ, & Ulloa O (2010) Novel lineages of Prochlorococcus thrive within the oxygen minimum zone of the eastern tropical South Pacific. Environ Microbiol Rep 2(6):728-738.

342. Strand A & Hov Ø (1996) A model strategy for the simulation of chlorinated hydrocarbon distributions in the global environment. Water, Air, and Soil Pollution 86(1-4):283-316.

343. Heid CA, Stevens J, Livak KJ, & Williams PM (1996) Real time quantitative PCR. Genome Research 6(10):986-994.

344. Lockey C, Otto E, & Long Z (1998) Real-time fluorescence detection of a single DNA molecule. BioTechniques 24(5):744-746.

345. Mullis KB & Faloona FA (1987) [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed

chain reaction. in Methods in Enzymology, pp 335-350.

346. Mullis KB (1990) The unusual origin of the polymerase chain reaction. Scientific American 262(4):56-65.

347. Powledge TM (2004) The polymerase chain reaction. Advances in physiology education 28(1-4):44-50.

348. Saiki RK, et al. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230(4732):1350-1354.

349. Pfaffl M (2006) Relative quantification. Real-time PCR, ed Dorak MT (Taylor & Francis, New York (USA)).

350. Higuchi R, Dollinger G, Walsh PS, & Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Bio/Technology 10(4):413-417.

351. Higuchi R, Fockler C, Dollinger G, & Watson R (1993) Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. Bio/Technology 11(9):1026-1030.

352. Bustin SA (2000) Absolute quantification of mrna using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25(2):169-193.

353. Morrison TB, Weis JJ, & Wittwer CT (1998) Quantification of lowcopy transcripts by continuous SYBR® green I monitoring during amplification. BioTechniques 24(6):954-962.

354. Schmittgen TD, et al. (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. Analytical Biochemistry 285(2):194-204.

355. Rajeevan MS, Vernon SD, Taysavang N, & Unger ER (2001) Validation of array-based gene expression profiles by real-time (Kinetic) RT-PCR. Journal of Molecular Diagnostics 3(1):26-31.

356. Tan BH, Lim EAS, Liaw JCW, Seah SGK, & Yap EPH (2004) Diagnostic value of real-time capillary thermal cycler in virus detection. Expert Review of Molecular Diagnostics 4(2):219-230.

357. Wittwer CT, Herrmann MG, Moss AA, & Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22(1):130-138.

358. Dorak MT (2006) Real-time PCR (Taylor & Francis, New York (USA)).

359. Ririe KM, Rasmussen RP, & Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry 245(2):154-160.

360. Pfaffl MW (2004) Quantification strategies in real-time PCR. A-Z of quantitative PCR, ed Bustin SA (International University Line, La Jolla, CA (USA)).

361. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. Journal of Molecular Endocrinology 29(1):23-39.

362. Cook NL, Vink R, Donkin JJ, & van den Heuvel C (2009) Validation of reference genes for normalization of real-time quantitative RT-PCR data in traumatic brain injury. Journal of Neuroscience Research 87(1):34-41.

363. Orlando C, Pinzani P, & Pazzagli M (1998) Developments in quantitative PCR. Clinical Chemistry and Laboratory Medicine 36(5):255-269.

364. Vandesompele J, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3(7).

365. Hellemans J, Mortier G, De Paepe A, Speleman F, & Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology 8(2).

366. Tichopad A, Didier A, & Pfaffl MW (2004) Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. Molecular and Cellular Probes 18(1):45-50.

367. Tichopad A, Dzidic A, & Pfaffl MW (2002) Improving quantitative real-time RT-PCR reproducibility by boosting primer-linked amplification efficiency. Biotechnology Letters 24(24):2053-2056.

368. Pfaffl MW, Horgan GW, & Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research 30(9).

369. Livak KJ & Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25(4):402-408.

370. Rasmussen RP (2001) Quantification on the LightCycler. Rapid Cycle Real-time PCR, ed Meuer S, Wittwer, C., Nakagawara, K. (Springer Press, Heidelberg).

371. Mackay IM, Arden KE, & Nitsche A (2002) Real-time PCR in virology. Nucleic Acids Research 30(6):1292-1305.

372. Steuerwald N, Cohen J, Herrera RJ, & Brenner CA (1999) Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. Molecular Human Reproduction 5(11):1034-1039.

373. Winer J, Jung CKS, Shackel I, & Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Analytical Biochemistry 270(1):41-49.

374. Wittwer CT & Garling DJ (1991) Rapid cycle DNA amplification: Time and temperature optimization. BioTechniques 10(1):76-78+80-83.

375. Galluzzi L, et al. (2004) Development of a Real-Time PCR Assay for Rapid Detection and Quantification of Alexandrium minutum (a Dinoflagellate). Appl Environ Microbiol 70(2):1199-1206.

376. Zhu F, Massana R, Not F, Marie D, & Vaulot D (2005) Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiology Ecology 52(1):79-92.

377. Countway PD & Caron DA (2006) Abundance and distribution of Ostreococcus sp. in the San Pedro Channel, California, as revealed by quantitative PCR. Appl Environ Microbiol 72(4):2496-2506.

378. Handy SM, Hutchins DA, Cary SC, & Coyne KJ (2006) Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: Capabilities and limitations. Limnol. Oceanogr. : Methods 4(JUN.):193-206.

379. John DE, Patterson SS, & Paul JH (2007) Phytoplankton-group specific quantitative polymerase chain reaction assays for RuBisCO mRNA transcripts in seawater. Mar Biotechnol (NY) 9(6):747-759.

380. John DE, et al. (2007) Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea CO_2 flux in the Mississippi River plume. ISME J 1(6):517-531.

381. Crosbie ND & Furnas MJ (2001) Abundance, distribution and flow-cytometric characterization of picophytoprokaryote populations in central (17°S) and southern (20°S) shelf waters of the Great Barrier Reef. Journal of Plankton Research 23(8):809-828.

382. Garczarek L, et al. (2007) High vertical and low horizontal diversity of Prochlorococcus ecotypes in the Mediterranean Sea in summer. FEMS Microbiol Ecol 60(2):189-206.

383. Holtzendorff J, et al. (2008) Genome streamlining results in loss of robustness of the circadian clock in the marine cyanobacterium Prochlorococcus marinus PCC 9511. Journal of Biological Rhythms 23(3):187-199.

384. Schon A (2002) Conserved and variable domains within divergent RNase P RNA gene sequences of Prochlorococcus strains. International Journal of Systematic and Evolutionary Microbiology 52(4):1383-1389.

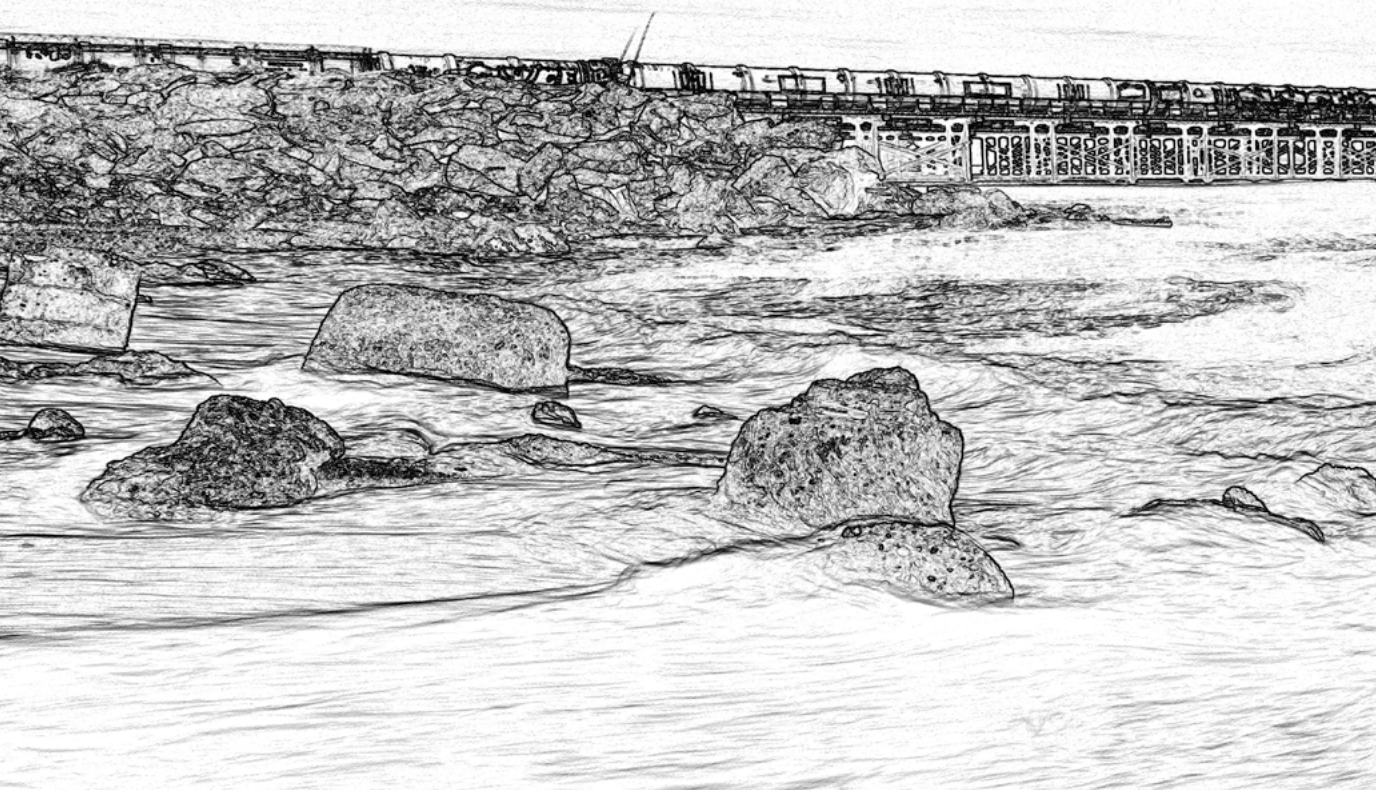
385. Steglich C, Mullineaux CW, Teuchner K, Hess WR, & Lokstein H (2003) Photophysical properties of Prochlorococcus marinus SS120 divinyl chlorophylls and phycoerythrin in vitro and in vivo. FEBS Letters 553(1-2):79-84.

386. Berg GM, et al. (2011) Responses of psbA, hli and ptox genes to changes in irradiance in marine Synechococcus and Prochlorococcus. Aquatic Microbial Ecology 65(1):1-14.

387. Zeidner G, et al. (2003) Molecular diversity among marine picophytoplankton as revealed by psbA analyses. Environmental Microbiology 5(3):212-216.

388. Duarte CM (2015) Seafaring in the 21st century: The Malaspina 2010 circumnavigation expedition. Limnol. Oceanogr. Bulletin 24(1):11-14.

Chapter II



Clade-specific quantitative analysis of photosynthetic gene expression in *Prochlorococcus*

PLoS ONE. 2015, 10(8)

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ABSTRACT

Newly designed primers targeting *rbc*L (CO_2 fixation), *psb*A (photosystem II) and *rnp*B (reference) genes were used in qRT-PCR assays to assess the photosynthetic capability of natural communities of *Prochlorococcus*, the most abundant photosynthetic organism on Earth and a major contributor to primary production in oligotrophic oceans. After optimizing sample collection methodology, we analysed a total of 62 stations from the Malaspina 2010 circumnavigation (including Atlantic, Pacific and Indian Oceans) at three different depths. Sequence and quantitative analyses of the corresponding amplicons showed the presence of high-light (HL) and low-light (LL) *Prochlorococcus* clades in essentially all 182 samples, with a largely uniform stratification of LL and HL sequences. *Synechococcus* cross-amplifications were detected by the taxon-specific melting temperatures of the amplicons. Laboratory exposure of *Prochlorococcus* MED4 (HL) and MIT9313 (LL) strains to organic pollutants (PAHs and organochlorine compounds) showed a decrease of *rbc*L transcript abundances, and of the *rbc*L to *psb*A ratios for both strains. We propose this technique as a convenient assay to evaluate effects of environmental stressors, including pollution, on the oceanic *Prochlorococcus* photosynthetic function.

II.1. INTRODUCTION

Oceanic phytoplankton are responsible for almost a half of the global net primary production (NPP) (1), whereas marine picocyanobacteria account for 32 to 80% of primary production in the oligotrophic oceans (2-5). *Prochlorococcus* is the smallest and most abundant photosynthetic organism known on Earth, ubiquitously found throughout the euphotic zone in tropical and subtropical oligotrophic oceans from 40° S to 40° N (6), with population abundances of about 10^5 cells/mL (7-13). Therefore, *Prochlorococcus* contributes significantly to the NPP in this latitudinal band (8, 14, 15), playing a relevant role in the global carbon cycle. There are two clades of *Prochlorococcus*, adapted to either high-light (HL) and low-light (LL) conditions (16). They differ in a number of genetic and ecophysiological characteristics, including their divinyl-chlorophyll chlb2 / chla2 ratios, ribosomal 16S rDNA sequences, and distributions in the water column (16-18). Multiple genetic and physiologically different *Prochlorococcus* lineages have been so far characterized (17, 19, 20), showing distinct adaptations to solar irradiance, nutrients availability, and, consequently, different horizontal and vertical distributions (11, 13, 21, 22). Preliminary data indicate that there are many *Prochlorococcus* lineages still to be characterized in the world oceans (23-29).

Although genetic variability of *Prochlorococcus* has been extensively studied in the ocean, these studies were mainly based on the distinct sequences of the 16S and 23S rDNAs and the 16S/23S rRNA internal transcribed spacer (ITS) (7, 20, 22, 28, 30, 31). In contrast, analyses of functional gene expression are usually performed in laboratory conditions using pure cultures and specific probes for the target strains (32-37). When functional genes have been studied in the field, the assessment has been restricted to a limited group of strains (38, 39).

In this paper, we focus on the photosynthetic activity of *Prochlorococcus* given its relevance as a primary producer in the global oceans. With this purpose, we designed new primers for two functional photosynthetic protein-coding genes, *rbc*L and *psb*A, and measured their mRNA abundances by quantitative PCR assays along a global circumnavigation sampling campaign. The gene *rbc*L encodes the large protein subunit of the RuBisCO enzyme, responsible of catalysing the rate-limiting step of

Calvin cycle. RbcL protein abundance has been considered a good representative for the activity of the entire Calvin cycle (40) and hence quantification of the *rbc*L mRNA levels is considered a useful proxy for the carbon fixation activity (38, 39, 41, 42). While its transcription is mainly regulated by light intensity (41, 43, 44), it also correlates with other variables like nitrogen concentration (45). The *psb*A gene encodes the photosystem II (PSII) core protein D1, which is the primary target of photo-inactivation and protect the cell from photo-oxidative stress (46). The damage of D1 protein results in photo-inhibition, decrease of the photosystem II efficiency, and a drop of photosynthetic carbon fixation (47, 48). Levels of the PsbA protein subunit have been shown to reflect the cellular PSII content (40). We therefore consider the *psb*A transcript concentration as a proxy of the functionality of the PSII in the cell. Although *psb*A expression is mainly affected by light intensity (32, 44, 49) and UV radiation (48), it is also sensitive to variables such as iron starvation and glucose availability (50). We selected the *rnp*B gene as endogenous standard of the quantification of the two target genes. This gene encodes the RNA component of RNaseP, a ubiquitous enzyme required for tRNA 5' end maturation in prokaryotes. It has been previously reported as a suitable reference gene for *Prochlorococcus* in qRT-PCR analyses (44, 51-53), since its levels of mRNA remain stable at different conditions of irradiation, iron, phosphate, glucose, UV radiation, nitrogen, or light quality (32-37, 43, 52, 54, 55). Aiming to integrate the high genetic variability of *Prochlorococcus* strains by a simple biomarker, we designed for each of the three tested genes, *rnp*B, *rbc*L and *psb*A, two separate sets of primers for HL and LL *Prochlorococcus*. A fundamental reason for the selection of these genes (and of the regions within them to be amplified) is their high sequence conservation among the different isolates of the genus, and their relative divergence from homologous sequences from other cyanobacteria. However, giving the close phylogenetic relationship between some LL *Prochlorococcus* and strains from the genus *Synechococcus*, at least some cross-amplification seemed a priori unavoidable (20, 28).

There are multiple environmental variables (light, nutrients, etc.) known to affect photosynthesis, and hence prone to alter the expression of both target genes (32, 41, 43, 44, 48, 49). In this work, we selected persistent organic pollutants (POPs) as anthropogenic stressors of *Prochlorococcus* photosynthesis capacity, since they are broadly distributed over the globe and reach remote oceanic waters (56-58). They

accumulate in marine phytoplankton (59-64) decreasing cyanobacterial growth rate and biomass, inhibiting the PSII, and causing cellular bleaching and death for *Prochlorococcus* (65, 66).

The objectives of the present work were i) to develop a simple and high-throughput amenable methodology to quantify and detect changes in the expression of *rbc*L and *psb*A genes in *Prochlorococcus*; ii) to test the applicability and specificity of this methodology to both laboratory axenic cultures and field samples collected during an oceanographic cruise; and iii) to analyse the potential effects of POPs, under controlled conditions on *Prochlorococcus* pure cultures. The main goal is to generate a molecular tool applicable to natural communities to study the effects of the influence of diverse environmental stressors, and particularly anthropogenic ones, on the photosynthetic capacity of *Prochlorococcus* natural communities and, ultimately, on the oceanic carbon cycle.

II.2. MATERIAL AND METHODS

II.2.1. OPTIMIZATION OF SAMPLE COLLECTION AND RNA EXTRACTION

We performed preliminary filtration tests using water samples from the North Western Mediterranean Sea (41 39.7 N 02 54.6 E). Based on previous estimation of *Prochlorococcus* mRNA half-live rates (67), we set a maximum operation time of 10 minutes from sampling to RNA stabilization, and all parameters were adjusted to this limit. We tested three different 47-mm-diameter, 0.2-µm-pore-size filters: Nucleopore polycarbonate (Whatman, Freiburg, GE), Durapore PVDF (Millipore, Billerica, MA) and Omnipore PTFE (Millipore). Finally, for each filter type we evaluated the saturation capacity, cell retention and ease of use for nucleic acids isolation.

II.2.2.DESIGN OF PRIMERS AND SPECIFICITY CHECKS USING AXENIC CULTURES

Primers for *Prochlorococcus rbc*L, *psb*A and *rnp*B genes were designed de novo for both HL and LL clades (Table II.1), starting from different known *Prochlorococcus* sequences (68), listed in Table AI.1) using Geneious 5.6.6, Biomatters (available from http://www.geneious.com/). We tested the specificity and efficiency of the designed primers by qRT-PCR analyses using total RNA from axenic cultures of *Prochlorococcus* MED4 and MIT9515 HL strains, MIT9313 and NATL2A LL strains, and *Synechococcus* WH7803 strain. Cultures were grown in artificial media for *Prochlorococcus* (AMP-J) (69), at 22 °C in a natural diel light cycle incubator (69) with a maximum irradiance of 100 µmol quanta m^{-2} s^{-1}. In addition, a culture of *Prochlorococcus* EQPAC1 strain (31) was obtained from the Roscoff Culture Collection and processed for nucleic acid analyses without further manipulation. All the strains were collected at approximately 4×10^5 cells/mL, similar to the mean abundance of natural *Prochlorococcus* communities (8, 9, 15, 25). For consistency, the *Synechococcus* WH7803 strain was collected at the same cell density, despite the

TABLE II.1 - SPECIFIC PRIMERS FOR *PROCHLOROCOCCUS*

Designed primers used for qRT-PCR analysis, and expected PCR fragment sizes.

Gene		Primer sequences (5'- 3')	Amplicon size (bp)
*rnp*B-HL	Fp	GTGTTGGCTAGGTAAACCCCG	*81*
	Rp	ATCTACTTTTAAGCGCCGCTTG	
*rbc*L-HL	Fp	ATGGTCATCCATGGGGTTCAGC	*104*
	Rp	GGTCGCGAAATCGAAAAAGAGAGT	
*psb*A-HL	Fp	ACCAGTTTCAGCAGCTTTCGCA	*128*
	Rp	TGTTTTCCAGGCAGAGCACAACA	
*rnp*B-LL	Fp	TGCCACAGAAAMACACCGC	*106*
	Rp	GCATCGAGAGGTGCTGGC	
*rbc*L-LL	Fp	GAAGATATCCGCTTCCCGATGGC	*140*
	Rp	AAGCCAAAGCTTGGCCTTTCTGG	
*psb*A-LL	Fp	TCTGGTGCTGTTGTTCCTTCCAG	*200*
	Rp	GTATGCGCCCTTGGATCTGTGT	

Fp: Forward primer; Rp: Reverse primer

fact that this genus is typically one or two orders of magnitude less abundant than *Prochlorococcus* (6, 70, 71). The collection and storage procedure was as used for field samples, which is explained in detail below.

II.2.3. FIELD SAMPLE COLLECTION AND STORAGE

A total of 182 samples of oceanic water were collected at 62 stations in the Atlantic, Indian and Pacific Oceans during the Malaspina 2010 circumnavigation, from 14 December 2010 to 14 July 2011 aboard the R/V BioHesperides (Fig. II.1 and Table AI.2). We sampled three depths at each station: 3 m depth, (Niskin bottle), deep chlorophyll maximum (DCM) depth, and DCM+40 m depth (both in Niskin bottles attached to a rosette - CTD system). Samples were collected between 8 h and 12 h am local time (10 h 45 min ± 46 min, S2 Table). This time period coincides with the peak of carbon fixation by *Prochlorococcus*, which occurs between dawn and midday (43, 72), as well as with the maximal expression of *rbc*L and *psb*A genes (44). One litre of seawater from each sample was transferred from the Niskin bottle to a glass bottle, previously rinsed with milli-Q water and a small volume of the sampled seawater, prefiltered onto a 20-µm-pore-size net, and finally filtered onto 47-mm-diameter, 0.2-µm-pore-size PTFE filter at 80 mbar vacuum pressure. Filters were split into two halves. One half was preserved in lysis buffer (50 mM Tris-HCl, 40 mM EDTA, 0.75 M Sucrose) and stored at -20 °C for genomic DNA extraction. The second half was preserved in RNAlater (Sigma-Aldrich, Saint Louis, MO) for RNA isolation analysis and preserved at -80 °C. In all cases, we kept rigorously the time limit of 10 min between sample collection and the storage of the filter.

II.2.4. EXPERIMENTAL EXPOSURE OF AXENIC CULTURES TO POLLUTANT MIXTURES

To test the sensitivity of the designed primers to the potential effects of environmental stressors of photosynthesis presumably inducing a small variation on the gene expression, we decided to perform experiments under controlled

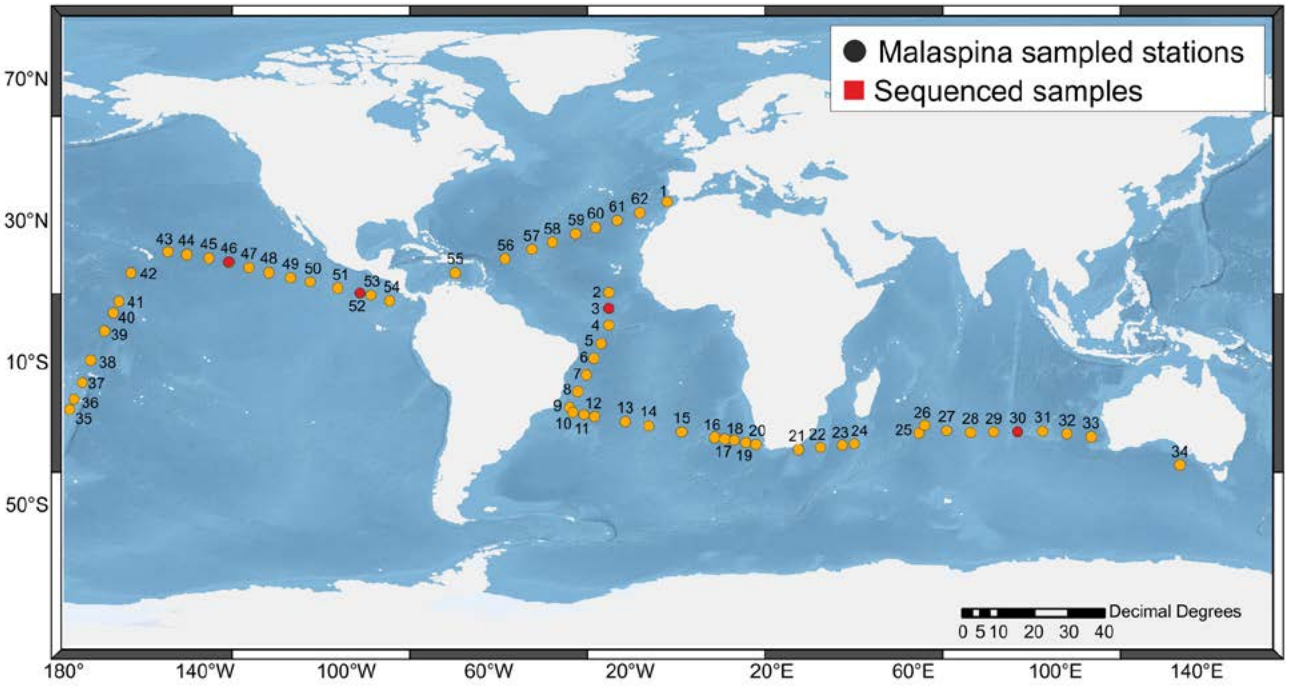


FIGURE II.1 - MALASPINA SAMPLED STATIONS

Oceanic stations from Malaspina 2010 circumnavigation selected for RNA analysis. Stations labelled in red correspond to those from which amplicon sequences were obtained.

conditions using axenic cultures and commercial mixtures of organic pollutants. We selected the well-characterized MED4 and MIT9313 *Prochlorococcus* strains as representatives of HL and LL clades, respectively, since they differ in size, nutritional requirements, preferences in light intensity, and genome size (73). We chose as stressors a mixture of the US Environmental Protection Agency 16 priority polycyclic aromatic hydrocarbons (PAHs) (74) (Dr. Ehrenstorfer, Augsburg, GE), and a mixture of five organochlorine pesticides (OClP), hexachlorobenzene (HCB), and hexachlorocyclohexane (HCH, and α, β, δ and γ isomers), given their toxicity, persistence, potential to bioaccumulate, and environmental relevance (57, 58, 75, 76). Growing MED4 and MIT9313 cultures were challenged with either pollutant mixtures. The PAH mixture was added to a final concentration of 700 ng/L, the approximated concentration estimated to reduce growth of natural populations of *Prochlorococcus* by 10% (LC10) (66). No equivalent toxicity data exist for the OClP mixture; thus, we used a final concentration of 500 ng/L that is known to have no effect on growth rate in the experimental conditions (not shown).

We performed 4 experiments, one for each combination of strain and pollutant mixture. For each experiment, we used a culture adapted previously to the experimental conditions, which was diluted to an approximate concentration of 10^5 cells/mL. After two days, once it had reached a concentration of 2-4*10^5 cells/ml, we split it into twelve 0.5 L flasks. 6 flasks where challenged with the pollutant mixture (treatments) and the other 6 where grown in regular conditions (controls). Three pairs of treatment and control flasks were collected after 30 minutes and the rest after 24 hours of exposure. Every experiment was started at 11 h local time, at the same point of the incubator diel cycle, when the radiation was still increasing before reaching the maximum, to avoid differences between experiments due to the effect of the diel cycle on the mRNA abundances of the target genes (44). Cell concentration was measured at the beginning and at the end of the experiment with a flow cytometer (Guava easyCyte, Millipore), using the characteristic red auto-fluorescence of *Prochlorococcus* (77). To analyse the content of chlorophyll *a*, a 50 mL aliquot of each culture was filtered through a GF/F filter (Whatman) under vacuum pressure and stored at -20 °C until analysis. Samples for molecular analyses were collected and preserved as described above for field samples.

II.2.5. DNA AND RNA ISOLATION

For DNA extraction, the corresponding half filters, once thawed, were incubated at 37 °C for 45 minutes with a 5 mg/mL lysozyme solution in lysis buffer. Then, 0.5 mg/mL proteinase K and 100 µL of 10% sodium dodecyl sulphate were added and further incubated at 55 °C for 1 h. DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1; pH 8), and once with chloroform-isoamyl alcohol (24:1). Genomic DNA from the aqueous phase was then precipitated adding ammonium acetate to 0.4 M and one volume of isopropyl alcohol. After 20 minutes of incubation at -20 °C, the precipitate was centrifuged and the supernatant was removed. The obtained DNA pellet was washed with 70% ethanol and finally suspended in 30 µL of TE (10 mM Tris, 1 mM EDTA; pH 8).

Total RNA was isolated from the RNA-stabilized filter using the mirVana kit (Ambion, Austin, TX) (78), after removing the excess of RNAlater. The final elution volume (about 100 µL) was concentrated by partial lyophilization to approximately 40 µL, and total RNA concentration was measured by spectrophotometric absorption at 260 nm with a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Delaware, DE). RNA quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was treated with DNase I (Ambion) to remove genomic DNA contamination and reverse transcribed to cDNA using First Strand cDNA Synthesis Kit (Roche, Mannheim, GE). Lastly, the resulting cDNA was stored at -20 °C for further analyses.

II.2.6. qRT-PCR ANALYSIS, CLONING AND SEQUENCING OF AMPLICONS FROM CULTURES AND FIELD SAMPLES

Aliquots of genomic DNA (2.2 ng for culture samples or 5 ng for field samples), or total RNA (2.2 ng for culture samples or 3.75 ng for field samples), were used to quantify specific transcripts by qRT-PCR in a LightCycler 480 (Roche Diagnostics, Indianapolis, IN) thermocycler using SYBR Green Mix (Takara Bio Inc., Siga, Japan). After thermal activation at 95 °C for 10 s, forty-five amplification cycles (95 °C for 5 s, 60 °C for 35 s), followed by a melting curve program (65 - 95 °C with a heating rate

of 0.11 °C/s) and a final extension step at 60 °C for 30 s. PCR efficiency values for the tested genes were calculated as described elsewhere (79). When necessary, we redesigned the primers until reaching efficiencies between 95% and 105%. Relative mRNA or genomic DNA abundances of the different genes were calculated using the second derivate maximum of their respective amplification curves (Cp). All samples were run by duplicate, ensuring that the difference between the replicates was less than 0.25 cycles. Cp values are inversely correlated with the logarithm of the initial number of copies of the amplified DNA sequence, N_0, following the equation

$$N_0 = k(1 + E)^{-Cp}$$

in which *E* is the efficiency of the reaction (equal to 1 if the primer efficiency is 100%), and *k* a coefficient related to the number of amplified molecules needed to detect the amplification product by the instrument. Therefore, high Cp values implicate low initial concentrations, and vice versa.

For relative gene expression analyses, we normalized the Cp values of the *rbc*L and *psb*A target genes (tg) to the corresponding values of the *rnp*B reference gene (ref) to obtain ΔCp values, $\Delta Cp = Cp_{ref} - Cp_{tg}$, to account for differential cell concentrations and sample processing when quantifying gene expression. The ratios between treatments and controls mRNA/DNA levels were calculated from these ΔCp values, as

$$\frac{\text{Copies}_{\text{Treatment}}}{\text{Copies}_{\text{Control}}} = 2^{\Delta Cp_{\text{Treatment}} - \Delta Cp_{\text{Control}}}$$

Similarly, the ratios between *rbc*L and *psb*A mRNA/DNA levels were calculated as

$$\frac{\text{Copies}_{rbcL}}{\text{Copies}_{psbA}} = 2^{\Delta Cp_{rbcL} - \Delta Cp_{psbA}}$$

Melting temperature (Tm) values for each amplified product from each sample was calculated as the negative first derivation curve of the fluorescence intensity curve over temperature set in the PCR protocol (-dF/dT curve) (80). Only amplifications with single peaks, denoting a single amplification product, were considered for

quantification. PCR products of MED4 and MIT9313 cultures and field samples from 4 oceanic stations (Table II.2) were cloned into the vector pTZ57R/T (InsTAclone PCR clone kit, Thermo Scientific, Waltham, MA) and propagated using XL-Blue competent cells. DNA sequencing was performed on 3730 DNA Analyzer (Applied Biosystems), and we compared the results to existing DNA sequences by the BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (81). We performed phylogenetic trees including the closest sequences identified in the BLAST analyses and the newly amplified sequences using Geneious 5.6.6, Biomatters (available from http://www.geneious.com) for sequence alignment and phylogenetic tree design. Final unrooted tree diagrams were drawn using the FigTree software (http://tree.bio.ed.ac.uk), and edited using the CorelDraw program (Corel Corporation, Ottawa, Ontario, Canada).

TABLE II.2 - MALASPINA SAMPLED STATIONS

Coordinates and collection data from four different oceanic stations sampled during Malaspina circumnavigation.

Sample Name	Station number	Location	Date (dd-mm-yy)	DCM (m)	MLD (m)	Collection local time (h)		
						3m	DCM	DCM+40
Atlantic	3	05 0.40 N. 26 1.59 W	30-12-10	120	76	8:16	10:35	10:35
Indian	30	29 40.27 S. 89 26.46 E	05-03-11	135	36	8:05	10:12	10:15
Pacific 1	46	18 4.30 N. 133 19.27 W	22-05-11	125	94	7:16	10:32	10:34
Pacific 2	52	9 26.42 N. 96 20.17 W	04-06-11	19	18	7:20	10:09	10:08

DCM: deep chlorophyll maximum depth; MLD: mixed layer depth

II.2.7. CHLOROPHYLL ANALYSES

Frozen filters were extracted in variable volumes (3 - 5 ml) of 95% acetone during 24 hours followed by sonication during 5 min at low temperature (4 °C). Extracts were then centrifuged at 4,000 rpm for 10 minutes to remove cell and filter debris. A 1.5-mL aliquot of the acetone extract was measured for chlorophyll using a UV-

spectrophotometer. We assumed that the absorbance at 665 nm was linearly correlated to the total amount of divinyl-chlorophyll *a* (82).

II.2.8. STATISTICAL TESTS

Statistical tests were performed using the SPSS 19 (SPSS Inc., Chicago, IL) package, with additional calculations performed using the R package (http://CRAN.R-project.org/). Normality of data distributions was checked by the Kolmogorov-Smirnov test. Differences on mRNA abundances at different sampling depths were tested by the ANOVA plus Tukey HSD post-hoc test. Effects on the *rbc*L and *psb*A gene expression or DNA abundance due to organic pollutant mixtures were tested by paired T-tests between treated and untreated cultures. A 3-way-ANOVA general linear model (GLM) was used to evaluate the differential effects of pollutants on cultures by comparison of the mean fold changes of the target genes of each treatment with respect to its control (mRNA $Copies_{Treatment}$ /$Copies_{Control}$). Three categorical predictors were used in the GLM to assess the variability on gene expression: Treatment (PAH mixture vs. OClP mixture), Strain (MED4 vs. MIT9313) and Time of exposure (0.5 h vs. 24 h). Significance levels were set to 0.05.

II.3. RESULTS

II.3.1. METHODOLOGICAL OPTIMIZATION

Sampling procedure

Comparison of filtration rates of PVDF, PTFE and PC filters under vacuum showed that only PVDF and PTFE filters maintained a filtration rate fast enough to filter 1 L of seawater in less than ten minutes, a time considered optimal to minimize changes on mRNA levels during sampling (graphs in Fig. AI.1, supporting information). In addition, PTFE filters were compatible with both nucleic acid stabilization reagents used in this work (RNAlater for RNA and DNA lysis buffer for DNA), and did not interfere with the corresponding RNA and DNA extraction methods. A typical RNA

extraction from 1 L of natural sea water yielded 100 to 3,000 ng of DNA/RNA (average 900 ng of DNA/RNA), an amount considered sufficient for the intended analyses. Whereas PVDF and PTFE showed similar (and fast) filtration rates (Fig. AI.1), PTFE filters showed better RNA extraction efficiency and cell retention (Figs. AI.2 and AI.3).

Specificity of designed primers

qRT-analysis of cDNA samples from axenic cultures of HL *Prochlorococcus* (MED4, MIT9515 and EQPAC1) and LL strains (MIT9313 and NATL2A) showed a strong specificity of the designed primers (Table AI.3). HL and LL strains amplified with the appropriate HL or LL primers showed Cp values at least 10 cycles lower than the values obtained with the non-appropriate LL or HL primers, which represents a 1,000 fold difference in amplification efficiency. Amplicons from MED4 and MIT9313 amplified with HL and LL primer pairs, respectively, were sequenced and the results corresponded to the expected *rbc*L, *psb*A and *rnp*B sequences when analysed by BLAST (Table II.3). LL *rnp*B primers showed detectable amplification products when challenged with *Synechococcus* templates with similar efficiency than LL *Prochlorococcus* templates (Table AI.3), whereas both LL *rbc*L and LL *psb*A primers gave Cp values more than 10 cycles higher for *Synechococcus* than for *Prochlorococcus* –again, a 1,000-fold lower efficiency. The LL *rbc*L amplification products of *Prochlorococcus* and *Synechococcus* differed in their melting temperature (Tm), which ranged from 81 °C to 86 °C for LL *Prochlorococcus*, and from 88 °C to 89 °C for *Synechococcus* strains (see Fig. AI.4 and Table AI.3 in Annex I), suggesting that this last amplicon corresponded to *Synechococcus*, although presenting a low-efficiency amplification. This characteristic can be used in field samples to detect the presence of *Synechococcus* or to evaluate sequence heterogeneity within a given sample. Fig. AI.4 illustrates this possibility, as it shows the Tm profile from a cDNA preparation that combined cDNA of *Prochlorococcus* strain MIT9313 and *Synechococcus* strain WH7803 at equal cell concentrations.

TABLE II.3 - BLAST ANALYSIS

Characterization by BLAST of sequenced amplicons obtained using the designed primers on total RNA samples from *Prochlorococcus* MED4 and MIT9313 pure cultures.

Primers	Specie/ Strain	Query coverage	Identity	Accession no.
MED4 (HL)				
*rnp*B-HL	*Pro*. MED4	98%	100%	BX548174.1
	Pro. PCC9511	98%	100%	AJ272223.1
*rbc*L-HL	*Pro*. MED4	100%	100%	BX548174.1
	Pro. MIT9215	100%	93%	CP000825.1
*psb*A-HL	*Pro*. MED4	100%	100%	BX548174.1
	Pro. MIT9515	100%	99%	AY599030.1
	Pro. MIT9312	100%	99%	AY599028.1
	Pro. MIT9116	100%	99%	AY599031.1
	Pro. MIT9301	100%	98%	CP000576.1
	Pro. MIT9302	100%	98%	AY599029.1
MIT9313 (LL)				
*rnp*B-LL	*Pro*. MIT9313	100%	99%	BX548175.1
	Pro. MIT9303	100%	98%	CP000554.1
	Syn. WH8102	100%	93%	BX569689.1
	Syn. WH7803	100%	91%	CT971583.1
	Syn. CC9311.	100%	91%	CP000435.1
*rbc*L-LL	*Pro*. MIT9313	100%	100%	BX548175.1
	Pro. MIT9303	100%	96%	CP000554.1
	Syn. PCC7009	95%	81%	AM701777.1
	Syn. CB0209	95%	81%	AY452729.1
*psb*A-LL	*Pro*. MIT9313	100%	99%	BX548175.1
	Pro. MIT9303	94%	98%	AY599035.1
	Syn. CC9902	100%	88%	CP000097.1
	Syn. WH8102	97%	88%	BX569693.1

Pro. *Prochlorococcus*; Syn.: *Synechococcus*.

II.3.2. APPLICABILITY OF THE DEVELOPED METHODOLOGY TO *PROCHLOROCOCCUS* NATURAL COMMUNITIES

Analysis of mRNA abundance in oceanic populations of *Prochlorococcus*

Analysis of *rbc*L, *psb*A and *rnp*B Cp values in samples from the vertical profiles of 62 oceanic stations showed the expected distribution of HL and LL sequences at the three sampled depths (Table II.4) (11, 18, 22). In 3 m depth samples, amplification

with HL primers occurred 5-10 cycles earlier than with LL primers. This indicates a concentration of the corresponding mRNAs 30 to 1,000 times higher, according to the reverse correlation between Cp values and the mRNA concentrations. The reverse occurs at both DCM and DCM+40, in which LL primers showed lower Cp values than HL primers (Table II.4). If we consider *rnp*B level as a direct indicator of the concentration of metabolically active cells, the data suggest that HL strains represent some 97% of the total *Prochlorococcus* cells at 3 m depth , a mere 6% at DCM and from 1 to 2% at DCM+40 (Table II.4). A characteristic of these data is their relatively low variation coefficient, less than 10% in most cases. If we consider that they correspond to samples from three oceans and collected in a period of seven months, this represents a remarkable homogeneity of *Prochlorococcus* populations across the globe.

Fig. II.2 shows the global distributions of relative abundances of *rbc*L and *psb*A, normalized by the reference gene *rnp*B, for the two clades and at the three sampled depths. The relative mRNA abundances of both HL genes decreased with the depth, being maximal at 3 m depth and minimal at DCM+40. On average, 3 m depth samples showed five times more HL *rbc*L, and three times more HL *psb*A mRNA copies than DCM+40 samples (Fig. II.2). LL genes showed the opposite tendency, although the effect was less clear. LL *rbc*L mRNA was about three times more abundant at DCM, or DCM+40, than at 3 m depth samples, whereas no significant differences were

TABLE II.4 - Cp VALUE ANALYSIS

Descriptive statistics of Cp values corresponding to the different primer pairs, stations, and depths sampled during Malaspina Circumnavigation.

	3 m			DCM			DCM+40		
	mean ± sd		n	mean ± sd		n	mean ± sd		n
*rnp*B-HL	20.98	± 2.34	61	22.65	± 2.73	61	26.17	± 2.57	60
*rbc*L-HL	21.09	± 2.35	61	24.05	± 2.80	61	28.71	± 2.33	60
*psb*A-HL	16.87	± 2.38	61	19.85	± 2.71	61	23.64	± 2.31	60
*rnp*B-LL	26.00	± 3.09	61	18.80	± 1.58	61	20.67	± 2.25	60
*rbc*L-LL	31.83	± 3.14	61	23.22	± 2.63	61	24.84	± 2.72	60
*psb*A-LL	25.07	± 3.85	61	17.38	± 2.51	61	20.02	± 2.85	60

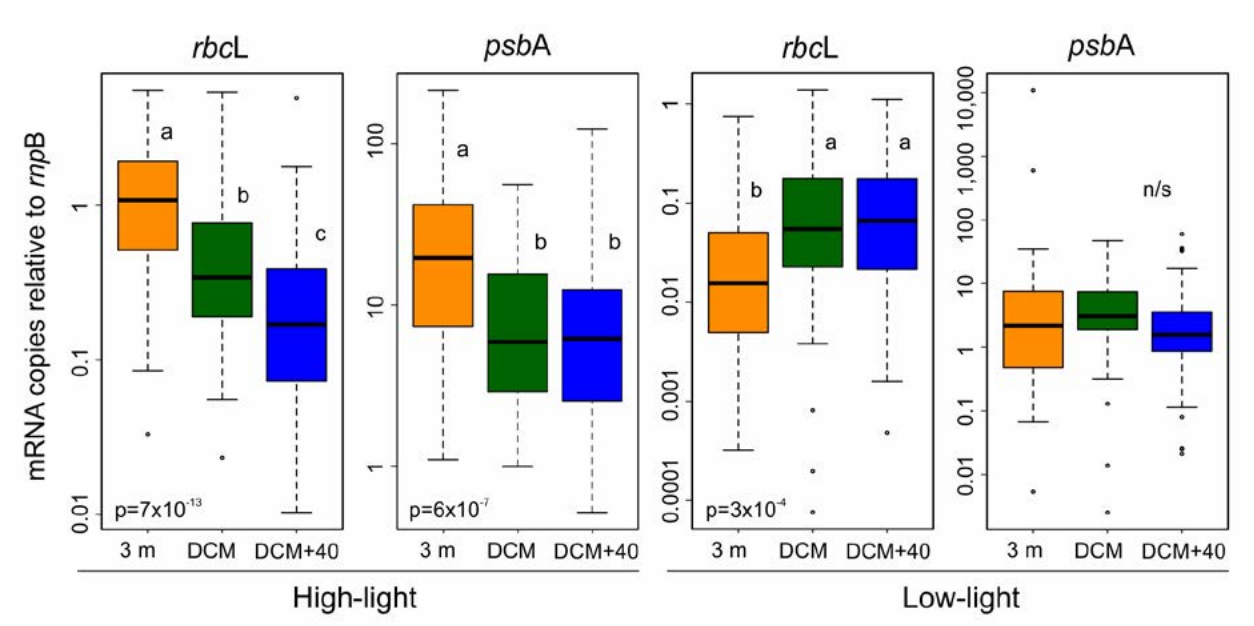


FIGURE II.2 - RELATIVE ABUNDANCES OF *rbc*L AND *psb*A

Relative expression of *rbc*L and *psb*A in surface (orange), DCM (green) and DCM+40 m (blue) samples for HL (left) and LL (right) clades. The boxes indicate data distribution parameters: range (whiskers), 25 to 75 percentiles (boxes) and median (thick horizontal bar). Circles represent outliers. Letters indicate statistically different distribution (ANOVA plus SD Tukey post hoc test); the corresponding p values are indicated at the bottom.

observed for LL *psb*A, in part due to the high dispersion of the values at 3 m depth (Table II.4).

Analysis of mRNA sequence heterogeneity in oceanic samples

A combined analysis of Tm values of the different amplicons and their corresponding Cp values was used to estimate the sequence heterogeneity (and hence, genetic variability) within each sample (Fig. II.3). *rnp*B and *psb*A HL amplicons showed an extreme homogeneity of Tm values for all 182 samples, irrespective of the depth or of their relative abundance (Fig. II.3). In fact, all samples showed essentially the same Tm value, 81 and 82 °C, respectively, within a margin of few tenths of °C (Table II.5). The situation was similar for HL *rbc*L amplicons, although some samples, particularly at DCM+40, showed clearly differentiated Tm values (Fig. II.3, central left panel). In this case, 13% of DCM+40 samples showed enough HL *rbc*L amplicon heterogeneity to be resolved in two Tm peaks, a phenomenon that was only episodic in samples from 3 m or DCM and unobserved with the other two HL amplicons at any depth (Table II.5). These atypical amplicons corresponded to samples with very low abundance in HL sequences – i.e., high Cp values (Fig. II.3). However, we consider that this Tm variability is still consistent with the natural variation of *Prochlorococcus* strains, as HL *rbc*L amplicons with a low Tm value were also observed for the cultured EQPAC1-C strain (marked as "EQ" in Fig. II.3, Table AI.3 in supplementary material).

LL amplicons showed much higher sequence heterogeneity than their HL counterparts (Table II.5, Fig. II.3, right panel). The corresponding graphs in Fig. II.3 show up to three distinct amplicon populations, particularly at 3 m depth. Also in this case, the highest variability corresponded to samples with low amplicon abundance (i.e., higher Cp values). We attributed a substantial fraction of this amplicon variability, at least at 3 m depth, to the presence of *Synechococcus*, as illustrated by the distribution of the LL *rbc*L amplicon in the central right panel of Fig. II.3. This particular panel shows a cluster of surface samples at high Cp values and high Tm values, similar to the ones observed for the cultured *Synechococcus* strain WH7803 (marked as WH in Fig. II.3, Table AI.3). However, the data also suggest the presence of some LL *Prochlorococcus* cells. Up to 26% of surface samples showed double Tm peaks for the LL *rbc*L amplicon, one of the peaks coinciding with the typical Tm values for

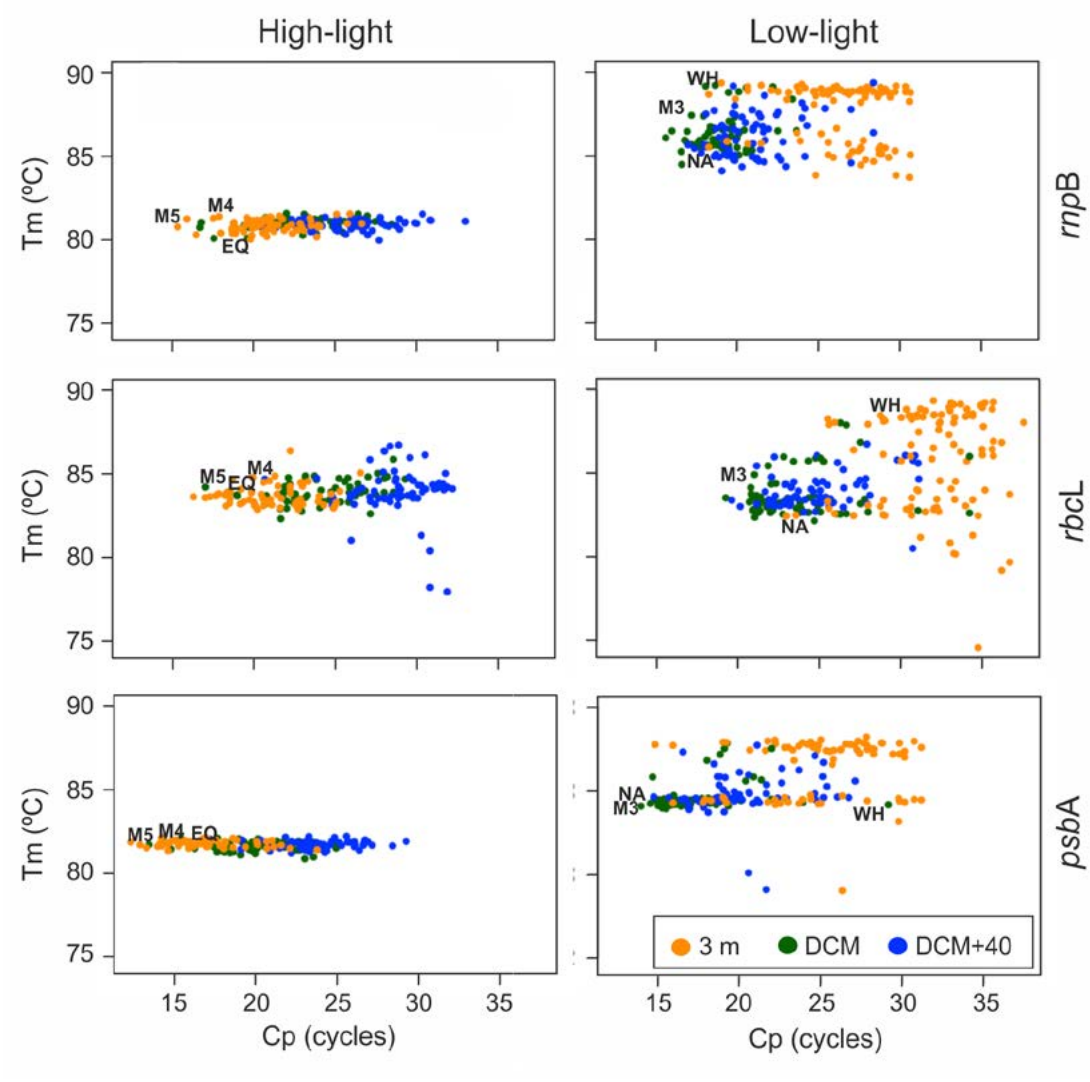


FIGURE II.3 - GENETIC VARIABILITY

Representation of amplicon melting temperature (Tm) values, in ^{o}C, plotted against Cp values (in cycles) obtained for field samples at the three sampled depths: Surface (orange), DCM (green) and DCM+40 (blue). For comparison, the graphs include the corresponding values from supplementary Table AI.3. HL amplicons: *Prochlorococcus* strains MED4 (M4), MIT9515 (M5) and EQPAC1-C (EQ). LL amplicons: *Prochlorococcus* strains MIT9313 (M3), NATL2A (NA), and the *Synechococcus* strain WH7803 (WH).

TABLE II.5 - TM VALUES ANALYSIS

Descriptive statistics of Tm values of amplicons from field samples.

	Cultured strains		**Field samples**									
	Tm values (ºC, x±sd)[a]				%Double peaks				% Non-average major peaks[b]			
Primers	*Pro.*	*Syn.*	Tm values (ºC, x±sd)	*n*	All	3 m	DCM	DCM+40	All	3 m	DCM	DCM+40
*rnp*B-HL	81.0 ± 0.3	81.3	80.9 ± 0.3	182	0.0	0.0	0.0	0.0	3.3	4.9	3.3	1.7
*rbc*L-HL	83.4 ± 2.4	84.4	83.8 ± 0.7	182	5.5	1.6	1.6	13.3	2.7	0.0	1.6	6.7
*psb*A-HL	81.7 ± 0.1	81.4	81.7 ± 0.2	182	0.0	0.0	0.0	0.0	3.8	0.0	8.2	3.3
*rnp*B-LL	87.5 ± 0.7	88.1	86.7 ± 1.5	182	0.5	1.6	0.0	0.0	0.0	0.0	0.0	0.0
*rbc*L-LL	83.2 ± 2.5	89.7	84.4 ± 2.0	182	8.8	26.2	0.0	0.0	7.7	23.0	0.0	0.0
*psb*A-LL	85.6 ± 2.3	82.4	85.3 ± 1.5	182	1.1	0.0	0.0	3.3	1.1	3.3	0.0	0.0

a) Separated values for each clade. Values from Table AI.2

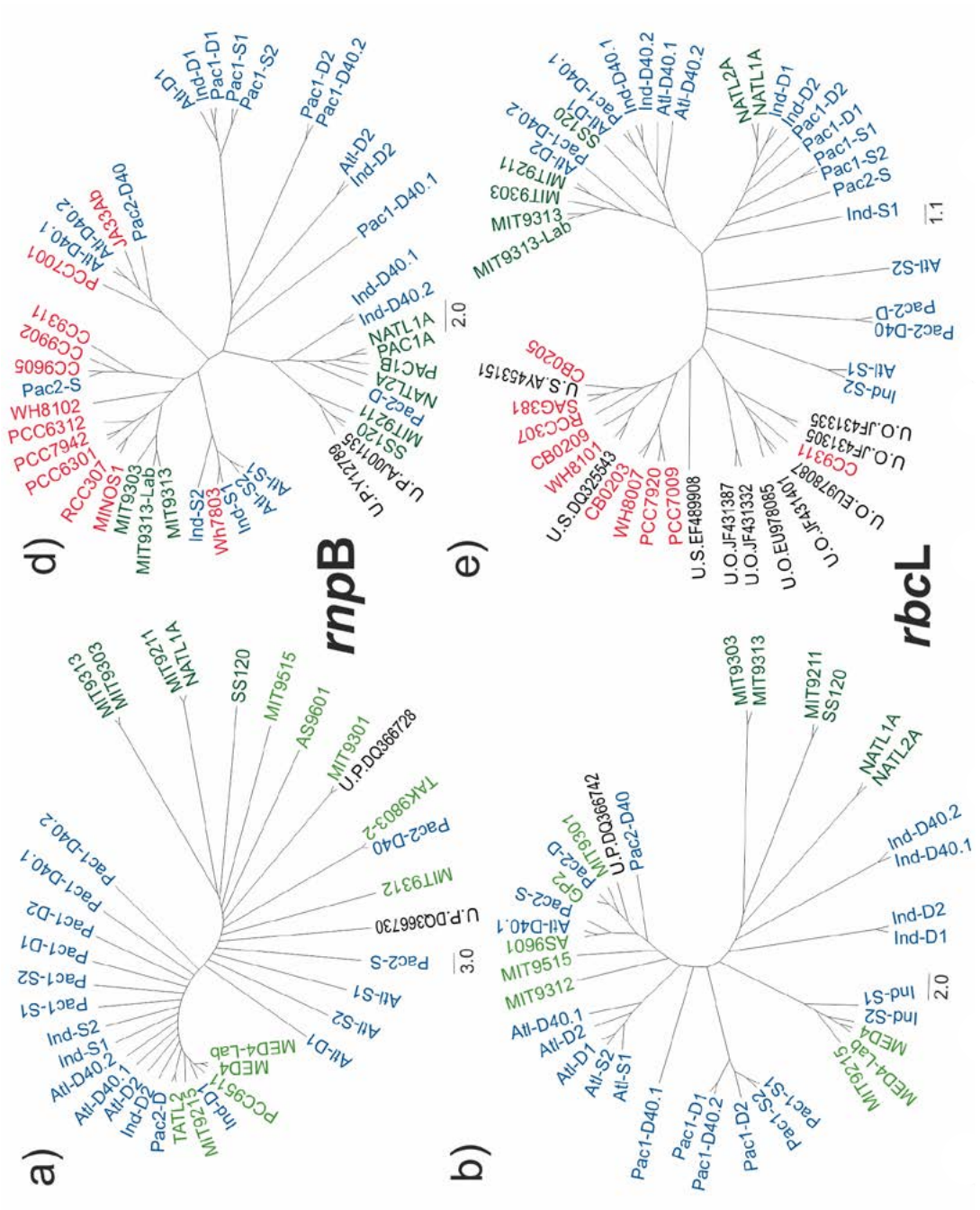
b) Percentage of major peaks statistically different (2*sd) from the corresponding average Tm values

Prochlorococcus (Table II.5). The other two LL genes do not allow distinction between the two genera, LL *Prochlorococcus* MIT9313 strain (M3 in Fig. II.3) shows amplicons with essentially the same Tm as their *Synechococcus* counterparts (Fig. II.3, Table AI.3 in Annex I).

Sequence analysis of amplicons from four different stations at the three sampled depths demonstrated that they all encoded the targeted genes (Fig. II.4 and supplementary Table AI.4). Sequences from HL amplicons were very similar in all cases to reported sequences from HL *Prochlorococcus* strains belonging to the eMED4 and eMIT9312 ecotypes (Table AI.4, supporting information). Similarly, amplicons from LL primers showed strong sequence similarities to LL *Prochlorococcus* strains included into the eMIT9313, eMIT9211, eNATL2A, and eSS120 ecotypes (20). However, ten out of the 21 sequenced LL amplicons from surface samples were identified as *Synechococcus* sequences (Table AI.4). This is keeping with the predominance of *Synechococcus* strains in conditions of high solar irradiance (10-12, 16-18, 22), and corroborates our interpretation of the amplicon Tm variability observed in Table II.5 and Fig. II.3.

The cladograms in Fig. II.4 compare the 126 sequenced amplicons with their closest sequences present in GenBank. For HL *rbc*L and *psb*A amplicons (left panels), field samples tend to form small clusters, loosely related to their geographical origin (Figs. II.4b and II.4c). The relative position of these clusters and bona fide HL *Prochlorococcus* sequences suggests that the variability observed among field samples is similar to the one observed in natural HL *Prochlorococcus* isolates. For example, Figs. II.4b and II.4c show most field sequences placed between MIT9301 and MED4. The same conclusion applies to HL *rnp*B sequences, despite the different topology of the corresponding cladogram (Fig. II.4a). Notice that sequences from LL *Prochlorococcus* strains (MIT9303, MIT9313, MIT9211, SS120, NATL1A and NATL2A) have been added to these HL cladograms as outgroups. They appear in separate branches in all three cladograms.

Cladograms for LL amplicons showed a clear distinction between *Synechococcus* and LL *Prochlorococcus* sequences for *rbc*L and *psb*A (Figs. II.4e and II.4f). Consistently with the BLAST analyses, most field samples clustered with known *Prochlorococcus* sequences, with the exception of some samples from 3 m depth, which appear


a)
rnpB
Alt-D1
Ind-D1
Pac1-D1
Pac1-S1
Pac1-S2
Pac1-D2
Pac1-D40.2
Alt-D2
Ind-D2
Pac1-D40.1
Ind-D40.1
Ind-D40.2
NATL1A
PAC1A
PAC1B
NATL2A
Pac2-D
MIT9211
SS120
U.P.AJ001135
U.P.Y12789
Alt-S1
Alt-S2
Ind-S1
Wh7803
Ind-S2
MIT9313
MIT9313-Lab
MIT9303
MINOS1
RCC307
PCC6301
PCC7942
PCC6312
WH8102
Pac2-S
CC9605
CC9902
CC9311
Pac2-D40
JA33AB
Alt-D40.2
Alt-D40.1
PCC7001
2.0
b)
rbcL
MIT9303
MIT9313
MIT9211
SS120
NATL1A
NATL2A
Ind-D40.2
Ind-D40.1
Ind-D2
Ind-D1
Ind-S1
Ind-S2
MED4
MED4-Lab
MIT9215
Pac1-S2
Pac1-S1
Pac1-D2
Pac1-D40.2
Pac1-D1
Pac1-D40.1
Alt-S1
Alt-S2
Alt-D1
Alt-D2
Alt-D40.1
MIT9312
MIT9515
AS9601
Alt-D40.1
Pac2-S
GP2
Pac2-D
MIT9301
U.P.DQ366742
Pac2-D40
2.0
c)
rpoC1
MIT9303
MIT9313
MIT9211
NATL1A
SS120
MIT9515
AS9601
MIT9301
U.P.DQ366728
TAK9803-2
Pac2-D40
MIT9312
U.P.DQ366730
Pac2-S
Alt-S1
Alt-S2
Alt-D1
MED4
MED4-Lab
PCC9511
Ind-D2
Ind-D1
MIT9215
TATL2
Pac2-D
Ind-D2
Alt-D2
Alt-D40.1
Ind-S1
Ind-S2
Pac1-S1
Pac1-S2
Pac1-D1
Pac1-D40.1
Pac1-D40.2
3.0
d)
rnpB
Alt-D1
Ind-D1
Pac1-D1
Pac1-S1
Pac1-S2
Pac1-D2
Pac1-D40.2
Alt-D2
Ind-D2
Pac1-D40.1
Ind-D40.1
Ind-D40.2
NATL1A
PAC1A
PAC1B
NATL2A
Pac2-D
MIT9211
SS120
U.P.AJ001135
U.P.Y12789
Alt-S1
Alt-S2
Ind-S1
Wh7803
Ind-S2
MIT9313
MIT9313-Lab
MIT9303
MINOS1
RCC307
PCC6301
PCC7942
PCC6312
WH8102
Pac2-S
CC9605
CC9902
CC9311
Pac2-D40
JA33AB
Alt-D40.2
Alt-D40.1
PCC7001
2.0
e)
rbcL
NATL2A
NATL1A
Ind-D1
Ind-D2
Pac1-D2
Pac1-D1
Pac1-S1
Pac1-S2
Pac2-S
Ind-S1
Alt-S2
Pac2-D
Pac2-D40
Alt-S1
Ind-S2
U.O.JF431335
U.O.JF431305
CC9311
U.O.EU978087
U.O.JF431401
U.O.EU978065
U.O.JF431387
U.O.JF431332
U.S.EF489908
PCC7007
PCC7920
WH8007
CB0203
U.S.DQ325543
CB0209
WH8101
RCC307
SAG381
U.S.AY453151
CB0205
MIT9313
MIT9303
MIT9313-Lab
MIT9211
MIT9215
Pac1-D40.1
Pac1-D40.2
Alt-D40.1
Alt-D40.2
Alt-S1
Alt-S2
Alt-D1
Alt-D2
Ind-D40.1
Ind-D40.2
1.1

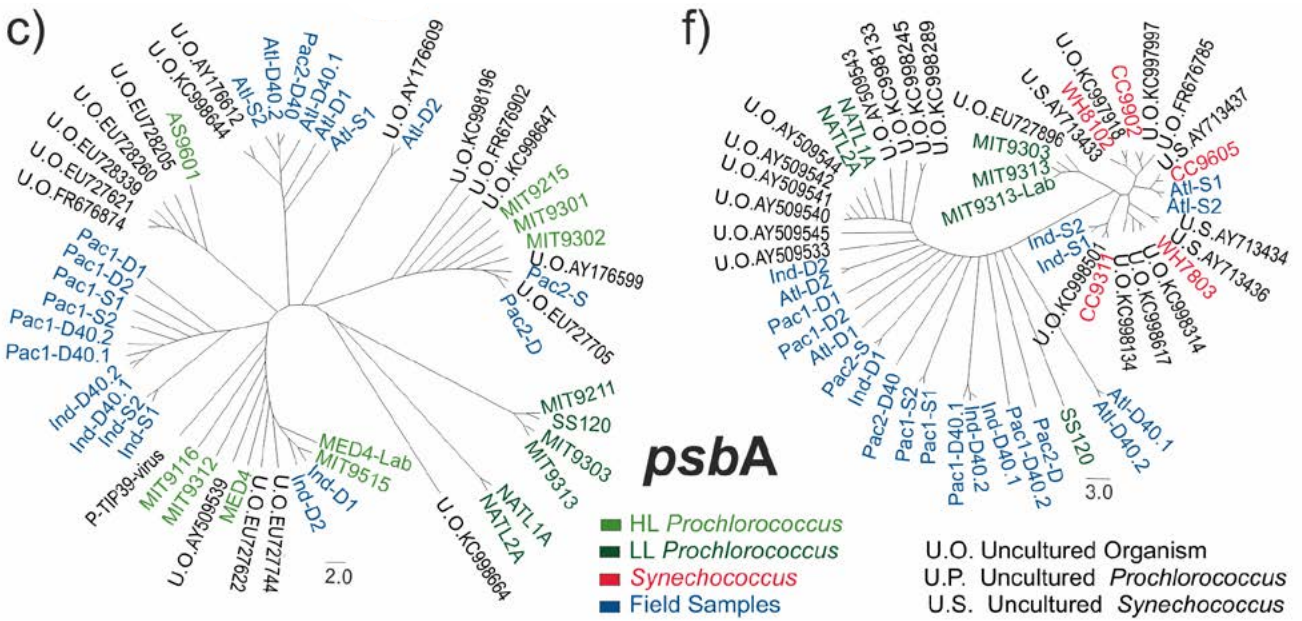


FIGURE II.4 - CLADOGRAMS

Cladograms of sequences from amplicons derived from field and laboratory samples compared to the closest sequences identified by BLAST. Two qRT-PCR products were sequenced for each sample from Atlantic, Indian and Pacific 1 stations, and only one for Pacific 2 samples (more information in supplementary Table AI.4). Amplicons from surface, DCM, and DCM+40 samples from the different stations are identified with “S”, “D” and “D40” letters. Panels a-c correspond to HL amplicons, whereas panels d-f correspond to LL amplicons. For each ecotype, cladograms corresponding to *rnp*B (a,d), *rbc*L (b,e) and *psb*A (c,f) genes are presented. The trees also include sequences from the GenBank. LL *Prochlorococcus*, HL *Prochlorococcus*, and *Synechococcus* (Syn.) sequences are indicated by dark green, light green and red colors, respectively. Amplicons from MIT9313 and MED4 strains grown in the lab were also sequenced and included in the analysis as MIT9313-Lab and MED4-Lab, respectively. Sequences for uncultured microorganism isolates are marked as U.P. (identified as *Prochlorococcus*), U.Syn. (identified as *Synechococcus*) or U.O, (not identified).

placed in *Synechococcus* branches. The topology of the LL *rnp*B cladogram differed from the other two, as it did not show a clear distinction between *Synechococcus* and *Prochlorococcus* sequences (Fig. II.4d). As a general conclusion, these cladograms indicate that all DCM and DCM+40 LL sequences corresponded to LL *Prochlorococcus* strains, whereas the results from 3 m samples showed a mixture of *Synechococcus* and LL *Prochlorococcus* sequences.

Effects of pollutant mixtures on *Prochlorococcus* gene expression

Exposure of experimental *Prochlorococcus* MED4 and MIT9313 cultures to either PAHs or OClP mixtures did not affect significantly growth rates or chlorophyll contents (Tables AI.5 and AI.6 in Annex I). However, treated cultures showed a decrease of the *rbc*L mRNA levels relative to control cultures, an effect not observed for *psb*A (Table II.6). Combining data for all treatments, *rbc*L mRNA levels were reduced by 20% relative to the reference gene, a moderate, but significant decrease (Table II.6). These data suggest that pollutants may alter the relative expression of photosynthetic

TABLE II.6 - CULTURE EXPERIMENT PAIRED T-TESTS

Mean results from paired t-tests between treatment and their respective controls ($\Delta Cp_{Treatment}$ vs. $\Delta Cp_{Control}$) and from the General Linear Model (GLM) performed with the fold changes (CopiesTreatment /CopiesControl), setting as fixed factors of GLM "Treatment" (PAH, OClP), "Strain" (MED4, MIT9313) and "Time" (0.5 h, 24 h) for each of the target genes and the ratio between them.

			***rbc*L**	***psb*A**	***rbc*L/*psb*A**
	Value/Factors	df		Mean ± SD[a]	
Paired T-test	Ratio (T/C)[b]	21	0.82 ± 0.41 **	1.09 ± 0.45	0.78 ± 0.29 ***
			F[a]		
GLM	Treatment	1	0.292	0.082	2.165
	Strain	1	0.083	0.410	0.338
	Time	1	0.727	0.137	4.762 *
	Treatment * Strain	1	0.130	0.110	0.006
	Treatment * Time	1	0.354	1.983	1.828
	Strain * Time	1	3.349	0.097	14.840 **
	Treatment * Strain * Time	1	0.163	0.039	1.153

a) *, p<0.05; **, p<0.01; ***, p<0.001

b) Treatment versus control

genes, an effect likely to be added to the influence of other natural effectors we observed in the natural oceanic populations sampled at different depths (Fig. II.2). The analysis of the influence of different parameters on the effects of pollutants in *rbc*L expression (3-way ANOVA) indicated "time of exposure" as the only single factor with significant between-subject effects, as well as an interaction between "time of exposure" and "strain" (Table II.6). These interactions can be visualized in Fig. II.5, which shows a decrease of the *rbc*L expression and of the *rbc*L/*psb*A ratio only after 24 hours of treatment for MIT9313, whereas for MED4 both values decreased during the first 0.5 h of exposure, recovering afterwards. While these results should be regarded only as indicative, they suggest that different *Prochlorococcus* strains may have different susceptibilities to organic pollutants, and that their temporal response to them may also differ.

None of these differences between treatments and controls were observed when DNA, rather than RNA, copies were evaluated (supplementary Table AI.7, compare to Table II.6), which confirms that the observed effects on mRNA levels were indeed reflecting changes in the expression of the target genes and not derived from gene alterations or cell abundances. We concluded that *Prochlorococcus* can be affected by relatively low concentrations of pollutants, and that *rbc*L expression and the *rbc*L/*psb*A ratio may be sensitive indicators of these effects even in conditions in which the overall growth and chlorophyll concentration remain unaffected.

II.4. DISCUSSION

The ubiquitous presence of *Prochlorococcus* in tropical and subtropical oceans makes it extremely important for oceanic and global change studies. However, any global study on this genus needs to overcome the striking genetic variability of *Prochlorococcus* strains. This is especially important when using DNA or RNA-based markers, for they require exact base sequences for adequate amplifications. Our strategy in designing qRT-PCR primers was based in three considerations. First, the targeted genes had to be representative of the main physiological/

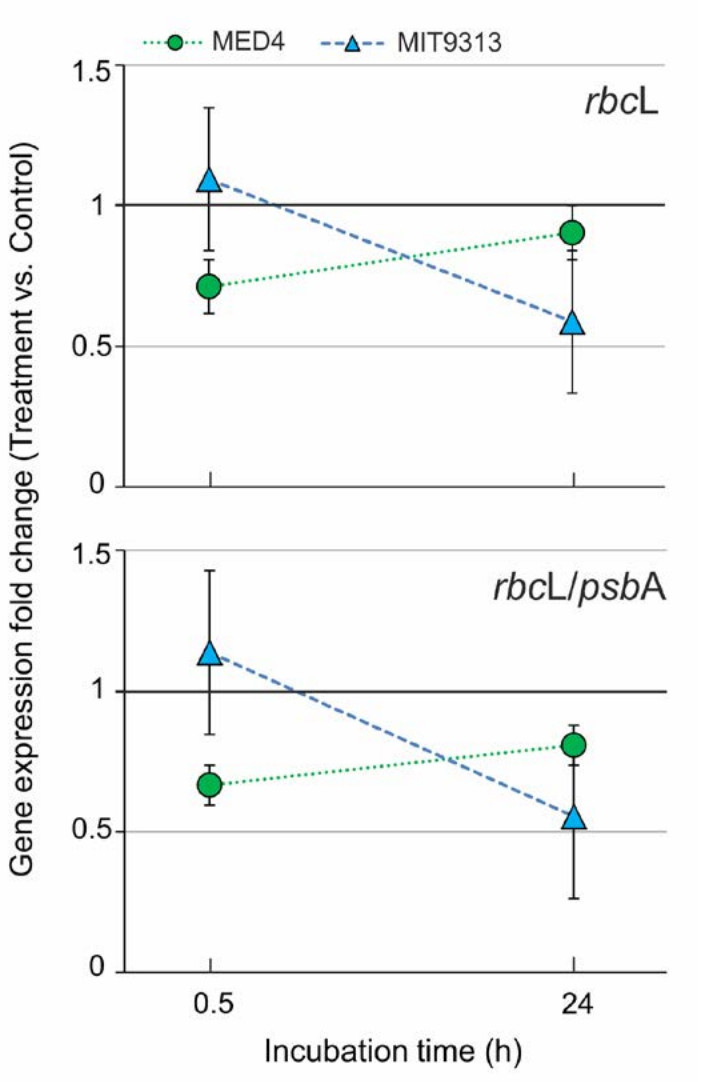


FIGURE II.5 - TEMPORAL RESPONSES TO POLLUTANTS

Graphic representation of the different temporal responses of MED4 (green circles) and MIT9313 (blue triangles) to pollutants. Results from PAH and OClP treatments are pooled together. Significant differences in mRNA abundance between treated and untreated cultures are marked with asterisks (*, $p<0.05$; **, $p<0.01$). Whiskers indicate 95% confidence limits.

ecological functions of *Prochlorococcus*. We selected photosystem functioning and carbon fixation, since these functions are intimately related to the major ecological function of *Prochlorococcus* as a main global primary producer, and because the photosynthetic machinery is at the core of the distinction between *Prochlorococcus* and other cyanobacteria. Secondly, we chose *psb*A and *rbc*L as representatives for PSII functionality and RuBisCO activity, respectively, as these two genes have been particularly well studied and sequenced for many cultured and non-cultured *Prochlorococcus* strains. Finally, we selected sequence regions that were particularly well conserved among *Prochlorococcus* strains and different from homologous regions in other cyanobacteria. However, the complexity of *Prochlorococcus* populations made it necessary to design two complete sets of primers targeting HL and LL ecotypes. Thus, we designed two sets of primers and tested them in real oceanic samples to analyse relative abundances and sequence heterogeneity. We tested the sensitivity of the developed markers in detecting physiological alterations induced by environmental pollutants in controlled experiments. Our ultimate goal was to generate a molecular tool applicable to natural communities to study the effects of global change vectors on the oceanic carbon cycle.

Unlike DNA, which is relatively stable and that can be found in cellular debris and other dead materials, RNA is easily degraded and only found in living cells. In addition, RNA synthesis (transcription) is a highly controlled process and usually reflects the environmental inputs on the cell metabolism. Therefore, analysis of RNA levels gives information not only of the presence of the cells, but also of their physiological status and metabolic activity. On the other hand, the very rapid turnover of mRNA in prokaryotes makes it difficult to obtain mRNA samples truly representative of the original (i.e., undisturbed) physiological state. To circumvent this problem, we limited manipulation times to an operational minimum and maximized the integrity of the collected RNA by the use of PTFE filters, relatively small volumes of water (1 L), and preserving filtered samples in RNAlater. This strategy allowed to obtain RNA preparations concentrated enough for qRT-PCR analyses and with no signs of RNA degradation.

As for most photosynthesis genes in *Prochlorococcus*, abundances of mRNA for *psb*A and *rbc*L exhibit significant periodicity over the light:dark photoperiod (44, 49).

*rbc*L mRNA peaks at sunrise, while *psb*A peaks at noon. Given these strong and not identical diel periodicities, we were careful to collect field and culture samples at the same time of day (morning). Consequently, *psb*A: *rnp*B, *rbc*L: *rnp*B, and/or *psb*A: *rbc*L ratio data from samples collected at other times of the day may thus not be directly comparable to our data, and will need to be interpreted with caution.

A major issue when analysing gene expression in *Prochlorococcus* in field communities is to differentiate their mRNA sequences from those from other cyanobacteria and, at the same time, to deal with the large genetic variability of the genus. The designed method allows quantification of mRNA levels of the selected genes belonging from a variety of *Prochlorococcus* strains, because the designed primers are general enough to amplify sequences of close related strains within the genus. As in previous works (28), *Synechococcus* cross-amplifications may occasionally occur, but it can be detected by using the differential Tm values of LL *rbc*L amplicons from both species. This improvement allows a fast and inexpensive method for the detection of cross-amplification that solves a major issue of the genetic similarity between these two genera, and simplifies the markers to just 6 pairs of primers instead of specific primers and/or probes for each strain (32-34, 37). This simplification is of great importance when using large sets of samples, as in our survey of samples from the Malaspina expedition.

More than 90% of the 126 amplicons sequenced from oceanic samples corresponded to *Prochlorococcus* strains, as judged by sequence homology. The only ten cases in which the amplified fragment encompassed *Synechococcus* sequences corresponded to 3 m depth samples amplified with LL primers, a combination that in almost all cases result in very high Cp values – that is, low specific mRNA levels. The analysis of Tm profiles of the amplified fragments showed that most samples presented single peaks with Tm values close to the expected *Prochlorococcus* values. Again, most exceptions belonged to surface samples amplified with LL primers, in which the appearance of Tm values corresponding to *Synechococcus* amplicons was very usual. Combining both types of results, we concluded that the designed primers amplified the expected *Prochlorococcus* sequences, with very little cross amplification, except for *Synechococcus* strains, found only in surface samples and only with LL primers.

Our results indicate variations in the expression of photosynthetic genes in response to external effectors. In natural HL populations, both *rbc*L and *psb*A showed their maximal relative mRNA levels in 3 m samples, where the conditions of light and oligotrophy were optimal, and minimal levels when they are most unfavourable – at DCM+40. The effect is not so clear for the LL clade, although the *rbc*L gene mRNA was significantly underrepresented in 3 m samples, an unfavourable environment for these strains. It is also noticeable the relationship between poor environmental conditions and sequence heterogeneity. In both clades, the minimal variability (at least in terms of Tm values, arguably a not particularly sensitive parameter) corresponded to the optimal growth conditions (3 m for HL, DCM for LL strains). If we interpret the reduction in photosynthetic gene expression as acclimatization to suboptimal light and/or temperature conditions, strains variability can similarly be interpreted as an adaptation phenomenon, in which different ecotypes may occupy specific niches in the water column.

On top of the natural variation in the relative gene expression of photosynthetic genes, the results from experimental exposures of *Prochlorococcus* to pollutant mixtures suggest that *rbc*L mRNA levels tend to decrease upon the presence of pollutants. This effect was observed in both the LL and HL cultured strains, which showed some differences on the timing and amplitude of the response. Our data also indicates that the ratio between *rbc*L and *psb*A mRNA levels is particularly sensitive to the presence of pollutants. A recent paper demonstrates that the ratio between RuBisCO (RbcL) and PSII (PsbA) molar ratio is strongly correlated to the capacity for electron transport away from PSII, suggesting a limitation of the electron transport rate by the RuBisCO to PSII ratio (40). This fact may determine the use of photosynthesis rate for carbon fixation or for ATP generation. While it is not possible to directly translate messenger levels to protein concentrations or activities, it is conceivable that the presence of pollutants may increase energy consumption for the cell (for example, for detoxification or extra metabolic activity), reducing its capacity to fix CO_2 and, therefore, the requirement for RuBisCO activity. Further experimental data is needed to test this hypothesis; however, we propose that both *rbc*L expression and the *rbc*L/*psb*A ratio may be useful indexes of the physiological status of the populations, likely responding to different environmental stressors.

We conclude that the proposed methodology allows not only quantifying the clade-specific photosynthetic potential of *Prochlorococcus* communities in the oceans, but also the assessment of changes in the gene expression due to environmental stressors, as light and/or nutrient conditions, and pollutants. The protocols for sample harvesting and preservation are adequate to ensure the integrity of RNA samples, and the qRT-PCR methodology allows processing of large number of samples at a reasonable cost. The developed methodology for quantifying the gene expression of the *rbc*L and *psb*A genes of *Prochlorococcus*, therefore, represents a first step for a future field and laboratory assessment of the different drivers and stressors affecting the photosynthetic function in *Prochlorococcus*, an essential issue for our understanding of the marine carbon cycle and its modulation in the current scenario of global change.

II.5. REFERENCES

1. Chavez FP, Messié M, & Pennington JT (2011) Marine Primary Production in Relation to Climate Variability and Change. Annual Review of Marine Science 3(1):227-260.
2. Li WK (1995) Composition of ultraphytoplankton in the central north Atlantic. Marine Ecology Progress Series 122(1-3):1-8.
3. Veldhuis MJW, Kraay GW, Van Bleijswijk JDL, & Baars MA (1997) Seasonal and spatial variability in phytoplankton biomass, productivity and growth in the northwestern Indian ocean: The southwest and northeast monsoon, 1992-1993. Deep-Sea Research Part I: Oceanographic Research Papers 44(3):425-449.
4. Goericke R & Welschmeyer NA (1993) The marine prochlorophyte *Prochlorococcus* contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. Deep-Sea Research Part I: Oceanographic Research Papers 40(11 /12):2283-2294.
5. Liu H, Nolla HA, & Campbell L (1997) *Prochlorococcus* growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. Aquatic Microbial Ecology 12(1):39-47.
6. Partensky F, Hess WR, & Vaulot D (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiology and Molecular Biology Reviews 63(1):106-127.
7. Bouman HA, et al. (2006) Oceanographic basis of the global surface distribution of *Prochlorococcus* ecotypes. Science 312(5775):918-921.
8. Campbell L, Liu H, Nolla HA, & Vaulot D (1997) Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean at Station ALOHA during the 1991-1994 ENSO event. Deep-Sea Research Part I: Oceanographic Research Papers 44(2):167-192.
9. Crosbie ND & Furnas MJ (2001) Abundance, distribution and flow-cytometric characterization of picophytoprokaryote populations in central (17°S) and southern (20°S) shelf waters of the Great Barrier Reef. Journal of Plankton Research 23(8):809-828.
10. Garczarek L, et al. (2007) High vertical and low horizontal diversity of *Prochlorococcus* ecotypes in the Mediterranean Sea in summer. FEMS Microbiol Ecol 60(2):189-206.
11. Johnson ZI, et al. (2006) Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science 311(5768):1737-1740.
12. Malmstrom RR, et al. (2010) Temporal dynamics of *Prochlorococcus* ecotypes in the Atlantic and Pacific oceans. ISME J 4(10):1252-1264.
13. Martiny AC, Kathuria S, & Berube PM (2009) Widespread metabolic potential for nitrite and nitrate assimilation among *Prochlorococcus* ecotypes. Proc Natl Acad Sci U S A 106(26):10787-10792.
14. Vaulot D, Marie D, Olson RJ, & Chisholm SW (1995) Growth of *Prochlorococcus*, a photosynthetic prokaryote, in the equatorial Pacific Ocean. Science 268(5216):1480-1482.

15. Durand MD, Olson RJ, & Chisholm SW (2001) Phytoplankton population dynamics at the Bermuda Atlantic Time-series station in the Sargasso Sea. Deep-Sea Research Part II: Topical Studies in Oceanography 48(8-9):1983-2003.

16. Moore LR, Rocap G, & Chisholm SW (1998) Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature 393(6684):464-467.

17. Moore LR & Chisholm SW (1999) Photophysiology of the marine cyanobacterium *Prochlorococcus*: Ecotypic differences among cultured isolates. Limnol. Oceanogr. 44(3 I):628-638.

18. West NJ & Scanlan DJ (1999) Niche-partitioning of *Prochlorococcus* populations in a stratified water column in the eastern North Atlantic Ocean? Appl Environ Microbiol 65(6):2585-2591.

19. Moore LR, Post AF, Rocap G, & Chisholm SW (2002) Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. Limnol. Oceanogr. 47(4):989-996.

20. Rocap G, Distel DL, Waterbury JB, & Chisholm SW (2002) Resolution of *Prochlorococcus* and *Synechococcus* Ecotypes by Using 16S-23S Ribosomal DNA Internal Transcribed Spacer Sequences. Appl Environ Microbiol 68(3):1180-1191.

21. Partensky F, La Roche J, Wyman K, & Falkowski PG (1997) The divinyl-chlorophyll a/b-protein complexes of two strains of the oxyphototrophic marine prokaryote *Prochlorococcus* - Characterization and response to changes in growth irradiance. Photosynthesis Research 51(3):209-222.

22. Zinser ER, et al. (2006) *Prochlorococcus* ecotype abundances in the North Atlantic Ocean as revealed by an improved quantitative PCR method. Appl Environ Microbiol 72(1):723-732.

23. Zeidner G, et al. (2003) Molecular diversity among marine picophytoplankton as revealed by *psb*A analyses. Environmental Microbiology 5(3):212-216.

24. West NJ, Lebaron P, Strutton PG, & Suzuki MT (2011) A novel clade of *Prochlorococcus* found in high nutrient low chlorophyll waters in the South and Equatorial Pacific Ocean. ISME J 5(6):933-944.

25. Malmstrom RR, et al. (2013) Ecology of uncultured *Prochlorococcus* clades revealed through single-cell genomics and biogeographic analysis. ISME Journal 7(1):184-198.

26. Huang S, et al. (2012) Novel lineages of *Prochlorococcus* and *Synechococcus* in the global oceans. ISME J 6(2):285-297.

27. Rusch DB, Martiny AC, Dupont CL, Halpern AL, & Venter JC (2010) Characterization of *Prochlorococcus* clades from iron-depleted oceanic regions. Proceedings of the National Academy of Sciences of the United States of America 107(37):16184-16189.

28. Martiny AC, Tai AP, Veneziano D, Primeau F, & Chisholm SW (2009) Taxonomic resolution, ecotypes and the biogeography of *Prochlorococcus*. Environ Microbiol 11(4):823-832.

29. Steglich C, Mullineaux CW, Teuchner K, Hess WR, & Lokstein H (2003) Photophysical properties of *Prochlorococcus* marinus SS120 divinyl chlorophylls and phycoerythrin in vitro and in vivo. FEBS Letters 553(1-2):79-84.

30. Ahlgren NA, Rocap G, & Chisholm SW (2006) Measurement of *Prochlorococcus* ecotypes using real-time polymerase chain reaction reveals different abundances of genotypes with similar light physiologies. Environ Microbiol 8(3):441-454.

31. West NJ, et al. (2001) Closely related *Prochlorococcus* genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides. Microbiology 147(7):1731-1744.

32. Berg GM, et al. (2011) Responses of *psb*A, *hli* and *ptox* genes to changes in irradiance in marine *Synechococcus* and *Prochlorococcus*. Aquatic Microbial Ecology 65(1):1-14.

33. Bibby TS, Mary I, Nield J, Partensky F, & Barber J (2003) Low-light-adapted *Prochlorococcus* species possess specific antennae for each photosystem. Nature 424(6952):1051-1054.

34. Coleman ML & Chisholm SW (2010) Ecosystem-specific selection pressures revealed through comparative population genomics. Proceedings of the National Academy of Sciences of the United States of America 107(43):18634-18639.

35. Gómez-Baena G, et al. (2008) Glucose uptake and its effect on gene expression in *Prochlorococcus*. PLoS ONE 3(10).

36. Kolowrat C, et al. (2010) Ultraviolet stress delays chromosome replication in light/dark synchronized cells of the marine cyanobacterium *Prochlorococcus* marinus PCC9511. BMC Microbiol 10:204.

37. Rangel OA, Gomez-Baena G, Lopez-Lozano A, Diez J, & Garcia-Fernandez JM (2009) Physiological role and regulation of glutamate dehydrogenase in *Prochlorococcus* sp. strain MIT9313. Environ Microbiol Rep 1(1):56-64.

38. John DE, Patterson SS, & Paul JH (2007) Phytoplankton-group specific quantitative polymerase chain reaction assays for RuBisCO mRNA transcripts in seawater. Mar Biotechnol (NY) 9(6):747-759.

39. John DE, et al. (2007) Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea CO(2) flux in the Mississippi River plume. ISME J 1(6):517-531.
40. Zorz JK, et al. (2015) The RUBISCO to photosystem ii ratio limits the maximum photosynthetic rate in picocyanobacteria. Life 5(1):403-417.
41. Pichard SL, Campbell L, Kang JB, Tabita FR, & Paul JH (1996) Regulation of ribulose bisphosphate carboxylase gene expression in natural phytoplankton communities. I. Diel rhythms. Marine Ecology Progress Series 139(1-3):257-265.
42. Pichard SL, et al. (1997) Analysis of ribulose bisphosphate carboxylase gene expression in natural phytoplankton communities by group-specific gene probing. Marine Ecology Progress Series 149(1-3):239-253.
43. Bruyant F, et al. (2005) Diel variations in the photosynthetic parameters of Prochlorococcus strain PCC 9511: Combined effects of light and cell cycle. Limnol. Oceanogr. 50(3):850-863.
44. Zinser ER, et al. (2009) Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, *Prochlorococcus*. PLoS ONE 4(4).
45. Tolonen AC, et al. (2006) Global gene expression of *Prochlorococcus* ecotypes in response to changes in nitrogen availability. Mol Syst Biol 2:53.
46. Mulo P, Sirpiö S, Suorsa M, & Aro E-M (2008) Auxiliary proteins involved in the assembly and sustenance of photosystem II. Photosynthesis research 98(1-3):489-501.
47. Clarke AK, Campbell D, Gustafsson P, & Oquist G (1995) Dynamic responses of Photosystem II and phycobilisomes to changing light in the cyanobacterium *Synechococcus* sp. PCC 7942. Planta 197(3):553-562.
48. Garczarek L, et al. (2008) Function and evolution of the *psb*A gene family in marine *Synechococcus*: *Synechococcus* sp. WH7803 as a case study. ISME Journal 2(9):937-953.
49. Garczarek L, et al. (2001) Differential expression of antenna and core genes in *Prochlorococcus* PCC 9511 (Oxyphotobacteria) grown under a modulated light-dark cycle. Environmental Microbiology 3(3):168-175.
50. García-Fernández JM, et al. (1998) Expression of the *psb*A gene in the marine oxyphotobacteria *Prochlorococcus* spp. Archives of Biochemistry and Biophysics 359(1):17-23.
51. Schon A (2002) Conserved and variable domains within divergent RNase P RNA gene sequences of *Prochlorococcus* strains. International Journal of Systematic and Evolutionary Microbiology 52(4):1383-1389.
52. Martiny AC, Coleman ML, & Chisholm SW (2006) Phosphate acquisition genes in *Prochlorococcus* ecotypes: evidence for genome-wide adaptation. Proc Natl Acad Sci U S A 103(33):12552-12557.
53. Gomez-Baena G, Rangel OA, Lopez-Lozano A, Garcia-Fernandez JM, & Diez J (2009) Stress responses in *Prochlorococcus* MIT9313 vs. SS120 involve differential expression of genes encoding proteases ClpP, FtsH and Lon. Res Microbiol 160(8):567-575.
54. Osburne MS, et al. (2010) UV hyper-resistance in *Prochlorococcus* MED4 results from a single base pair deletion just upstream of an operon encoding nudix hydrolase and photolyase. Environ Microbiol 12(7):1978-1988.
55. Steglich C, Futschik M, Rector T, Steen R, & Chisholm SW (2006) Genome-wide analysis of light sensing in *Prochlorococcus*. J Bacteriol 188(22):7796-7806.
56. Jurado E & Dachs J (2008) Seasonality in the "grasshopping" and atmospheric residence times of persistent organic pollutants over the oceans. Geophysical Research Letters 35(17).
57. Jurado E, et al. (2005) Wet deposition of persistent organic pollutants to the global oceans. Environ Sci Technol 39(8):2426-2435.
58. Lohmann R, Breivik K, Dachs J, & Muir D (2007) Global fate of POPs: current and future research directions. Environ Pollut 150(1):150-165.
59. Berrojalbiz N, et al. (2011) Biogeochemical and physical controls on concentrations of polycyclic aromatic hydrocarbons in water and plankton of the Mediterranean and Black Seas. Global Biogeochemical Cycles 25(4):n/a-n/a.
60. Galbán-Malagón C, Berrojalbiz N, Ojeda MJ, & Dachs J (2012) The oceanic biological pump modulates the atmospheric transport of persistent organic pollutants to the Arctic. Nature communications 3:862.
61. Galbán-Malagón CJ, et al. (2013) Polychlorinated biphenyls, hexachlorocyclohexanes and hexachlorobenzene in seawater and phytoplankton from the Southern Ocean (Weddell, South Scotia, and Bellingshausen Seas). Environ Sci Technol 47(11):5578-5587.
62. Frouin H, et al. (2013) Partitioning and bioaccumulation of pcbs and pbdes in marine plankton from the strait of Georgia, British Columbia, Canada. Progress in Oceanography 115:65-75.
63. Wallberg P & Andersson A (1999) Determination of adsorbed and absorbed polychlorinated biphenyls (PCBs) in seawater microorganisms. Marine Chemistry 64(4):287-299.

64. Del Vento S & Dachs J (2002) Prediction of uptake dynamics of persistent organic pollutants by bacteria and phytoplankton. Environmental Toxicology and Chemistry 21(10):2099-2107.

65. Echeveste P, Agusti S, & Dachs J (2010) Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations. Environ Pollut 158(1):299-307.

66. Echeveste P, Dachs J, Berrojalbiz N, & Agusti S (2010) Decrease in the abundance and viability of oceanic phytoplankton due to trace levels of complex mixtures of organic pollutants. Chemosphere 81(2):161-168.

67. Steglich C, et al. (2010) Short RNA half-lives in the slow-growing marine cyanobacterium *Prochlorococcus*. Genome Biol 11(5):R54.

68. Kelly L, Huang KH, Ding H, & Chisholm SW (2012) ProPortal: a resource for integrated systems biology of *Prochlorococcus* and its phage. Nucleic Acids Res 40(Database issue):D632-640.

69. Morris JJ, et al. (2011) Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface. PLoS ONE 6(2).

70. Mella-Flores D, et al. (2011) Is the distribution of *Prochlorococcus* and *Synechococcus* ecotypes in the Mediterranean Sea affected by global warming? Biogeosciences 8(9):2785-2804.

71. Zwirglmaier K, et al. (2008) Global phylogeography of marine *Synechococcus* and *Prochlorococcus* reveals a distinct partitioning of lineages among oceanic biomes. Environ Microbiol 10(1):147-161.

72. Claustre H, et al. (2002) Diel variations in *Prochlorococcus* optical properties. Limnol. Oceanogr. 47(6):1637-1647.

73. Rocap G, et al. (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature 424(6952):1042-1047.

74. UNECE (1998) The 1998 Aarhus Protocol on Persistent Organic Pollutants (POPs). Protocol to the 1979 Convention on Long-Range Transboundary Air Pollution on Persistent Organic Pollutants. (United Nations Economic Commission for Europe).

75. Berrojalbiz N, et al. (2011) Persistent organic pollutants in Mediterranean seawater and processes affecting their accumulation in plankton. Environ Sci Technol 45(10):4315-4322.

76. US Environmental Protection Agency. USEPA (http://www.epa.gov.

77. Chisholm SW, et al. (1988) A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334(6180):340-343.

78. Lindell D, Jaffe JD, Johnson ZI, Church GM, & Chisholm SW (2005) Photosynthesis genes in marine viruses yield proteins during host infection. Nature 438(7064):86-89.

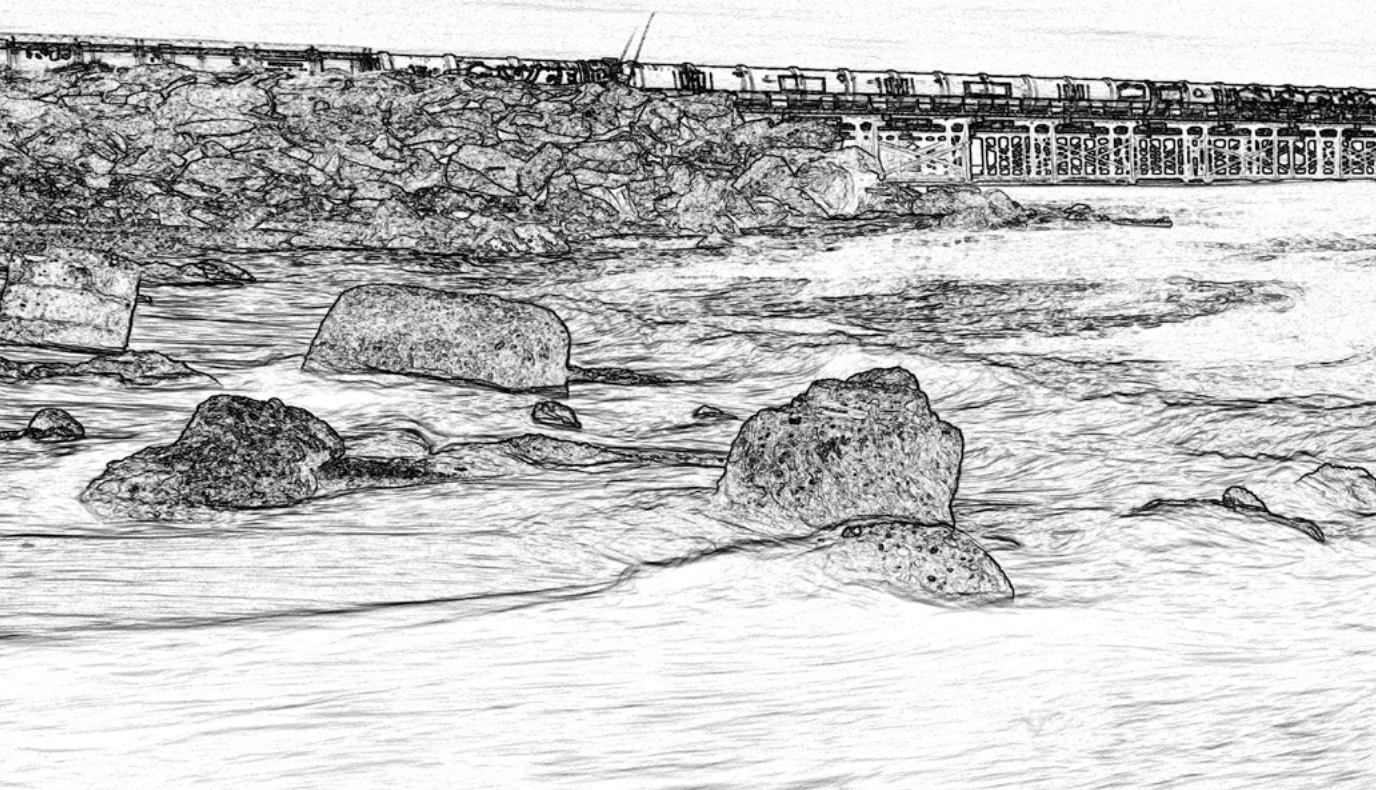
79. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9).

80. Anonymous (2007) Real-time PCR (Taylor & Francis).

81. Altschul SF, Gish W, Miller W, Myers EW, & Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215(3):403-410.

82. Jeffrey SW & Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher-plants, algae and natural phytoplankton. Biochemie Und Physiologie Der Pflanzen 167(2):191-194.

Chapter III



Dysregulation of photosynthetic genes in oceanic *Prochlorococcus* populations exposed to organic pollutants

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ABSTRACT

Persistent organic pollutants (POPs) constitute a major vector of global change, but their impact on ecosystems has not been adequately evaluated yet. To monitor their effects in oceanic populations of the cyanobacterium *Prochlorococcus*, the most abundant known photosynthetic organism on Earth, we quantified the expression of two photosynthetic genes, *rbc*L (RuBisCO large subunit) and *psb*A (PSII D1protein) in water samples from the Atlantic, Indian and Pacific Oceans. On-deck maintained *Prochlorococcus* populations showed the expected diel cycle variations on expression of photosynthetic genes. In addition, exposure to POP complex mixtures at concentrations only 2-fold above the environmental levels resulted in a decrease of expression of both genes, suggesting an effect on the photosynthetic function. While POP effects on marine phytoplankton have been already demonstrated at the cellular level, this is the first report showing alterations of a global ecosystem function by complex mixtures of anthropogenic organic pollutants at the molecular level.

III.1. INTRODUCTION

Persistent organic pollutants (POPs) reach the global oceans by long-range atmospheric transport and deposition (1-3). Once in the water column, POPs accumulate in planktonic organisms due to their hydrophobicity, thus entering into the food web (1, 4-6). Concentrations of hydrophobic organic pollutants, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), are 10^4 to 10^7 times higher in phytoplankton than in seawater (7-10). It is known that the accumulation of POPs in phytoplankton is a key biogeochemical control on the POPs occurrence and sinks in the marine environment (6, 11-13). However, the potential effects of POPs on the major processes driving the carbon cycle are still uncharacterised. Recently, several studies have showed that mixtures of POPs can exert a toxic effect on marine phytoplankton, reducing their abundance and viability at concentrations one order of magnitude higher than those found in oceanic waters (14-17). Nevertheless, the possible effects of POPs, at environmental relevant concentrations on phytoplankton ecological functions, such as photosynthesis, remain unexplored. Phytoplankton is responsible for as much as 50% of worldwide inorganic carbon fixation through photosynthesis (18, 19) and any perturbation of this activity would have an important impact on the carbon cycle. This potential coupling of occurrence and cycling of POPs in the oceans and the oceanic photosynthesis would help determinate the effects of chemical pollution at a global scale. This constitutes an already identified, but still unquantified, vector of global change (20).

The cyanobacterium genera *Prochlorococcus* and *Synechococcus* are responsible of 32-80% of net primary productivity (NPP) in oligotrophic oceans (21-23). *Prochlorococcus* dominates phytoplankton communities under highly oligotrophic conditions (as the central oceanic gyres) and stratified waters, which makes them the most abundant photosynthetic organism known, ubiquitous in all oceans in the latitude band from 40 °S to 40 °N, and in the water column from surface to 200 m depth, presenting abundances of 10^5 cells/mL (24-28). *Prochlorococcus* is classified into two genetic and physiologically different groups. The high-light (HL) clade comprises strains adapted to high light intensities, usually distributed in the first 100 m depth, whereas the low-light (LL) clade consists of strains adapted to low light intensities and usually found between 80 m and 200 m depth (29-33).

Prochlorococcus has been described as a particularly sensitive organism to organic pollutants and to other environmental stressors such as UV radiation, hydroxyl radicals, or copper, at the physiological level (14, 15, 34-36). This is likely related to its tiny size and spherical shape, which confer it a large surface/volume ratio and therefore a higher exposure to pollutants and a faster bioconcentration kinetics (7).

Toxic effects of both simple and complex organic pollutant mixtures have been reported at cellular level, appearing complex mixtures more toxic than simple mixtures of their analysed components. These effects include decreased cell abundance and growth of *Prochlorococcus*, as well as a reduction on chlorophyll *a* concentrations (15, 17). The recent development of a quantitative method to assess the expression of the photosynthetic genes *rbc*L (large subunit of RuBisCO) and *psb*A (D1 protein) of *Prochlorococcus* allowed identifying a specific perturbation of these genes in *Prochlorococcus* axenic cultures when exposed to simple mixtures of polycyclic aromatic hydrocarbons (PAHs) or organochlorine pesticides (OClPs) at sublethal levels (37). It remains unknown whether or not complex mixtures of POPs at the ultra-trace levels found in the ocean could exert such an influence on natural *Prochlorococcus* populations. Not all the organic pollutants present in seawater are known, nor can they be adequately quantified (38). It is now becoming evident that the known compounds only explain a small fraction of the toxicity of fresh – and sea-waters. For example, only 1% of the observed effects produced by organic pollutants in an environmental sample can be explained by the known and detected compounds (39) or less than 1% of the oxidative stress measured in continental waters was due to known compounds (40).

Our working hypothesis is that the ubiquitous complex mixture of organic pollutants found in oceans may represent an environmental impact for oceanic organisms, such as *Prochlorococcus*, and therefore affect photosynthesis as their main ecological function. Therefore, the objectives were to analyse the effects of both simple and complex mixtures of POPs found in ocean at environmental relevant levels on the photosynthetic capacity of *Prochlorococcus*. This is accomplished by measuring the expression of two photosynthetic genes in natural communities of *Prochlorococcus* from the Atlantic, Pacific and Indian Oceans (Fig. III.1) after exposure

to POP mixtures. A total of 13 experiments were performed assessing the effects of PAHs, OClPs and a complex mixture of organic pollutants.

III.2. MATERIAL AND METHODS

III.2.1. EXPERIMENTS WITH NATURAL COMMUNITIES

During the Malaspina circumnavigation cruise, from December 14, 2010 to July 14, 2011 on board the R/V Hesperides, we performed a total of 13 experiments in different locations of the Indian, Pacific and Atlantic Oceans (Fig. III.1 and Table AII.1). Experiments were carried out by challenging natural populations from the deep chlorophyll maximum (DCM) with three different mixtures of POPs (see below).

All the experiments started between 10 and 12:30 h local time, coinciding with the peak of carbon fixation by *Prochlorococcus*, which occurs between dawn and midday (41, 42). The incubations were performed in baked 1-L glass bottles. The pollutant mixtures were added to the treatment bottles and the solvent (acetone) to the control bottles 1 hour before adding the seawater to allow for evaporation and avoid potential toxic effects of the solvents used to prepare the POP mixtures. One litre of seawater from DCM was added, and the bottles immediately placed in an incubator located on the deck of the vessel that maintained the DCM temperature. The sunlight radiation at the sampled depth was simulated using a net covering the bottles. Each experiment consisted of 4 different exposure times: 0.5 h, 2 h, 6 h and 24 h, using one pair of treatment and control for each time point. We collected samples to study *Prochlorococcus* gene expression at every exposure time following the protocol described below. Additional samples were taken at 0.5 h and 24 h to analyse the concentration of dissolved-phase POPs, and picoplankton cell abundance by flow cytometry.

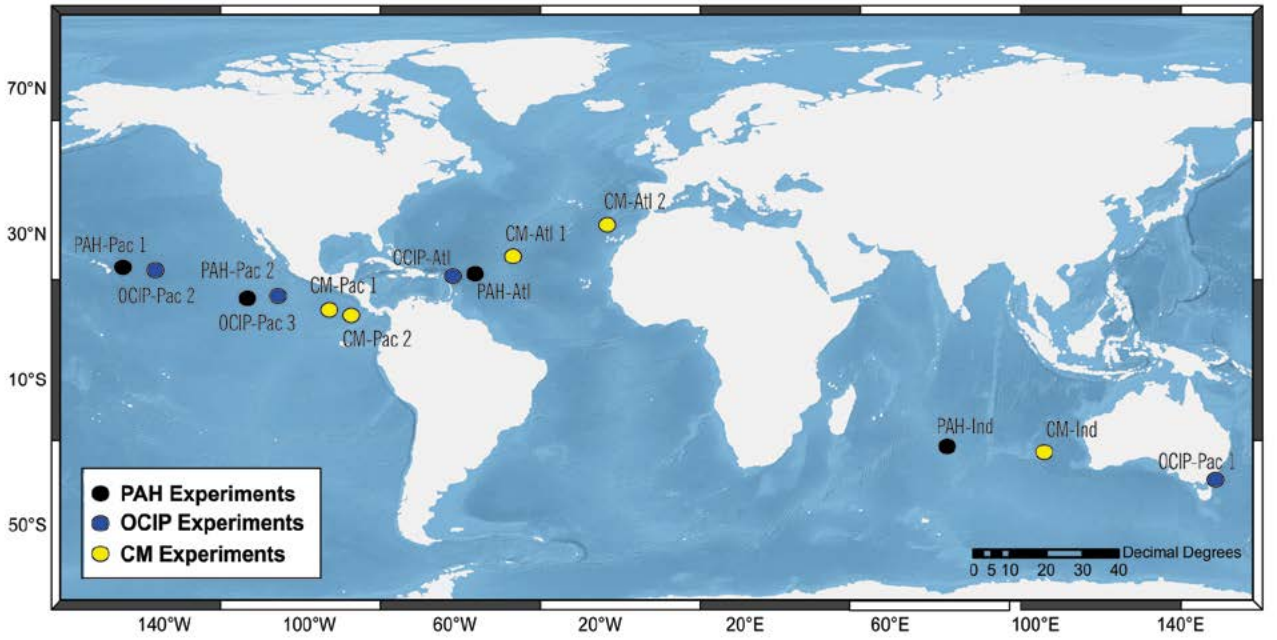


FIGURE III.1 - LOCATION OF THE EXPERIMENTS

During Malaspina circumnavigation on board of R/V Hesperides, natural communities from DCM were challenged with the three different pollutant mixtures, Polycyclic aromatic hydrocarbons (PAHs, black), organochlorine pesticides (OClP, blue), and complex chemical mixtures (CM,yellow).

III.2.2. PREPARATION OF POLLUTANT SPIKE SOLUTIONS

We used two simple mixtures of pollutants obtained from pure standards, and a complex mixture of pollutants directly obtained from seawater. The first simple mixture contained the 16 polycyclic aromatic hydrocarbons (PAHs) regulated by the US Environmental Protection Agency (EPA) (acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i) perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene and pyrene) spiked at 700 ng/L, the approximated concentration estimated to reduce growth of natural populations of *Prochlorococcus* by 10% (LC10) (15). No equivalent toxicity data exist for the second simple mixture containing organochlorine pesticides (OClP), so it was spiked at a nominal concentration of 500 ng/L, that is known to have no effect on *Prochlorococcus* growth rate (37). The OClPs were, hexachlorobenzene (HCB) and α, β, δ and γ isomers of hexaclorocyclohexane (HCH). Both PAHs and OClPs are ubiquitously found in oceanic waters and plankton (1, 5, 43).

The third pollutant spike solution was a seawater extract containing every organic pollutant found in the surface ocean (4 m depth) at a nominal concentration of 10-fold their surface concentrations. It will be referred to as the complex mixture (CM). Briefly, the CM was obtained by sampling oceanic seawater, in a previous campaign in the oligotrophic NE Atlantic, as usually performed for POP analysis (5, 43). The 200 L of seawater concentrated on a XAD-2 adsorbent were eluted, concentrated and fractionated on an alumina column to separate the polar and non-polar compounds. The latter fraction contains most of the chemicals with properties typical of POPs, such as PCBs, PAHs and other hydrocarbons, OClPs, polybrominated diphenyl ethers (PBDEs), polychlorinated dibenzo-p-dioxins and dibenzofurans, dechlorane plus, chlorinated naphthalene, among other POPs that have been previously described in the marine environment (38). These chemical mixtures also contain unknown POPs which have not been described yet in the literature due to lack of appropriate analytical methods (44), and which can contribute significantly to the overall CM toxicity as found in other studies (39, 45). The concentrations in the CM of PAHs, PCBs and OClPs were measured prior to their use in the experiments, using the

methods described elsewhere (4, 43), and the amount of each contaminant added to the experimental samples is reported in Table AII.2.

III.2.3. ANALYSIS OF DISSOLVED PHASE CONCENTRATIONS OF ORGANIC POLLUTANTS

After sample collection for gene expression analyses by filtering onto a 0.2-µm pore-size filter, we added to the filtrated water a surrogate standard mix containing five deuterated PAHs (naphtalene-d10, acenaphthene-d10, phenanthrene-d10, chrysene-d12 and perylene-d12), one n-alkane (tetracosane-d50) and two PCBs (PCB 65 and PCB 200). The filtrated water was then pre-concentrated in a solid-phase extraction 6 cc Oasis HLB cartridge (Waters, Montevideo, Uruguay) containing 500 mg of sorbent, using a vacuum manifold. The SPE cartridges had been previously conditioned with 5 mL of hexane followed by 5 mL of dichloromethane/hexane (2:1), 5 mL of dichloromethane/methanol (2:1) and 5 mL of HPLC-grade water. The Oasis cartridges were eluted with 5 mL of hexane, 10 mL of dichloromethane/hexane (2:1), and 5 mL of dichloromethane/methanol (2:1). Any aqueous residual in the extract was purified on a glass funnel filled with 50-60 g of anhydrous sodium sulfate. The extract was concentrated to 0.5-1 mL by vacuum rotary evaporation, transferred to a 1.5 mL amber vial, and evaporated to 50-100 µL under a gentle nitrogen stream. We analysed a number of model organic pollutants in the water from the experiments performed with CM: PAHs, n-alkanes, organochlorine pesticides (HCHs and HCB) and PCBs. These POPs cover a wide range of physical chemical properties (solubility, vapor pressure, hydrophobicity, and persistence) and are used here as surrogates of the POPs present in seawater, as it is unfeasible to analyse all of them. In the experiments challenged with the simple mixtures of PAHs and OClPs, we analysed only the family of pollutants that was fortified. Before the instrumental analysis for the quantification of PAHs, n-alkanes and PCBs, we added to the extract an internal standard mix containing 50 ng of anthracene-d10, pyrene-d10, P-therphenyl-d14 and benzo(b)fluoranthene-d12; 12 µg of nonadecane-d40; and 2 ng of the PCBs congeners 30 and 142. PAHs and n-alkanes were quantified by gas-chromatography

coupled to mass spectrometry, and PCBs, HCB and HCHs by gas-chromatography coupled to an electron capture detector as described elsewhere (46-48).

III.2.4. CELL ABUNDANCE ESTIMATION

Cell concentration of *Prochlorococcus*, *Synechococcus* and picoeukaryotes was measured in vivo on board of the ship in subsamples of 1 mL by flow cytometry using a FACSCalibur (Becton Dickinson Biosciences, San Jose, California, USA) as explained elsewhere (49).

III.2.5. *PROCHLOROCOCCUS* GENE EXPRESSION ANALYSES

Sample collection consisted on the filtration of 990 mL of seawater onto 47-mm-diameter, 0.2-µm pore-size PTFE filters (Millipore, Billerica, MA) under low vacuum pressure. Each filter was split into two halves, one was place into RNAlater (Sigma-Aldrich, Saint Louis, MO) at -80 °C to preserve RNA, and the other one into lysis buffer (50 mM Tris HCl, 40 mM EDTA, 0.75 M Sucrose) at -20 °C to preserve DNA (37).

For RNA isolation, we extracted the half-filter samples in RNAlater using the mirVana™ kit (Ambion, Austin, TX), after removing the storage reagent by centrifugation. We concentrated total RNA by partial lyophilization to approximately 40 µL, measured its concentration by a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Delaware, DE). Quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was treated with DNase I (Ambion) to remove genomic DNA contamination and reverse transcribed to cDNA using First Strand cDNA Synthesis Kit (Roche, Mannheim, GE). The resulting cDNA preparation was stored at -80 °C until quantitative real-time PCR (qRT-PCR) analysis.

The target genes selected were *rbc*L, which encodes the large subunit of RuBisCO, responsible of carbon fixation, and *psb*A, that encodes the core protein D1 of photosystem II, the primary target of photoinactivation. To normalize the expression

of these genes we used *rnp*B as reference gene, that encodes the RNA component of the RNaseP, and has been described as a suitable reference gene in qRT-PCR analysis (50-53). Two set of specific primers to target HL and LL *Prochlorococcus* clades were used as described elsewhere (37).

Aliquots of 3.75 ng of total cDNA were amplified by qRT-PCR in a LightCycler 480 (Roche Diagnostics, Indianapolis, IN) thermocycler using SYBR Green Mix (Takara Bio Inc., Siga, Japan) with the following parameters: activation at 95 °C for 10 s, forty-five amplification cycles (95 °C for 5 s, 60 °C for 35 s), followed by a melting curve program (65-95 °C with a heating rate of 0.11 °C /s) and a final extension at 60 °C for 30 s. All samples were run in duplicates, and the amplifications of target and reference genes of the same sample were performed in the same plate in order to minimize systematic errors.

We used the second derivate maximum of the amplification curves (Cp) to calculate the relative quantity of mRNA of each gene. Cp values for the target genes (tg) *rbc*L and *psb*A were normalized to reference gene (ref) *rnp*B to obtain ΔCp values ($\Delta Cp = Cp_{ref} - Cp_{tg}$) as explained elsewhere (37). The ratios between treatments and controls mRNA/DNA levels were calculated from these ΔCp values, as

$$\frac{Copies_{Treatment}}{Copies_{Control}} = 2^{\Delta Cp_{Treatment} - \Delta Cp_{Control}} \quad (37).$$

The PCR efficiency for the tested genes were calculated as described in (54).

III.2.6. STATISTICAL ANALYSIS

Given the experimental design, in which each experiment started from a freshly (few hours) obtained DCM water sample split into pairs of independent containers, one treated and the other left as control, comparisons between treated and untreated cultures were performed using paired t-test assays for both qRT-PCR and chemical data. Time variations were analysed using the non-parametric

Kruskal-Wallis test, as it is more robust against outliers than the parametric ANOVA counterpart. Comparisons between T/C (treated versus control) ratios for qRT-PCR or pollutant concentration data were also analysed by non parametric (Spearman' s) tests, to account for their intrinsic non-linearity and their different data structure. Concentrations of organic pollutants in the dissolved phase presenting values below the method detection limit (MDL) were replaced with MDL/2, and missing data values were substituted with median concentrations. Statistical tests and figures were performed using the SPSS 19 (SPSS Inc., Chicago, IL) package. Figures were edited using CorelDraw X6 (Corel Corporation, Ottawa, Ontario, Canada). Additional statistical analyses (e.g., heatmap) were performed using the R package (http:// CRAN.R-project.org/).

III.3. RESULTS AND DISCUSSION

III.3.1. CONCENTRATIONS OF ORGANIC POLLUTANTS

The measured organic pollutant concentrations in treatments were higher than in the controls for all experiments performed in the Atlantic, Indian and Pacific Oceans during the Malaspina circumnavigation cruise (Fig. III.1, Table AII.1). The PAHs treatment represented an increase of about 150 times over the environmental levels ($C/C_{control}$), although this figure was reduced to 48% of initial values after 24 hours of exposure ($p<0.01$) (Table AII.3). The OClP treatment increased in a range from 260 to 470 fold the concentrations found in the different water samples. No differences in OClP concentrations were found after 24 h of incubation (Table AII.4). Finally, addition of chemical mixtures (CM) represented an increase of 1.3 fold in PAHs concentrations and 1.8 fold of even-carbon number alkane concentrations (i.e., anthropogenic n-alkanes, Table AII.5) (55, 56). After 24h of exposure no significant differences were observed in PAH concentrations, but n-alkanes were reduced to 54% of initial concentrations ($p<0.01$) (Table AII.3). The concentrations of OClPs and PCBs in experiments with the CM were below limit detection. The

average amount added to 1 L of treatment of PCBs and OClPs was of 137 pg, 8.31 pg and 3.24 pg for PCBs, HCB and HCHs, respectively. These amounts correspond to the amount found in 10 L of surface seawater in the NE oligotrophic Atlantic (Table AII.2), thus the nominal increase ($C/C_{Control}$) was of 10, even though the real $C/C_{Control}$ in the incubations may be lower than the nominal concentrations. The $C/C_{Control}$ values for other POPs, not measured and/or unknown may be of the similar order of magnitude, between 1.3 and 10 times, as n-alkanes, PAHs and PCBs cover the range of properties of most POPs (1).

The PAH and OClP mixtures added to two sets of experiments included organic pollutants ubiquitously found in the marine environment, but their concentrations were significantly higher than those found in the ocean. Nevertheless, the $C/C_{control}$ ration between 70 and 360 found at the end of incubations for PAHs and OClPs, respectively, did not induce a noticeable decrease in *Prochlorococcus*, *Synechococcus* and picoeukaryote abundances (Table AII.6). This lack of lethality of a PAH mixture is consistent with the results of Echeveste and coworkers (15), who estimated that a 10 % decrease (LC10) of the *Prochlorococcus* and *Synechococcus* abundance, would require PAH concentrations 660 and 810 times above current oceanic levels, respectively.

III.3.2. DIEL CYCLE VARIATIONS OF PHOTOSYNTHETIC GENE EXPRESSION IN NATURAL *PROCHLOROCOCCUS* COMMUNITIES

Analysis of RNA abundances of *rbc*L and *psb*A genes in both HL and LL clades showed the expected variations during the diel cycle (52, 57). *rbc*L mRNA levels dropped at dusk (6 h after starting of the incubation) by more than 90% compared to noon or early afternoon values (0.5, 2 and 24 h times, Fig. III.2a, mean the log2 scale). In contrast, *psb*A mRNA values did not show any significant variation during the same period. Whereas *psb*A levels should in principle be lower in light conditions than in darkness, the reported changes, two-fold variation or less (52), are probably too weak for being observed under our experimental conditions. Exposure to organic pollutants did not alter this temporal expression pattern (Fig. III.2b) The diel cycle had little or no effect in the relative differences on expression of either gene

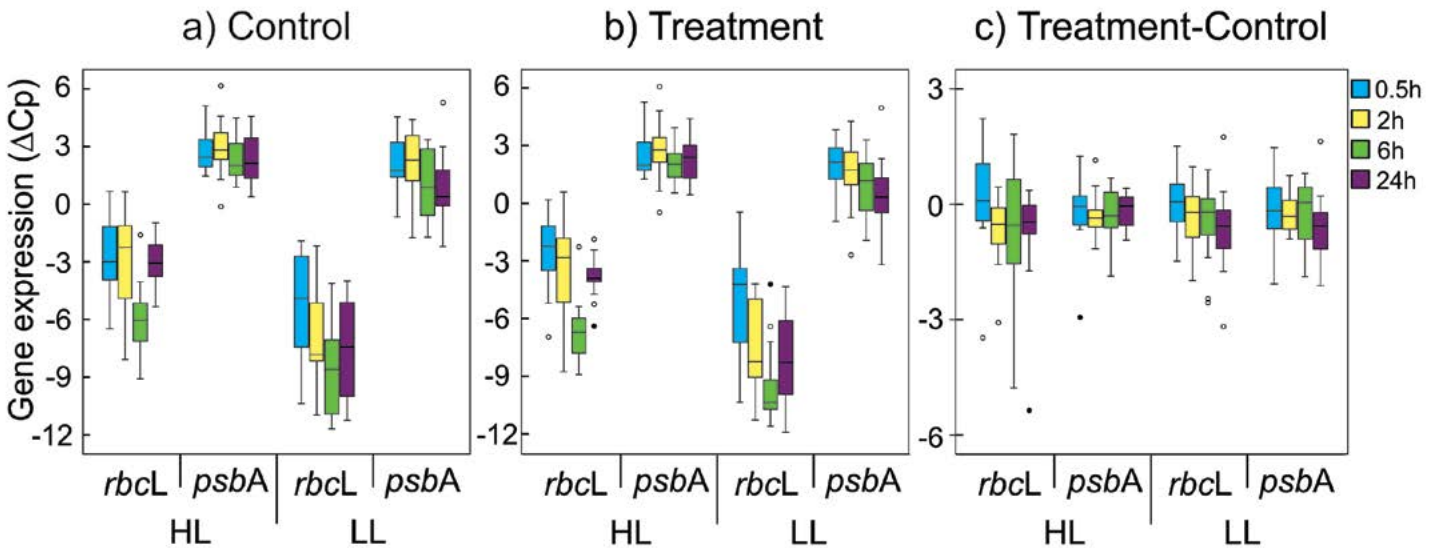


FIGURE III.2 - EXPRESSION TIME COURSE OF THE PHOTOSYNTHETIC *rbc*L AND *psb*A GENES.

Expression of both target genes was measured for HL and LL *Prochlorococcus* separately in every sample of each experiment. This figure shows the time course of each gene in a) control (no-treated) samples, b) treatments (treated with any of the pollutant mixture), and also c) the differential expression between treatments and controls. p values associated to the different gene expression data distributions (Kruskal-Wallis) are indicated. n/s, non-significant (p>0.05)

between treated and non-treated (control) cultures (ΔΔCp values, Fig. III.2c). Flow cytometry showed mild variations in *Prochlorococcus* abundance for most samples during the 24 h the cultures were maintained on deck, although important changes (one order of magnitude in both senses) were indeed observed for a limited number of experiments (Table AII.6). Even in these cases no major variations were observed between treated and untreated samples (Table AII.6). We thus concluded that field populations behaved very similarly for treated and untreated cultures during the experiments.

The observation of a clear diel cycle for *rbc*L expression levels is a first-time confirmation of this phenomenon in nature, to our knowledge only seen before in highly controlled laboratory conditions (52, 57). This illustrates the utility of on-board ship experiments of natural microorganism populations to allow experimental setups similar to those performed in laboratory conditions using pure cultures, but taking into account the natural genetic and community variability present in nature.

III.3.3. EFFECTS OF ORGANIC POLLUTANTS ON *PROCHLOROCOCCUS rbc*L AND *psb*A GENE EXPRESSION

Exposure of *Prochlorococcus* natural populations to complex mixtures of organic pollutants (CM) resulted in a decrease of *rbc*L expression for both clades (paired t-test, $p<0.05$), whereas exposure to the PAH mixture resulted in a decrease of *psb*A mRNA levels for the LL clade (paired t-test, $p<0.01$). No significant effects were observed when natural populations of *Prochlorococcus* were exposed to OClPs (Fig. III.3). We found temporal differences in the response gene expression to exposure of organic pollutants. This was more evident in populations exposed to the CM, as the maximal effects occurred after 2 h of exposure (Table III.1 and Fig. AII.1). This can be described as an acute toxic effect, which tended to decrease as cells went acclimated to the presence of the mixture. Conversely, PAH exposure resulted in a significant reduction of LL *psb*A mRNA levels at all incubation times, except for the 2 h incubation, reaching the maximum effect (45% decrease) after 24 h of expression, following a rather chronic toxicity pattern. Analysis of our data (see, for example, Fig. III.2) suggests that LL expression values did not recover completely

their initial levels after 24 h on-deck, even when the light conditions were similar to the initial ones, and this may be aggravated by the presence of pollutants. Although our current data do not allow a rigorous analysis of this complex effect, it is likely

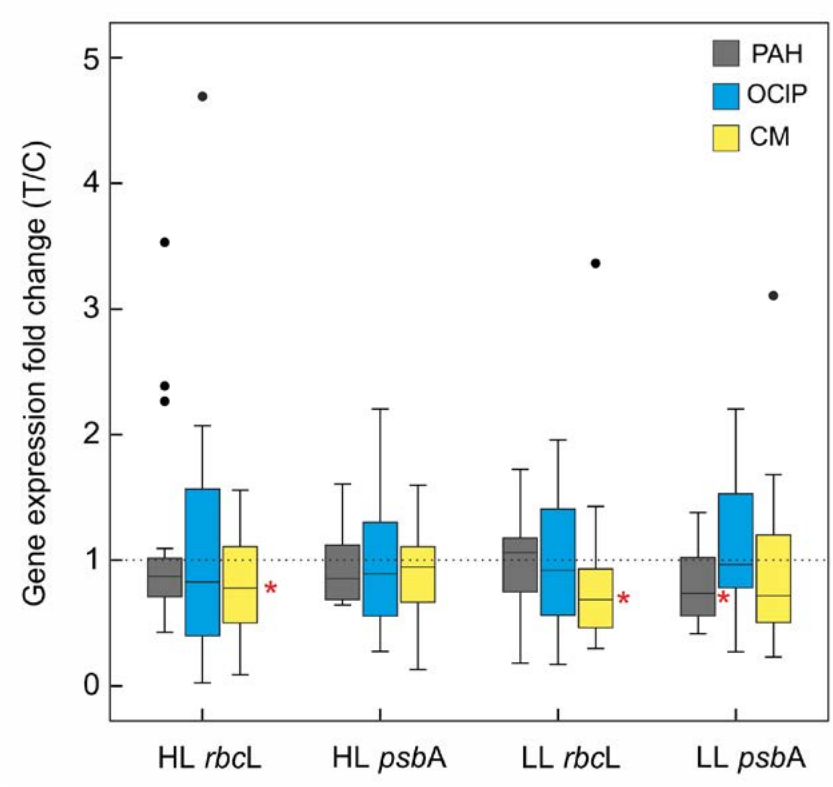


FIGURE III.3 - EFFECTS OF ORGANIC POLLUTANTS

Distribution of gene expression changes (T/C) between the different Treated/Control samples for all treatments and incubation times. Significant differences (p-values) were calculated using paired t-tests (*, p<0.05; **, p<=0.025; ***, p<0.01).

that the presence of pollutants may contribute to increase the stress of LL cells in unfavourable conditions.

TABLE III.1 - FIELD EXPERIMENT PAIRED T-TESTS

Paired t-test treatment vs. control for the different experiments and exposure time.

		High-light genes				Low-light genes			
		***rbc*L**		***psb*A**		***rbc*L**		***psb*A**	
		Fold Change ± sd	*p*	Fold Change ± sd	*p*	Fold Change ± sd	*p*	Fold Change ± sd	*p*
PAHs	0.5 h	1.05 ± 1.27	0.555	0.84 ± 0.77	0.145	0.94 ± 1.01	0.398	0.74 ± 0.62	0.025*
	2 h	0.78 ± 0.65	0.034*	0.91 ± 0.86	0.288	1.16 ± 1.08	0.812	1.15 ± 0.91	0.935
	6 h	1.31 ± 2.35	0.696	1.02 ± 1.05	0.546	0.70 ± 1.18	0.240	0.61 ± 0.61	0.039*
	24 h	0.78 ± 0.75	0.110	0.86 ± 0.74	0.126	0.91 ± 0.84	0.288	0.56 ± 0.53	0.018*
OCIPs	0.5 h	1.97 ± 2.72	0.929	1.15 ± 1.01	0.836	1.10 ± 1.03	0.709	1.28 ± 1.45	0.802
	2 h	0.90 ± 1.01	0.343	0.91 ± 1.19	0.399	1.11 ± 1.34	0.636	0.90 ± 0.98	0.335
	6 h	0.24 ± 0.83	0.088	0.55 ± 0.73	0.083	0.70 ± 1.41	0.277	0.97 ± 1.59	0.476
	24 h	0.34 ± 1.40	0.157	0.84 ± 0.91	0.246	0.56 ± 0.60	0.039*	0.88 ± 0.79	0.192
CM	0.5 h	1.20 ± 1.38	0.740	1.14 ± 1.37	0.666	1.33 ± 1.67	0.800	1.15 ± 1.66	0.637
	2 h	0.40 ± 0.60	0.029*	0.66 ± 0.62	0.018*	0.49 ± 0.53	0.012*	0.62 ± 0.50	0.001*
	6 h	0.70 ± 0.94	0.149	0.95 ± 0.92	0.374	0.71 ± 0.71	0.054	0.91 ± 1.12	0.362
	24 h	0.72 ± 0.85	0.117	1.04 ± 0.94	0.602	0.50 ± 1.22	0.144	0.71 ± 1.29	0.238

Significant correlations are marked with asterisk *

III.3.4. PATTERNS OF ORGANIC POLLUTANTS FROM CM AND THEIR RELATIONSHIP WITH *rbc*L AND *psb*A GENE EXPRESSION

The relative influence of the different components of the CM mixtures on the expression of photosynthetic *Prochlorococcus* genes after two hours of incubation were studied by correlation analyses (Fig. III.4). The analysis show significant negative correlations between both LL genes and some PAHs with 3 and 4 aromatic rings: fluorene, phenanthrene, fluoranthene, pyrene, and chrysene, and the C21 alkane heneicosane (Fig. III.4). These compounds are predominant in the PAH mixtures found in marine waters' samples (4, 56, 58). In contrast, only few positive correlations (i.e., the higher concentration change, the higher expression) were found for HL clade genes (Fig. III.4). Whereas the results for LL *rbc*L and *psb*A are consistent with the negative effect of PAHs in gene expression of LL genes (Fig. III.3, Table III.1), our data suggest that the components of the CM mixture responsible for the observed inhibition of both HL and LL genes (Fig. III.3, Table III.1) were not included in the chemical analysis. The observed positive correlations between gene expression and pollutant concentration may well be related to their known dual role as toxicant and carbon – and/or nutrient – sources.

Whereas the PAH incubation experiment did represent a substantial increase on PAH concentrations, it is unclear which component of CM mixtures may be responsible for the observed effects. The concentrations of organic pollutants measured in the dissolved phase of the incubations with CM reflect the general pattern of PAHs and n-alkanes found in seawater, with high predominance of three- and four-ringed parental PAHs and their alkylated derivatives (43, 56). For analogous CM of organic pollutants, Echeveste (15) estimated that a 10% decrease of *Prochlorococcus* and *Synechococcus* abundance would be observed at $C/C_{Control}$ values of 21 and 27, respectively, significantly higher than the factor 1.3 – 2 measured here. However, it is not unlikely that the added extracts contained much higher amounts of other uncharacterised compounds, and that these were the triggers of the inhibitory effects seen in our experiments. In any case, we consider that the observed effects on gene expression cannot result from the rather modest addition

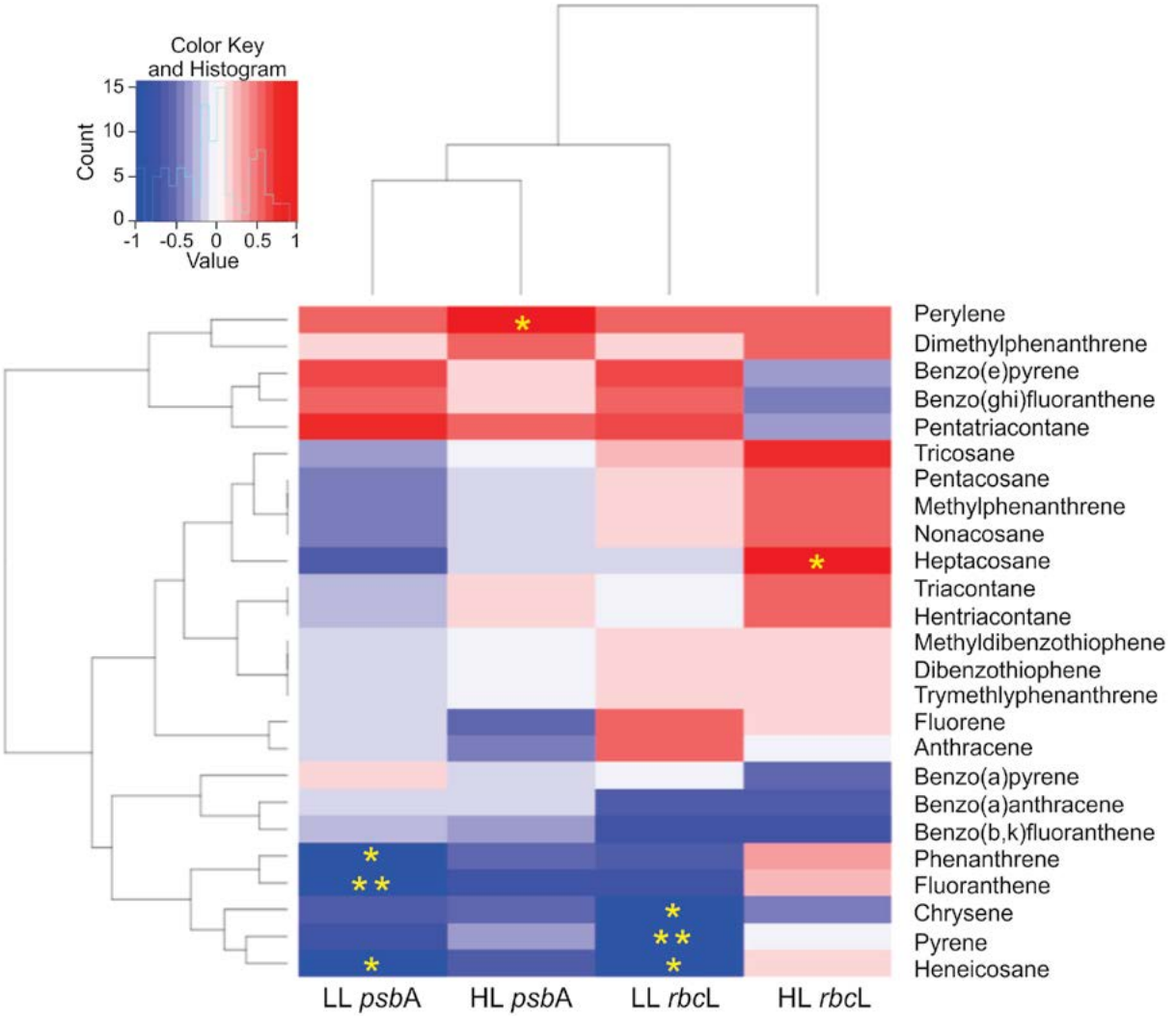


FIGURE III.4 - CORRELATIONS BETWEEN GENE EXPRESSIONS AND ORGANIC POLLUTANT CONCENTRATIONS

Correlations between changes in gene expression after 2 h exposure and the increase on concentration of the different components of the CM mixtures. Both magnitudes are expressed as fold changes (treated versus control). Spearman correlation heatmap. Significant correlations are indicated by asterisks (*, p<0.05; **, p<0.01).

of pollutants we quantified by chemical analyses. Instead the analysed compounds can be considered tracers of the whole mixture, as POPs with similar properties behave similarly (38). These results are not only consistent with those described for lethality effects on oceanic *Prochlorococcus* (15), but to other toxicological studies performed with different toxicity endpoints and for continental waters, in which the toxicity of known compounds is a small fraction of the total toxicity detected in the sample (39, 40). Finally, we cannot exclude synergistic effects of complex mixtures, although we have no direct proof for that.

III.4. CONCLUSION

This work supports the importance of the study of the organic pollutant toxicity as a whole (complete chemical mixture) instead for each compound individually, the importance of performing studies using natural communities with an intrinsic variability of different strains, and the impact of the incorporation of advanced, high sensitivity-high selectively analytical tools to the analysis of the anthropogenic impact on the Biosphere. The extrapolation of the results of these experiments to oceanic waters is difficult due to the multiple processes involved. While decrease in transcript levels not necessarily means a decrease of the corresponding protein concentration in the cell or an impairment of a given physiological function, it is clear from studies in different organisms that dysregulation of key genes is a powerful indicator of the presence of stressors in natural populations (59, 60). Obviously, photosynthesis by *Prochlorococcus* will respond to other key variables such as nutrient availability, radiation and various environmental stressors, but our findings suggest that organic pollutants might modulate this response, an issue requiring further research. The results reported here provide the first evidence that small variations (less than 2 fold) of the seawater concentrations of organic pollutants can induce an effect on the expression of *Prochlorococcus* photosynthetic genes. This modulation of such relevant global function by the myriad of organic pollutants present in the oceans may affect the carbon fluxes mediated by phytoplankton. Whereas this

perturbation may be small in magnitude, it may contribute to the temporal trends in phytoplankton abundance and productivity reported for the Anthropocene (61, 62).

III.5. REFERENCES

1. Lohmann R, Breivik K, Dachs J, & Muir D (2007) Global fate of POPs: current and future research directions. Environ Pollut 150(1):150-165.
2. Gioia R, et al. (2008) Polychlorinated biphenyls (PCBs) in air and seawater of the Atlantic Ocean: Sources, trends and processes. Environ Sci Technol 42(5):1416-1422.
3. Galbán-Malagón C, Cabrerizo A, Caballero G, & Dachs J (2013) Atmospheric occurrence and deposition of hexachlorobenzene and hexachlorocyclohexanes in the Southern Ocean and Antarctic Peninsula. Atmospheric Environment 80:41-49.
4. Berrojalbiz N, et al. (2011) Persistent organic pollutants in Mediterranean seawater and processes affecting their accumulation in plankton. Environ Sci Technol 45(10):4315-4322.
5. Galbán-Malagón CJ, Del Vento S, Berrojalbiz N, Ojeda MJ, & Dachs J (2013) Polychlorinated biphenyls, hexachlorocyclohexanes and hexachlorobenzene in seawater and phytoplankton from the Southern Ocean (Weddell, South Scotia, and Bellingshausen Seas). Environ Sci Technol 47(11):5578-5587.
6. Galbán-Malagón C, Berrojalbiz N, Ojeda MJ, & Dachs J (2012) The oceanic biological pump modulates the atmospheric transport of persistent organic pollutants to the Arctic. Nature communications 3:862.
7. Del Vento S & Dachs J (2002) Prediction of uptake dynamics of persistent organic pollutants by bacteria and phytoplankton. Environmental Toxicology and Chemistry 21(10):2099-2107.
8. Berrojalbiz N, et al. (2009) Accumulation and cycling of polycyclic aromatic hydrocarbons in zooplankton. Environ Sci Technol 43(7):2295-2301.
9. Gerofke A, Kömp P, & McLachlan MS (2005) Bioconcentration of persistent organic pollutants in four species of marine phytoplankton. Environmental Toxicology and Chemistry 24(11):2908-2917.
10. Koelmans AA (2014) Limited reversibility of bioconcentration of hydrophobic organic chemicals in phytoplankton. Environ Sci Technol 48(13):7341-7348.
11. Galbán-Malagón CJ, Berrojalbiz N, Gioia R, & Dachs J (2013) The "degradative" and "biological" pumps controls on the atmospheric deposition and sequestration of hexachlorocyclohexanes and hexachlorobenzene in the North Atlantic and Arctic Oceans. Environ Sci Technol 47(13):7195-7203.
12. Morales L, et al. (2015) Oceanic sink and biogeochemical contrlols on the accumulation of polychlorinated dibenzo-P-dioxins, dibenzofurans and dibenzofurans in plankton. Environ Sci Technol 49 (23), 13853-13861
13. Everaert G, De Laender F, Goethals PLM, & Janssen CR (2015) Multidecadal Field Data Support Intimate Links between Phytoplankton Dynamics and PCB Concentrations in Marine Sediments and Biota. Environ Sci Technol 49 (14), 8704-8711
14. Echeveste P, Agusti S, & Dachs J (2010) Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations. Environ Pollut 158(1):299-307.
15. Echeveste P, Dachs J, Berrojalbiz N, & Agusti S (2010) Decrease in the abundance and viability of oceanic phytoplankton due to trace levels of complex mixtures of organic pollutants. Chemosphere 81(2):161-168.
16. Echeveste P, Agusti S, & Dachs J (2011) Cell size dependence of additive versus synergetic effects of UV radiation and PAHs on oceanic phytoplankton. Environ Pollut 159(5):1307-1316.
17. Everaert G, De Laender F, Goethals PLM, & Janssen CR (2015) Relative contribution of persistent organic pollutants to marine phytoplankton biomass dynamics in the North Sea and the Kattegat. Chemosphere 134:76-83.

18. Field CB (1998) Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. Science 281(5374):237-240.

19. Longhurst A, Sathyendranath S, Platt T, & Caverhill C (1995) An estimate of global primary production in the ocean from satellite radiometer data. Journal of Plankton Research 17(6):1245-1271.

20. Rockström J, et al. (2009) A safe operating space for humanity. Nature 461(7263):472-475.

21. Goericke R & Welschmeyer NA (1993) The marine prochlorophyte *Prochlorococcus* contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. Deep-Sea Research Part I: Oceanographic Research Papers 40(11 /12):2283-2294.

22. Li WK (1995) Composition of ultraphytoplankton in the central north Atlantic. Marine Ecology Progress Series 122(1-3):1-8.

23. Liu H, Nolla HA, & Campbell L (1997) *Prochlorococcus* growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. Aquatic Microbial Ecology 12(1):39-47.

24. Partensky F, Hess WR, & Vaulot D (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiology and Molecular Biology Reviews 63(1):106-127.

25. Ting CS, Rocap G, King J, & Chisholm SW (2002) Cyanobacterial photosynthesis in the oceans: The origins and significance of divergent light-harvesting strategies. Trends in Microbiology 10(3):134-142.

26. Campbell L, Liu H, Nolla HA, & Vaulot D (1997) Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean at Station ALOHA during the 1991-1994 ENSO event. Deep-Sea Research Part I: Oceanographic Research Papers 44(2):167-192.

27. Campbell L, et al. (1998) Response of microbial community structure to environmental forcing in the Arabian Sea. Deep-Sea Research Part II: Topical Studies in Oceanography 45(10-11):2301-2325.

28. Crosbie ND & Furnas MJ (2001) Abundance, distribution and flow-cytometric characterization of picophytoprokaryote populations in central (17°S) and southern (20°S) shelf waters of the Great Barrier Reef. Journal of Plankton Research 23(8):809-828.

29. Johnson ZI, et al. (2006) Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science 311(5768):1737-1740.

30. Moore LR, Rocap G, & Chisholm SW (1998) Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature 393(6684):464-467.

31. Moore LR & Chisholm SW (1999) Photophysiology of the marine cyanobacterium *Prochlorococcus*: Ecotypic differences among cultured isolates. Limnol. Oceanogr. 44(3 I):628-638.

32. West NJ & Scanlan DJ (1999) Niche-partitioning of *Prochlorococcus* populations in a stratified water column in the eastern North Atlantic Ocean? Appl Environ Microbiol 65(6):2585-2591.

33. Zinser ER, et al. (2006) *Prochlorococcus* ecotype abundances in the North Atlantic Ocean as revealed by an improved quantitative PCR method. Appl Environ Microbiol 72(1):723-732.

34. Llabrés M, Dachs J, & Agustí S (2012) Transference of atmospheric hydroxyl radical to the ocean surface induces high phytoplankton cell death. Photochemistry and Photobiology 88(6):1473-1479.

35. Mann EL, Ahlgren N, Moffett JW, & Chisholm SW (2002) Copper toxicity and cyanobacteria ecology in the Sargasso Sea. Limnol. Oceanogr. 47(4):976-988.

36. Llabrés M & Agustí S (2006) Picophytoplankton cell death induced by UV radiation: Evidence for oceanic Atlantic communities. Limnol. Oceanogr. 51(1 I):21-29.

37. Fernández-Pinos MC, et al. (2015) Clade-specific quantitative analysis of photosynthetic gene expression in *Prochlorococcus*. PloS one, 10(8), e0133207

38. Dachs J & Méjanelle L (2010) Organic Pollutants in Coastal Waters, Sediments, and Biota: A Relevant Driver for Ecosystems During the Anthropocene? Estuaries and Coasts 33(1):1-14.

39. Tang JYM, et al. (2013) Mixture effects of organic micropollutants present in water: Towards the development of effect-based water quality trigger values for baseline toxicity. Water Research 47(10):3300-3314.

40. Escher BI, Van Daele C, Dutt M, Tang JYM, & Altenburger R (2013) Most oxidative stress response in water samples comes from unknown chemicals: The need for effect-based water quality trigger values. Environ Sci Technol 47(13):7002-7011.

41. Bruyant F, et al. (2005) Diel variations in the photosynthetic parameters of *Prochlorococcus* strain PCC 9511: Combined effects of light and cell cycle. Limnol. Oceanogr. 50(3):850-863.

42. Claustre H, et al. (2002) Diel variations in *Prochlorococcus* optical properties. Limnol. Oceanogr. 47(6):1637-1647.

43. Berrojalbiz N, et al. (2011) Biogeochemical and physical controls on concentrations of polycyclic aromatic hydrocarbons in water and plankton

of the Mediterranean and Black Seas. Global Biogeochemical Cycles 25(4):n/a-n/a.

44. Muir DCG & Howard PH (2006) Are there other persistent organic pollutants? A challenge for environmental chemists. Environ Sci Technol 40(23):7157-7166.

45. Smith KEC, et al. (2013) Baseline toxic mixtures of non-toxic chemicals: "solubility addition" increases exposure for solid hydrophobic chemicals. Environ Sci Technol 47(4):2026-2033.

46. González-Gaya B, Zúñiga-Rival J, Ojeda MJ, Jiménez B, & Dachs J (2014) Field measurements of the atmospheric dry deposition fluxes and velocities of polycyclic aromatic hydrocarbons to the global oceans. Environ Sci Technol 48(10):5583-5592.

47. Dachs J, Eisenreich SJ, Baker JE, Ko FC, & Jeremiason JD (1999) Coupling of phytoplankton uptake and air-water exchange of persistent organic pollutants. Environ Sci Technol 33(20):3653-3660.

48. Cabrerizo A, et al. (2011) Factors influencing the soil-air partitioning and the strength of soils as a secondary source of polychlorinated biphenyls to the atmosphere. Environ Sci Technol 45(11):4785-4792.

49. Marie D, Simon N, & Vaulot D (2005) Phytoplankton Cell Counting by Flow Cytometry. Algal Culturing Techniques, ed Andersen R (Elsevier, Amsterdam), pp 253-267.

50. Lindell D, Jaffe JD, Johnson ZI, Church GM, & Chisholm SW (2005) Photosynthesis genes in marine viruses yield proteins during host infection. Nature 438(7064):86-89.

51. Martiny AC, Coleman ML, & Chisholm SW (2006) Phosphate acquisition genes in *Prochlorococcus* ecotypes: evidence for genome-wide adaptation. Proc Natl Acad Sci U S A 103(33):12552-12557.

52. Zinser ER, et al. (2009) Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, *Prochlorococcus*. PLoS ONE 4(4).

53. Gomez-Baena G, Rangel OA, Lopez-Lozano A, Garcia-Fernandez JM, & Diez J (2009) Stress responses in *Prochlorococcus* MIT9313 vs. SS120 involve differential expression of genes encoding proteases ClpP, FtsH and Lon. Res Microbiol 160(8):567-575.

54. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9).

55. Dachs J, Bayona JM, Fowler SW, Miquel JC, & Albaigés J (1998) Evidence for cyanobacterial inputs and heterotrophic alteration of lipids in sinking particles in the Alboran Sea (SW Mediterranean). Marine Chemistry 60(3-4):189-201.

56. Dachs J, Bayona JM, Fillaux J, Saliot A, & Albaigés J (1999) Evaluation of anthropogenic and biogenic inputs into the western Mediterranean using molecular markers. Marine Chemistry 65(3-4):195-210.

57. Garczarek L, et al. (2001) Differential expression of antenna and core genes in *Prochlorococcus* PCC 9511 (Oxyphotobacteria) grown under a modulated light-dark cycle. Environmental Microbiology 3(3):168-175.

58. Nizzetto L, et al. (2008) PAHs in Air and Seawater along a North–South Atlantic Transect: Trends, Processes and Possible Sources. Environ Sci Technol 42(5):1580-1585.

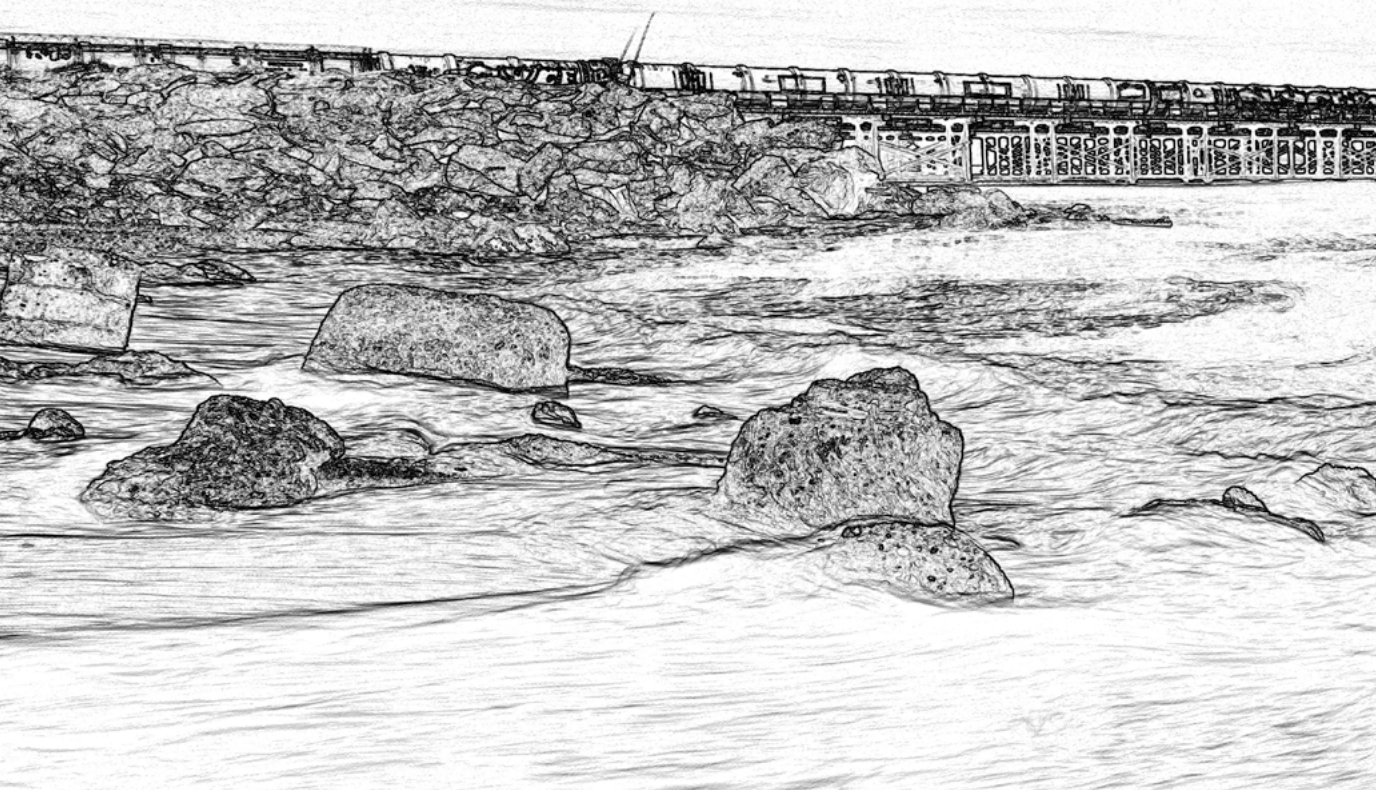
59. Piña B, Casado M, & Quirós L (2007) Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. TrAC Trends in Analytical Chemistry 26(11):1145-1154.

60. Piña B & Barata C (2011) A genomic and ecotoxicological perspective of DNA array studies in aquatic environmental risk assessment. Aquatic Toxicology 105(3–4, Supplement):40-49.

61. Boyce DG, Lewis MR, & Worm B (2010) Global phytoplankton decline over the past century. Nature 466(7306):591-596.

62. Henson SA, et al. (2010) Detection of anthropogenic climate change in satellite records of ocean chlorophyll and productivity. Biogeosciences 7(2):621-640.

Chapter IV



Photosynthetic gene expression responses of *Prochlorococcus* wild population to changing environmental factors: the role of persistent organic pollutants

MC Fernández-Pinos, B Piña, M Vila-Costa, M Casado and J Dachs

ABSTRACT

The cyanobacterium *Prochlorococcus* accounts for a substantial fraction of oceanic primary production. Here, we present for the first time a comprehensive assessment of the influence of physical, biological and chemical variables on the photosynthetic function of *Prochlorococcus*. We measured a total of 158 variables from seawater samples at three different depths from the Malaspina circumnavigation expedition, including several families of persistent organic pollutants (POPs), one of the less studied drivers of global change. The influence of these variables was evaluated using a high- throughput methodology based on the expressions of functional genes related to photosynthesis. The model resulted consistent with the known physiology of the high-light (HL) and low-light (LL) clades of *Prochlorococcus*, being the water temperature a determinant factor for the presence of the genus, and the radiation a limiting factor for both clades at and below the deep chlorophyll maximum (DCM). The preference of LL for deeper water was remarkable, showing less dependence on radiation than HL. LL photosynthetic function correlated positively with the oxygen concentration at DCM+40 m depth, and a negative correlation with the heterotrophic bacterial production. Besides the strong influence of these environmental factors, POP concentrations also affected *Prochlorococcus* photosynthetic capability. Several dissolved polycyclic aromatic hydrocarbons (PAHs) and lead (Pb) correlated negatively with the carbon fixation proxy (*rbc*L gene expression) for *Prochlorococcus* and the maximum yield of photosystem II for the entire photosynthetic community. Concentrations of different POPs on average explained the 20% of *Prochlorococcus rbc*L expression and even a higher share of its abundance. This is the first reported field evidence of a relevant impact of POP mixtures on the oceanic photosynthetic capability of a major primary producer, which may be extended to other photosynthetic organisms, and potentially to the global marine primary production.

IV.1. INTRODUCTION

Photosynthesis converts sunlight, water and carbon dioxide into energy-rich organic carbon molecules and oxygen. Basically, this chemical process fuels life on Earth and regulates atmospheric carbon dioxide and oxygen concentrations that, in turn, affect climate and biogeochemical cycles. Marine primary production accounts for approximately half of the global biospheric production (1, 2). Moreover, it is the first step of the biological pump, which has absorbed about 30% of human carbon dioxide emissions since industrialization (3), making the ocean the second largest sink for anthropogenic carbon dioxide after the atmosphere itself (4). Thus, detecting the impact of the different global change vectors on marine primary production is an essential task for understanding the Earth system.

Oceanic photosynthesis is conducted mainly by single-celled phytoplanktonic organisms that inhabit the euphotic zone. One of these phytoplankton is the cyanobacterium *Prochlorococcus*, the smallest and most abundant known photosynthetic organism on Earth (5). It dominates the oligotrophic waters of tropical and subtropical world's oceans between 40 °N and 40 °S latitude (5-7), and contributes up to 50–80% of primary production in these regions (5, 8). *Prochlorococcus* genus is broadly divided into two major groups differentially adapted to a range of light intensities, high-light (HL) and low-light (LL) adapted clades (9). HL are usually found in surface waters, while LL have their abundance maximum in the base of the euphotic zone, reaching depths of 150- 200 m (5, 10-12).

In the euphotic zone nutrients required for the synthesis and growth of cells occur in relatively low concentrations, being the main limiting factor of marine primary production (13). Its supply at high latitudes depends on winter mixing and thermocline erosion processes, while at low latitude, upwelling and thermocline shoaling are the main supply processes (14). An alteration of these processes is predicted as results of global climate change. Increasing temperatures and changes in wind patterns and magnitude will affect mixing and stratification processes in the surface ocean, limiting the nutrient supply to the euphotic zone at low latitude, and consequently potentially reducing primary production (15). On the contrary, at high latitudes, where light limits phytoplankton growth during winter, decreased mixings

may result in increased primary production (16, 17). These changes in the seasonality of oceanic primary production, in combination of alterations of phytoplankton distributions due to increased temperatures, are expected to affect carbon export and storage (18, 19).

Global climate change may be implicated with the recent observed changes in oceanic chlorophyll and primary production, and a decrease on total phytoplankton abundance over the past century (20, 21). However, they cannot be unequivocally attributed just to the impact of climate change (20, 21), and there are also other drivers of global change that could be related to these changes in marine photosynthesis. For instance, persistent organic pollutants (POPs) and other organic pollutants constitute one of the less studied drivers of global change (22). It has been demonstrated that organic pollutants at ultra-trace concentrations can reduce phytoplankton growth, abundance and cell viability (23, 24). POPs are more toxic as smaller the phytoplankton cell size is (25), resulting Prochlorococcus one of the most sensitive phytoplanktonic organisms to organic pollutants (25, 26). Most POPs are prone to long-range transportation by atmospheric circulation (27, 28), reaching remote open sea areas by atmospheric deposition (28, 29). Hydrophobic POPs bioaccumulate and biomagnify through food webs (30-33), with a biogeochemical cycle coupled with that of the oceanic carbon cycle (34-36).

Although there is an increasing number of toxicity tests and risk assessments of POPs, most of them focus mainly on the effects of single compounds in laboratory conditions (37), whereas in their natural environment organisms are in fact exposed to complex mixtures of chemicals (38-42), interacting with a number of changing environmental variables. Indeed, some studies on effects of real pollutant mixtures in the ocean have shown that non-toxic chemicals can become toxic in a mixture (43-45). Toxic effects of complex mixtures of pollutants can be up to a thousand times more toxic than a single compound (26). Moreover, synergistic effects of POPs with environmental variables occur (46-49). These effects have also been observed with *Prochlorococcus*, which was much more sensitive to complex mixtures of organic pollutants from seawater than to single pollutants separately (26), and when exposed to increased UV radiation (48).

Traditionally, studies on global oceanic photosynthesis have measured chlorophyll concentration as indicator, but its relation to phytoplankton carbon changes substantially with species composition, light intensity, and nutrient availability (50). In addition, most toxic effects on oceanic cyanobacteria have been measured at cellular level (25, 26, 48), whereas photosynthetic function may be affected or accommodated without associated cellular damages. Hence, effects of POPs on phytoplanktonic photosynthesis may be underestimated due to the current methodologies used in this field, and investigations on the consequences of the cocktail of POPs accumulated in oceanic waters must be faced by more integrative and sensitive techniques.

Functional gene expression has been demonstrated to be a useful and comprehensive technique to assess effects on the photosynthetic function of *Prochlorococcus* of a variety of environmental variables, including POPs (51-56). Therefore, the main objective of this work is assessing the role of POPs, as one of the drivers of global change, in primary production occurring in the tropical and subtropical oceans, by using a previously developed method based on gene expression proxies to measure the photosynthetic capability of *Prochlorococcus* (56). If this effect is observed, then there is the potential for POPs to have affected the marine carbon cycle over the past century (20, 21). The high- throughput methodology used in this work allows detecting changes in the expression of two genes involved in photosynthesis, *rbc*L and *psb*A genes, for HL and LL clades separately (56). The *rbc*L gene encodes the large subunit of RuBisCO, responsible of carbon fixation, and the *psb*A gene encodes the core protein D1 of photosystem II, the primary target of photoinactivation. To normalize the expression of these genes, we used *rnp*B as reference gene, which encodes the RNA component of the RNase P (56). Multivariate analysis of gene expression data in relation to a number of environmental variables, including POP concentrations in dissolved and particulate phase of water, and bioaccumulated in plankton, have been subsequently used to assess correlation between biological, physical, and pollution data.

IV.2. MATERIAL AND METHODS

IV.2.1. SAMPLE COLLECTION

We sampled a total of 62 stations for *Prochlorococcus* gene expression along the Atlantic, Indian and Pacific Oceans during the Malaspina 2010 Expedition (Fig. IV.1), which took place from December 2010 to July 2011 on board the R/V BioHesperides. At each station, samples from 3 m, deep chlorophyll maximum (DCM) and DCM+40 m were collected with Niskin bottles, manually at 3 m, and attached to a rosette sampler system at DCM and DCM+40 m. The rosette was fitted with a calibrated Seabird 911 Plus CTD probe that measured a number of physical variables along the vertical profiles. All seawater samples were collected between 8 h and 12 h am local time, coinciding with the peak of carbon fixation and the maximum *rbc*L and *psb*A expressions of *Prochlorococcus* (55, 57, 58). Seawater samples were directly siphoned from Niskin bottles into 1 L glass bottles, and immediately filtered onto a 20-µm-pore-size net and a 47- mm-diameter, 0.2-µm-pore-size PTFE filter (Millipore, Billerica, MA) at 80 mbar vacuum pressure. A filter half of each sample was stored in RNAlater (Sigma-Aldrich, Saint Louis, MO) at -80 °C for later RNA analysis. The time spent between sampling and storing was set in a maximum of 10 minutes to minimize expression changes and RNA degradation (56, 59).

IV.2.2. ANALYSIS OF *rbc*L AND *psb*A EXPRESSIONS IN *PROCHLOROCOCCUS* WILD POPULATIONS

RNA was isolated using the mirVanaTM kit (Ambion, Austin, TX), after removing the storage reagent by centrifugation. When necessary, total RNA was lyophilized for concentrating it. RNA concentration was measured using a NanoDrop ND_8000 spectrophotometer (NanoDrop Technologies, Delaware, DE), and its quality was checked by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was treated with DNase I (Ambion) and reverse transcribed to cDNA using First Strand cDNA Synthesis Kit (Roche, Mannheim, GE). Then, aliquots of 3.75 ng of total cDNA were amplified by qRT-PCR in a LightCycler 480 (Roche Diagnostics,

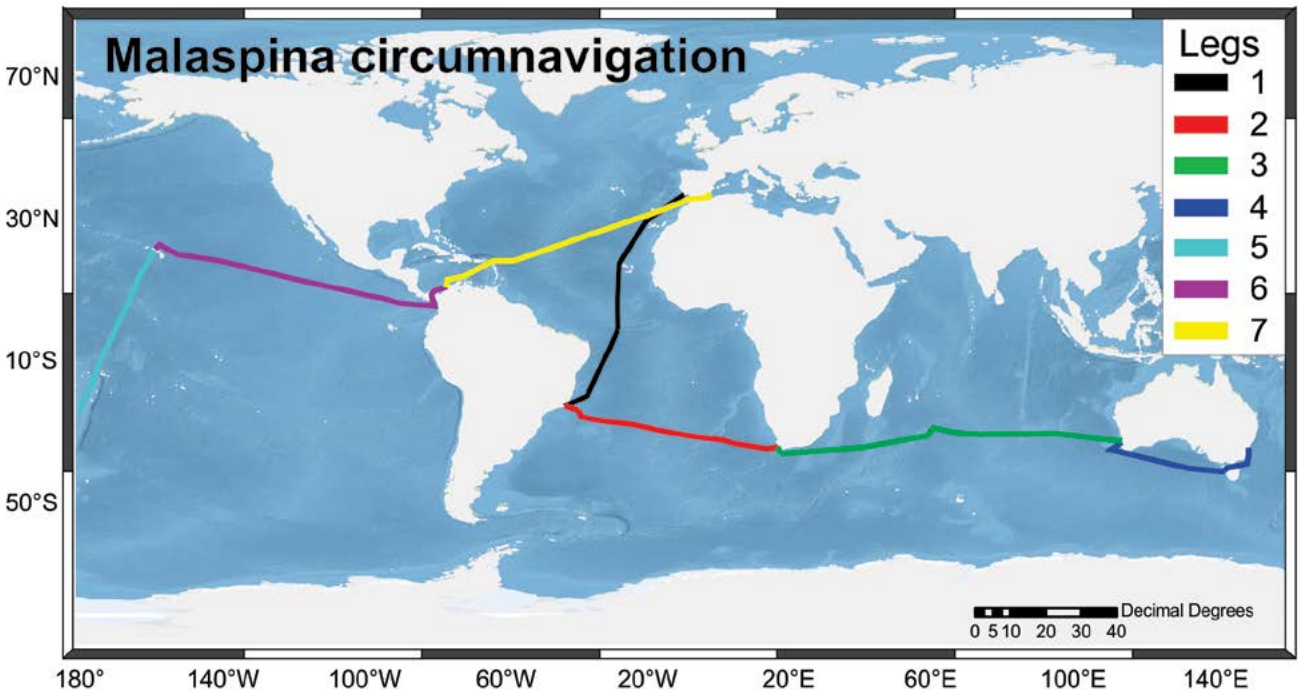


FIGURE IV.1 - THE MALASPINA CIRCUMNAVIGATION

Representation of the Malaspina circumnavigation course. Colours indicate the seven different legs that formed the cruise.

Indianapolis, IN) thermocycler using SYBR Green Mix (Takara Bio Inc., Siga, Japan). We used the same PCR protocol and the specific primers for both target genes *rbc*L and *psb*A, and the reference gene *rnp*B that in Fernandez-Pinos et al (56). All samples were run in duplicates, and the amplifications of target and reference genes of the same sample were performed in the same plate in order to minimize systematic errors.

Relative gene expressions were calculated using the ΔC mathematical model (60, 61):

$$\Delta Cp = Cp_{reference} - Cp_{target}$$

where Cp is the PCR crossing point calculated as the second derivate maximum of the amplification curve of a sample, $Cp_{reference}$ is the crossing point corresponding to the reference gene, i.e. *rnp*B, and Cp_{target} is the crossing point corresponding to the target gene, i.e. either *rbc*L or *psb*A.

IV.2.3. MULTIVARIATE ANALYSIS OF *rbc*L AND *psb*A GENE EXPRESSION IN RELATION TO ENVIRONMENTAL VARIABLES

As different environmental variables were measured in surface, DCM and DCM+40 m, three data sets were assessed separately according to depth. A complete list of the physical, biological and chemical variables included in each data set, their units and a brief description is available in Table AIII.1. To correct the negative correlation between Cp values and specific mRNA concentrations, Cp values for *rnp*B are represented as cycles above background levels, 30-Cp. This assumes 30 as the Cp value corresponding to non-specific amplification, based on our empiric observations when using these primers in natural oceanic samples. Concentration data were normalized by logarithmic transformation.

Pearson bivariate between quantified biological and physical variables and *rnp*B Cp values, and relative expressions of *rbc*L and *psb*A, were used to analyse the correspondence between our molecular proxies and the photosynthetic activity of *Prochlorococcus* in the ocean, and to analyse the main environmental variables that influence the expression of the selected genes. Separate analyses were carried out

for each sampled depth, surface, DCM and DCM+40. Given the vertical distribution of the two major *Prochlorococcus* clades (5, 56), at surface, only gene expression for the HL clade was taken into account, whereas data for both HL and LL clades were used for DCM. At deep waters (DCM+40) only LL data was considered. False discovery rate (fdr) was used for correction of multiple correlation data, at a significance level of 0.05 (62). Unless otherwise specified, only variables with at least one significant correlation with the gene expression data (30-*rnp*B Cp, *rbc*L ΔCp or *psb*A ΔCp) were included in graphs and tables.

To assess the possible effects of organic pollutants on *Prochlorococcus* photosynthetic capability we ran a partial least squares regression (PLSR) analysis with all the available oceanic variables, including chemical measurements. PLSR was selected for being a statistical method for relating two data matrices, an X matrix of predictors (environmental variables) and a Y matrix of responses (gene expressions), by a linear multivariate model. This approach was usefulness in this study thanks to its ability to analyse data with many, noisy, collinear, and even incomplete variables in both X and Y (63).

The R package (http://CRAN.R-project.org/) was used for running all the statistical analysis and performing the figures, which were edited using CorelDraw X6 (Corel Corporation, Ottawa, Ontario, Canada).

IV.3. RESULTS AND DISCUSSION

IV.3.1. GENE EXPRESSION AND ENVIRONMENTAL DATA IN SURFACE SAMPLES

Bivariate correlation analysis of all measured variables in surface waters (target gene expressions, physical, biological and chemical variables) showed significant correlations among 24 environmental variables (out of 186) and expression values for at least one of the target genes for surface samples (Fig. IV.2, only HL clade). Hierarchical clustering revealed that each gene grouped with a different subset of variables. HL-*rnp*B Cp values (expressed as cycles above background, note that this

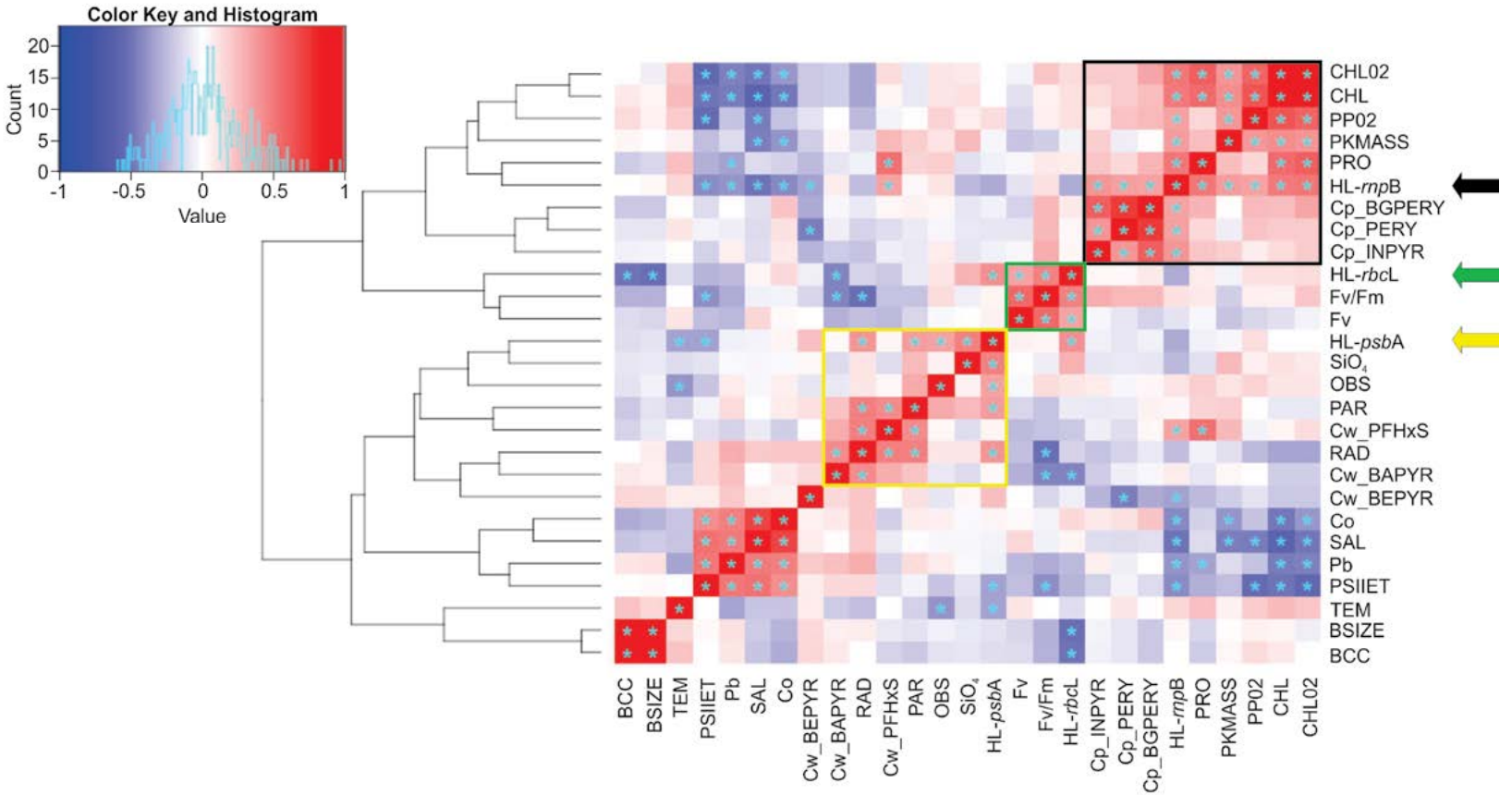


FIGURE IV.2 - SURFACE PEARSON CORRELATIONS

Pearson's correlations between target gene expressions and environmental variables for Surface samples. The hierarchical cluster analysis is shown on the left. Only those variables showing significant correlations with gene expression data (marked with arrows on the right) are displayed. Significant clusters including target genes are framed. Blue and red denote negative and positive correlations, respectively. Cyan asterisks indicate significant Pearson's correlation values ($p<0.05$, fdr).

parameter is positively correlated with RNA abundance) clustered with a number of parameters related to *Prochlorococcus* abundance (CHLO2, PRO, PPO2, black frame in Fig. IV.2). No correlation was found between HL-*rnp*B Cp values and *Synechococcus* abundance, which confirmed the specificity of HL-*rnp*B primers and that no cross-amplification between both genera occurred, consistent with previous observations (56). These results demonstrate that HL-*rnp*B abundance reflects the actual concentration of *Prochlorococcus* gene sequences in the environmental RNA sample and, therefore, that it is an adequate reference gene for gene expression. The significant correlation between HL-*rnp*B Cp values and *Prochlorococcus* abundance determined by fluorescence measurements in all surface samples is shown in Fig. AIII.1.

HL-*rbc*L relative expression showed a strong positive correlation with the maximum yield of Photosystem II (Fv/Fm) from the total photosynthetic community (Fig. IV.2), whereas HL-*psb*A was included in a cluster together with two parameters related to light intensity, solar radiation and photosynthetically active radiation (RAD and PAR, Fig. IV.2, yellow frame). These results show that whereas HL-*rnp*B Cp indicated the relative abundance of *Prochlorococcus* cells in the samples, both HL-*rbc*L and HL-*psb*A levels were related with different aspects of photosynthesis, namely the efficiency of the photosynthetic function and light intensity, respectively. In addition, HL-*rbc*L showed a strong negative correlation with the dissolved benzo(a) pyrene concentration (Fig.AIII.2)

IV.3.2. GENE EXPRESSION AND ENVIRONMENTAL DATA IN DCM SAMPLES

Similar bivariate correlation analysis for DCM samples, now including HL and LL clade data, showed also 14 environmental parameters with at least one significant correlation with one of the tested gene expression values (six variables in total) (Fig. IV.3). No correlation was observed between *rnp*B Cp data (either clade) and the observed *Prochlorococcus* abundance, probably because fluorescence values aggregate both clades and the PCR data quantifies them separately. The nature of the PCR data prevents calculating aggregate values by pure addition of the two

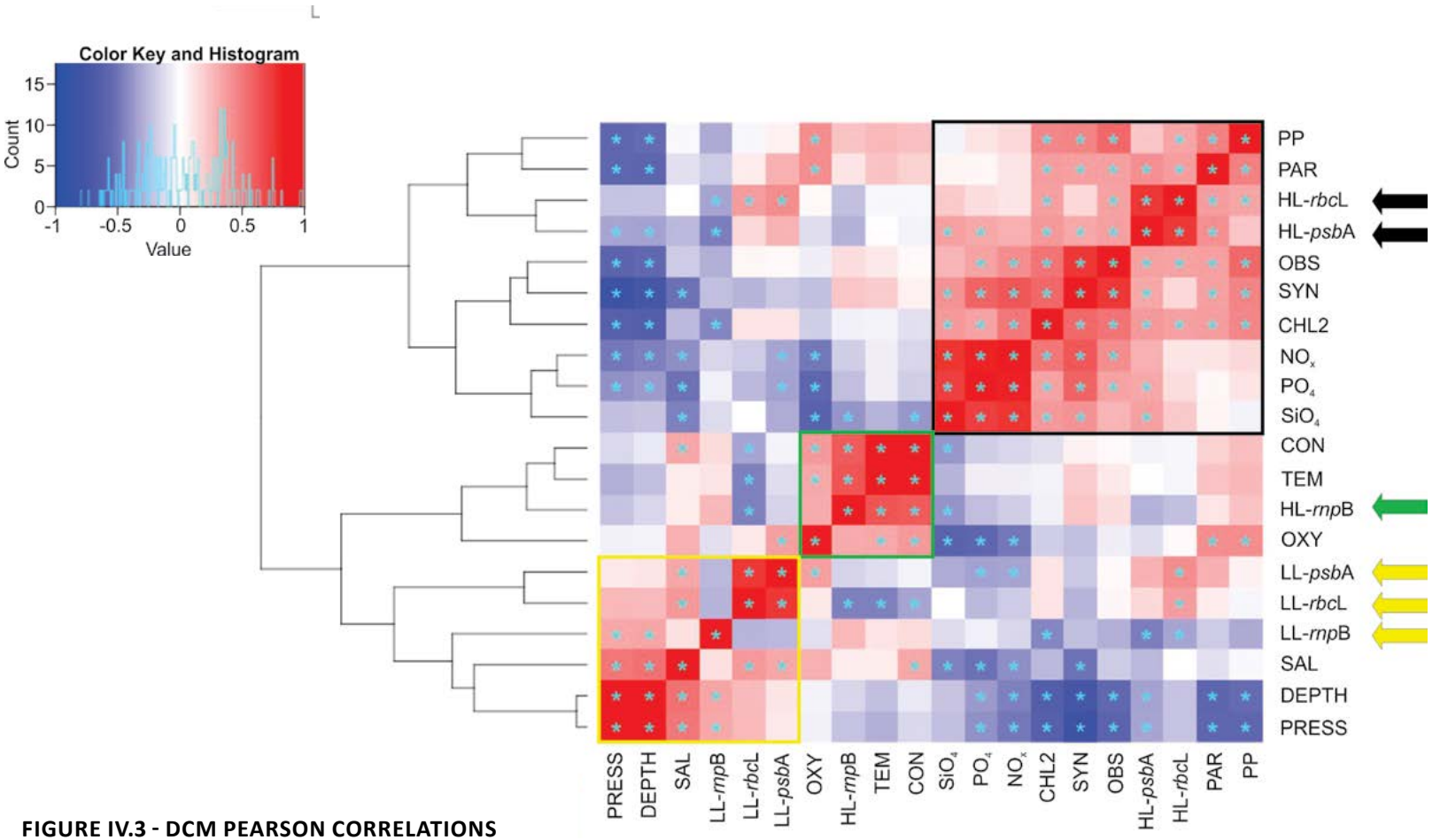


FIGURE IV.3 - DCM PEARSON CORRELATIONS

Pearson's correlations between target gene expressions and environmental variables from the DCM samples. The hierarchical cluster analysis is shown on the left. Only those variables showing significant correlations with gene expressions (marked with arrows on the right) are displayed. Significant clusters including these data are framed. Blue and red denote negative and positive correlations, respectively. Cyan asterisks indicate significant Pearson's correlation values ($p<0.05$, fdr).

separate values. Rather, HL-*rnp*B Cp data showed strong positive correlations with two environmental parameters, temperature and conductivity (TEM, CON), that may be determinant for the *Prochlorococcus* high-light clade survival at DCM (Fig. IV.3, green square). These results are consistent with previous works that revealed a significant correspondence between the distribution of *Prochlorococcus* ecotypes and environmental factors, being temperature a key ecological determinant (64, 65). On the other hand LL-*rnp*B Cp data showed strong positive correlations with depth, also consistent with the environmental requirements of low-light *Prochlorococcus* (12, 66)

Relative expression of *rbc*L and *psb*A clustered together for each clade, suggesting a differential response of both clades to DCM environmental conditions involving both light harvesting and CO_2 fixation processes. HL-*rbcL* and HL-*psb*A relative expression values clustered together with light intensity parameters (PAR, OBS) and other photosynthetically relevant parameters such as chlorophyll *a* concentration of size fraction 2- 20 µm and total primary production rate (CHL2, PP, Fig. IV.3, yellow square). This is consistent with the dependence of the HL clade (and of other photosynthetic species, like *Synechococcus*) on the radiation (57, 64, 67). In contrast, their LL counterparts (and the *rnp*B LL data) clustered with depth and pressure, suggesting that LL clades thrive in oceanic conditions of comparatively deep DCM levels and relatively low radiation conditions.

These results suggest that LL clade photosynthesis is not primarily dependent on light intensity, at least at DCM. The cluster also includes some physical data, like pressure, depth and salinity concentration, which may indicate that the physicochemical conditions of water, rather than light availability, may be the main driver of LL clade photosynthetic function at DCM. Other photosynthesis-related parameters, like photosystem II (PSII) efficiency, were not available for DCM. Fig. AIII.3 shows the different responses of both clades HL and LL to PAR, and the correlation of cell abundance with temperature and pressure, respectively.

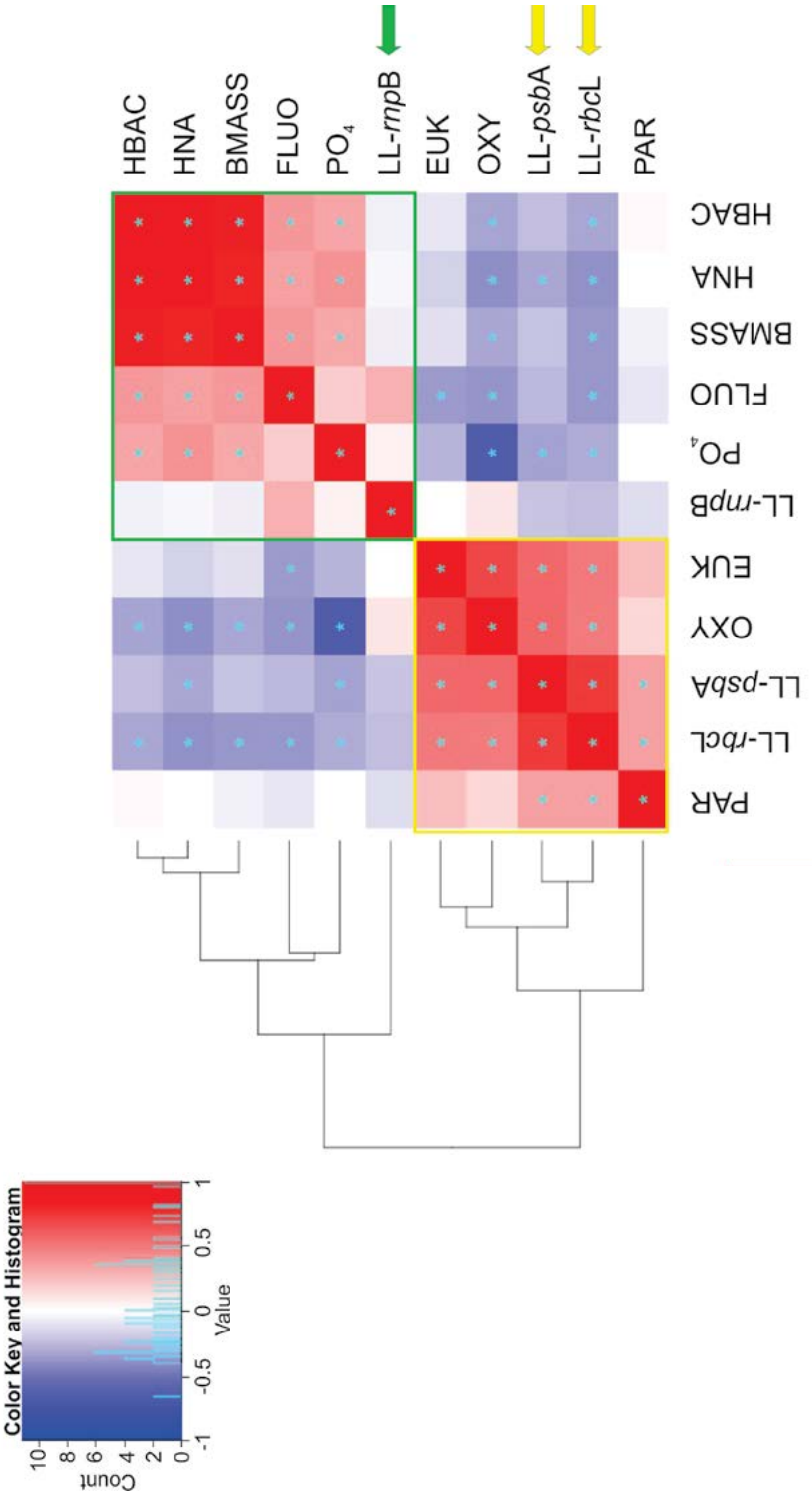


FIGURE IV.4 - DCM+40 PEARSON CORRELATIONS

Pearson's correlations between target gene expressions and environmental variables from DCM+40 samples. Only those variables showing significant correlations with target gene expressions (marked with arrows on the right) are displayed. Significant clusters including these data are framed. Blue and red denote negative and positive correlations, respectively. Cyan asterisks indicate significant Pearson's correlation values ($p<0.05$, fdr).

IV.3.3. GENE EXPRESSION AND ENVIRONMENTAL DATA IN DCM+40 SAMPLES

A similar analysis performed for DCM+40 samples (only LL clade), showed only 8 variables significantly correlated with gene expression data (Fig. IV.4). Hierarchical cluster analysis showed two major clusters, with strong negative correlation between them (Fig. IV.4, green and yellow frames). Contrasting with results from DCM, relative expression of LL-*rbc*L and LL-*psb*A clustered together with photosynthetically active radiation (PAR), oxygen (OXY) and picoeukaryote (EUK) abundance (Fig. IV.4, yellow frame). In contrast, LL-*rnp*B Cp values clustered with a weak correlation with some indicators of heterotrophic bacterial activity (HBAC, HNA), total chlorophyll a fluorescence (FLUO) and total bacterial biomass (Fig. IV.4, green frame). We propose that these data reflects the heterogeneity of DCM+40 environments, from a light-rich ones, in which *Prochlorococcus* thrives, and darker, nutrient-rich ones, in which heterotrophic bacteria proliferate. Some relevant correlations are shown in Fig. AIII.4.

IV.3.4. MULTIVARIATE ANALYSIS

The availability of a large number of variables for the 62 surface samples allowed us to study the effect of pollutants on *Prochlorococcus* abundance and metabolism using PLSR multivariate analysis. Only data corresponding directly to pollutants' occurrence at surface (i.e., no data from water column, like pollutant in plankton which integrate the photic zone) were added to the model as predictive variables (X-data). These variables were used to explain surface parameters related to either *Prochlorococcus* abundance or total photosynthesis functioning. To facilitate the analysis, only parameters showing significant correlation with at least one the gene expression data (Pearson correlation, p=0.05) were introduced in the model, which included 25 X-variables (pollutant concentration data) and 10 biological variables (Y-data, Table IV.1).

Three components explained about 50% of X-variation and predicted about 33% of Y-variation (Table IV.1). Correlation analyses (Table IV.1) suggested that PC1

showed a strong positive correlation with HL-*rbc*L expression and chlorophyll *a* concentration of size fraction 0.2- 2 µm (CHL02). It also was correlated with at least two photosystem II (PSII) functioning parameters, the maximum yield of PSII (Fv/Fm) and PSII turnover time (TAU) measured at 4 m depth (Table IV.1). Both PC2 and PC3 explained most of the HL-*rnp*B Cp variability, along with the measured *Prochlorococcus* abundance (PRO, PC2) and the energy transfer between PSII reaction centres measured at 4 m (PSIIET, Table IV.1).

The mutual correlation between X- and Y-loads for the different parameters and X-scores for samples can be analysed in Fig. IV.5. The plot shows some of the individual correlations already observed in the bivariate analysis, like the direct

TABLE IV.1 - SURFACE PLSR MODEL

Fraction of X- and Y-variability explained by the first 3 components of the model. Correlations between x-scores and biological data (yt correlations), shown as Pearson's r coefficients. Asterisks indicate significant correlation values ($p<0.01$). No further significant correlations were discovered after adding up to 10 components to the model.

	t1	t2	t3
	% Exp. Var.		
R2X	0.195	0.232	0.086
R2Xcum	0.195	0.428	0.513
R2Y	0.151	0.093	0.081
R2Ycum	0.151	0.244	0.325
	y/t correlation		
30 - HL-*rnp*B	0.280	0.462*	0.421*
HL-*rbc*L	0.437*	-0.155	-0.124
HL-*psb*A	-0.064	-0.102	0.112
PP02	0.196	0.073	0.277
Fv	0.341	-0.185	-0.123
Fv/Fm	0.595*	-0.121	-0.032
PSIIET	-0.399	-0.052	-0.497*
TAU	-0.565*	0.409	0.017
PRO	0.237	0.632*	0.305
CHL02	0.444*	0.246	0.409
Y-data	30 – HL-*rnp*B, HL-*rbc*L, HL-*psb*A, PP2, Fv, Fv/Fm, PSIIET, TAU, PRO, CHL02		
X-data	Pb, Co, Cw_PFHxS, Cw_PFOA, Cw_PFOS, Cw_PFDA, Cw_DBT, Cw_MDBT, Cw_MPHE, Cw_FLUORANT, Cw_PYR, Cw_MPYR, Cw_BAANT, Cw_MCHRY, Cw_BBKFLUORANT, Cw_BEPYR, Cw_BAPYR, Cw_PERY, Cw_BGPERY, Cp_DMDBT, Cp_PERY, Cp_INPYR, Cp_DBANT, Cp_BGPERY		

correlation between HL-*rbc*L expression and the maximum yield of PSII (Fv/Fm), or between HL-*rnp*B Cp values and *Prochlorococcus* abundance (compare Figs. IV.2, AIII.1, and AIII.2 to the plot in Fig. IV.5). In addition, the plot suggests strong negative interactions between HL-*rbc*L expression and HL-*rnp*B Cp values on one side, and levels of many different PAHs and Pb in water. The space distribution of the different samples (dots in Fig. IV.5) suggests regional differences in both the chemical and the biological parameters introduced in the method, although it also suggests that

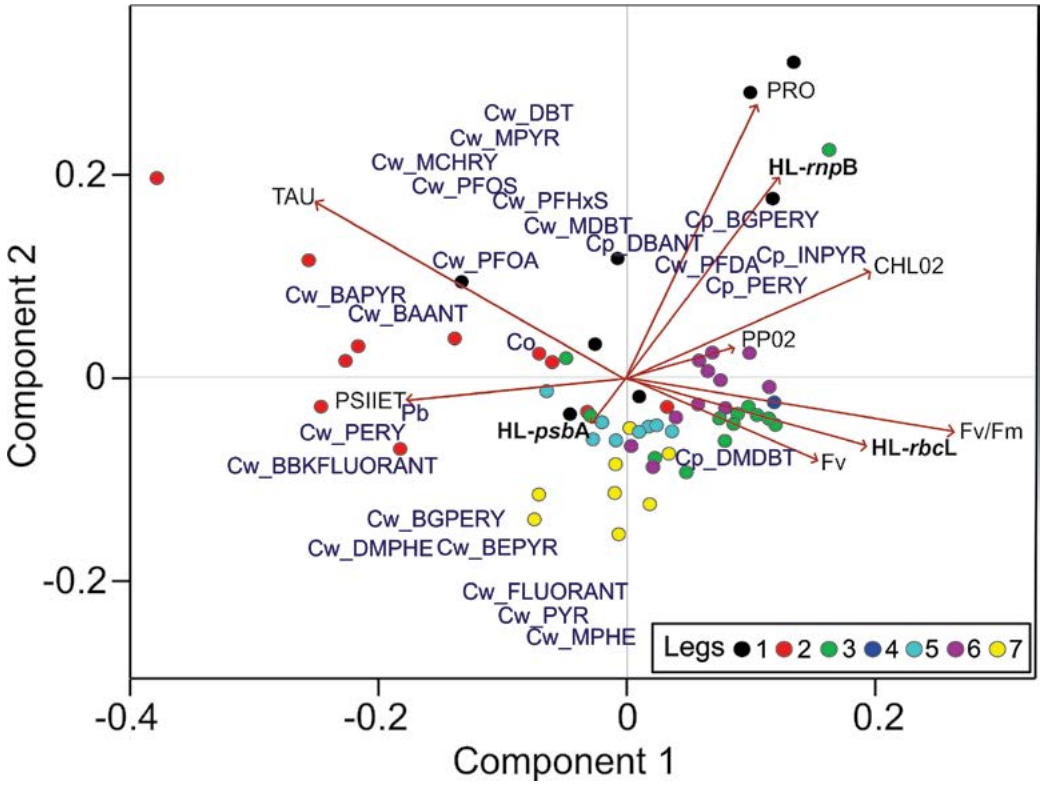


FIGURE IV.5 - SURFACE PLSR

Graphic representation of surface PLSR results. Dots represent score plots for the 62 stations colour-coded by leg (see Fig. IV.1). Loadings for the X-parameters are represented in blue characters, loadings for the Y-parameters are marked by red arrows. Values have been scaled to fit in the general graph. Only the two first PLSR components are represented.

samples from most legs are participating in the model (Fig. IV.5, mean the colour coding for the different legs).

The relative importance ("weight") of each pollutant in the different components is displayed in Fig. IV.6. Only parameters actually contributing to the model (VIPs, Variables Important for Prediction) are displayed; for consistency, the graph includes all VIPs corresponding to at least one of the three displayed components. Component 1 includes (negative) contributions of several PAHs in the dissolved phase (Cw_BGFLUORANT, Cw_BAPYR, Cw_PERY, and Cw_BAANT, only to mention the ones with the highest weights) and Pb. Note that only dissolved pollutant levels (not particulate levels) were selected by the model as VIPs. The dissolved phase PAHs are those that are bioavailable for *Prochlorococcus*, while particle phase PAHs correspond to concentrations of the different pools of organic carbon (not only

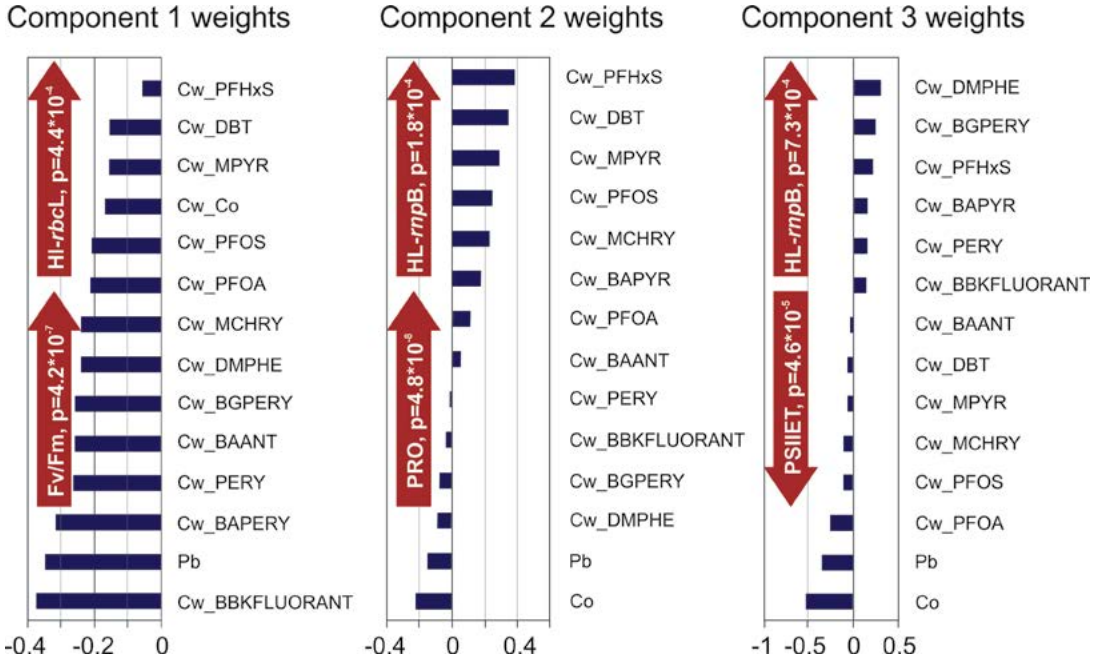


FIGURE IV.6 - SURFACE PLSR VIPs

Graphic representation of weight values of the different VIPs for the first three components of the model performed with surface samples. Arrows indicate correlations of each component with different biological, Y-variables (see also Fig. IV.5). Direction of the arrows indicates positive (upwards) or negative (downwards) correlations, the corresponding p-values are indicated.

Prochlorococcus. The PAHs with stronger negative contributions to component 1 are mainly pyrogenic.

PC1 in strongly (and positively) correlated with HL-*rbc*L expression ($p= 4 \times 10-4$, red arrow in Fig. IV.6), so the analysis suggests a negative correlation between CO_2 fixation capability and PAH and Pb pollutant levels. The graph also suggests that the same pollutants also affect the photosynthetic machinery, as the maximum yield of PSII (Fv/Fm) appears also negatively correlated with pollutant levels.

Both component 2 and 3 showed both positive and negative contributions for different pollutants, corresponding the two most negative contributions to two metals, Pb and Co. Both metals showed negative correlations with 30 - HL-*rnp*B Cp values and *Prochlorococcus* abundance, and a positive one with the energy transfer between PSII reaction centres (PSIIET, Fig. IV.6). It is unclear the meaning of positive correlations of *Prochlorococcus* abundance parameters with some pollutants, although it may be related with different pollutant sources or other physical parameters not included in the model. For example, positive contributions for components 1 and 2 (Figs. IV.5-6) correspond to particle phase PAHs, which are more abundant close to continents (sources of particles) as well as *Prochlorococcus* is more abundant in richer waters, in terms of nutrients, adjacent to continents.

PLSR analysis allowed to predict the theoretical values of Y variables from the observed X parameters, in this case pollutant levels. A summary of such prediction is displayed in Fig. AIII.5.

Using only the first three components, the model predicts between 40 and 50% of the variability associated to *Prochlorococcus* and chlorophyll *a* concentration corresponding to picophytoplankton (CHL02), HL-*rnp*B Cp values and the energy transfer between PSII reaction centres (PSIIET) and the photosystem II turnover (TAU). Lower levels of prediction, albeit still significant ones, were obtained for HL-*rbc*L expression levels (23%, $p<10^{-4}$) and Fv/Fm (37%, $p< 10^{-6}$). No significant predictive values were obtained for HL-*psb*A expression and primary production (Fig. AIII.5). Analysis of the geographical distribution of the different samples (colour codes in the plots in Fig. AIII.5) shows some variability among the different legs, although, as in the case of the score plots in Fig. IV.2, the graph suggests that samples from all legs participated in the final regression.

A relatively trivial explanation for the observed correlation might be that they reflect secondary interactions of any of the two most relevant physical parameters revealed by the binary analysis, light and salinity (Fig. IV.2). Salinity is strongly correlated with Pb and Co concentrations, so it is conceivable that salt levels, and not metals, may be the inhibitory factor suggested by components 2 and 3 of our model (Fig. IV.6). Light (either as total solar radiation or photosynthetically active radiation) has a strong influence in all photosystem functioning parameters, and it can also determine the synergy with some pollutants (48). The influence of both parameters on the correlation between x-scores and biological values (yt correlations) was analysed by partial regression, separately using RAD and SAL as control variables (Table IV.2).

The results indicate that neither parameter affected decisively in the yt correlations for PC 1 and 2, whereas salinity essentially eliminated all significant correlations between biological data and x-scores for PC 3 (Table IV.2). A likely explanation for that is that PC 3 grouped all X-data variation linked to salinity – obviously including Pb and Co water concentrations. However, as PC 2 also includes a strong contribution from these two pollutants, one can conclude that at least a part of HL-*rnp*B Cp values and of *Prochlorococcus* abundance is negatively associated to pollutant levels, disregarding the effect of salinity values present in each sampling point. Similarly, the negative correlation between HL-*rbc*L expression, chlorophyll *a* concentration from the size fraction 0.2- 2 µm, the maximum yield of PSII and some PAHs seemed very little affected by either salinity or radiation (Table IV.2).

A similar multivariate analysis could not be performed for DCM or DCM+40 samples, for no direct pollutant concentration in water or particulate are available for these sampling points. Rather, we designed a PLSR model in which PCR data for both clades (HL-*rnp*B Cp, LL-*rnp*B Cp, HL-*rbc*L, HL-*psb*A, LL-*rbc*L and LL-*psb*A,) were set as Y- (predictable) data and the rest of available data (physicochemical and biological parameters) were use as predictors, X-data (Table IV.3).

The relative importance (“weight”) of each predictor in the model is displayed in Fig. IV.7 (only VIPs, Variables important for prediction, for at least one of the three displayed components). Component 1 includes positive weights for Depth and Pressure, and negative contributions of nanophytoplankton chlorophyll *a*

TABLE IV.2 - SURFACE PARTIAL CORRELATIONS

Results from partial correlation analysis performed with surface samples.

	Initial YT Correlation			Control for Salinity			Control for Radiation		
Y-Variable	Pearson r	p value		estimate	p value		estimate	p value	
Component 1									
30 – HL-*rnp*B	0.280	2.9E-02	*	0.168	2.0E-01		0.316	1.4E-02	*
HL-*rbc*L	0.437	4.4E-04	***	0.469	1.6E-04	***	0.444	3.8E-04	***
HL-*psb*A	-0.064	6.2E-01		-0.081	5.4E-01		0.087	5.1E-01	
PP02	0.196	1.3E-01		0.076	5.6E-01		0.217	9.5E-02	
Fv	0.341	7.1E-03	**	0.405	1.3E-03	**	0.308	1.7E-02	*
Fv/Fm	0.595	4.2E-07	***	0.605	3.0E-07	***	0.566	2.4E-06	***
PSIIET	-0.399	1.4E-03	**	-0.309	1.6E-02	*	-0.451	3.0E-04	***
TAU	-0.565	2.1E-06	***	-0.544	7.1E-06	***	-0.480	1.1E-04	***
PRO	0.237	6.6E-02		0.209	1.1E-01		0.330	1.0E-02	*
CHL02	0.444	3.4E-04	***	0.367	4.0E-03	**	0.445	3.6E-04	***
Component 2									
30 – HL-*rnp*B	0.462	1.8E-04	***	0.588	7.9E-07	***	0.469	1.6E-04	***
HL-*rbc*L	-0.155	2.3E-01		-0.160	2.2E-01		-0.142	2.8E-01	
HL-*psb*A	-0.102	4.3E-01		-0.098	4.6E-01		-0.218	9.4E-02	
PP02	0.073	5.8E-01		0.130	3.2E-01		0.072	5.9E-01	
Fv	-0.185	1.5E-01		-0.202	1.2E-01		-0.152	2.5E-01	
Fv/Fm	-0.121	3.5E-01		-0.117	3.7E-01		-0.069	6.0E-01	
PSIIET	-0.052	6.9E-01		-0.116	3.8E-01		-0.041	7.5E-01	
TAU	0.409	1.1E-03	**	0.401	1.5E-03	**	0.341	7.7E-03	**
PRO	0.632	4.8E-08	***	0.653	1.6E-08	***	0.617	1.5E-07	***
CHL02	0.246	5.6E-02		0.335	8.9E-03	**	0.279	3.1E-02	*
Component 3									
30 – HL-*rnp*B	0.421	7.3E-04	***	0.090	4.9E-01		0.420	8.4E-04	***
HL-*rbc*L	-0.124	3.4E-01		-0.131	3.2E-01		-0.117	3.7E-01	
HL-*psb*A	0.112	3.9E-01		0.111	4.0E-01		0.079	5.5E-01	
PP02	0.277	3.1E-02	*	-0.117	3.7E-01		0.277	3.2E-02	*
Fv	-0.123	3.4E-01		-0.017	9.0E-01		-0.108	4.1E-01	
Fv/Fm	-0.032	8.1E-01		-0.103	4.4E-01		-0.009	9.5E-01	
PSIIET	-0.497	4.6E-05	***	-0.194	1.4E-01		-0.495	5.7E-05	***
TAU	0.017	8.9E-01		0.228	8.0E-02		-0.032	8.1E-01	
PRO	0.305	1.7E-02	*	0.303	1.8E-02	*	0.293	2.3E-02	*
CHL02	0.409	1.1E-03	**	0.078	5.6E-01		0.422	7.8E-04	***

* < 0.05; ** < 0.01; *** < 0.001

and *Synechococcus* cell abundance, among others. This component was positively correlated to LL-*rnp*B Cp values and negatively correlated to HL-*psb*A relative gene expression (Fig. IV.7). Conversely, component 2 showed strong positive contributions from temperature and weaker, negative ones from pressure or salinity. In this case, the predicted variable with highest correlation was HL-*rnp*B Cp, whereas LL-*rbc*L relative expression showed a somewhat weaker negative correlation. Finally, relative expression of LL-*psb*A and LL and HL-*rbc*L showed significant positive correlations with PC 3, which showed photosynthetically active radiation (PAR) as stronger positive component and inorganic nutrients (PO_4 and NO_x) as the strongly negative ones (Fig. IV.7). The data suggest that the depth at which the maximum chlorophyll occurs in the water column and its temperature defines the relative abundance of HL and LL clades, described from the corresponding *rnp*B Cp data, whereas the efficiency

TABLE IV.3 - DCM PLSR MODEL

Summary of PLSR analysis for DCM samples. Fraction of X- and Y-variability explained by the first 3 components of the model. Correlations between x-scores and biological data (yt correlations), shown as Pearson's r coefficients. Asterisks indicate significant correlation values ($p<0.01$). No further significant correlations were discovered after adding up to 10 components to the model.

	t1	t2	t3
% Exp. Var.			
R2X	0.232	0.125	0.103
R2Xcum	0.232	0.356	0.460
R2Y	0.077	0.109	0.109
R2Ycum	0.077	0.186	0.295
y/t correlation			
30- HL-*rnp*B	0.043	0.570*	0.122
30- LL-*rnp*B	0.357*	0.142	-0.272
HL-*rbc*L	-0.311	-0.140	0.337*
HL-*psb*A	-0.441*	-0.052	0.103
LL-*rbc*L	0.125	-0.506*	0.388*
LL-*psb*A	0.167	-0.165	0.538*
Y-data	30 – HL-*rnp*B, 30 – LL-*rnp*B, HL-*rbc*L, HL, *psb*A, LL-*rbc*L, LL- *psb*A		
X-data	FLUO, PP02, PP, PRO, SYN, EUK, CHL, CHL20, CHL2 CHL02, BACT, LNA, HNA, HBAC, BSIZE, BCC, BMASS, BP, SGR, TD, PKMASS, DEPTH, MLD, OXY, SAL, PRESS, TEM, CON, PAR, OBS, BAT, SiO_4, PO_4, NO_X, Cw_PFBS, Cw_PFHpA, Cw_PFHxS, Cw_PFOA, Cw_PFHpS, Cw_PFNA, Cw_PFOS, Cw_PFDA		

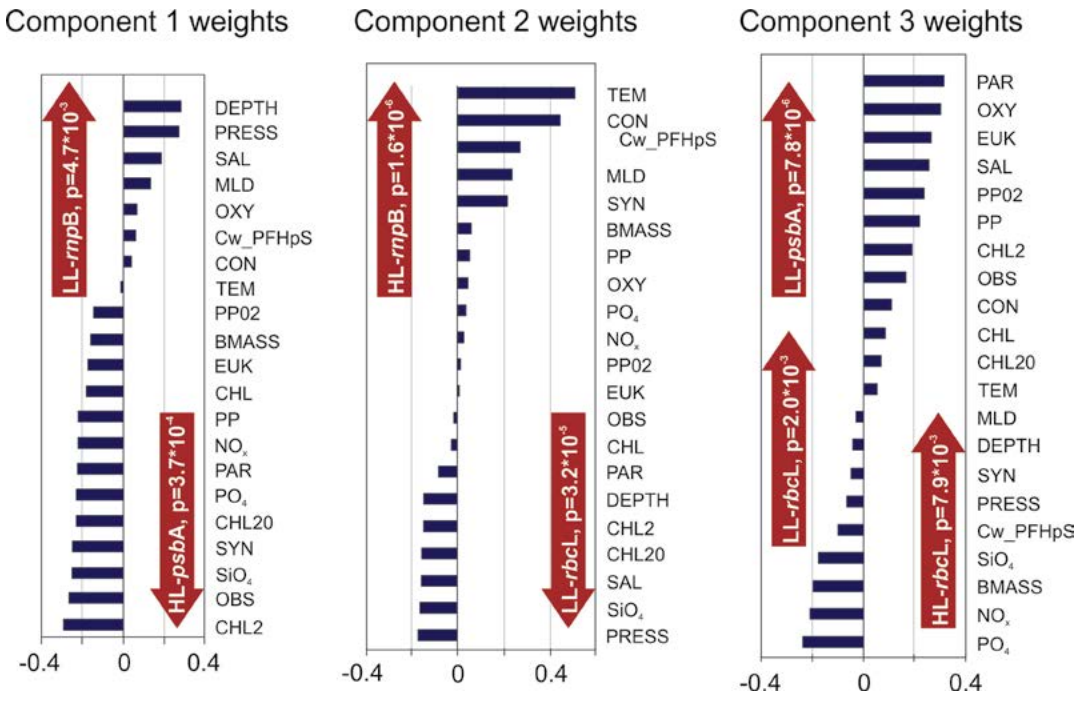


FIGURE IV.7 - DCM PLSR VIPs

Graphic representation of weight values of the different VIPs for the first three components of the model performed with DCM samples. Arrows indicate correlations of each component with different PCR, Y-variables (see also Fig. IV.3). Direction of the arrows indicates positive (upwards) or negative (downwards) correlations, the corresponding p-values are indicated.

of photosynthesis is driven by different parameters, like photosynthetically active radiation, particularly for the HL clade.

Environmental data for DCM+40 samples was still more limited than for DCM, as only 29 physicochemical and biological data were available as predictors for the LL clade PCR data (Table IV.4). As in the other two cases, three components included all significant correlations with X-parameters. PC 1 and PC 2 showed positive and negative correlations (respectively) with both LL-*rbc*L and LL-*psb*A relative expression data, whereas PC 3 was positively correlated to the abundance of LL-*rnp*B transcripts (Table IV.4).

Analysis of weights assigned for the different VIPs in these three components (Fig. IV.8) showed a positive contribution of oxygen, eukaryote abundance and

photosynthetically active radiation (PAR) for PC 1, and a negative one for HNA bacteria abundance. PC 2 showed strong positive correlation with conductivity and temperature, whereas eukaryote abundance, SiO_4, turbidity (OBS) and PAR appeared as negative contributors (Fig. IV.8). PC 3 showed a positive contribution from chlorophyll *a* fluorescence data and a negative one from SiO_4. A simple explanation of these data is that LL *Prochlorococcus* clades represent a strong contribution to the total fluorescence values at DCM+40 (and hence, the correlation between this parameter and LL-*rbc*L transcript abundance), whereas the actual efficiency of *Prochlorococcus* photosynthesis depends upon the PAR and inorganic nutrients, in a similar way as other, non-*Prochlorococcus* photosynthetic organisms.

Photosynthetically active radiation (PAR) appears as a major driver explaining the relative expression of both *rbc*L and *psb*A for both clades, in a depth-dependent manner (Table IV.5). Whereas no significant correlation was found between PAR and

TABLE IV.4 - DCM+40 PLSR MODEL

Summary of PLSR analysis for DCM+40 samples. Fraction of X- and Y-variability explained by the first 3 components of the model. Correlations between x-scores and biological data (yt correlations), shown as Pearson's r coefficients. Asterisks indicate significant correlation values ($p<0.01$). No further significant correlations were discovered after adding up to 10 components to the model.

	t1	t2	t3
% Exp. Var.			
R2X	0.262	0.144	0.064
R2Xcum	0.262	0.407	0.470
R2Y	0.213	0.114	0.087
R2Ycum	0.213	0.327	0.414
y/t correlation			
30- LL-*rnp*B	0.071	0.144	0.469*
LL-*rbc*L	0.579*	-0.435*	-0.185
LL-*psb*A	0.547*	-0.363*	-0.073
Y-data	30 – LL-*rnp*B, LL-*rbc*L, LL-*psb*A		
X-data	PRO, SYN, EUK, CHL, BACT, LNA, HNA, HBAC, BSIZE, BCC, BMASS, BP, SGR, TD, PKMASS, DEPTH, MLD, OXY, SAL, PRESS, TEM, CON, FLUO, PAR, OBS, BAT, SiO_4, PO_4, NO_X		

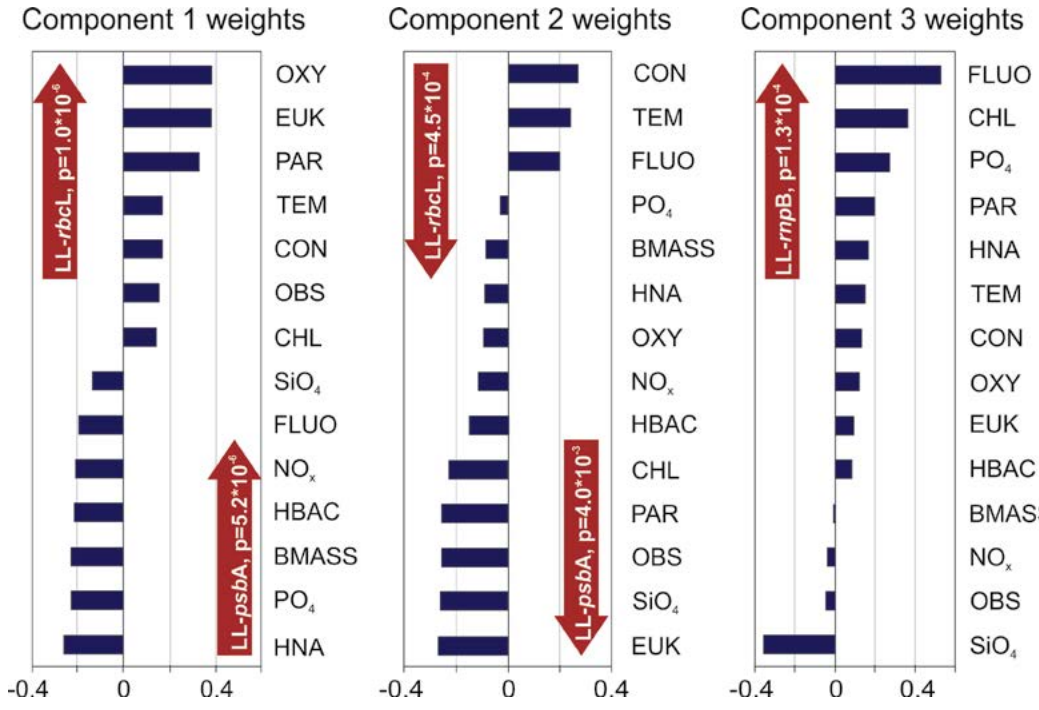


FIGURE IV.8 - DCM+40 PLSR VIPS

Graphic representation of weight values of the different VIPs for the first three components of the model performed with DCM+40 samples. Arrows indicate correlations of each component with different PCR, Y-variables. Direction of the arrows indicates positive (upwards) or negative (downwards) correlations, the corresponding p-values are indicated.

TABLE IV.5 - PAR vs. GENE EXPRESSION

Pearson's correlations between PAR and gene expressions at different depths

	Surface	DCM	DCM+40
30 - HL-*rnp*B	0.035	0.092	ND
30 - LL-*rnp*B	-0.071	0.362***	ND
HL-*rbc*L	0.369***	0.414***	ND
HL-*psb*A	ND	-0.194	0.038
LL-*rbc*L	ND	0.073	0.482***
LL-*psb*A	ND	0.291*	0.412***

ND: non detected, * < 0.05, ** < 0.01, *** < 0.001

*rnp*B transcript abundance for either clade in any sample, *rbc*L and *psb*A relative expression was strongly correlated at DCM (only for the HL clade) and at DCM+40 (LL clade, Table IV.5). This suggests that light intensity is a limiting factor for the photosynthetic activity of HL *Prochlorococcus* strains at DCM and even for LL strains at the DCM+40 levels. The apparent correlation between PAR and *psb*A relative expression at surface is likely related to the protective role of *psb*A protein against photoinactivation of *Prochlorococcus* cells (55, 68-70)

We conclude that even though *Prochlorococcus* photosynthesis responds to key environmental variables such as light, temperature or nutrients, organic pollutants also have a role in its modulation. Our data suggest that at least 20% of *rbc*L expression levels and even a higher proportion of *Prochlorococcus* total abundance (calculated either as cell counts or expressed as *rnp*B Cp values for a given amount of total RNA in an specific volume of sea water) are globally related to the concentration of different pollutants in ocean water, independently from other physical parameters. To our knowledge, these are the first field evidences of a relevant relationship between POPs concentrations and *Prochlorococcus* photosynthetic capability. As these parameters are directly related to the ability of *Prochlorococcus* to fix CO_2, the observed inhibitory effects may influence the entire carbon cycle. More alarmingly, some of our results, like the effects on the maximum yield of PSII and the chlorophyll *a* concentration corresponding to picophytoplankton, suggest that many other primary producers could be also affected by the increasing presence of thousands of POPs in the environment.

IV.4. REFERENCES

1. Field CB (1998) Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. Science 281(5374):237-240.
2. Jennings S, et al. (2008) Global-scale predictions of community and ecosystem properties from simple ecological theory. Proceedings of the Royal Society B: Biological Sciences 275(1641):1375-1383.
3. Lee K, et al. (2003) An updated anthropogenic CO2 inventory in the Atlantic Ocean. Global Biogeochemical Cycles 17(4).
4. Iglesias-Rodriguez MD, et al. (2008) Phytoplankton calcification in a high-CO2 world. science 320(5874):336-340.
5. Partensky F, Hess WR, & Vaulot D (1999) Prochlorococcus, a marine photosynthetic prokaryote of global significance. Microbiology and Molecular Biology Reviews 63(1):106-127.
6. Chisholm SW, et al. (1988) A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334(6180):340-343.

7. Olson RJ, Chisholm SW, Zettler ER, Altabet MA, & Dusenberry JA (1990) Spatial and temporal distributions of prochlorophyte picoplankton in the North Atlantic Ocean. Deep Sea Research Part A, Oceanographic Research Papers 37(6):1033-1051.

8. Liu H, Nolla HA, & Campbell L (1997) Prochlorococcus growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. Aquatic Microbial Ecology 12(1):39-47.

9. Moore LR & Chisholm SW (1999) Photophysiology of the marine cyanobacterium Prochlorococcus: Ecotypic differences among cultured isolates. Limnol. Oceanogr. 44(3 I):628-638.

10. West NJ & Scanlan DJ (1999) Niche-partitioning of Prochlorococcus populations in a stratified water column in the eastern North Atlantic Ocean? Appl Environ Microbiol 65(6):2585-2591.

11. West NJ, et al. (2001) Closely related Prochlorococcus genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides. Microbiology 147(7):1731-1744.

12. Johnson ZI, et al. (2006) Niche partitioning among Prochlorococcus ecotypes along ocean-scale environmental gradients. Science 311(5768):1737-1740.

13. Elser JJ, et al. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecology letters 10(12):1135-1142.

14. Pennington JT, et al. (2006) Primary production in the eastern tropical Pacific: a review. Progress in Oceanography 69(2):285-317.

15. Sarmiento JL, et al. (2004) Response of ocean ecosystems to climate warming. Global Biogeochemical Cycles 18(3):GB3003 3001-3023.

16. Bopp L, et al. (2001) Potential impact of climate change on marine export. Global Biogeochemical Cycles 15(1):81-99.

17. Doney SC (2006) Oceanography: Plankton in a warmer world. Nature 444(7120):695-696.

18. Lutz MJ, Caldeira K, Dunbar RB, & Behrenfeld MJ (2007) Seasonal rhythms of net primary production and particulate organic carbon flux to depth describe the efficiency of biological pump in the global ocean. Journal of Geophysical Research: Oceans (1978–2012) 112(C10).

19. Henson S, Cole H, Beaulieu C, & Yool A (2013) The impact of global warming on seasonality of ocean primary production. Biogeosciences 10(6):4357-4369.

20. Henson SA, et al. (2010) Detection of anthropogenic climate change in satellite records of ocean chlorophyll and productivity. Biogeosciences 7(2):621-640.

21. Boyce DG, Lewis MR, & Worm B (2010) Global phytoplankton decline over the past century. Nature 466(7306):591-596.

22. Rockström J, et al. (2009) A safe operating space for humanity. Nature 461(7263):472-475.

23. Hjorth M, Forbes VE, & Dahllöf I (2008) Plankton stress responses from PAH exposure and nutrient enrichment. Marine Ecology Progress Series 363:121-130.

24. Gilde K & Pinckney JL (2012) Sublethal Effects of Crude Oil on the Community Structure of Estuarine Phytoplankton. Estuaries and Coasts 35(3):853-861.

25. Echeveste P, Agusti S, & Dachs J (2010) Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations. Environ Pollut 158(1):299-307.

26. Echeveste P, Dachs J, Berrojalbiz N, & Agusti S (2010) Decrease in the abundance and viability of oceanic phytoplankton due to trace levels of complex mixtures of organic pollutants. Chemosphere 81(2):161-168.

27. Lohmann R, Breivik K, Dachs J, & Muir D (2007) Global fate of POPs: current and future research directions. Environ Pollut 150(1):150-165.

28. Wania F & Mackay D (1996) Tracking the distribution of persistent organic pollutants. Environ Sci Technol 30(9):390A-397A.

29. Galbán-Malagón C, Cabrerizo A, Caballero G, & Dachs J (2013) Atmospheric occurrence and deposition of hexachlorobenzene and hexachlorocyclohexanes in the Southern Ocean and Antarctic Peninsula. Atmospheric Environment 80:41-49.

30. Del Vento S & Dachs J (2002) Prediction of uptake dynamics of persistent organic pollutants by bacteria and phytoplankton. Environmental Toxicology and Chemistry 21(10):2099-2107.

31. Roche H, et al. (2009) Organochlorines in the Vaccarès Lagoon trophic web (Biosphere Reserve of Camargue, France). Environ Pollut 157(8-9):2493-2506.

32. Berrojalbiz N, et al. (2009) Accumulation and cycling of polycyclic aromatic hydrocarbons in zooplankton. Environ Sci Technol 43(7):2295-2301.

33. Johnson-Restrepo B, Kannan K, Addink R, & Adams DH (2005) Polybrominated diphenyl ethers and polychlorinated biphenyls in a marine foodweb of coastal Florida. Environ Sci Technol 39(21):8243-8250.

34. Persson NJ, et al. (2002) Soot-carbon influenced distribution of PCDD/Fs in the marine environment of the Grenlandsfjords, Norway. Environ Sci Technol 36(23):4968-4974.

35. Lohmann R, Macfarlane JK, & Gschwend PM (2005) Importance of black carbon to sorption of native PAHs, PCBs, and PCDDs in Boston and New York Harbor sediments. Environ Sci Technol 39(1):141-148.

36. Dachs J, et al. (2002) Oceanic biogeochemical controls on global dynamics of persistent organic pollutants. Environ Sci Technol 36(20):4229-4237.

37. Anonymous (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/ 45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155 EEC, 93/67/EEC. (European Union).

38. Altenburger R & Greco WR (2009) Extrapolation Concepts for dealing with multiple contamination in environmental risk assessment. Integrated Environmental Assessment and Management 5(1):62-68.

39. Syberg K, Jensen TS, Cedergreen N, & Rank J (2009) On the use of mixture toxicity assessment in reach and the water framework directive: A review. Human and Ecological Risk Assessment 15(6):1257-1272.

40. Spurgeon DJ, et al. (2010) Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. Science of the Total Environment 408(18):3725-3734.

41. Backhaus T & Faust M (2012) Predictive environmental risk assessment of chemical mixtures: A conceptual framework. Environ Sci Technol 46(5):2564-2573.

42. McCarty LS & Borgert CJ (2006) Review of the toxicity of chemical mixtures containing at least one organochlorine. Regulatory Toxicology and Pharmacology 45(2):104-118.

43. Smith KEC, et al. (2013) Baseline toxic mixtures of non-toxic chemicals: "solubility addition" increases exposure for solid hydrophobic chemicals. Environ Sci Technol 47(4):2026-2033.

44. Tang JYM, et al. (2013) Mixture effects of organic micropollutants present in water: Towards the development of effect-based water quality trigger values for baseline toxicity. Water Research 47(10):3300-3314.

45. Escher BI, Van Daele C, Dutt M, Tang JYM, & Altenburger R (2013) Most oxidative stress response in water samples comes from unknown chemicals: The need for effect-based water quality trigger values. Environ Sci Technol 47(13):7002-7011.

46. 46. Petersen DG & Dahllöf I (2007) Combined effects of pyrene and UV-light on algae and bacteria in an arctic sediment. Ecotoxicology 16(4):371-377.

47. Grote M, Schüürmann G, & Altenburger R (2005) Modeling photoinduced algal toxicity of polycyclic aromatic hydrocarbons. Environ Sci Technol 39(11):4141-4149.

48. Echeveste P, Agusti S, & Dachs J (2011) Cell size dependence of additive versus synergetic effects of UV radiation and PAHs on oceanic phytoplankton. Environ Pollut 159(5):1307-1316.

49. Diamond SA (2003) Photoactivated toxicity in aquatic environments. UV Effects in Aquatic Organisms and Ecosystems. New York: Johm Wiley & Sons, Inc:219-250.

50. Geider RJ (1987) Light and temperature dependence of the carbon to chlorophyll a ratio in microalgae and cyanobacteria: implications for physiology and growth of phytoplankton. New Phytologist:1-34.

51. John DE, Patterson SS, & Paul JH (2007) Phytoplankton-group specific quantitative polymerase chain reaction assays for RuBisCO mRNA transcripts in seawater. Mar Biotechnol (NY) 9(6):747-759.

52. John DE, et al. (2007) Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea CO(2) flux in the Mississippi River plume. ISME J 1(6):517-531.

53. Pichard SL, Campbell L, Kang JB, Tabita FR, & Paul JH (1996) Regulation of ribulose bisphosphate carboxylase gene expression in natural phytoplankton communities. I. Diel rhythms. Marine Ecology Progress Series 139(1-3):257-265.

54. Pichard SL, et al. (1997) Analysis of ribulose bisphosphate carboxylase gene expression in natural phytoplankton communities by group-specific gene probing. Marine Ecology Progress Series 149(1-3):239-253.

55. Zinser ER, et al. (2009) Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, Prochlorococcus. PLoS ONE 4(4).

56. Fernández-Pinos MC, et al. (2015) Clade-specific quantitative analysis of photosynthetic gene expression in prochlorococcus. PLoS ONE 10(8).

57. Bruyant F, et al. (2005) Diel variations in the photosynthetic parameters of Prochlorococcus strain PCC 9511: Combined effects of light and cell cycle. Limnol. Oceanogr. 50(3):850-863.

58. Claustre H, et al. (2002) Diel variations in Prochlorococcus optical properties. Limnol. Oceanogr. 47(6):1637-1647.

59. Steglich C, et al. (2010) Short RNA half-lives in the slow-growing marine cyanobacterium Prochlorococcus. Genome Biol 11(5):R54.

60. Livak KJ & Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25(4):402-408.

61. Pfaffl M (2006) Relative quantification. Real-time PCR, ed Dorak MT (Taylor & Francis, New York (USA)).

62. Benjamini Y & Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57(1):289-300.

63. Wold S, Sjöström M, & Eriksson L (2001) PLS-regression: a basic tool of chemometrics. Chemometrics and intelligent laboratory systems 58(2):109-130.

64. Zinser ER, et al. (2007) Influence of light and temperature on Prochlorococcus ecotype distributions in the Atlantic Ocean. Limnol. Oceanogr. 52(5):2205-2220.

65. Kent AG, Dupont CL, Yooseph S, & Martiny AC (2016) Global biogeography of Prochlorococcus genome diversity in the surface ocean. The ISME Journal.

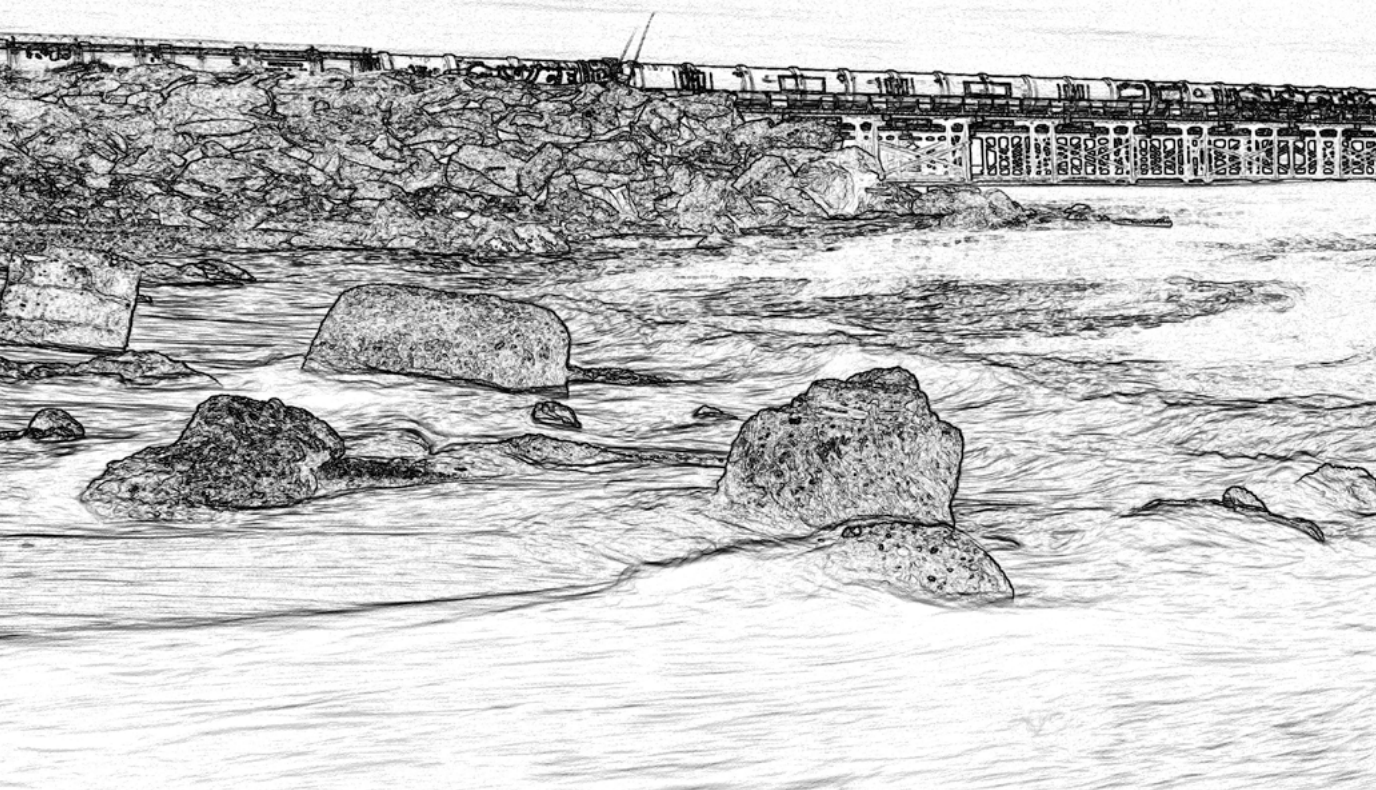
66. Garczarek L, et al. (2007) High vertical and low horizontal diversity of Prochlorococcus ecotypes in the Mediterranean Sea in summer. FEMS Microbiol Ecol 60(2):189-206.

67. Moore LR, Goericke R, & Chisholm SW (1995) Comparative physiology of Synechococcus and Prochlorococcus: Influence of light and temperature on growth, pigments, fluorescence and absorptive properties. Marine Ecology Progress Series 116(1-3):259-276.

68. Berg GM, et al. (2011) Responses of psbA, hli and ptox genes to changes in irradiance in marine Synechococcus and Prochlorococcus. Aquatic Microbial Ecology 65(1):1-14.

69. Mulo P, Sirpiö S, Suorsa M, & Aro E-M (2008) Auxiliary proteins involved in the assembly and sustenance of photosystem II. Photosynthesis research 98(1-3):489-501.

70. García-Fernández JM, Hess WR, Houmard J, & Partensky F (1998) Expression of the psbA gene in the marine oxyphotobacteria Prochlorococcus spp. Archives of Biochemistry and Biophysics 359(1):17-23.



Chapter V

General conclusions and recommendations for future research

V.1. GENERAL CONCLUSIONS

This thesis contributes to the understanding of the interactions between POPs and carbon cycles, focusing specially in the effects of organic pollutants found in seawater on the marine primary production. The use of *Prochlorococcus* as sentinel organism allowed us to assess these interactions at a global level, given the genus distribution and abundance. This assessment was possible due to the development of specific molecular methods coping with the variety of *Prochlorococcus* strains. These methods are based on quantifying the expression of two functional genes needed for photosynthesis and that can be used as proxies of the CO_2 fixation and the functionality of the PSII in the cell, respectively. Using this approach, we were able to detect changes in *Prochlorococcus* photosynthetic capability at sublethal level when cells were exposed to a number of environmental conditions, including the presence of organic pollutants. The application of the method to both laboratory cultures and wild populations showed a remarkable influence of organic pollutants on the photosynthesis modulation of *Prochlorococcus*. This influence of organic pollutant was higher as more complex was the mixture of organic pollutants present in seawater. There are also evidences of a probable widespread impact of organic pollutants on the rest of the marine photosynthetic community.

In summary, the most important lesson we have learned from this thesis is that beyond the well-known determinants of photosynthesis such as radiation and nutrients, organic pollutants are also modulating the magnitude of this important ecological function. In the scenario of current Global Change, little attention has been paid to organic pollutants up to now. However, our results indicate that, if we want to achieve a comprehensive understanding of the multiple anthropogenic perturbations of the Earth system, we must consider organic pollutants as a driver of environmental change. Hence, the present work opens new ground by showing a methodological approach using target genes of photosynthesis, and pointing out to interactions between organic pollutants and photosynthesis, which should trigger further research in the future. These future studies should aim to a more integrative and comprehensive approach of the different perturbations of the key ecosystem functions in the field.

The main conclusions obtained from this work are summarized below:

1. Development of a feasible and inexpensive collection methodology to test Prochlorococcus field populations suitable for qRT-PCR analysis:
   - We designed a feasible and simple collection methodology under the conditions of a seven-months circumnavigation campaign.
   - This collection methodology ensured minimal degradation or changes on mRNA levels during sampling and guaranteed the integrity of nucleic acid for several months.
2. Design of a high-throughput mRNA analysis method to quantify the photosynthetic capability of *Prochlorococcus*:
   - We generated a simple, quantitative, and low cost mRNA analysis method useful to detect physiological alterations induced by environmental variables on *Prochlorococcus* photosynthetic capability.
   - This method resulted to be fairly specific for both cultured and wild Prochlorococcus strains, and allowed us to detect possible *Synechococcus* cross amplification.
3. Organic pollutant effects on *Prochlorococcus* photosynthetic capability tested in pure cultures of MED4 and MIT9313 strains:
   - Despite the exposure to organic pollutants did no affect cellular parameters or genomic DNA, it caused a decrease of 20% in the CO_2 fixation capability of MED4 and MIT9313 strains.
   - We detected some differences in the timing and amplitude of this decrease in the CO_2 fixation capability between both strains.
   - We also found a decrease in the *rbc*L/*psb*A ratio of both strains when exposed to pollutants, which may mean a limitation of the electron transport.
4. Effects of organic pollutant mixtures on wild *Prochlorococcus* populations under controlled conditions:

- When natural communities from DCM were exposed to either a PAH mixture, an OClP mixture or a complex mixture (CM), none of the treatments induced cellular death of *Prochlorococcus*, *Synechococcus* or picoeukaryotes.
- No significant effects on *Prochlorococcus* photosynthetic capability were observed with the addition of OClPs at concentrations between 260 and 470 times the environmental concentrations.
- An increase of PAHs between 70 and 150 times over the environmental levels caused a chronic toxicity pattern in the PSII functionality of *Prochlorococcus*.
- A small increase lower than 2-fold over the environmental concentration of the complex mixture of organic pollutants present in seawater resulted in an acute toxic effect on the CO_2 fixation capability of *Prochlorococcus*.
- These results suggest the existence of uncharacterised organic pollutants in the CM that could contribute to the inhibitory effects, and support the hypothesis about synergistic effects by every organic compounds being part of the CM.

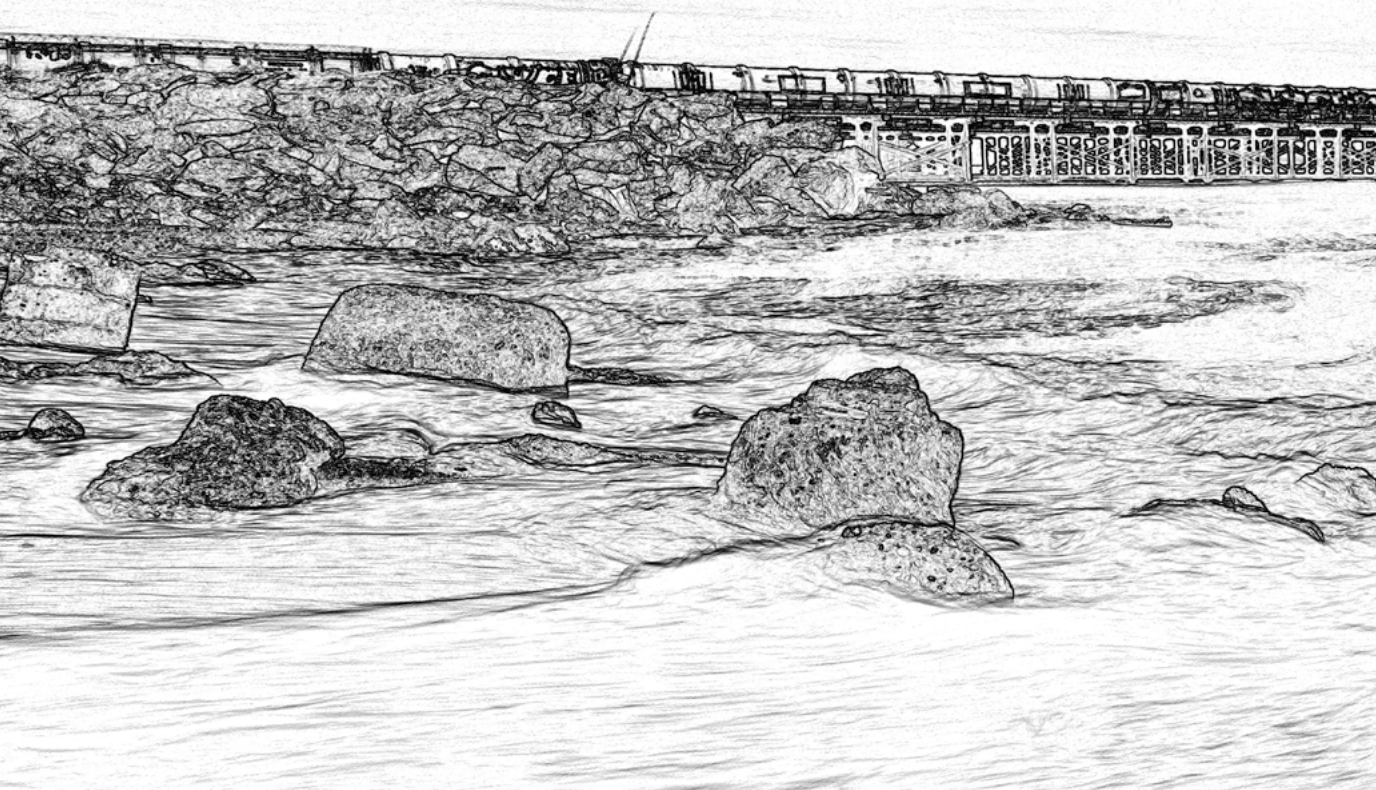
5. Influence of physical, biological and chemical environmental variables on *Prochlorococcus* photosynthetic capability in wild populations sampled during the Malaspina circumnavigation
   - Light conditions highly influenced *Prochlorococcus* photosynthetic capability at the three sampled depths, although it was more limiting for HL strains that for LL strains.
   - The contribution of *Prochlorococcus* to the total photosynthesis was especially important at 3 m and DCM+40 m.
   - The positive correlation between HL-*rnp*B Cp values and *Prochlorococcus* cell abundance at 3 m corroborated the effectiveness of *rnp*B as reference gene.
   - Organic pollutants present in seawater also had an important role on the modulation of its photosynthetic capability, reducing up to 20% the CO_2 fixation capability of *Prochlorococcus*.

- Several bioavailable PAHs resulted negatively correlated to both *Prochlorococcus* gene expression data and some parameters from the entire photosynthetic communities. These result suggest that the inhibitory effects of organic pollutants on photosynthesis may be not only limited to *Prochlorococcus* but they could affect other primary producers.
- This work demonstrates for the first time the potential of the organic pollutant mixture already present in seawater to alter the oceanic primary production at a global scale, becoming a relevant driver of Global Change to be concerned about.

V.2. RECOMMENDATIONS FOR FUTURE RESEARCH

This thesis should be useful as a point of departure to achieve a comprehensive view about the influence of organic pollutants present in seawater on the oceanic primary production. However some improvements can be helpful for a better understanding of this interaction:

- More experimental efforts are needed to quantify the concentration of tracer organic pollutants deeper in the water column, as current available data sets focus on surface concentrations.
- Additional works on the characterization of unknown organic compounds being part of the complex mixture of organic pollutants present in the environment would help to understand its toxicity mechanism.
- Further experiments to assess the possible synergy between the organic pollutants found in seawater are needed for a better understanding of organic pollutants effects, and how these interact with other environmental variables.
- Using novel molecular tools such as metatranscriptomics could generate much more information about the physiological state of the whole picophytoplanctonic community.



Annex I

Supporting information of chapter II

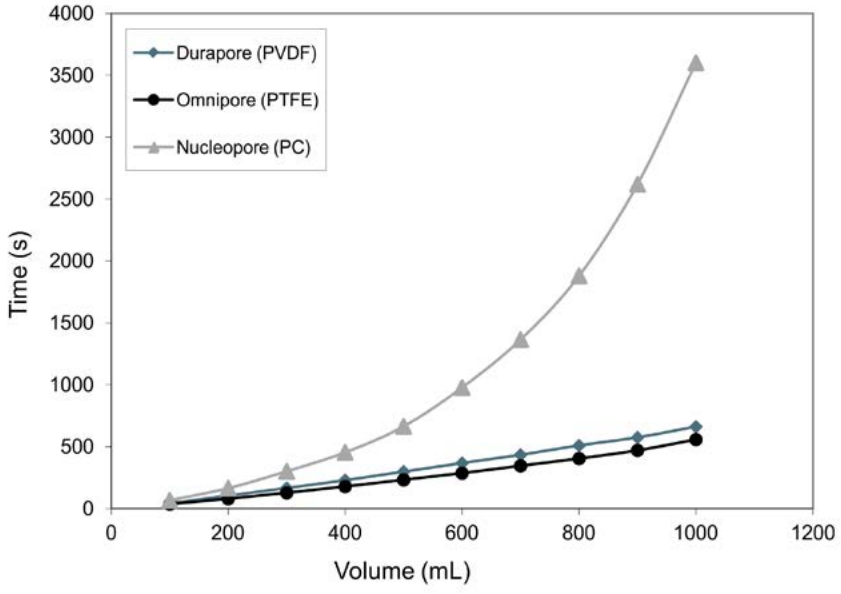


FIGURE AI.1 - PERMEABILITY TESTS

Three filters of 0.2 µm pore-size and 47 mm of diameter made of different materials were used. Aliquots of 1 L of a seawater sample collected on 08/11/10 in Mediterranean Sea (41 39.7 N 02 54.6) were filtered onto each filter.

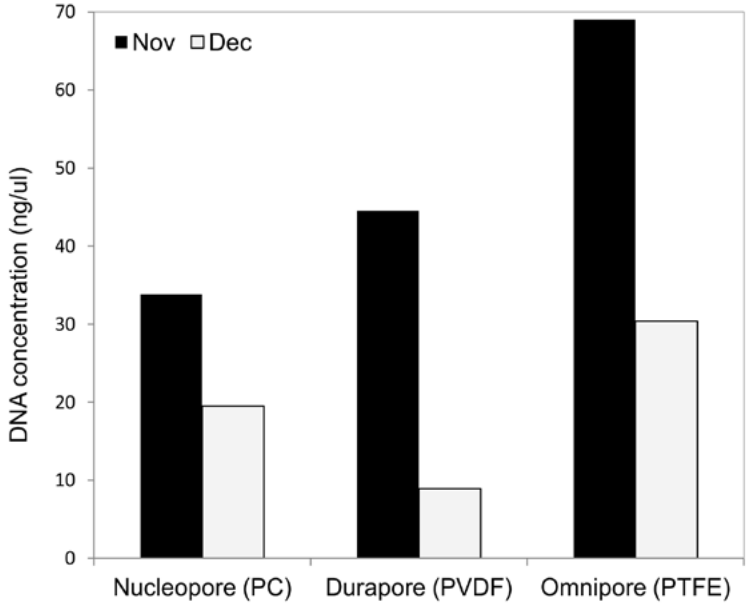


FIGURE AI.2 - DNA RECOVERY

Two water samples of Mediterranean Sea (41 39.7 N 02 54.6) were collected on 11/11/2010 (Nov) and 16/12/2010 (Dec), respectively, using three filters of 0.2 µm pore size and 47 mm of diameter made of different materials. 1 L of water was filtered through each filter.

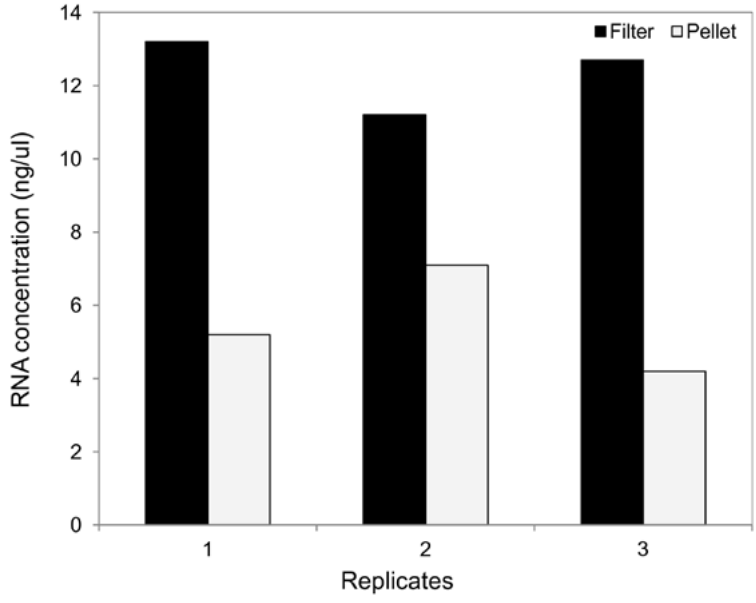


FIGURE AI.3 - RNA RECOVERY

RNA isolated from both the filter and the pellet resulting from centrifugation of RNAlater where the filter was immersed. Three replicates of 1 L of Mediterranean Sea (41 39.7 N 02 54.6) water filtered onto Ominipore (PTFE) filters are shown. RNA concentration obtained from Omnipore (PTFE) filter is between two and three fold that obtained from the pellet.

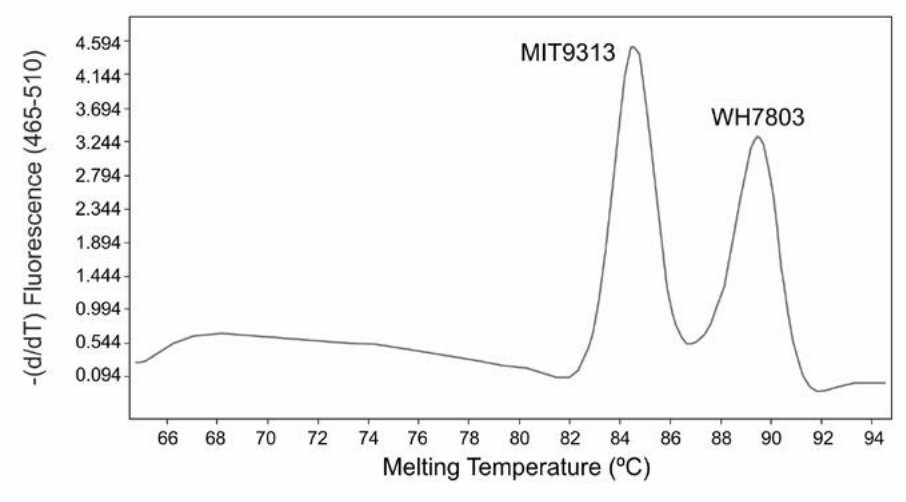


FIGURE AI.4 - GENETIC MARKER FOR *SYNECHOCOCCUS*

The graphs shows melting temperature curves of amplicons using rbcL LL primers. Melting peaks for *Prochlorococcus* str. MIT9313 and *Synechococcus* str. WH7803 in the same sample are shown

TABLE AI.1 - *PROCHLOROCOCCUS* AND *SYNECHOCOCCUS* STRAINS

Prochlorococcus and *Synechococcus* strains used to design specific primers for *Prochlorococcus* of the selected genes by sequences alignments using Software Geneious.

Strain	Species/ Ecotype	GenBank accession ID
AS9601	*Prochlorococcus*: High-light	CP000551
MED4	*Prochlorococcus*: High-light	BX548174
MIT9301	*Prochlorococcus*: High-light	CP000576
MIT9312	*Prochlorococcus*: High-light	CP000111
MIT9515	*Prochlorococcus*: High-light	CP000552
MIT9215	*Prochlorococcus*: High-light	CP000825
MIT9313	*Prochlorococcus*: Low-light	BX548175
MIT9303	*Prochlorococcus*: Low-light	CP000554
MIT9211	*Prochlorococcus*: Low-light	CP000878
NATL1A	*Prochlorococcus*: Low-light	CP000553
NATL2A	*Prochlorococcus*: Low-light	CP000095
SS120	*Prochlorococcus*: Low-light	AE017126
CC9311	*Synechococcus*	CP000435
CC9902	*Synechococcus*	CP000097
RCC307	*Synechococcus*	CT978603
CC9605	*Synechococcus*	CP000110
WH7803	*Synechococcus*	CT971583

TABLE AI.2 - MALASPINA SAMPLED STATIONS

Coordinates and collection data of field samples collected during Malaspina cruise.

Station Number	Longitude (DD)	Latitude (DD)	Collection Date (dd-mm-yy)	DCM Depth (m)	Collection local time (3 m depth sample)	Collection local time (DCM & DCM+40 samples)
1	-9.5	35.2	16-12-10	130	9:44	11:43
2	-26.0	9.6	28-12-10	70	8:50	11:11
3	-26.0	5.0	30-12-10	120	8:16	10:35
4	-26.0	0.3	01-01-11	62	8:30	11:05
5	-28.2	-4.8	03-01-11	110	9:30	12:00
6	-30.2	-9.1	05-01-11	150	9:30	11:50
7	-32.4	-13.7	07-01-11	150	9:23	12:09
8	-34.7	-18.4	09-01-11	130	9:20	11:55
9	-37.0	-23.0	11-01-11	150	9:32	12:10
10	-36.2	-24.3	19-01-11	119	8:00	11:00
11	-33.1	-24.9	20-01-11	120	8:40	11:22
12	-30.1	-25.4	21-01-11	120	9:00	10:30
13	-21.4	-26.9	24-01-11	125	8:45	11:15
14	-14.8	-28.1	26-01-11	150	8:40	11:04
15	-5.4	-29.7	29-01-11	110	8:45	11:27
16	3.7	-31.3	01-02-11	70	8:00	12:00
17	6.8	-31.8	02-02-11	85	9:00	11:00
18	9.4	-32.1	03-02-11	72	8:45	11:45
19	12.7	-32.8	04-02-11	48	8:45	11:45
20	15.5	-33.3	05-02-11	44	8:00	10:00
21	27.5	-34.8	14-02-11	96	8:30	11:52
22	33.7	-34.2	16-02-11	75	7:52	9:40
23	39.9	-33.5	18-02-11	125	8:26	9:42
24	43.2	-33.2	19-02-11	110	7:46	9:57
25	61.5	-30.1	24-02-11	130	8:15	10:22
26	63.2	-28.0	25-02-11	112	8:38	10:38
27	69.4	-29.4	27-02-11	130	9:11	11:04
28	76.1	-29.9	01-03-11	140	8:12	10:48

29	82.6	-29.8	03-03-11	135	8:12	10:08
30	89.5	-29.7	05-03-11	120	8:05	10:12
31	96.4	-29.6	07-03-11	114	8:09	10:41
32	103.3	-30.3	09-03-11	100	8:13	10:29
33	110.2	-31.2	11-03-11	90	8:16	10:24
34	135.2	-39.2	24-03-11	70	8:30	11:02
35	-178.2	-23.4	20-04-11	110	8:40	11:22
36	-176.9	-20.6	21-04-11	110	–	11:21
37	-174.5	-15.9	23-04-11	105	8:05	9:41
38	-172.3	-9.5	26-04-11	115	7:20	10:20
39	-168.4	-1.3	30-04-11	65	7:20	10:32
40	-165.8	3.8	02-05-11	80	7:15	9:43
41	-164.4	7.0	03-05-11	80	8:50	8:12
42	-160.9	15.0	06-05-11	140	10:05	9:51
43	-150.4	21.1	16-05-11	105	7:17	10:29
44	-145.2	20.3	18-05-11	–	7:05	–
45	-139.0	19.3	20-05-11	130	7:19	10:42
46	-133.3	18.1	22-05-11	125	7:16	10:32
47	-127.6	16.6	24-05-11	100	9:25	10:04
48	-122.0	15.3	26-05-11	137	7:22	10:26
49	-115.8	13.8	28-05-11	90	7:20	10:05
50	-110.4	12.5	30-05-11	125	7:19	11:36
51	-102.4	10.8	02-06-11	37	7:33	9:52
52	-96.3	9.4	04-06-11	19	7:20	10:09
53	-93.1	8.8	05-06-11	24	7:18	11:20
54	-87.9	7.2	07-06-11	20	8:34	10:11
55	-69.3	15.1	22-06-11	95	7:08	10:52
56	-55.2	19.0	27-06-11	140	7:20	10:58
57	-47.8	21.7	30-06-11	120	7:46	11:04
58	-41.9	23.7	02-07-11	130	7:18	10:04
59	-35.3	26.1	04-07-11	150	7:15	10:07
60	-29.7	28.0	06-07-11	140	7:13	10:13
61	-23.7	30.0	08-07-11	100	7:20	10:23
62	-17.3	32.1	10-07-11	110	7:14	10:20

TABLE AI.3 - qRT-PCR ANALYSIS

Specificity of the designed primers checked by qRT-PCR using pure cultures of some *Prochlorococcus* and *Synechococcus* strains at equal cell concentrations and verification of the method validity in natural communities*.

			High-light primers							
			*rnp*B			*rbc*L			*psb*A	
			Cp	Tm_1	Tm_2	Cp	Tm_1	Tm_2	Cp	
Axenic cultures	HL *Prochlorococcus*	MED4	17.38	81.14	n/d	20.04	84.96	n/d	15.72	
		MIT9515	14.86	81.05	n/d	18.41	84.53	n/d	13.18	
		EQPAC1-C	19.26	80.52	n/d	18.90	84.82	n/d	17.09	
	LL *Prochlorococcus*	MIT9313	32.75	80.96	n/d	33.45	87.36	81.59	27.98	
		NATL2A	30.11	80.95	n/d	31.37	84.97	n/d	29.67	
	Synechococcus	WH7803	32.81	81.05	n/d	33.14	84.41	81.90	28.66	
	MIT9313 + WH7803 (equal cell concentration)		33.48	81.29	n/d	33.52	87.47	87.57	29.91	
Field samples	Atlantic	3 m	21.08	81.31	n/d	21.49	84.27	n/d	18.82	
		DCM	21.21	81.08	n/d	21.59	83.77	n/d	17.87	
		DCM+40	27.84	81.03	n/d	29.00	84.08	n/d	25.26	
	Indian	3 m	20.76	81.29	n/d	21.42	83.78	n/d	15.87	
		DCM	23.38	81.34	n/d	25.78	84.75	n/d	21.69	
		DCM+40	30.39	81.54	n/d	31.78	85.03	n/d	25.56	
	Pacific 1	3 m	20.99	81.09	n/d	20.05	83.09	n/d	16.78	
		DCM	22.59	80.77	n/d	25.83	83.31	n/d	21.59	
		DCM+40	26.05	81.30	n/d	29.04	83.71	n/d	23.44	
	Pacific 2	3 m	18.88	80.38	n/d	17.67	83.69	n/d	15.04	
		DCM	19.59	80.09	n/d	18.97	83.69	n/d	14.29	
		DCM+40	23.75	80.48	n/d	26.30	83.99	n/d	20.28	

* Second derivate maximum of the amplification curves (Cp; number of cycle) are shown as calculated for 1.1 ng/ml of cDNA per qPCR reaction by averaging of triplicates. Cp values were calculated by the Second Derivate Maximum method. Melting temperatures (Tm; °C) are shown for the main PCR product (Tm_1) and the minor one (Tm_2), in the case that two different products (i.e. different sequences) are amplified. When Tm > 88 °C for *rbc*L LL primers the PCR product is considered to belong to *Synechococcus* (red asterisk *). n/d means value not detected.

Low -light primers										
*psb*A		*rnp*B			*rbc*L			*psb*A		
Tm_1	Tm_2	Cp	Tm_1	Tm_2	Cp	Tm_1	Tm_2	Cp	Tm_1	Tm_2
81.75	n/d	34.09	77.00	n/d	37.80	78.00	n/d	31.89	87.11	84.32
81.60	n/d	33.49	87.92	84.28	31.52	84.73	n/d	33.14	87.32	83.90
81.58	n/d	30.85	84.88	87.87	37.93	81.14	78.90	34.95	83.43	77.54
87.99	n/d	15.40	87.98	n/d	18.29	84.92	n/d	14.08	84.69	n/d
81.59	n/d	18.09	86.90	n/d	21.02	81.30	n/d	14.14	84.92	n/d
81.35	n/d	17.83	88.01	n/d	28.27	89.62*	n/d	28.26	84.53	78.92
81.43	n/d	15.49	88.03	n/d	23.14	84.60	89.38*	16.25	86.82	n/d
81.99	n/d	26.56	88.80	n/d	34.02	85.75	88.88*	28.67	87.85	n/d
81.42	n/d	20.87	85.31	n/d	23.83	82.92	n/d	17.72	84.1	n/d
81.67	n/d	24.12	84.95	87.83	27.44	84.61	n/d	22.56	85.45	n/d
81.50	n/d	29.06	88.98	85.46	31.97	89.38*	86.54	25.73	86.87	n/d
81.87	n/d	18.46	85.97	n/d	22.42	83.09	n/d	16.41	84.32	n/d
82.22	n/d	19.35	84.99	n/d	24.99	83.61	n/d	18.56	84.57	n/d
81.76	n/d	26.06	86.63	89.15	27.94	83.11	87.96	22.96	84.30	87.59
81.68	n/d	18.55	85.74	n/d	23.74	83.21	n/d	17.80	84.35	n/d
81.63	n/d	19.15	85.06	n/d	24.07	83.45	n/d	18.55	84.49	n/d
82.00	n/d	22.05	88.90	n/d	32.02	88.52*	82.93	24.98	88.03	84.22
81.99	n/d	20.50	89.07	85.36	34.18	82.64	86.04	29.12	84.15	n/d
82.00	n/d	18.03	87.49	n/d	24.80	86.09	n/d	16.49	87.30	n/d

TABLE AI.4 - FIELD SAMPLE SEQUENCING

Results from sequencing of single amplicons from samples collected at three different depths and three sampled station during Malaspina circumnavigation.

Station[a]	Depth	Amp.[b]	rnpB_HL	Iden. (%)[c]	rbcL_HL	Iden. (%)[c]	psbA_HL
Atlantic	3 m	1	MIT9312 (HL)	100	MIT9515 (HL)	100	MIT9215, MIT9301, MIT9302 (HL)
		2	MIT9312 (HL)	100	MIT9515 (HL)	100	MIT9215, MIT9301, MIT9302 (HL)
	DCM	1	MIT9312 (HL)	100	MIT9515 (HL)	98	MIT9215, MIT9301, MIT9302 (HL)
		2	MIT9312 (HL)	100	MIT9515 (HL)	100	MIT9215, MIT9301, MIT9302 (HL)
	DCM40	1	MIT9312 (HL)	98	MIT9515 (HL)	100	MIT9215, MIT9301, MIT9302 (HL)
		2	MIT9312 (HL)	100	AS9601 (HL)	98	MIT9215, MIT9301, MIT9302 (HL)
Indian	3 m	1	MIT9312 (HL)	98	MIT9215, MIT9515 (HL)	98	MED4, AS9601, MIT9312, MIT9116 (HL)
		2	MIT9312 (HL)	100	MED4 (HL)	98	MED4, MIT9116 (HL)
	DCM	1	MIT9212 (HL)	96	MED4(HL)	97	MED4, MIT9312, MIT9116 (HL)
		2	MIT9312 (HL)	94	MIT9515 (HL)	95	MED4, MIT9312, MIT9116 (HL)
	DCM40	1	No amplification		MIT9515 (HL)	90	MIT9215, MIT9301, MIT9302 (HL)
		2	No amplification		MIT9515 (HL)	98*	MIT9215, MIT9301, MIT9302 (HL)
Pacific1	3 m	1	MIT9312 (HL)	100	MIT9515 (HL)	98	MIT9215, MIT9301, MIT9302 (HL)
		2	MIT9312 (HL)	100	MIT9515 (HL)	98	MIT9215, MIT9301, MIT9302 (HL)
	DCM	1	MIT9312 (HL)	100	MIT9515 (HL)	98	MIT9215, MIT9301, MIT9302 (HL)
		2	MIT9312 (HL)	100	MIT9515 (HL)	98	MIT9215, MIT9301, MIT9302 (HL)
	DCM40	1	MIT9312 (HL)	100	MIT9515 (HL)	97	MIT9215, MIT9301, MIT9302 (HL)
		2	MIT9312 (HL)	100	MIT9515 (HL)	94	MIT9215, MIT9301, MIT9302 (HL)
Pacific2	3 m	1	MIT9301, AS9601, MIT9312 (HL)	99-100	AS9601 (HL)	99	MIT9215, MIT9301, MIT9302 (HL)
	DCM	1	AS9601, TATL2 (HL)	93	MIT9301 (HL)	98	MIT9301, MIT9302(HL)
	DCM40	1	TAK9803 (HL)	99	MIT9301, MIT9515, AS9601 (HL)	94	MIT9215, MIT9301, MIT9312, MIT9302 (HL)

a) Station data in are shown in Table II.2. b) Two qRT-PCR products were sequenced for each sample from Atlantic, Indian and Pacific1 stations, and only one for Pacific2 samples. c) Results of maximum score from BLAST are shown. All sequences showed a coverage

Iden. (%) [c]	rnpB_LL	Iden. (%) [c]	rbcL_LL	Iden. (%) [c]	psbA_LL	Ident (%) [c]
99	No similarity found		*Syn.* CB0103, CB0102	93	*Syn*. RSS9907	95
96	MIT9313, MIT9211, MIT9303 (LL); *Syn.*: WH7803, CC9311, CC9605, CC9902, WH8102, PCC7001	87*	*Syn.* CB0205	89	*Syn*. RSS9907	94
98	NATL2A (LL)	95	SS120 (LL)	89	NATL2A, NATL1A (LL)	98
98	NATL2A (LL)	94	MIT9313 (LL)	89	NATL2A, NATL1A (LL)	98
96	MIT9303 (LL)	85*	SS120 (LL)	88	MIT9211 (LL)	88
98	MIT9303 (LL)	85	MIT9313 (LL)	90	MIT9211 (LL)	87
98	NATL2A. NATL1A, PAC1B, PAC1A (LL)	96*	NATL2A, NATL1A (LL)	85	*Syn*. WH8109, RSS9907	97
94	MIT9313, MIT9211, MIT9303 (LL); *Syn.*: WH7803, CC9311, CC9605, CC9902, WH8102, PCC7001	92	*Syn*. WH7803, WH8102, WH8108, WH8104, WH8103, WH8008, WH8006, WH7805	95*	*Syn*. WH8109, RSS9907	96
98	NATL2A. NATL1A, PAC1B, PAC1A (LL)	92-98	NATL2A, NATL1A (LL)	91	NATL2A, NATL1A (LL)	96
97	NATL2A. NATL1A, PAC1B, PAC1A (LL)	98	NATL2A, NATL1A (LL)	92	NATL2A, NATL1A (LL)	97
91	NATL2A. NATL1A, PAC1B, PAC1A (LL)	96	MIT9313 (LL)	88	NATL2A, NATL1A (LL)	96
92	NATL2A. NATL1A, PAC1B, PAC1A (LL)	95	MIT9313 (LL)	91	NATL2A, NATL1A (LL)	94
98	NATL2A. NATL1A, PAC1B, PAC1A (LL)	84	NATL2A, NATL1A (LL)	95	NATL2A, NATL1A (LL)	96
97	NATL2A. NATL1A, PAC1B, PAC1A (LL)	94	NATL2A, NATL1A (LL)	92	NATL2A, NATL1A (LL)	92
99	NATL2A. NATL1A, PAC1B, PAC1A (LL)	93	NATL2A, NATL1A (LL)	92	NATL2A, NATL1A (LL)	96
98	NATL2A. NATL1A, PAC1B, PAC1A (LL)	94	NATL2A, NATL1A (LL)	93	NATL2A, NATL1A (LL)	98
94	NATL2A. NATL1A, PAC1B, PAC1A (LL)	92	MIT9313 (LL)	91	NATL2A, NATL1A (LL)	93
97	NATL2A. NATL1A, PAC1B, PAC1A (LL)	95	MIT9313 (LL)	89	NATL2A, NATL1A (LL)	94
98	*Syn*: CC9605, CC9902	99	NATL2A, NATL1A (LL)	93	NATL2A, NATL1A (LL)	97
99	NATL2A. NATL1A, PAC1B, PAC1A (LL)	95	MIT9313, MIT9303 (LL)	89	NATL2A, NATL1A (LL)	97
97	MIT9211, MIT9303 (LL), SYN.WH8102	85	MIT9313 (LL)	87	NATL2A, NATL1A (LL)	89

higher than 70% except the marked with " * ", that showed a coverage between 58-69%.
d) *Synechococcus* strains are indicated as "Syn".

TABLE AI.5 - CELL CONCENTRATIONS ANALYSIS

Paired-sample Wilcoxon signed rank tests achieved with control-treatment pairs of cell concentration measured

Strain	Treatment	Cell concentration Paired-Sample Wilcoxon Signed Rank Test	
		N	Sig.
MED4	PAHs	6	0.753
	OCIP	6	0.917
MIT9313	PAHs	6	0.917
	OCIP	6	0.345

TABLE AI.6 - CHLOROPHYLL ANALYSIS

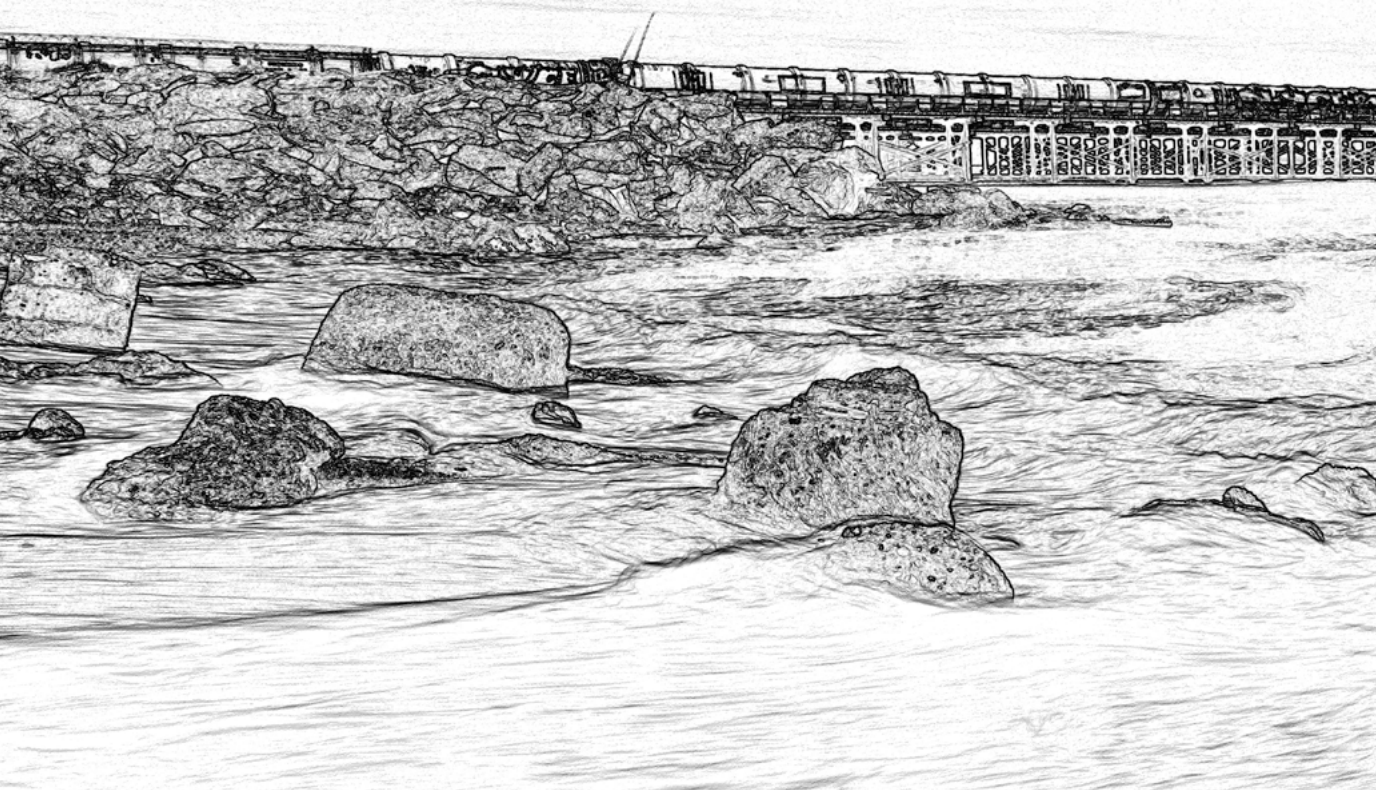
Results from comparing means test achieved with control- treatment pairs of chlorophyll *a* fluorescence

		Fluorescence Paired T-test	
		df	Sig. (2-tailed)
All experiments	All	20	0.679
Strain	MED4	10	0.341
	MIT9313	10	0.765
Treatment	PAHs	8	0.888
	OCIP	11	0.508
Exposure time	0.5h	9	0.898
	24h	11	0.458

TABLE AI.7 - GENOMIC DNA ANALYSIS

Relative changes in DNA abundance for different genes in MIT9313 and MED4 *Prochlorococcus* cultures when challenged with organic pollutant treatments (PAHs or OCIP), evaluated at two different incubation times (0.5 and 24 hours). Results from four different paired t-tests are shown: one of the whole data set ("All samples") and one of each data subset ("Strain", "Treatment" and "Incubation time").

		Average fold change (95% confidence limits)[a]			n^b
Ratios		***rbc*L**	***psb*A**	***rbc*L/*psb*A**	
All samples		0.97 (0.92- 1.03)	0.98 (0.91- 1.06)	0.99 (0.92- 1.07)	22
Strain	MIT9313	0.92 (0.84- 1.01)	1.00 (0.86- 1.17)	0.92 (0.80- 1.05)	10
	MED4	1.03 (0.97- 1.09)	0.97 (0.90- 1.04)	1.06 (1.00- 1.13)	12
Treatment	PAHs	0.98 (0.91- 1.06)	1.03(0.93- 1.14)	0.95 (0.86- 1.06)	12
	OCIP	0.96 (0.86- 1.07)	0.90 (0.83- 0.97)*	1.07 (0.97- 1.17)	10
Incubation time	0.5h	0.98 (0.88- 1.08)	1.01 (0.88- 1.16)	0.97 (0.87- 1.08)	11
	24h	0.97 (0.90- 1.04)	0.96 (0.87- 1.06)	1.01 (0.89- 1.15)	11



Annex II

Supporting information of chapter III

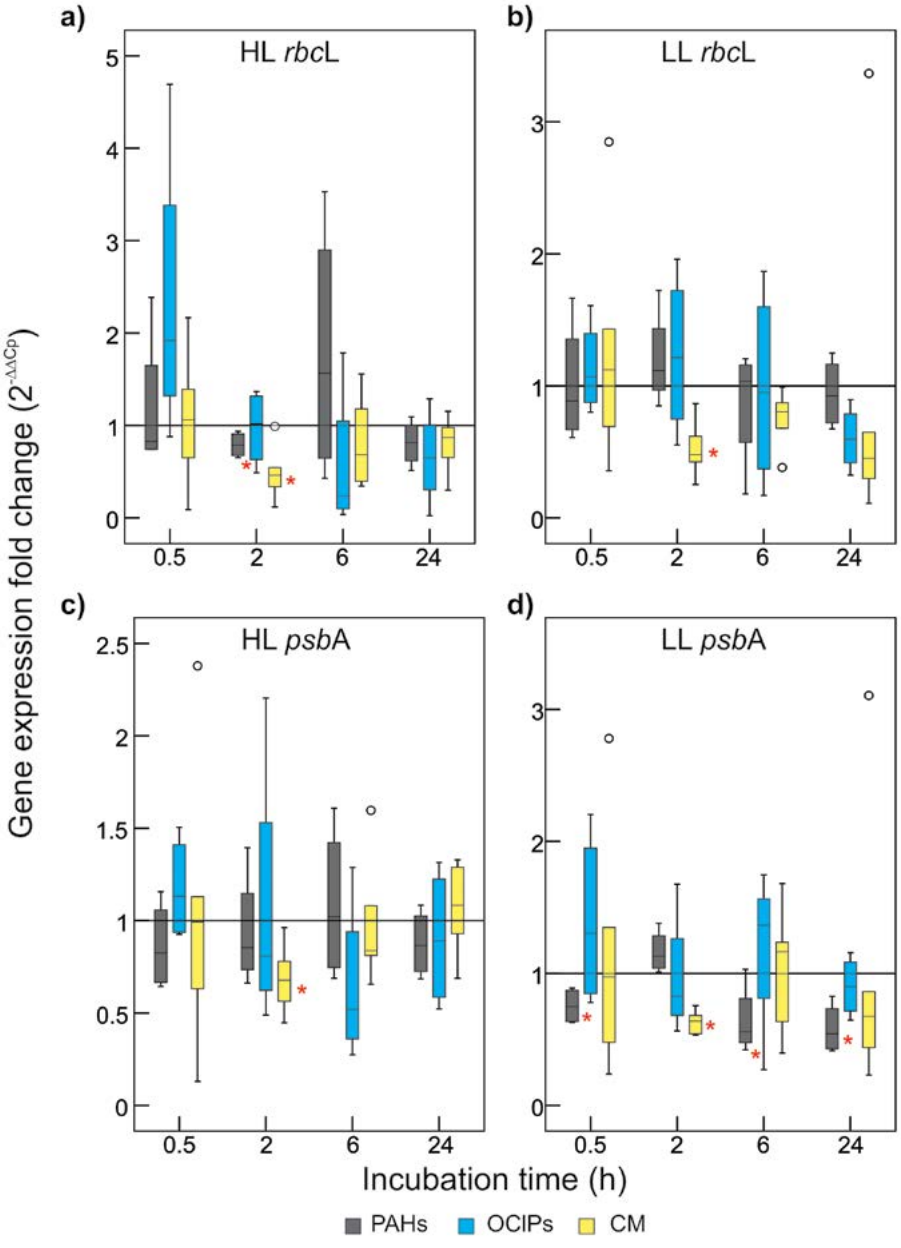


FIGURE AII.1 - GENE EXPRESSION ANALYSIS OF *PROCHLOROCOCCUS*

Gene expression of *Prochlorococcus rbc*L (a,b) and *psb*A (c,d) genes of treatments and controls were analysed by paired t-tests for the four incubation times tested. LL *Prochlorococcus* (b, d) showed more significant effects of pollutants (p-value < 0.05 are marked with "*") than HL *Prochlorococcus* (a,c). The most toxic pollutant mixture was the complex mixture (CM, yellow), that produced a significant decrease of LL *rbc*L (b) and *psb*A expression from both clades (c,d) after 2 h of exposure. In contrast, PAH mixture (grey) only affected significantly the expression of LL *psb*A after 0.5 h and 24 h (d), and the OCIP mixture that did not affect significantly gene expression of any target gene.

TABLE AII.1 - MALASPINA SAMPLED STATIONS FOR EXPERIMENTS

Position and depth of the stations where the natural phytoplankton communities where sampled, date and local time of sampling, and type of pollutant mixture used to perform each experiment. PAH = polyaromatic hidrocarbon mixture; OClP = Organochlorine pesticide mixture; and CM = complex mixture

Name	Date	Pollutants	Ocean	Latitude	Longitude	DCM depth (m)
PAH_Ind	1-Mar-11	PAH	Indian	-29.892	76.066	140
CM_Ind	9-Mar-11	CM	Indian	-30.327	103.32	100
OClP_Pac1	28-Mar-11	OClP	South Pacific	-38.699	150.436	60
PAH_Pac1	16-May-11	PAH	North Pacific	21.063	-150.442	105
OClP_Pac2	19-May-11	OClP	North Pacific	19.917	-141.635	125
PAH_Pac2	29-May-11	PAH	North Pacific	13.187	-113.267	70
OClP_Pac3	31-May-11	OClP	North Pacific	11.978	-108.032	59
CM_Pac1	5-Jun-11	CM	North Pacific	8.809	-93.143	24
CM_Pac2	7-Jun-11	CM	North Pacific	7.207	-87.9	20
OClP_Atl	25-Jun-11	OClP	North Atlantic	17.427	-59.833	90
PAH_Atl	28-Jun-11	PAH	North Atlantic	20.014	-52.691	130
CM_Atl1	2-Jul-11	CM	North Atlantic	23.766	-41.918	130
CM_Atl2	10-Jul-11	CM	North Atlantic	32.084	-17.286	110

TABLE AII.2 - COMPLEX MIXTURE POLLUTANTS

Amounts of the measured organic pollutants that were added to the seawater in the experiments challenged with complex mixtures of pollutants

		Amount of pollutant added to 1L of experimental sample (pg)					
	Organic pollutant	CM_Ind	CM_Pac1	CM_Pac2	CM_Atl1	CM_Atl2	**Average**
OClP	HCB	3.86E-01	7.60E+00	7.60E+00	9.03E+00	9.03E+00	8.31E+00
	α-HCH	0.00E+00	1.11E+00	1.11E+00	1.54E+00	1.54E+00	1.32E+00
	γ-HCH	7.29E-02	4.33E-01	4.33E-01	0.00E+00	0.00E+00	2.16E-01
	δ-HCH	3.76E-02	1.11E+00	1.11E+00	2.29E+00	2.29E+00	1.70E+00
DDT-like compuonds	4,4-DDD	1.08E-01	1.78E+00	1.78E+00	3.96E+00	3.96E+00	2.87E+00
	4,4'-DDD	0.00E+00	1.63E+00	1.63E+00	4.71E+00	4.71E+00	3.17E+00
	2,4'-DDT	0.00E+00	1.06E+00	1.06E+00	2.86E+00	2.86E+00	1.96E+00
	4,4'-DDT	8.47E-02	4.57E+00	4.57E+00	7.44E+00	7.44E+00	6.01E+00
PCBs	PCB 18	2.80E-01	3.03E+00	3.03E+00	0.00E+00	0.00E+00	1.51E+00
	PCB 17	4.00E-02	3.85E-01	3.85E-01	0.00E+00	0.00E+00	1.92E-01
	PCB 31	3.11E-01	1.78E+00	1.78E+00	2.56E+00	2.56E+00	2.17E+00
	PCB 28	2.85E-01	2.69E+00	2.69E+00	3.74E+00	3.74E+00	3.22E+00
	PCB 33	0.00E+00	2.69E+00	2.69E+00	5.77E+00	5.77E+00	4.23E+00
	PCB 52	2.59E-01	2.79E+00	2.79E+00	1.47E+01	1.47E+01	8.73E+00
	PCB 49	2.02E-01	2.12E+00	2.12E+00	0.00E+00	0.00E+00	1.06E+00
	PCB 44	0.00E+00	3.10E+01	3.10E+01	0.00E+00	0.00E+00	1.55E+01
	PCB 74	0.00E+00	1.73E+00	1.73E+00	0.00E+00	0.00E+00	8.65E-01
	PCB 70	2.05E-01	3.27E+00	3.27E+00	3.66E+00	3.66E+00	3.46E+00
	PCB 95	3.67E-01	6.68E+00	6.68E+00	9.25E+00	9.25E+00	7.97E+00
	PCB 99+PCB 1	8.14E-01	1.35E+01	1.35E+01	1.02E+01	1.02E+01	1.18E+01
	PCB 87	1.65E-01	1.73E+00	1.73E+00	5.68E+00	5.68E+00	3.71E+00
	PCB 110	1.69E-01	2.40E+00	2.40E+00	3.39E+00	3.39E+00	2.90E+00
	PCB 82	1.55E-01	3.41E+00	3.41E+00	5.77E+00	5.77E+00	4.59E+00
	PCB 151	0.00E+00	2.79E+00	2.79E+00	0.00E+00	0.00E+00	1.39E+00
	PCB 149	2.31E-01	7.26E+00	7.26E+00	7.93E+00	7.93E+00	7.59E+00
	PCB 118	2.99E-01	8.75E+00	8.75E+00	1.44E+01	1.44E+01	1.16E+01
	PCB 153	3.06E-01	9.33E+00	9.33E+00	1.35E+01	1.35E+01	1.14E+01

	PCB 132	1.44E-01	2.07E+00	2.07E+00	3.48E+00	3.48E+00	2.77E+00
	PCB 105	8.00E-02	1.20E+00	1.20E+00	3.61E+00	3.61E+00	2.41E+00
	PCB 138	3.65E-01	6.92E+00	6.92E+00	3.08E+00	3.08E+00	5.00E+00
	PCB 158	3.76E-02	1.06E+00	1.06E+00	5.07E+00	5.07E+00	3.06E+00
	PCB 187	1.91E-01	4.04E+00	4.04E+00	0.00E+00	0.00E+00	2.02E+00
	PCB 183	0.00E+00	1.54E+00	1.54E+00	0.00E+00	0.00E+00	7.69E-01
	PCB 177	0.00E+00	7.21E-01	7.21E-01	0.00E+00	0.00E+00	3.61E-01
	PCB 171+PCB 15	0.00E+00	1.39E+00	1.39E+00	1.32E+00	1.32E+00	1.36E+00
	PCB 180	1.08E-01	3.03E+00	3.03E+00	1.49E+01	1.49E+01	8.96E+00
	PCB 191	0.00E+00	6.01E+00	6.01E+00	0.00E+00	0.00E+00	3.00E+00
	PCB 170	0.00E+00	9.13E-01	9.13E-01	1.01E+00	1.01E+00	9.63E-01
	PCB 201+PCB 19	0.00E+00	2.07E+00	2.07E+00	0.00E+00	0.00E+00	1.03E+00
	PCB 195	4.24E-02	4.81E-01	4.81E-01	4.41E-01	4.41E-01	4.61E-01
	PCB 194	0.00E+00	4.81E-01	4.81E-01	7.93E-01	7.93E-01	6.37E-01
	Fluorene	2.12E+04	7.06E+03	7.06E+03	1.77E-02	1.77E-02	7.06E+03
	Dibenzothiophene	3.07E+03	1.02E+03	1.02E+03	2.56E-03	2.56E-03	1.02E+03
	Metyldibenzothiopene	8.72E+03	2.91E+03	2.91E+03	7.27E-03	7.27E-03	2.91E+03
	Phenanthrene	2.87E+04	9.57E+03	9.57E+03	2.39E-02	2.39E-02	9.57E+03
	Anthracene	3.62E+03	1.21E+03	1.21E+03	3.02E-03	3.02E-03	1.21E+03
	Metylphenanthrene	1.89E+04	6.30E+03	6.30E+03	1.58E-02	1.58E-02	6.30E+03
	Dimetylphenanthrene	9.39E+03	3.13E+03	3.13E+03	7.83E-03	7.83E-03	3.13E+03
	Fluoranthene	3.16E+04	1.05E+04	1.05E+04	2.63E-02	2.63E-02	1.05E+04
	Pyrene	2.24E+04	7.48E+03	7.48E+03	1.87E-02	1.87E-02	7.48E+03
PAHs	Benzo[a]anthracene	7.58E+02	2.53E+02	2.53E+02	6.32E-04	6.32E-04	2.53E+02
	Crysene	1.41E+03	4.70E+02	4.70E+02	1.17E-03	1.17E-03	4.70E+02
	Benzo[b]fluoranthene	2.42E+02	8.08E+01	8.08E+01	2.02E-04	2.02E-04	8.08E+01
	Benzo[k]fluoranthene	8.29E+01	2.76E+01	2.76E+01	6.91E-05	6.91E-05	2.76E+01
	Benzo[e]pyrene	1.15E+02	3.83E+01	3.83E+01	9.58E-05	9.58E-05	3.83E+01
	Benzo[a]pyrene	9.95E+01	3.32E+01	3.32E+01	8.29E-05	8.29E-05	3.32E+01
	Perylene	4.42E+01	1.47E+01	1.47E+01	3.68E-05	3.68E-05	1.47E+01
	Indeno[1,2,3-cd]pyrene	1.06E+03	3.53E+02	3.53E+02	8.82E-04	8.82E-04	3.53E+02
	Dibenzo[a,h]anthracene	1.03E+03	3.43E+02	3.43E+02	8.57E-04	8.57E-04	3.43E+02
	Benzo[ghi]perylene	3.79E+02	1.26E+02	1.26E+02	3.16E-04	3.16E-04	1.26E+02

TABLE AII.3 - TRACER POLLUTANT CONCENTRATIONS IN PAH EXPERIMENTS

Relative concentrations (C/Ccontrol) of PAHs measured in the PAH experiments after 0.5 h and 24 h of incubation.

	PAH_Ind		PAH_Pac1		PAH_Pac2		PAH_Atl		**Average**	
	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h
Fluorene	12.43	9.53	16.71	7.64	45.13	13.94	22.59	39.40	24.21	17.63
Phenanthrene	20.04	32.81	9.59	16.48	37.15	24.47	18.12	56.31	21.22	32.52
Antracene	47.70	9.69	123.42	9.95	393.94	2.89	211.44	29.68	194.12	13.05
Fluoranthene	17.43	57.82	102.89	299.31	257.40	65.74	159.44	188.32	134.29	152.80
Pyrene	15.65	43.61	161.03	173.12	350.63	82.54	93.29	70.59	155.15	92.47
Benzo(a)-anthracene	22.37	4.50	317.12	178.11	488.37	33.26	799.86	249.37	406.93	116.31
Chrysene	59.46	6.65	280.21	338.38	370.58	74.79	680.96	458.46	347.80	219.57
Benzo(byk)-fluoranthene	19.53	4.62	48.54	18.78	27.07	3.71	24.41	11.36	29.89	9.61
Benzo(a)pyrene	74.18	13.65	51.27	12.90	64.02	2.50	41.31	8.32	57.69	9.34
Average	32.09	20.32	123.42	117.19	226.03	33.76	227.94	123.53	152.37	73.70

TABLE AII.4 - TRACER POLLUTANT CONCENTRATIONS IN OCLP EXPERIMENTS

Relative concentrations (C/Ccontrol) of HCB and HCH isomers measured in the OClP experiments after 0.5 h and 24 h of incubation.

	OClP_Pac1		OClP_Pac2		OClP_Pac3		OClP_Atl		**Average**	
	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h
HCB	0.99	1.09	19.37	19.37	11.03	22.80	20.55	19.22	12.98	15.62
αHCH	5.02	6.40	61.63	61.63	27.05	31.73	71.05	123.77	41.19	55.88
γHCH	734.49	1067.46	1341.01	1341.01	1108.62	879.80	690.81	1033.06	968.73	1080.33
βHCH	233.30	163.86	1152.82	1152.82	1152.82	459.03	1152.82	918.06	922.94	673.44
δHCH	827.04	483.43	1654.09	1654.09	1654.09	1315.22	1654.09	1315.22	1447.33	1191.99
Average	360.17	344.45	845.78	845.78	790.72	541.71	717.86	681.87	678.63	603.45

TABLE AII.5 - TRACER POLLUTANT CONCENTRATIONS IN CM EXPERIMENTS

Relative concentrations (C/Ccontrol) of PAHs and alkanes measured as surrogate in the complex mixture experiments after 0.5 h and 24 h of incubation. N/A = Not available, N/D = compound non detected in the control.

		CM_Ind		CM_Pac1		CM_Pac2		CM_Atl1		CM_Atl2		**Average**	
		0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h
PAHs	Fluorene	1.31	1.31	1.15	1.33	0.86	0.79	0.70	1.80	0.80	1.02	0.97	1.25
	Dibenzothiophene	1.21	1.21	0.91	1.30	0.89	1.74	1.49	1.37	0.71	0.75	1.04	1.27
	Methyldibenzothiophene	1.19	1.19	0.91	1.37	0.54	1.70	1.40	1.42	0.42	0.85	0.89	1.31
	Phenanthrene	1.50	1.50	0.79	1.23	0.91	0.95	1.27	2.24	1.03	0.96	1.10	1.38
	Methylphenanthrene	1.32	1.32	0.97	1.41	1.07	1.72	1.30	1.48	0.53	0.77	1.04	1.34
	Dimethylphenanthrene	1.11	1.11	0.92	1.32	1.11	1.42	1.73	1.55	0.21	0.65	1.02	1.21
	Trymethylphenanthrene	1.07	1.07	1.05	0.72	0.55	1.87	1.27	1.32	0.28	1.00	0.84	1.19
	Anthracene	1.35	1.35	1.17	1.29	1.02	1.07	1.05	3.31	0.82	0.74	1.08	1.55
	Fluoranthene	2.01	2.01	0.64	1.45	1.06	1.13	1.25	1.51	1.71	0.88	1.34	1.40
	Pyrene	1.39	1.39	0.60	1.49	1.08	0.98	1.47	0.34	7.85	0.89	2.48	1.02
	Benzo(ghi)fluoranthene	1.12	1.12	1.38	0.91	0.27	2.30	1.17	1.13	0.26	1.03	0.84	1.30
	Benzo(a)anthracene	1.12	1.12	1.23	0.94	0.99	0.55	2.16	1.92	2.00	0.88	1.50	1.08
	Chrysene	1.11	1.11	1.07	0.95	0.95	0.34	3.36	2.39	6.13	0.92	2.52	1.14
	Benzo(byk)fluoranthene	1.11	1.11	1.11	0.72	0.67	1.67	1.32	4.09	2.22	0.99	1.29	1.72
	Benzo(e)pyrene	1.01	1.01	1.35	0.97	1.16	1.70	1.01	1.12	0.01	1.00	0.91	1.16
	Benzo(a)pyrene	1.34	1.34	1.38	0.87	0.54	1.14	1.57	0.93	0.71	1.00	1.11	1.05
	Perylene	0.91	0.91	0.95	0.71	1.05	2.75	1.00	1.06	0.32	0.79	0.85	1.24
	Average	1.25	1.25	1.03	1.12	0.87	1.40	1.44	1.70	1.53	0.89	1.22	1.27

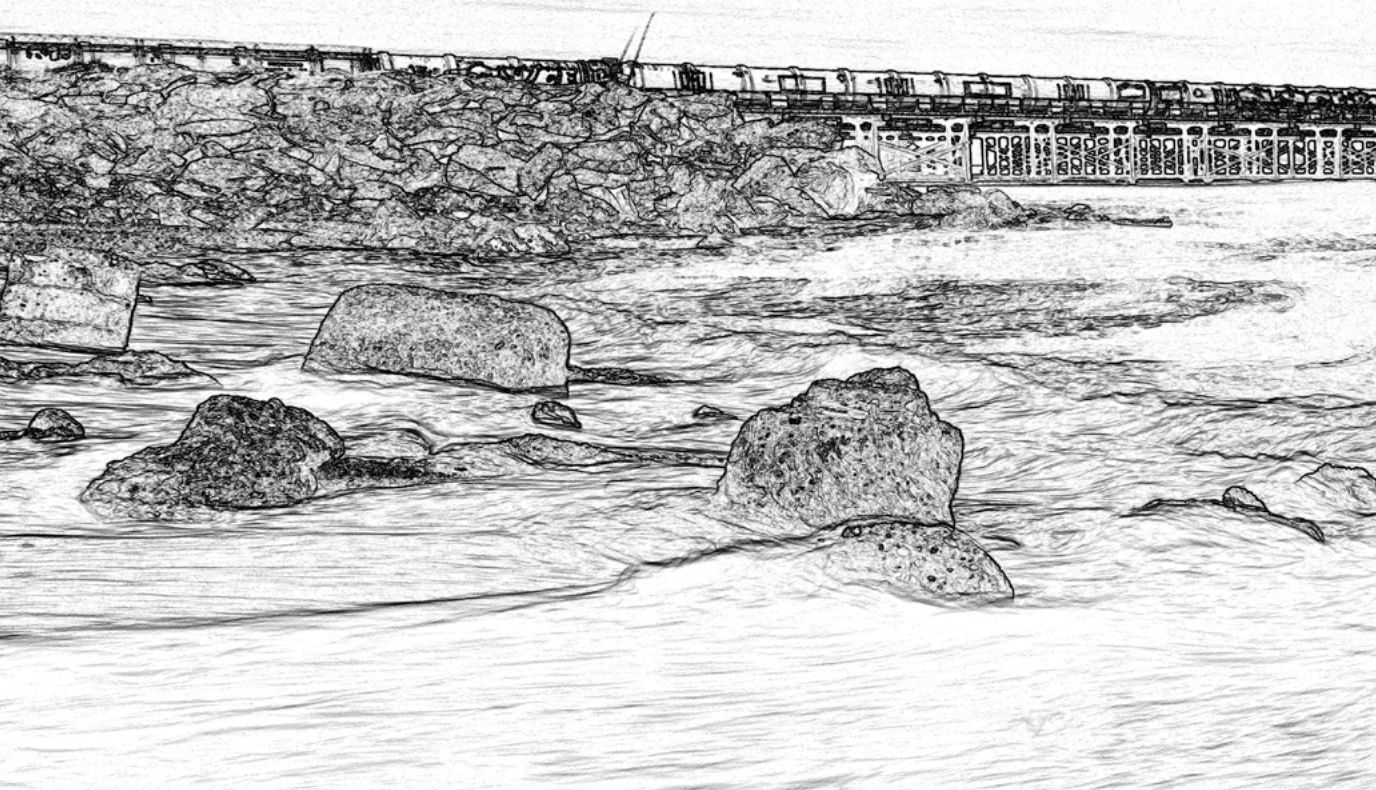
Alkanes	Heneicosane	4.53	4.53	1.22	2.20	3.36	1.00	3.54	2.03	15.68	1.73	5.67	2.30
	Tricosane	5.93	5.93	1.10	1.89	2.42	1.53	2.28	0.79	0.73	1.14	2.49	2.26
	Pentacosane	5.05	5.05	1.07	1.66	2.33	1.88	2.39	0.68	0.44	0.51	2.26	1.96
	Heptacosane	7.90	7.90	1.12	1.64	2.21	1.37	2.03	0.92	1.15	1.28	2.88	2.62
	Nonacosane	3.67	3.67	1.12	1.47	1.45	1.34	2.01	0.89	0.14	1.15	1.68	1.71
	Hentriacontane	1.78	1.78	0.99	0.99	1.06	1.13	1.84	1.17	0.19	1.22	1.17	1.26
	Tritriacontane	1.21	1.21	0.88	1.09	0.94	0.76	1.49	1.02	0.79	2.00	1.06	1.22
	Pentatriacontane	1.00	1.00	2.00	2.01	1.00	1.00	1.02	1.01	0.94	1.02	1.19	1.21
	Average	3.88	3.88	1.19	1.62	1.85	1.25	2.07	1.06	2.51	1.26	2.30	1.82

TABLE AII.6 - FLOW CYTOMETER ANALYSIS

Flow cytometer results for cyanobacteria and picoeukaryotes for the different experiment. Samples after 0.5 and 24 h on deck.

Experiment		Time (h)	Cell Counts (cell/mL)								
			Prochlorococcus			*Synechococcus*			Picoeukaryotes		
			Control sample	Treated sample	p-value (T/C)[1]	Control sample	Treated sample	p-value (T/C)[1]	Control sample	Treated sample	p-value (T/C)[1]
PAHs	29-May-11	0.5	2.14E+05	3.17E+05	0.615	1.46E+03	1.69E+03	0.780	1.92E+04	2.25E+04	0.638
	29-May-11	24	2.51E+04	1.29E+04		1.78E+03	1.84E+03		5.60E+03	1.25E+03	
	28-Jun-11	0.5	1.70E+04	2.36E+03		6.59E+02	5.22E+02		1.97E+05	1.96E+05	
	28-Jun-11	24	1.48E+05	1.47E+05		1.75E+03	1.46E+03		3.57E+03	3.07E+03	
OCIP	31-May-11	0.5	3.50E+05	3.66E+05	0.458	2.03E+04	2.14E+04	0.207	1.83E+04	1.85E+04	0.484
	31-May-11	24	2.54E+05	2.64E+05		1.91E+04	2.31E+04		1.17E+04	1.14E+04	
	25-Jun-11	0.5	ND	ND		2.56E+03	ND		1.74E+03	ND	
	25-Jun-11	24	1.37E+05	1.30E+05		4.81E+03	5.46E+03		3.49E+03	3.34E+03	
CM	5-Jun-11	0.5	8.85E+05	7.20E+04	0.322	8.59E+05	8.41E+05	0.395	1.04E+03	1.04E+05	0.348
	5-Jun-11	24	9.99E+04	6.95E+04		1.12E+06	1.29E+06		1.31E+05	1.39E+05	
	7-Jun-11	0.5	1.40E+05	1.50E+05		7.43E+05	7.55E+05		1.90E+05	1.89E+05	
	7-Jun-11	24	1.09E+05	1.20E+05		4.69E+05	4.64E+05		1.09E+05	1.03E+05	
	2-Jul-11	0.5	2.00E+05	2.07E+05		3.91E+02	3.52E+02		7.81E+03	6.80E+03	
	2-Jul-11	24	1.09E+05	1.14E+05		6.40E+02	2.64E+02		7.18E+03	4.29E+03	
	10-Jul-11	0.5	2.52E+05	1.17E+05		6.35E+02	7.08E+02		1.15E+05	6.12E+03	

1) Paired t-test, control vs. treated.
ND, no data available.



Annex III

Supporting information of chapter IV

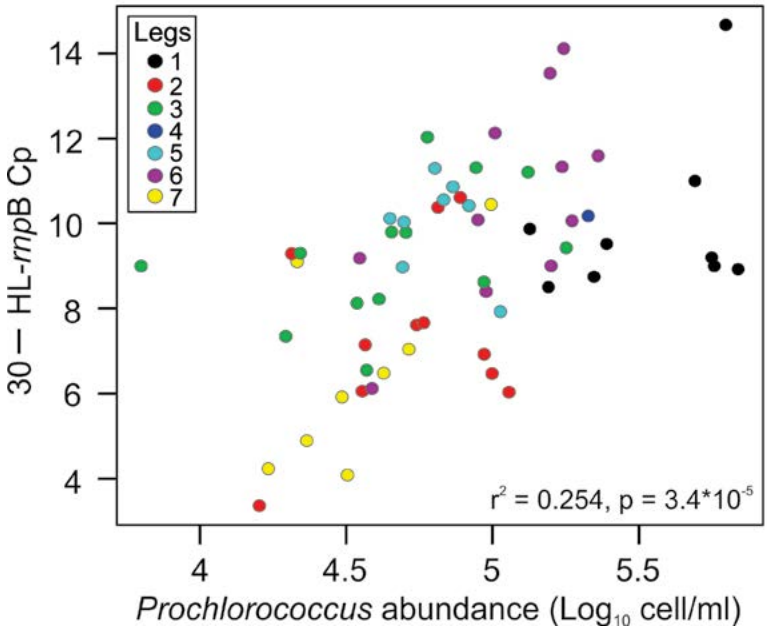


FIGURE AIII.1 - HL-*rnp*B CP VALUES VS. *PROCHLOROCOCCUS* ABUNDANCE

Pearson correlations between HL-*rnp*B Cp values (expressed as cycles above background) and *Prochlorococcus* abundance determined by fluorescence measurements in surface samples. Colours indicate the different legs of Malaspina cruise (Fig. IV.1).

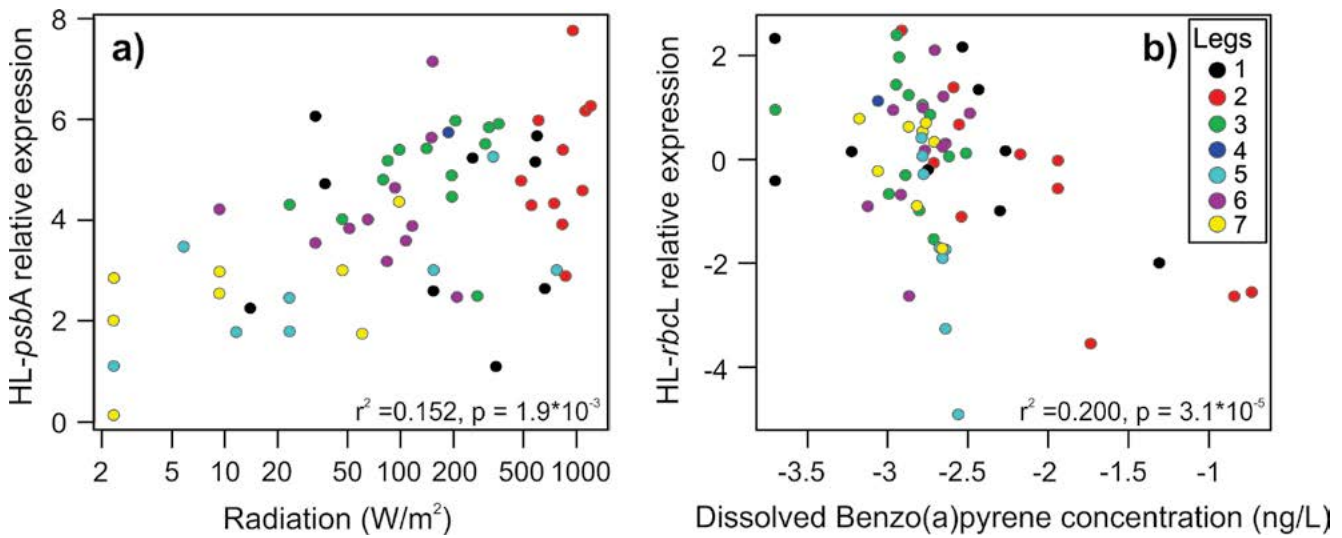
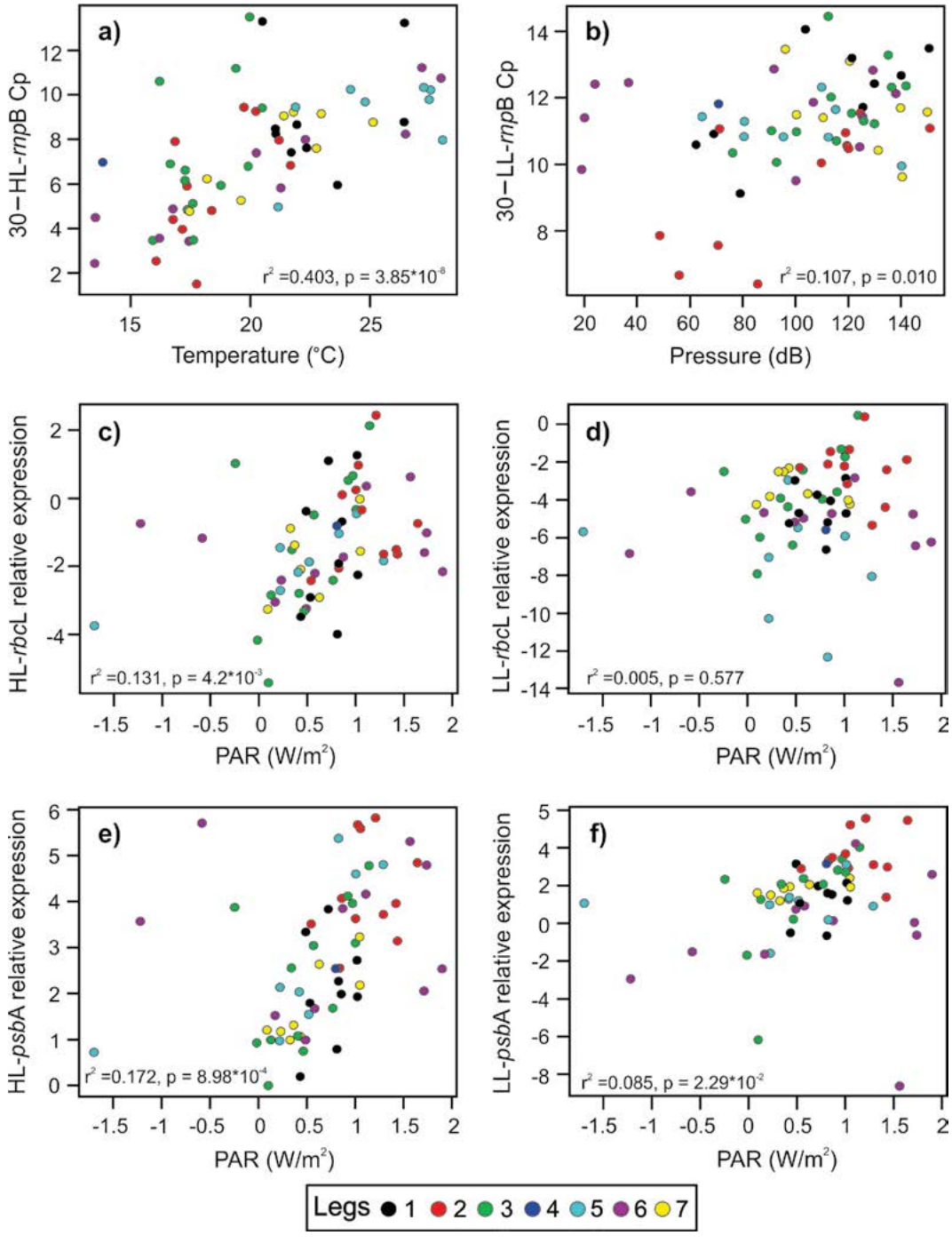


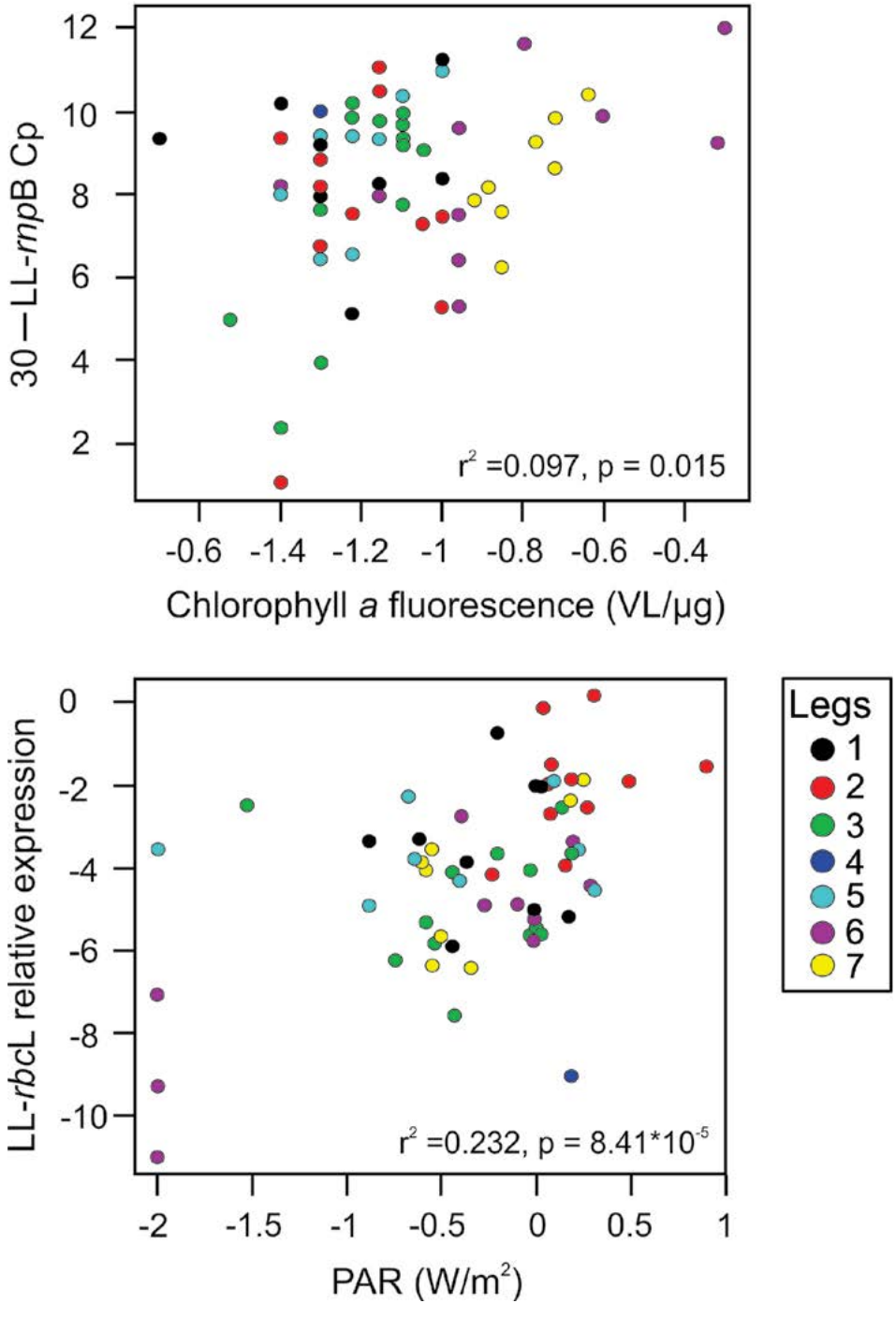
FIGURE AIII.2 - SURFACE PEARSON CORRELATIONS

Pearson correlations between relative expression of a) HL-*psb*A and b) HL-*rbc*L and dissolved benzo(a) pyrene concentration and solar radiation (RAD), respectively, measured at surface. Colours indicate the different legs of Malaspina cruise (Fig. IV.1).

FIGURE AIII.3 - DCM PEARSON CORRELATIONS

Pearson correlations between relative expression of HL (left panels) and LL (right panels) target gene expressions and different environmental variables in DCM samples. a) and b) the correlation between temperature and HL-cell abundance, and pressure and LL-cell abundance, respectively. c) and d) show the differential responses of *rbc*L expression to photosynthetically active radiation (PAR) in each clade: HL- *rbc*L relative expression is significantly positive correlated to PAR, while LL- *rbc*L is not correlated. e) and f) show a similar result in relation to *psb*A expression, showing a weaker correlation with PAR for LL than HL, that turn to no correlation when data are corrected by false discovery rate (fdr, Fig. IV.3). Colors indicate the different legs of Malaspina expedition (Fig. IV.1)


a)
30−HL-rnpB Cp
Temperature (°C)
r² =0.403, p = 3.85*10⁻⁸
b)
30−LL-rnpB Cp
Pressure (dB)
r² =0.107, p = 0.010
c)
HL-rbcL relative expression
PAR (W/m²)
r² =0.131, p = 4.2*10⁻³
d)
LL-rbcL relative expression
PAR (W/m²)
r² =0.005, p = 0.577
e)
HL-psbA relative expression
PAR (W/m²)
r² =0.172, p = 8.98*10⁻⁴
f)
LL-psbA relative expression
PAR (W/m²)
r² =0.085, p = 2.29*10⁻²
Legs 1 2 3 4 5 6 7


30 — LL-rnpB Cp
12
10
8
6
4
2
r² =0.097, p = 0.015
-0.6 -1.4 -1.2 -1 -0.8 -0.6 -0.4
Chlorophyll a fluorescence (VL/µg)
LL-rbcL relative expression
0
-2
-4
-6
-8
-10
r² =0.232, p = 8.41*10⁻⁵
-2 -1.5 -1 -0.5 0 0.5 1
PAR (W/m²)
Legs
1
2
3
4
5
6
7

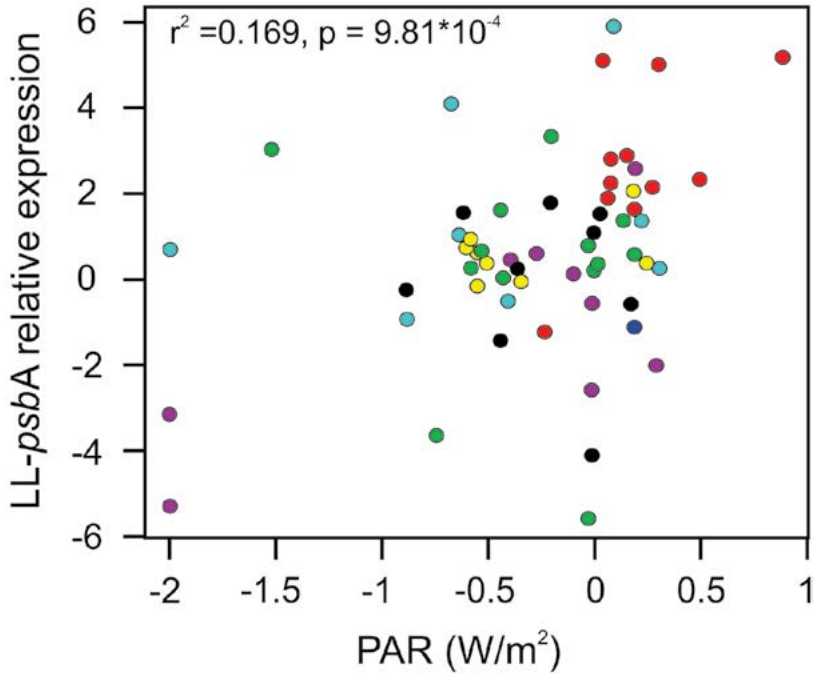
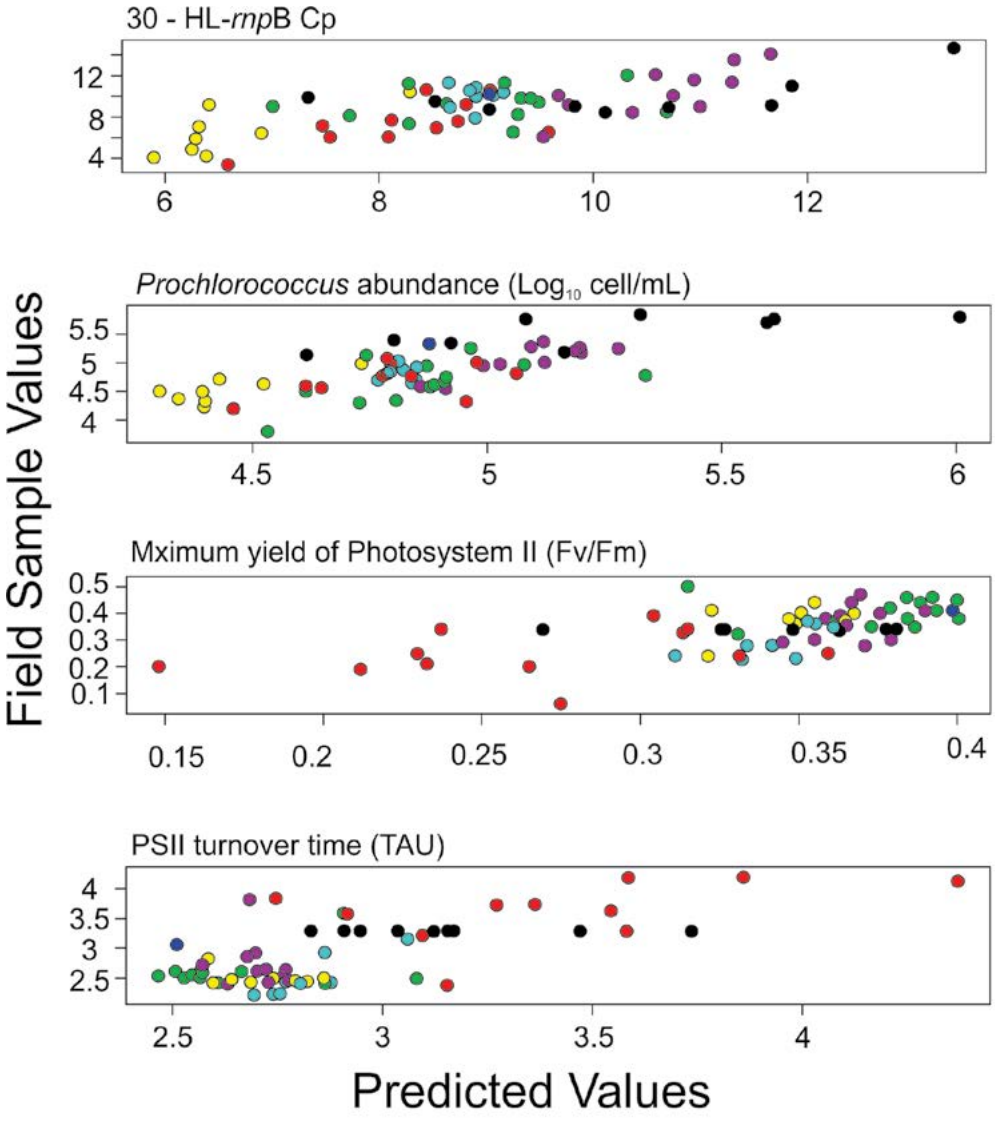


FIGURE AIII.4 - DCM+40 PEARSON CORRELATIONS

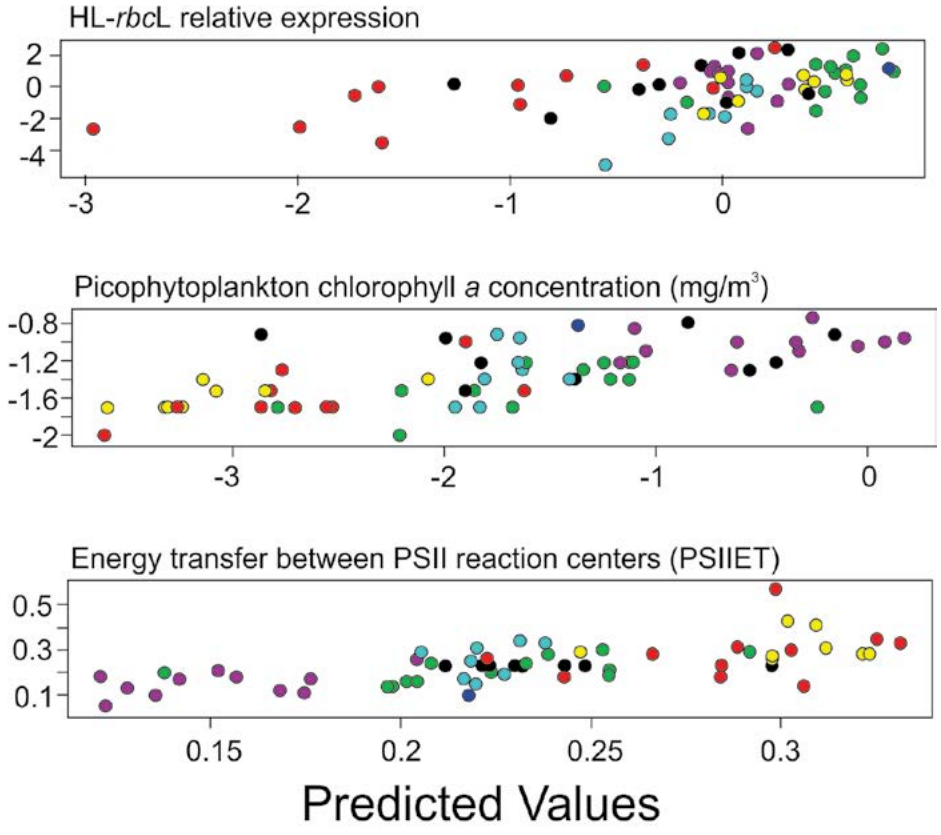
Pearson correlations between relative expression of LL-*rbc*L (a) and LL-*psb*A (b) expression and the photosynthetically active radiation at DCM+40, respectively. Colours indicate the different legs of Malaspina expedition (Fig. IV.1)



	HL-*rnp*B	HL-*rbc*L	HL-*psb*A	PP02	Fv/Fm	
r^2	0.469	0.229	0.027	0.120	0.370	
p	$1.2*10^{-9}$	$9.3*10^{-5}$	0.205	$6.1*10^{-3}$	$2.0*10^{-7}$	

FIGURE AIII.5 - SURFACE PLSR MODEL

Prediction results for Y-data using the first three components of the PLSR model. The table corresponds to the regressions between predicted and observed values. The graphs show several of these different correlations. Each dot corresponds to a single sample, colour-code by leg as in Fig. IV.1.



	PSIIET	TAU	PRO	CHL02
	0.410	0.486	0.549	0.425
	$2.9*10^{-8}$	$4.3*10^{-10}$	$8.9*10^{-12}$	$1.3*10^{-8}$

TABLE AIII.1 - ENVIRONMENTAL VARIABLES

Physical, biological and chemical variables measured during Malaspina cruise.

Type of variable	Variable	Units	Description	Measurement method
PHYSICAL	RAD	W m^{-2}	Solar radiation at 5 m	Seabird 911Plus CTD probe (1)
	OXY	mL L^{-1}	Dissolved oxygen	
	SAL	PSU	Salinity	
	PRESS	dB	Pressure	
	TEM	°C	Temperature	
	CON	S m^{-1}	Conductivity	
	FLUO	V L μg^{-1}	Chlorophyll *a*.fluorescence	
	PAR	W m^{-2}	Photosynthetically active radiation (irradiance)	
	OBS	FTU	Turbidity	
	BAT	1 m^{-1}	Beam Attenuation	
	SiO_4	µg L^{-1}	Dissolved silicate concentration	(1, 2)
	PO_4	µg L^{-1}	Dissolved phosphate concentration	
	NO_x	µg L^{-1}	Dissolved nitrogen-oxides concentration	
	Mo	nM	Dissolved molybdenum concentration at 3 m	Towed-fish (3)
	Cd	pM	Dissolved cadmium concentration at 3 m	
	Pb	pM	Dissolved lead concentration at 3 m	
	V51	nM	Dissolved vanadium-51 concentration at 3 m	
	Fe	nM	Dissolved iron concentration at 3 m	
	Co	pM	Dissolved cobalt concentration at 3 m	
	Ni60	nM	Dissolved nickel-60 concentration at 3 m	
	Cu63	nM	Dissolved cupper-63 concentration at 3 m	

BIOLOGICAL	PP02	mg C m^{-3} h^{-1}	Primary production rate, size fraction 0.2-2 µm (picoplankton, including *Prochlorococcus*)	$14CO_2$-uptake technique (1)
	PP	mg C m^3 h^1	Total (all size fractions) primary production rate	
	Fo	DL*	Initial value of chlorophyll fluorescence yield before light saturation at 4 m	Fast Repetition Rate Fluorometry (FRRF) (4)
	Fm	DL*	Final value of chlorophyll fluorescence yield after saturation of photosynthetic reaction centers at 4 m	
	Fv	DL*	Variable fluorescence (Fm - Fo) at 4 m	
	Fv/Fm	DL*	Normalized variable fluorescence, equivalent to (Fm-Fo)/Fm or maximum yield of PSII (ΦPSII) at 4 m	
	SIGMA	DL*	Photosystem II (PSII) functional absorption cross-section at 4 m	
	PSIIET	DL*	Energy transfer between PSII reaction centers at 4 m	
	TAU	DL*	Photosystem II turnover time at 4 m	
	PRO	cel mL^{-1}	*Prochlorococcus* abundance	FACSCalibur (5)
	SYN	cel mL^{-1}	*Synechococcus* abundance	
	EUK	cel mL^{-1}	Picoeukaryotes abundance	
	CHL	mg m^{-3}	Total (all size fractions) chlorophyll a (Chl*a*) concentration	(6)
	CHL20	mg m^{-3}	Chl*a* concentration, size fraction ≥ 20 µm (microphytoplankton)	
	CHL2	mg m^{-3}	Chl*a* concentration, size fraction 2- 20 µm (nanophytoplankton)	
	CHL02	mg m^{-3}	Chl*a* concentration, size fraction 0.2- 2 µm (picophytoplankton)	
	BACT	pmol Leu L^{-1} h^{-1}	Bacterial activity	(7, 8)
	LNA	cel mL^{-1}	DAPI-corrected LNA bacteria abundance	
	HNA	cel mL^{-1}	DAPI-corrected HNA bacteria abundance	
	HBAC	cel mL^{-1}	Heterotrophic bacteria (LNA+HNA) abundance	
	BSIZE	μm^3	Bacterial cell size	(9, 10)
	BCC	fg C $cell^{-1}$	Bacterial cell carbon (C) concentration	
	BMASS	µg C L^{-1}	Bacterial biomass	
	BP	µg C L^{-1} d^{-1}	Bacterial production	
	SGR	d^{-1}	Bacterial specific growth rate	
	TD	d	Bacterial doubling time	
	PKMASS	µg L^{-1}	Plankton biomass (≤ 50 µm) from DCM+20 m to surface	(1)

TABLE AIII.1 - (Continuation)

Type of variable	Variable	Units	Description	Measurement method
DISSOLVED POLYFLUORINATED ALKYL SUBSTANCES	Cw_PFBS	pg μL^{-1}	Dissolved perfluorobutanesulfonic acid concentration	(11)
	Cw_PFHpA	pg μL^{-1}	Dissolved perfluoroheptanoic acid concentration	
	Cw_PFHxS	pg μL^{-1}	Dissolved perfluorohexanesulfonic acid concentration	
	Cw_PFOA	pg μL^{-1}	Dissolved perfluorooctanoic acid concentration	
	Cw_PFHpS	pg μL^{-1}	Dissolved perfluoroheptane sulfonate concentration	
	Cw_PFNA	pg μL^{-1}	Dissolved perfluorononanoate concentration	
	Cw_PFOS	pg μL^{-1}	Dissolved perfluorooctane sulfonate concentration	
	Cw_PFDA	pg μL^{-1}	Dissolved perfluorodecanoic acid concentration	
DISSOLVED POLYCYCLIC AROMATIC HYDROCARBONS	Cw_FLUORE	ng L^{-1}	Dissolved fluorene concentration	(12)
	Cw_DBT	ng L^{-1}	Dissolved dibenzothiophene concentration	
	Cw_MDBT	ng L^{-1}	Dissolved methyldibenzothiophene concentration	
	Cw_DMDBT	ng L^{-1}	Dissolved dimethyldibenzothiopene concentration	
	Cw_PHE	ng L^{-1}	Dissolved phenanthrene concentration	
	Cw_MPHE	ng L^{-1}	Dissolved methylphenantrene concentration	
	Cw_DMPHE	ng L^{-1}	Dissolved dimethylphenanthrene concentration	
	Cw_ANT	ng L^{-1}	Dissolved anthracene concentration	
	Cw_FLUORANT	ng L^{-1}	Dissolved fluoranthene concentration	
	Cw_PYR	ng L^{-1}	Dissolved pyrene concentration	
	Cw_MPYR	ng L^{-1}	Dissolved methylpyrene concentration	
	Cw_DMPYR	ng L^{-1}	Dissolved dimethylpyrene concentration	
	Cw_BGFLUORANT	ng L^{-1}	Dissolved benzo(g,h,i)fluoranthene concentration	
	Cw_BAANT	ng L^{-1}	Dissolved benzo(a)anthracene concentration	
	Cw_CHRY	ng L^{-1}	Dissolved chrysene concentration	
	Cw_MCHRY	ng L^{-1}	Dissolved methylchrysene concentration	
	Cw_BBKFLUORANT	ng L^{-1}	Dissolved benzo(b+k)fluoranthene concentration	

	Cw_BEPYR	ng L^{-1}	Dissolved benzo(e)pyrene concentration	
	Cw_BAPYR	ng L^{-1}	Dissolved benzo(a)pyrene concentration	
	Cw_PERY	ng L^{-1}	Dissolved perylene concentration	
	Cw_INPYR	ng L^{-1}	Dissolved indeno(1,2,3-cd)pyrene concentration	
	Cw_DBANT	ng L^{-1}	Dissolved dibenzo(a,h)anthracene concentration	
	Cw_BGPERY	ng L^{-1}	Dissolved benzo(g,h,i)perylene concentration	
PARTICULATE POLYCYCLIC AROMATIC HYDROCARBONS	Cp_FLUORE	ng L^{-1}	Particulated fluorene concentration	
	Cp_DBT	ng L^{-1}	Particulated dibenzothiophene concentration	
	Cp_MDBT	ng L^{-1}	Particulated methyldibenzothiophene concentration	
	Cp_DMDBT	ng L^{-1}	Particulated dimethyldibenzothiopene concentration	
	Cp_PHE	ng L^{-1}	Particulated phenanthrene concentration	
	Cp_MPHE	ng L^{-1}	Particulated methylphenantrene concentration	
	Cp_DMPHE	ng L^{-1}	Particulated dimethylphenanthrene concentration	
	Cp_ANT	ng L^{-1}	Particulated anthracene concentration	
	Cp_FLUORANT	ng L^{-1}	Particulated fluoranthene concentration	
	Cp_PYR	ng L^{-1}	Particulated pyrene concentration	
	Cp_MPYR	ng L^{-1}	Particulated methylpyrene concentration	
	Cp_DMPYR	ng L^{-1}	Particulated dimethylpyrene concentration	(12)
	Cp_BGFLUORANT	ng L^{-1}	Particulated benzo(g,h,i)fluoranthene concentration	
	Cp_BAANT	ng L^{-1}	Particulated benzo(a)anthracene concentration	
	Cp_CHRY	ng L^{-1}	Particulated chrysene concentration	
	Cp_MCHRY	ng L^{-1}	Particulated methylchrysene concentration	
	Cp_BBKFLUORANT	ng L^{-1}	Particulated benzo(b+k)fluoranthene concentration	
	Cp_BEPYR	ng L^{-1}	Particulated benzo(e)pyrene concentration	
	Cp_BAPYR	ng L^{-1}	Particulated benzo(a)pyrene concentration	
	Cp_PERY	ng L^{-1}	Particulated perylene concentration	
	Cp_INPYR	ng L^{-1}	Particulated indeno(1,2,3-cd)pyrene concentration	
	Cp_DBANT	ng L^{-1}	Particulated dibenzo(a,h)anthracene concentration	
	Cp_BGPERY	ng L^{-1}	Particulated benzo(g,h,i)perylene concentration	

TABLE AIII.1 - (Continuation)

Type of variable	Variable	Units	Description	Measurement method
BIOACCUMULATED POLYCYCLIC AROMATIC HYDROCARBONS IN PLANKTON FROM DCM+20 M DEPTH TO SURFACE	P_NAPH	ng g^{-1}	Planktonic naphthalene concentration	
	P_DMNAPH	ng g^{-1}	Planktonic dimethylnaphtalene concentration	
	P_MNAPH	ng g^{-1}	Planktonic methylnaphtalene concentration	
	P_TMNAPH	ng g^{-1}	Planktonic trimethylnaphtalene concentration	
	P_ACEYL	ng g^{-1}	Planktonic acenaphthylene concentration	
	P_ACEEN	ng g^{-1}	Planktonic acenaphthene concentration	
	P_FLUORE	ng g^{-1}	Planktonic fluorene concentration	
	P_MDBT	ng g^{-1}	Planktonic methyldibenzothiophene concentration	
	P_DMDBT	ng g^{-1}	Planktonic dimethyldibenzothiopene concentration	
	P_PHE	ng g^{-1}	Planktonic phenanthene concentration	
	P_MPHE	ng g^{-1}	Planktonic methylphenantrene concentration	
	P_DMPHE	ng g^{-1}	Planktonic dimethylphenanthene concentration	
	P_ANT	ng g^{-1}	Planktonic anthracene concentration	
	P_FLUORANT	ng g^{-1}	Planktonic fluoranthene concentration	(12)
	P_PYR	ng g^{-1}	Planktonic pyrene concentration	
	P_MPYR	ng g^{-1}	Planktonic methylpyrene concentration	
	P_DMPYR	ng g^{-1}	Planktonic dimethylpyrene concentration	
	P_BGFLUORANT	ng g^{-1}	Planktonic benzo(g,h,i)fluoranthene concentration	
	P_BAANT	ng g^{-1}	Planktonic benzo(a)anthracene concentration	
	P_CHRY	ng g^{-1}	Planktonic chrysene concentration	
	P_MCHRY	ng g^{-1}	Planktonic methylchrysene concentration	
	P_BBKFLUORANT	ng g^{-1}	Planktonic benzo(b+k)fluoranthene concentration	
	P_BEPYR	ng g^{-1}	Planktonic benzo(e)pyrene concentration	
	P_BAPYR	ng g^{-1}	Planktonic benzo(a)pyrene concentration	
	P_PERY	ng g^{-1}	Planktonic perylene_pescas concentration	
	P_DBANT	ng g^{-1}	Planktonic dibenzo(a,h)anthracene concentration	
	P_BGPERY	ng g^{-1}	Planktonic benzo(g,h,i)perylene concentration	

BIOACCUMULATED DIOXINS AND DIOXIN-LIKE PCBS IN PLANKTON FROM DCM+20 M DEPTH TO SURFACE	P_TCDF	pg g^{-1}	Planktonic 2,3,7,8-TCDF	
	P_1PeCDF	pg g^{-1}	Planktonic 1,2,3,7,8-PeCDF	
	P_2PeCDF	pg g^{-1}	Planktonic 2,3,4,7,8-PeCDF	
	P_1HxCDF	pg g^{-1}	Planktonic 1,2,3,4,7,8-HxCDF	
	P_2HxCDF	pg g^{-1}	Planktonic 1,2,3,6,7,8-HxCDF	
	P_3HxCDF	pg g^{-1}	Planktonic 2,3,4,6,7,8-HxCDF	
	P_4HxCDF	pg g^{-1}	Planktonic 1,2,3,7,8,9-HxCDF	
	P_1HpCDF	pg g^{-1}	Planktonic 1,2,3,4,6,7,8-HpCDF	
	P_2HpCDF	pg g^{-1}	Planktonic 1,2,3,4,7,8,9-HpCDF	
	P_OCDF	pg g^{-1}	Planktonic OCDF	
	P_TCDD	pg g^{-1}	Planktonic 2,3,7,8-TCDD	
	P_PeCDD	pg g^{-1}	Planktonic 1,2,3,7,8-PeCDD	
	P_1HxCDD	pg g^{-1}	Planktonic 1,2,3,4,7,8-HxCDD	
	P_2HxCDD	pg g^{-1}	Planktonic 1,2,3,6,7,8-HxCDD	
	P_3HxCDD	pg g^{-1}	Planktonic 1,2,3,7,8,9-HxCDD	(13)
	P_HpCDD	pg g^{-1}	Planktonic 1,2,3,4,6,7,8-HpCDD	
	P_OCDD	pg g^{-1}	Planktonic OCDD	
	P_PCB81	pg g^{-1}	Planktonic PCB-81	
	P_PCB77	pg g^{-1}	Planktonic PCB-77	
	P_PCB123	pg g^{-1}	Planktonic PCB-123	
	P_PCB118	pg g^{-1}	Planktonic PCB-118	
	P_PCB114	pg g^{-1}	Planktonic PCB-114	
	P_PCB105	pg g^{-1}	Planktonic PCB-105	
	P_PCB126	pg g^{-1}	Planktonic PCB-126	
	P_PCB167	pg g^{-1}	Planktonic PCB-167	
	P_PCB156	pg g^{-1}	Planktonic PCB-156	
	P_PCB157	pg g^{-1}	Planktonic PCB-157	
	P_PCB169	pg g^{-1}	Planktonic PCB-169	
	P_PCB189	pg g^{-1}	Planktonic PCB-189	

AIII.1. REFERENCES

1. Moreno-Ostos E (2012) Expedición de circunnavegación Malaspina 2010: cambio global y exploración de la biodiversidad del océano. Libro blanco de métodos y técnicas de trabajo oceanográfico.
2. Hansen HP & Koroleff F (2007) Determination of nutrients. Methods of Seawater Analysis, (Wiley-VCH Verlag GmbH), pp 159-228.
3. Tovar-Sánchez A (2012) Comprehensive sampling and sample preparation. Comprehensive Sampling and Sample Preparation : Analytical Techniques for Scientists, ed Elsevier (Pawliszyn, Janusz), pp 317-334.
4. Royer SJ, Mahajan A, Galí M, Saltzman E, & Simó R (2015) Small-scale variability patterns of DMS and phytoplankton in surface waters of the tropical and subtropical Atlantic, Indian, and Pacific Oceans. Geophysical Research Letters 42(2):475-483.
5. Marie D, Simon N, & Vaulot D (2005) Phytoplankton Cell Counting by Flow Cytometry. Algal Culturing Techniques, ed Andersen R (Elsevier, Amsterdam), pp 253-267.
6. Yentsch CS & Menzel DW (1963) A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep Sea Research and Oceanographic Abstracts, (Elsevier), pp 221-231.
7. Gasol JM & Arístegui J (2007) Cytometric evidence reconciling the toxicity and usefulness of CTC as a marker of bacterial activity.
8. Gasol JM & Del Giorgio PA (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Scientia Marina 64(2):197-224.
9. Gasol JM (1999) How to measure bacterial activity and production with the uptake of radiolabeled leucine. (http://www.cmima.csic.es/pub/gasol/Manuals/ProdBact/Leucine.htm).
10. Kirchman D (1993) Leucine incorporation as a measure of biomass production by heterotrophic bacteria. Handbook of methods in aquatic microbial ecology. Lewis:509-512.
11. González-Gaya B, Dachs J, Roscales JL, Caballero G, & Jiménez B (2014) Perfluoroalkylated substances in the global tropical and subtropical surface oceans. Environ Sci Technol 48(22):13076-13084.
12. Berrojalbiz N, et al. (2011) Biogeochemical and physical controls on concentrations of polycyclic aromatic hydrocarbons in water and plankton of the Mediterranean and Black Seas. Global Biogeochemical Cycles 25(4):n/a-n/a.
13. Morales L, et al. (2014) Background concentrations of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in the global oceanic atmosphere. Environ Sci Technol 48(17):10198-10207.

This thesis contributes to the understanding of the interactions between POPs and carbon cycles, focusing specially in the effects of organic pollutants found in seawater on the marine primary production. The use of *Prochlorococcus* as sentinel organism allowed to assess these interactions at a global level, given the genus distribution and abundance. This assessment was possible due to the development of specific molecular methods coping with the variety of *Prochlorococcus* strains. These methods are based on quantifying the expression of two functional genes needed for photosynthesis and that can be used as proxies of the CO_2 fixation and the functionality of the PSII in the cell, respectively. Using this approach, we were able to detect changes in *Prochlorococcus* photosynthetic capability at sublethal level when cells were exposed to a number of environmental conditions, including the presence of organic pollutants.

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