Universidade de Aveiro
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Departamento de Biologia

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SENSITIVITY OF FRESHWATER DIATOMS TO HERBICIDES AND METALS: GEOGRAPHY, PHYLOGENY AND BIOCHEMISTRY

SENSIBILIDADE DE DIATOMÁCEAS DE ÁGUA DOCE A HERBICIDAS E METAIS: GEOGRAFIA, FILOGENIA E BIOQUÍMICA

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Salomé Fernandes Pinheiro de Almeida, Professora auxiliar do Departamento de Biologia e GeoBioTec da Universidade de Aveiro, Doutora Etelvina Maria Paula de Almeida Figueira, Professora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro, e Doutor Fréderic Rimet, engenheiro de investigação do Institut National de la Recherche Agronomique, França

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palavras-chave diatomáceas, herbicida inibidores do PSII, metal, sensibilidade, sinal filogenético, biogeografia, variabilidade intra-específica, bioquímica

resumo

Os ambientes aquáticos estão contaminados por muitos poluentes, incluindo pesticidas e metais. Tem sido demonstrado que as diatomáceas são sensíveis a tais micropoluentes. Estudos anteriores demonstraram grupos monofiléticos de diatomáceas com tolerância homogénea a herbicidas, pressupondo-se que a sensibilidade de um determinado grupo taxonómico é homogénea.

Esta hipótese foi investigada com *Nitzschia palea* e *Achnanthidium* spp, espécies com sensibilidades distintas à poluição, bem como a diversidade filogenética, taxonómica e intra-específica. A hipótese original de sensibilidade do grupo monofilético aos herbicidas também foi testada. Trinta e nove estirpes, incluindo 11 de *Achnanthidium* spp e 14 de *N. palea*, foram submetidas a ensaios biológicos para avaliar a sua sensibilidade aos herbicidas atrazina, terbutrina, diurão e isoproturão. As estirpes de *N. palea* também foram testadas quanto à sua sensibilidade a metais, tais como o cádmio e cobre. Culturas mono-específicas foram expostas a estas substâncias durante 96 horas, após as quais se avaliou a inibição do crescimento. A sensibilidade das estirpes foi combinada com a sua filogenia para verificar a existência de uma ligação entre ambos. Para *N. palea* e *Achnanthidium* spp, a origem das estirpes também foi ponderada para estabelecer se a distância geográfica desempenhava um papel relevante na sensibilidade de estirpes de uma mesma espécie. Numa segunda fase, uma estirpe sensível e uma tolerante de *N. palea* foram expostas a atrazina, diurão, cádmio e cobre, e analisadas a nível bioquímico.

Observou-se uma correlação entre a sensibilidade das espécies para todos os pesticidas, com excepção da atrazina. Quanto às espécies analisadas intraspecificamente, não se encontrou qualquer correlação para as estirpes de *N. palea* e *Achnantidium* spp quando expostas à atrazina, no entanto, para *Achnantidium* spp exposto aos outros herbicidas e *N. palea* exposta a metais verificou-se correlação entre isolados da mesma espécie. A distância geográfica entre isolados não contribuiu significativamente para a variação observada na sensibilidade, com excepção dos ensaios com metais. Ao nível bioquímico, o isolado tolerante e o sensível apresentaram diferentes estratégias, não havendo um parâmetro que se destacasse como possível biomarcador.

keywords

diatom, PSII-inhibitor herbicide, metal, sensitivity, phylogenetic signal, biogeography,intraspecific variability, biochemistry

abstract

Aquatic environments are impacted by many pollutants, including pesticides and metals. It has been shown that diatoms are sensitive to such micropollutants. Previous studies have shown clades of diatoms with homogeneous herbicide tolerance, making the assumption that a given taxon's sensitivity is homogeneous.

This hypothesis was investigated with *Nitzschia palea* and *Achnanthidium* spp, species with distinct sensitivity to pollution, as well as taxonomic and intraspecific phylogenetic diversity. The original clade sensitivity to herbicides hypothesis was also tested. Thirty-nine strains, comprising 11 of *Achnanthidium* spp and 14 of *N. palea*, were bioassayed to assess their sensitivity to the herbicides atrazine, terbutryn, diuron and isoproturon. The *N. palea* strains were also tested for their sensitivity to cadmium and copper. Single strain cultures were exposed to these substances for 96 hours, after which growth inhibition was assessed. Strains' sensitivity was matched to its phylogeny to verify the existence of a link between both. For *N. palea* and *Achnanthidium* spp, strains' origin was also weighted in to establish if the geographic distance played a role in the sensitivity of strains from the same species. In a second phase, one sensitive and one tolerant *N. palea* strain were chosen to be exposed to atrazine, diuron, cadmium and copper and analysed at a biochemical level.

A correlation between species' sensitivity was observed for all the pesticides, except for atrazine. As for the species analysed intraspecifically, while no correlation was found for the *N. palea* strains or *Achnanthidium* spp when exposed to atrazine, it was present for *Achnanthidium* spp, when exposed to the other herbicides and *N. palea* exposed to metals. Geographic distance among each strain isolates did not account significantly for the sensitivity variation observed, with exception of the metal assays. At a biochemical level, tolerant and sensitive strain often presented different strategies, with no parameter standing out as a potential biomarker

Contents

Abreviations

Atr	atrazine
CAT	catalase
Cd	cadmium
Chl *a*	chlorophyll *a*
Chl *c*	chlorophyll *c*
Cu	copper
Diu	diuron
EC_{50}	median effective concentration
GP*x*	glutathione peroxidase
GSH	reduced glutathione
GSTs	glutathione-S-transferase
LPO	lipid peroxidation
N. palea	*Nitzschia palea*
PCO	principal coordinates ordination
Prot	protein
ROS	reactive oxygen species
SOD	superoxide dismutase
TCC	Thonon culture collection

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1 General Introduction

Life on Earth is very abundant in diversity. Less than one fifth of all species have been described, but the number of eukaryotic species already rises above 8 million (Sweetlove, 2011). Different species vary in their ecological preferences, a result of the importance of ecophysiological divergence and local adaptation in the speciation process (Weisse, 2008). Understanding species ecology is extremely valuable, once it can be used for monitoring ecosystems. Species composition and abundance is a tool used by the Water Framework Directive (European Parliament, 2000) to assess the quality of aquatic ecosystems. Organisms from the phytoplankton, macrophytes and phytobenthos, benthic invertebrate fauna and fish are used as biological indicators, in order to assess water quality (European Union, 2000). Diatoms are used as good representative of the vast phytobenthic community (Kelly et al., 2008), and routinely used to evaluate organic, nutrient (e.g. Rimet, 2012a) and metal pollution (e.g. Luís et al., 2009).

Diatoms are ubiquitous in the environment and present both species and morphological diversity (Mann & Vanormelingen, 2013). The multiplicity of valve morphologies is used to distinguish and identify species (Round et al., 1990). Scanning electron microscopy (SEM) allowed a better demarcation of identical aspects under the conventional light microscopy (LM) techniques (Morales et al., 2001). Nonetheless, it was the application of molecular methods to diatom identification that allowed the discovery of several (pseudo)cryptic species (Beszteri et al., 2005; Kermarrec et al., 2013). Since cryptic species have similar morphology but distinct genetics, their finding lead to changes in classification (L. K. Medlin et al., 1993; Williams & Kociolek, 2011). Some of those (pseudo)cryptic species may even present ecological differences (Potapova & Hamilton, 2007; Trobajo et al., 2009), conducting to taxonomical inflation, .and ecological characterization and biomonitoring hindrance (Rimet & Bouchez, 2012).

Knowledge on niche conservatism (Wiens et al., 2010), and possible links between phylogeny and ecology may help to unravel some of the questions (pseudo)cryptic species raised. Some data is available for diatoms' growth forms and niche habitats (Nakov et al., 2015) and phylogeny of ecological preferences (Keck et al., 2015). However, the lengthy and strenuous assays required to define

species niche in the context of micropollutants deems the task rather demanding. Even so, the presence of micropollutants in the aquatic systems, in particular pesticides and metals (Loos et al., 2009; Rimet, 2012b), constitute a risk to the environment and society, increasing the importance of species niche definition.

In the scope of this work, the relation among the sensitivity of 39 freshwater diatom strains to 4 herbicides (atrazine, terbutryn, diuron and isoproturon) and 2 metals (cadmium and copper), their origin and phylogeny was tested. The main goals were: I) to assess if the differences in sensitivity among species were quantifiable phylogenetically. II) to assess if the sensitivity differences within one species were measurable at a phylogenetic level; III) in affirmative case, if those differences can be explained by the contamination at the sampling site and, finally, IV) to assess if the intraspecific differences were detectable at a cellular level.

2 Genetic and geographic signal

2.1 Introduction

2.1.1 Biogeography

Microorganisms' small dimensions and high growth rates allow them to colonize numerous environments. Their distribution is assumed to follow the Baas-Becking hypothesis: "everithing is everywhere: but, the environment selects" (Baas-Becking, 1934; De Wit & Bouvier, 2006) and, therefore, not having a biogeographic signal (Finlay et al., 2004). Also, in the case of protists, the rapid growth and wide dispersion would not be suitable for allopatric speciation or high number of global morphospecies (Finlay et al., 2004).

In order to colonize habitats, species must pass a series of nested filters, which consist of habitat characteristics that will shape the likelihood a taxon has to enter and endure as a community member (Hillebrand & Blenckner, 2002; Poff, 1997). Filters differ from macro to microorganisms, only affected by biotic and abiotic factors (Fig. 1). Species traits' must be in accordance with the filter constrains, so that the species manage to pass the filter and join the community.

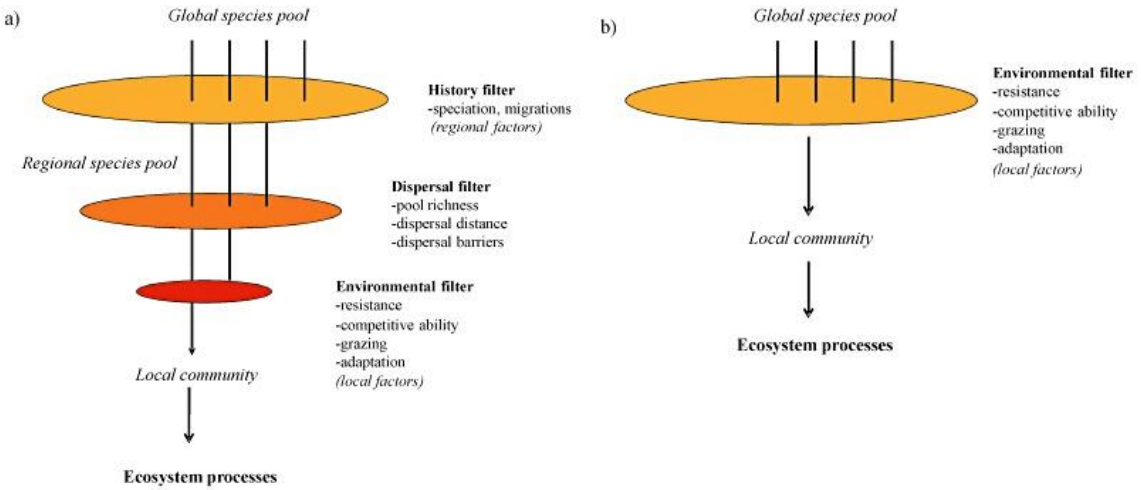

Figure 1 Model of local community assembly process through a series of nested filters, for macroorganisms (a) and ubiquitous microorganisms (b). [Extracted from Soininen (2012)]

Diatoms can be found in aquatic systems over a wide range of temperature, nutrients, salt and pH (Kociolek, 2011), which render them the title of “the ubiquitous diatoms” (Lohman, 1960). Diatoms, however, can be regulated to the same process that condition macroorganisms distribution (Kilroy et al. 2007; reviewed: Soininen 2007; 2012) and have numerous morphospecies as well as endemic species. Among them, benthic diatoms, have managed to specialize in some freshwater microhabitats (Soininen & Eloranta, 2004). Mann & Vanormelingen (2013) theorized gene flow did not occur at a rate high enough over the short-term, favoring parapatric speciation. This hypothesis is in line with the findings of Hillebrand et al. (2001), that geographical distance diminishes communities similarities.

A simpler explanation for endemism lies within the sampling process: if the sampled environment fulfils the species’ habitat requirements, sample size is large and the species is locally abundant, endemism is almost assured. In addition, under-report of rare taxa and the under-sample of adequate environments undermine biogeographical studies (Finlay et al., 2002).

Diatoms have an important role in monitoring, with certain species considered reference in environments from pristine to highly polluted. Several indices for ecological status assessment are based on diatoms’ ecology (e.g. Biological Diatom Index (BDI, Coste et al., 2009; Lenoir & Coste, 1996), Trophic Diatom Index (TDI, Kelly & Whitton, 1995), therefore proper knowledge of autecology will help improve aquatic system evaluation.

2.1.2 Phylogenetic signal

Assessing one species'. sensitivity to a certain chemical is usually based in laboratory bioassays, where responses to chemical exposure are observed and registered. Nonetheless, due to diatoms' high diversity, testing all existing species-chemical combination is unrealistic. For this reason, a monitoring tool that could help predicting a species' sensitivity would improve water management. If we assume chemical sensitivity as a result of traits subject to evolutionary adaptations, the effects of these traits could be modelled into a phylogenetic signal (Guénard et al., 2014). The phylogenetic signal is "a tendency for related species to resemble each other more than they resemble species drawn at random from the [phylogenetic] tree" (Blomberg & Garland, 2002). Two of the most commonly molecular markers used to study phylogenetic relations are the ribosomal 18S (Kooistra & Medlin, 1996), and the plastidial rbcL (Daugbjerg & Andersen, 1997).

The small sub-unit ribosomal DNA (18S rDNA) is one of the most used regions due to its low substitution rate (Kooistra & Medlin, 1996), slow evolution (Mann & Evans, 2007) and hypervariable regions , such as V4 (Elwood et al., 1985; Zimmermann et al., 2011). This gene also presents the advantage of being present in the nuclear genome in numerous copies. The rbcL, in contrast, is present in single copy per chloroplast. The gene coding for the big sub-unit of ribulose-1,5-bisphosphate carboxylase has been previously used in phylogenetic studies along with ribosomal genes (Daugbjerg & Andersen, 1997). Once it is not as conserved as the 18S, rbcL allows a better discrimination at species level, though not powerful enough to distinguish all diatoms (MacGillivary & Kaczmarska, 2011)

Recently, species' phylogenetic data and sensitivity were combined, in order to predict the sensitivity of related species to a certain chemical, using the phylogenetic signal. The existence of a phylogenetic signal has already been investigated in algae (Wängberg & Blanck, 1988), macroinvertebrates (Buchwalter et al., 2008) aquatic insects (Carew et al., 2011), fish (Jeffree et al., 2010) and amphibians (Hammond et al., 2012), which presented variations in strength according to the pollutant tested. Concerning the freshwater diatoms, Larras, Keck et al (2014) conducted a preliminary essay with the purpose of using the use of phylogenetic signal to survey diatoms' sensitivity to herbicides. An

interspecific phylogenetic signal was found (Larras, Keck et al., 2014), which lead to the assumption of intraspecific homogeneity.

The existence of homogeny is a crucial hypothesis in diatoms, given the important number of cryptic species (Mann et al., 2010) and that within one species the signal tends to be feeble (Ashton, 2004). Conversely, it was shown in amphibians that intraspecific variation to pesticide sensitivity was larger than the interspecific variation (Bridges & Semlitsch, 2000; Hammond et al., 2012). If sensitivity variations happen to be large at intraspecific levels in diatoms, this may jeopardize hopes based on the use of the phylogenetic signal to simplify sensitivity assessment, as proposed by Larras, Keck et al. (2014).

Environmental monitoring uses data from species' sensitivity assays to: *a posteriori* association of stressor and respective effect and, once the effects are known, *a priori* prediction of a pollutants impact. However, said assays are performed on model species, which are not representatives of the whole ecosystem. This gap may be filled by means of the phylogenetic signal, through extension of link between phylogenetic and sensitivity.

2.2 Material and Methods

Thirty-nine strains belonging to the Thonon Cell Culture (TCC, available at https://www6.inra.fr/carrtel-collection_eng/Culture-search/List-of-microalgal-cultures) were selected for testing, based on their ecology, origin and physiological state, (Table 1). Strains TCC912, TCC851, TCC852, TCC854 and TCC855 were added to the collection by the University of Aveiro and strains TCC907, TCC911, TCC910, TCC906, TCC908 and TCC909 were added to the collection by the Royal Botanic Garden Edinburgh and Institute for Agri-Food Research and Technology.

Table 1 Strain cultures used in the tests

ID number	Species
TCC849	*Achnanthidium catenatum* (Bily & Marvan) Lange-Bertalot 1999
TCC564	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC667	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC676	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC688	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC696	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC746	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC748	*Achnanthidium minutissimum* (Kützing) Czarnecki 1994
TCC832	*Achnanthidium pyrenaicum* (Hustedt) H.Kobayasi 1997
TCC831	*Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot 1999
TCC833	*Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot 1999
TCC107	*Craticula accomoda* (Hustedt) D.G. Mann ex Round et al.1990
TCC535	*Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot, 1997
TCC752	*Fragilaria perminuta* Lange-Bertalot 2000
TCC653	*Gomphonema parvulum* Kützing 1849
TCC892	*Luticola ventricosa* (Kützing) D.G.Mann, 1990
TCC893	*Luticola ventricosa* (Kützing) D.G.Mann, 1990
TCC897	*Mayamaea permitis* (Hustedt) K.Bruder & Medlin 2008
TCC899	*Mayamaea permitis* (Hustedt) K.Bruder & Medlin 2008
TCC366	*Mayamaea permitis* (Hustedt) K.Bruder & Medlin 2008
TCC139-1	*Nitzschia palea* (Kützing) W.Smith 1856
TCC583	*Nitzschia palea* (Kützing) W.Smith 1856
TCC762	*Nitzschia palea* (Kützing) W.Smith 1856
TCC764	*Nitzschia palea* (Kützing) W.Smith 1856
TCC766	*Nitzschia palea* (Kützing) W.Smith 1856
TCC851	*Nitzschia palea* (Kützing) W.Smith 1856
TCC852	*Nitzschia palea* (Kützing) W.Smith 1856
TCC854	*Nitzschia palea* (Kützing) W.Smith 1856
TCC855	*Nitzschia palea* (Kützing) W.Smith 1856
TCC906	*Nitzschia palea* (Kützing) W.Smith 1856
TCC907	*Nitzschia palea* (Kützing) W.Smith 1856
TCC908	*Nitzschia palea* (Kützing) W.Smith 1856
TCC909	*Nitzschia palea* (Kützing) W.Smith 1856
TCC912	*Nitzschia palea* (Kützing) W.Smith 1856
TCC894	*Nitzschia pusilla* Grunow 1862
TCC898	*Nitzschia pusilla* Grunow 1862
TCC896	*Nitzschia pusilla* Grunow 1862
TCC524	*Sellaphora minima* (Grunow) Mann 1990
TCC635	*Ulnaria acus* (Kützing) M.Aboal, 2003

2.2.1 Cell culture

The strains were grown using variations of two adapted Chu number 10 medium (Chu, 1943). Strains for the herbicide assay were kept in DVII, a modified medium from Hughes & Lund (1962) (available at http://www6.inra.fr/carrtel-collection/Milieux-de-culture/Composition-des-milieux-de-cultures). Strains to be used in the metals assay were grown in a medium where vitamins, salt and EDTA were replaced with f/2 (Guillard & Ryther, 1962) medium nutrients, to avoid chelation

Cells were kept at a temperature of 20°C, light intensity of 100 $\mu E.m^{-2}.s^{-1}$ and a photoperiod of 16h light / 8h darkness.

2.2.2 Cell number assessment

For the toxicity assays, the number of cells was assessed by calibrating the fluorescence measured using a Fluoroskan instrument (Fluoroskan Ascent, Thermo-Scientific, Finland), set with a 430nm excitation and a 680nm emission filter, with the Malassez haemocytometer (Malassez, 1873). The fluorescence against function of the number of cells was plotted for each strain tested and the best fitting curve was calculated. This calibration curve was used to estimate the number of cells after fluorescence readings.

2.2.3 DNA extraction and amplification

A 45 µm filter, used to collect the cells and placed in a 15 mL centrifuge tube, was rinsed with 300 µL of elution buffer (0.01M Tris HCl, 1mM EDTA, pH=8). The cells were then transferred to a 1.5 mL tube, to which was added 200 µL of lysis buffer (0.05M Tris HCl, 0.32M EDTA, 0.75M sucrose). The mixture was then submitted to a temperature shock: 15 minutes at -80 °C followed by 2 minutes at 55 °C and 20 seconds in the ultrasonic bath. After addition of 25 µL of 20% SDS and 10 µL of proteinase K, the sample was incubated with agitation for one hour at 37 °C followed by enzyme inactivation in a 55 °C water bath for 20 minutes. Frustules and cellular debris were then separated by centrifugation for 3 minutes at 13000 rpm, at 4 °C. The supernatant was placed in a 2 mL tube. The DNA present in the supernatant was precipitated by adding 1 µL of GenElute™-LPA (Sigma-Aldrich), 0.1 times the volume of the sample of 3M sodium acetate. Samples were incubated at room temperature during 5-10

minutes, and 1 volume of isopropanol was added. DNA was recovered by centrifugation at 12000 rpm, at 4 °C, for 10 minutes and washed at least twice, with 200µL of 80% ethanol. The remaining alcohol was removed by evaporation in the Speedvac for 20 minutes and the pellet was resuspended in elution buffer.

DNA concentration was assessed by measuring the fluorescence of the sample at 260 and 280 nm. Derived solutions with a final concentration of 25 ng/µL were prepared and the samples were stored at -20 °C, until use.

Polymerase chain reaction (PCR) was used to amplify the two genes of interest for subsequent sequencing of the 28S (large ribosomal unit) V4 region, and rbcL (ribulose-1,5-bisphosphate carboxylase/ oxygenase), both forward and reverse sequences Primer sequences can be found in Table 2.

Table *2* Primer sequences used in amplification

Primer	Sequence (sense 5'->3')	Reference
18S_1F	AACCTGGTTGATCCTGCCAG	Medlin et al., 1988
18S_528F	GCGGTAATTCCAGCTCCAA	Elwood et al., 1985
18S_1528R	CTTCTGCAGGTTCACCTAC	Medlin et al., 1988
rbcL_1F	AAGGAGGAADHHATGTCT	Daugbjerg & Andersen, 1997
rbcL_7R	AAASHDCCTTGTGTWAGTYTC	Daugbjerg & Andersen, 1997

To assess the quality of the PCR, an agarose gel was made. The gel was made adding 1.5g agarose to 100 mL of 0.5% TBE buffer, two drops of ethidium bromide were added to mark the DNA. After polymerisation, 2.5 µL of marker, 4 µL of each sample, positive and negative control with loading buffer were loaded in the wells and run with a voltage of 100V for 40 minutes.

The gels were then analysed using the gel reader and the samples which presented well-defined bands were sent for sequencing, by the Sanger method (Sanger & Coulson, 1975), in GATC Biotech (Konstanz, Germany).

2.2.4 Toxicity assays

The toxicity assays were carried out, in accordance with the Standard Guide for Conducting Static Toxicity Tests with Microalgae (ASTM, 1998), at a temperature of 20 °C, light intensity of 100 $\mu E.m^{-2}.s^{-1}$, a photoperiod of 16h light /8h dark and an initial concentration of 10,000 cells/mL, for 96 hours. The tests were conducted, in triplicate, on 12-well culture plates, containing in each well 1

mL of cell culture and 1 mL of toxic (atrazine, diuron, isoproturon or terbutryn) stock solution.

2.2.5 Data modelling and Effective Concentration (EC) calculation

Each strain tested was fitted and modelled using R software and the *"drc"* version 2.5-12 (Ritz & Streibig, 2005) package. In the case of monotonically decreasing functions, a log-logistic model (Equation 1) was used to fit the data.

Equation 1: $f\big(x,(b,c,d,e)\big) = c + \frac{d-c}{1+\exp(b(\log(x)-\log(e)))}$

where *b* is the relative slope around the EC_{50}; *c* and *d* are the lower and upper limits, respectively; and *e*, the EC_{50} value (Ritz & Streibig, 2005). In the cases where hormesis was observed, the Cedergreen-Ritz-Streibig model (Equation 2) fitted the data better, since it describes an inverted U or J-shaped curve (E. Calabrese & Baldwin, 2002). In this model, *f* describes the stimulation phase, increasing with the hormesis effect.

Equation 2: $f\big(x,(b,c,d,e)\big) = c + \frac{d-c+f exp(-\frac{1}{x^{\propto}})}{1+\exp(b(\log(x)-\log(e)))}$

For the strains whose ECs appeared to be unusual or were not calculated by the software, the ECs were calculated manually, using the equation of the tendency curve connecting the inhibitions immediately before and after the point of interest.

2.2.6 Phylogenetic analysis

The alignment, was made using the MUSCLE algorithm (Edgar, 2004) accessed from SeaView (Gouy et al., 2010). The 'maximum likelihood' phylogenetic trees were constructed using the graphical front-end RAxMLGUI (Silvestro & Michalak, 2011; Stamatakis, 2006). After setting the genes partitions, the GTR model (Tavaré, 1986) was applied, for 20 runs and a thorough bootstrap with 100 repetitions.

The best tree produced was matched with strains' estimated EC_{50}, using the R (R Development Core Team, 2011) packages "*ape" v*ersion 3.3 (Paradis et al., 2004), to read the data, and *"phylobase"* version 0.6.8 (Hackathon et al., 2014), to pair the phylogenetic and sensitivity data. Package *"adephylo"* version 1.1-6 (Jombart & Dray, 2010) was used to measure the phylogenetic signal,

employing Abouheif's test based on Moran's I (Pavoine et al., 2008) and plot the phylogeny/sensitivity diagram.

2.2.7 Mantel's test

The sampling site coordinates (Table 3), sensitivity and phylogenetic data were computed into a distance matrix using the "*ade4*" and "***ape"*** packages. The matrices' correlation, sampling site vs. phylogenetic and sampling site vs. sensitivity, was then compared using the Mantel's test (Mantel, 1967).

Table 3 Location of the *Nitzschia palea* and *Achnanthidium* strains' sampling sites

ID number	Species	Location	Latitude (°)	Longitude (°)
TCC139-1	*Nitzschia palea*	Lake Geneva, France	46.4005	6.565387
TCC583	*Nitzschia palea*	Chiers river, France	49.5328	5.7929
TCC762	*Nitzschia palea*	Chamberonne estuary, Switzerland	46.5034	6.6402
TCC764	*Nitzschia palea*	Chamberonne estuary, Switzerland	46.5034	6.6402
TCC766	*Nitzschia palea*	Venoge estuary, Switzerland	46.5105	6.5959
TCC851	*Nitzschia palea*	Aveiro, Portugal	40.6375	-8.6604
TCC852	*Nitzschia palea*	Casal da Misarela, Portugal	40.2050	-8.3621
TCC854	*Nitzschia palea*	Miranda do Corvo, Portugal	40.0915	-8.3286
TCC855	*Nitzschia palea*	Mogofores, Portugal	40.4507	-8.4623
TCC906	*Nitzschia palea*	Northumberland, England	55.3671	-2.2017
TCC907	*Nitzschia palea*	Northumberland, England	55.3671	-2.2017
TCC908	*Nitzschia palea*	Royal Botanic Garden Edinburgh, Scotland	55.9654	-3.2059
TCC909	*Nitzschia palea*	Royal Botanic Garden Edinburgh, Scotland	55.9654	-3.2059
TCC912	*Nitzschia palea*	Aveiro, Portugal	40.6375	-8.6604
TCC564	*Achnanthidium minutissimum*	Sainte Suzanne river, Réunion Island, France (Indian Ocean)	-20.9070	55.6058
TCC667	*Achnanthidium minutissimum*	Sûre river, Luxembourg	49.9065	5.9591
TCC676	*Achnanthidium minutissimum*	Trentino river, Italy	46.1848	11.3280
TCC688	*Achnanthidium minutissimum*	Piemonte river, Italy	45.1333	7.1500
TCC696	*Achnanthidium minutissimum*	Adige river, Italy	45.4452	10.9928
TCC746	*Achnanthidium minutissimum*	Foron estuary, France	46.3426	6.3792
TCC748	*Achnanthidium minutissimum*	Foron estuary, France	46.3426	6.3792
TCC831	*Achnanthidium straubianum*	Thonon-les-Bains, France	46.3694	27.4407
TCC832	*Achnanthidium pyrenaicum*	Edian river, France	46.2596	6.7255
TCC833	*Achnanthidium straubianum*	Thonon-les-Bains, France	46.3694	27.4407
TCC849	*Achnanthidium catenatum*	Lake Geneva, France	46.4005	6.565387

2.3 Results

2.3.1 Phylogeny

The maximum Likelihood tree (Figure 3), inferred from the concatenated 18S rRNA V4 region and rbcL sequences, comparing 39 diatoms tested over 736 nucleotide positions. The picoplanktonic heterokont *Bolidomonas pacifica* (rbcL: Genbank accession no. HQ912421, and 18S: Genbank accession no. HQ912557) was used as an outgroup taxon, due to its genetic proximity to diatoms. The pennate diatoms were divided into eight clades, Achnanthidium, Stauroneidaceae, Mayamaea, Sellaphoraceae, Fragilariophyceae (araphid),

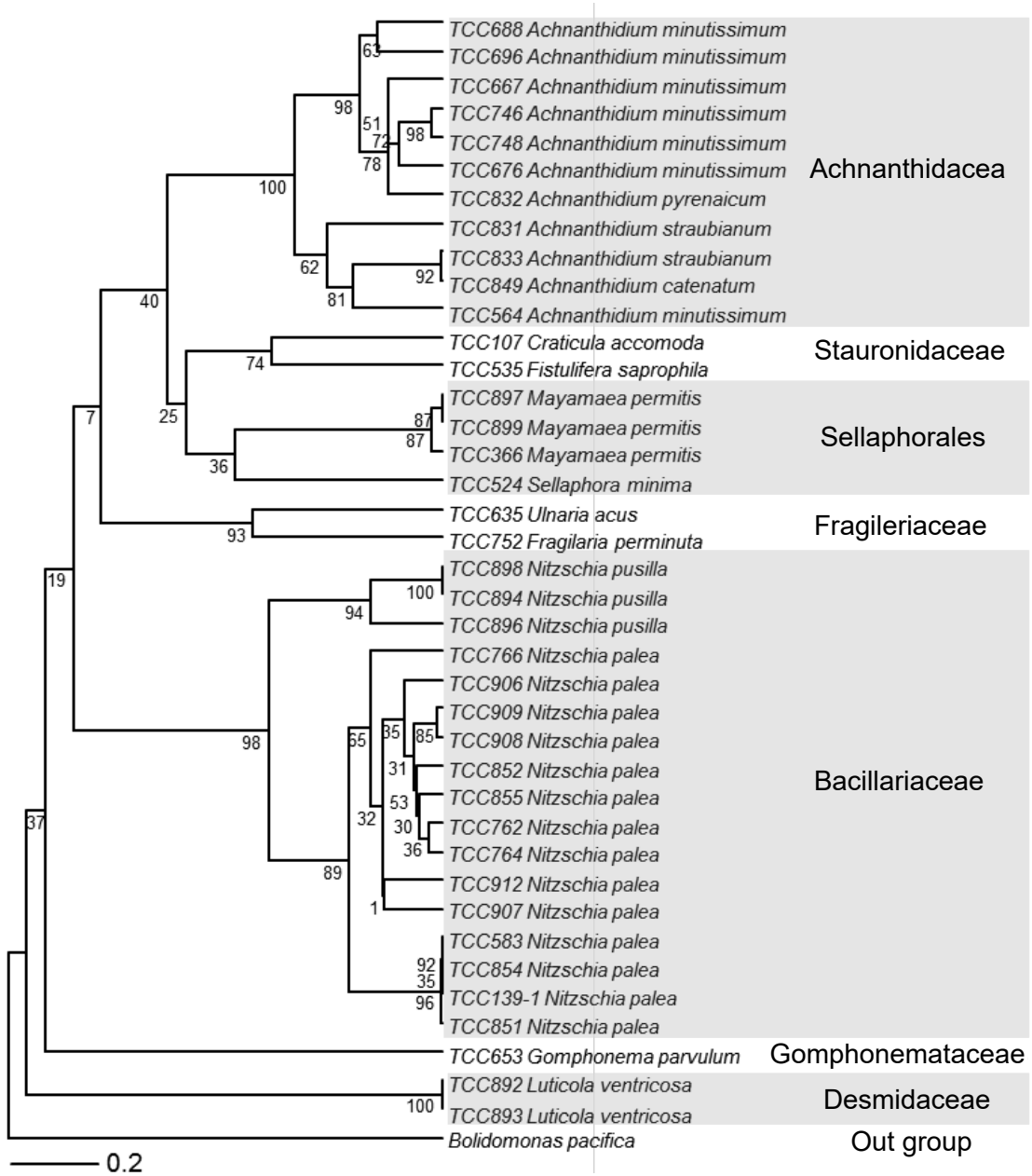

Figure 2 Maximum Likelihood tree of the 18S and rbcL concatenated genes and bootstrap values, from the 39 diatom strains tested

Bacillariales (with a canal raphe system), Gomphonemataceae and Diadesmidaceae.

The two araphid diatoms studied were sister species to the Achnanthidium, Stauroneidaceae and Sellaphoraceae families.

The *A. minutissimum* strains, formed a paraphyletic clade, without the separation of the species *A. pyrenaicum*, *A. straubianum* and *A. catenatum*. Though some of the external nodes had little support, this clade had a 100% support.

All the *N. palea* strains were grouped together, in a monophyletic clade but only a quarter of the branches were well supported (> 70%). The supposition of a shared a common ancestor with *N. pusilla* was well-supported (98%) and the same occurred for the Bacillariaceae ancestor (> 70%). Most of the internal nodes had very little support (< 50%).

2.3.2 Toxicity tests

The dose-response curves from the strains tested displayed very different behaviours, from logistical to inverted-U shaped curves (not shown). In the cases where growth inhibition was higher than 50%, the highest concentration tested was assumed to be the EC_{50}. The strains that have an abnormally high EC_{50} value presented a nearly flat slope and the effective concentration was extrapolated by the fitting model.

2.3.2.1 Herbicides

Table 4 EC_{50} of Atrazine, Diuron, Isoproturon, and Terbutryn affecting the 39 strains tested.

ID number	Species	Atrazine ($mg.L^{-1}$)	Diuron ($mg.L^{-1}$)	Isoproturon ($mg.L^{-1}$)	Terbutryn ($mg.L^{-1}$)
TCC849	*Achnanthidium catenatum*	0.1794	0.0059	0.0130	0.0016
TCC564	*Achnanthidium minutissimum*	0.1547	0.0080	0.0192	0.0076
TCC667	*Achnanthidium minutissimum*	0.2669	0.0369	0.0427	0.0041
TCC676	*Achnanthidium minutissimum*	0.0996	0.0116	0.0182	0.0071
TCC688	*Achnanthidium minutissimum*	0.0998	0.0025	0.0052	0.0009
TCC696	*Achnanthidium minutissimum*	0.1530	0.0105	0.0396	0.0068
TCC746	*Achnanthidium minutissimum*	0.2149	0.0131	0.0400	0.0076
TCC748	*Achnanthidium minutissimum*	0.2203	0.0174	0.0430	0.0078
TCC832	*Achnanthidium pyrenaicum*	0.1944	0.0213	0.0409	0.0072
TCC831	*Achnanthidium straubianum*	0.2290	0.0419	0.0415	0.0114
TCC833	*Achnanthidium straubianum*	0.1100	0.0088	0.0173	0.0028
TCC107	*Craticula accomoda*	3.0700	0.0454	0.1571	0.0110
TCC535	*Fistulifera saprophila*	0.4665	0.0516	0.1293	0.0465
TCC752	*Fragilaria perminuta*	0.5021	0.0200	0.0239	0.0082
TCC653	*Gomphonema parvulum*	0.4347	0.0454	0.0608	0.0013
TCC892	*Luticola ventricosa*	1.0259	0.3127	0.2156	0.0780
TCC893	*Luticola ventricosa*	1.0576	0.2906	0.8678	0.0567
TCC366	*Mayamaea permitis*	0.7400	0.0837	0.1728	0.0398
TCC897	*Mayamaea permitis*	0.4531	0.0315	0.0488	0.0089
TCC899	*Mayamaea permitis*	0.1585	0.0478	0.0147	0.0076
TCC139-1	*Nitzschia palea*	0.1508	0.0021	0.0094	0.0027
TCC583	*Nitzschia palea*	0.4969	0.0379	0.0458	0.0080
TCC762	*Nitzschia palea*	1.2465	0.0379	0.1337	0.0420
TCC764	*Nitzschia palea*	3.3677	0.0484	0.0450	0.0065
TCC766	*Nitzschia palea*	0.4429	0.0322	0.0299	0.0037
TCC851	*Nitzschia palea*	0.3087	0.0303	0.0099	0.0063
TCC852	*Nitzschia palea*	0.4969	0.0379	0.0458	0.0080
TCC854	*Nitzschia palea*	0.7011	0.0427	0.0146	0.0088
TCC855	*Nitzschia palea*	0.0205	0.0361	0.0402	0.0168
TCC906	*Nitzschia palea*	0.1439	0.0173	0.0437	0.0096
TCC907	*Nitzschia palea*	0.1299	0.0092	0.0094	0.0066
TCC908	*Nitzschia palea*	0.3644	0.1299	0.0199	0.0076
TCC909	*Nitzschia palea*	4.0000	0.0257	0.0451	0.1160
TCC912	*Nitzschia palea*	0.1451	0.0052	0.0120	0.0017
TCC894	*Nitzschia pusilla*	0.1581	1.6746	1.1923	0.0089
TCC896	*Nitzschia pusilla*	0.7630	0.0294	0.2207	0.0100
TCC898	*Nitzschia pusilla*	1.5000	0.0375	0.0501	0.0132
TCC524	*Sellaphora minima*	0.1651	0.0155	0.0108	0.0074
TCC635	*Ulnaria acus*	0.0627	0.0138	0.0001	0.0010

In the toxicity assays, the EC_{50} ranged from a few micrograms per litre to some grams per litre (Table 4). The most and least sensitive species varied with

the herbicide tested. For Atrazine, the most sensitive species was TCC855 *Nitzschia palea* (0.0200mgL^{-1}) and the least sensitive TCC764 *Nitzschia palea* (3.368mgL^{-1}). For Diuron, the most and the least sensitive were TCC139-1 *Nitzschia palea* (0.0020 mgL^{-1}) and TCC895 *Mayamaea permitis* (4.0000mgL^{-1}), respectively. TCC894 *Nitzschia pusilla* was the least sensitive to Isoproturon (1.192mgL^{-1}) and TCC635 *Ulnaria acus* (0.0001mgL^{-1}) the most sensitive. *TCC909 Nitzschia palea* was the least sensitive to Terbutryn (0.116mgL^{-1}) and TCC688 *Achnanthidium minutissimum* (0.0009mgL^{-1}) the most sensitive.

2.3.2.2 Metals

Concerning the *N. palea* strains submitted to the metals (Table 5), the cadmium tolerance ranged, from a few micrograms (TCC852: 0.0061mgL-1) to a few grams litre(TCC762: 1.9325mgL-1), while for copper the difference remained at only two orders of magnitude (TCC583: 0.0014mgL-1, TCC909: 0.4073mgL-1).

Table 5 EC_{50} of Cadmium and Copper affecting the N. palea strains tested

***N. palea* strains**	**Cadmium (mg.L^{-1})**	**Copper (mg.L^{-1})**
TCC139-1	0.9493	0.0121
TCC583	0.0096	0.0014
TCC762	1.9325	0.0412
TCC764	0.8797	0.0161
TCC766	0.5365	0.0151
TCC851	0.0087	0.0095
TCC852	0.0061	0.0351
TCC854	0.0091	0.0191
TCC855	0.0371	0.0474
TCC906	0.0126	0.0470
TCC907	0.0433	0.0105
TCC908	0.0149	0.0045
TCC909	0.0484	0.4073
TCC910	0.1035	0.1618
TCC911	0.0465	0.0237
TCC912	0.0209	0.0279

2.3.3 Differences between of Achnanthidium spp. and *N. palea*

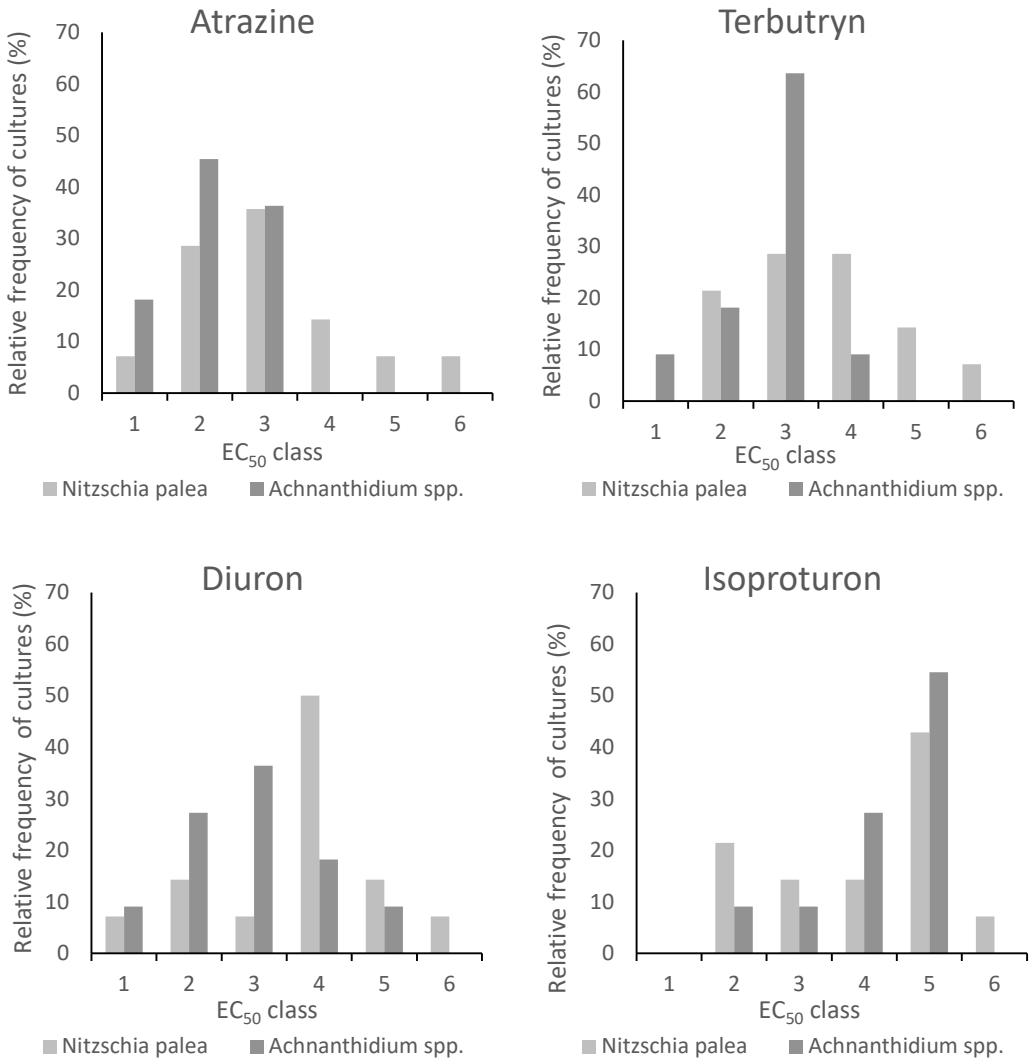

Figure 3 Frequency histogram for the EC_{50} of *Achnanthidium* spp. and *Nitzschia palea*. No differences of EC_{50} were observed between both taxa except for Diuron ($p < 0,05$). Class boundaries: Atrazine: class1 < 0.1 ≤ class2 < 0.2 ≤ class3 < 0.5 ≤ class4 < 2 ≤ class5 < 4 ≤ class6 < 10, Terbutryn: class1 <0.001 ≤ class2 < 0.004 ≤ class3 < 0.008 ≤ class4 < 0.016 ≤ class5 < 0.05 ≤ class6 <0.15, Diuron: class1 < 0.005 ≤ class2 < 0.01 ≤ class3 < 0.02 ≤ class4 < 0.04 ≤ class5 <0.1 ≤ class6 < 0.2, Isoproturon: class1 < 0.005 ≤ class2 < 0.01 ≤ class3 < 0.015 ≤ class4 < 0.03 ≤ class5 < 0.06 ≤ class6 < 0.15

EC_{50} standard deviations and averages of the different strains of *Achnanthidium* spp. and *Nitzschia. palea* were compared (Table 6) and a frequency histogram of EC_{50} of both species is given in (Figure 3). Standard deviations are highly significantly different for Atrazine, Terbutryn and Diuron, weakly significant for Isoproturon: Nitzschia palea shows a higher heterogeneity of EC_{50} than *Achnanthidium* spp. When comparing EC_{50} averages, Atrazine and Diuron show a weak significant difference, *Nitzschia palea* is therefore more

resistant than Achnanthidium spp. No significant differences are observed for Terbutryn and Isoproturon.

Table 6 Comparison of EC_{50} standard deviations and average for *Achnanthidium* spp. and *Nitzschia palea* strains

p-values	Standard deviations comparison (Fisher)	Averages comparison (Mann-Whitney)
Atrazine	< 0.0001	0.067
Terbutryn	< 0.0001	0.22
Diuron	0.008	0.067
Isoproturon	0.016	0.52

2.3.4 Phylogenetic signal

2.3.4.1 Herbicides

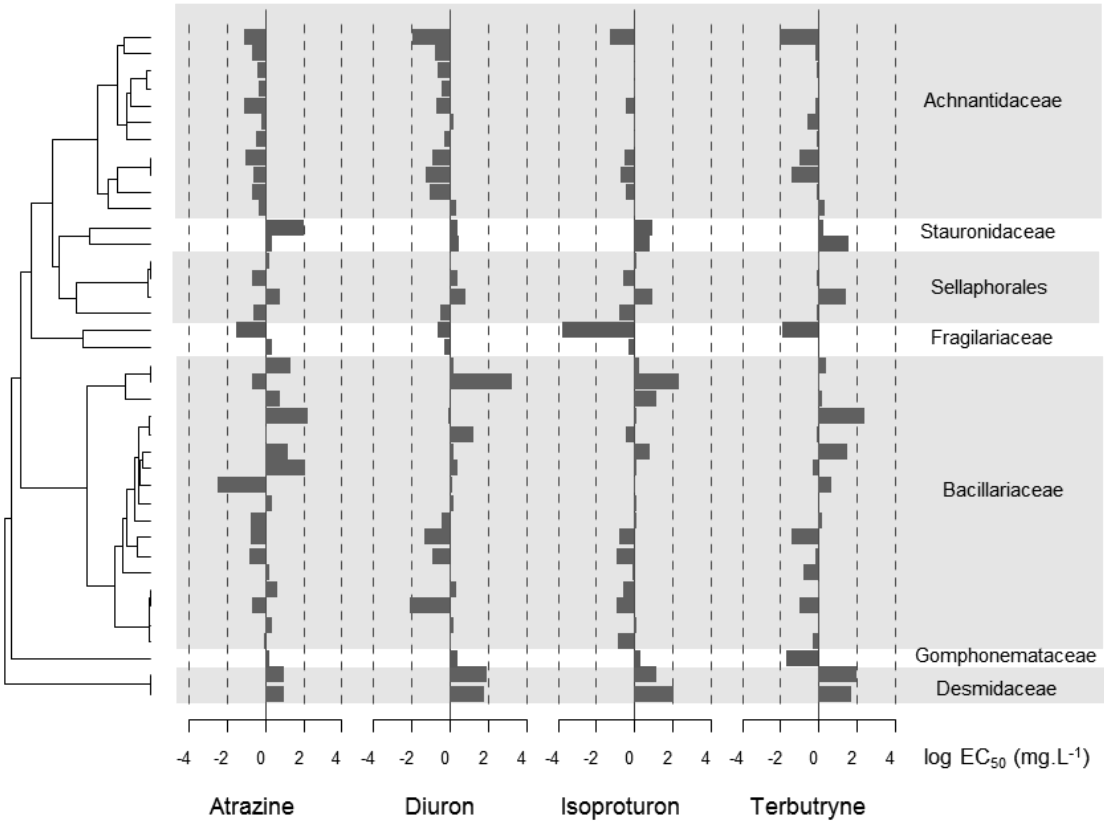

Figure 4 EC_{50} species scores of the 39 diatom species and strains mapped onto the phylogenetic tree. The size of the bar is proportional to the species log EC_{50}

When considering all the species, the phylogenetic signal tested was significant ($p < 0.05$) for the herbicides, except for Atrazine (Fig. 4 and Table 7).

Table 7 Results of the Mantel test for matrix correlation between phylogeny and geographic origin, and geographic origin and sensitivity of *Nitzschia palea* and *Achnanthidium* spp strains to herbicides. Statistically significant values (p <005) are marked in bold.

EC_{50}	**Abouheif's C_{mean} value**		
	All strains	*Achnanthidium* spp.	*Nitzschia palea*
Atrazine	0.099	0.289	0.115
Diuron	**0.264**	**0.372**	-0.039
Isoproturon	**0.251**	**0.347**	0.196
Terbutryn	**0.245**	**0.343**	-0.003
Cadmium	-	-	0.977
Copper	-	-	0.739

When considering *Nitzschia. palea* no phylogenetic signal was observed for the four herbicides. On the other hand, for *Achnanthidium* strains were a significant phylogenetic signal was observed for Diuron, Isoproturon and Terbutryn, but not for Atrazine.

2.3.4.2 Metals

The *Nitzschia. palea* strains exposed to metal did not show any phylogenetic signal (Fig 5 and Table 7).

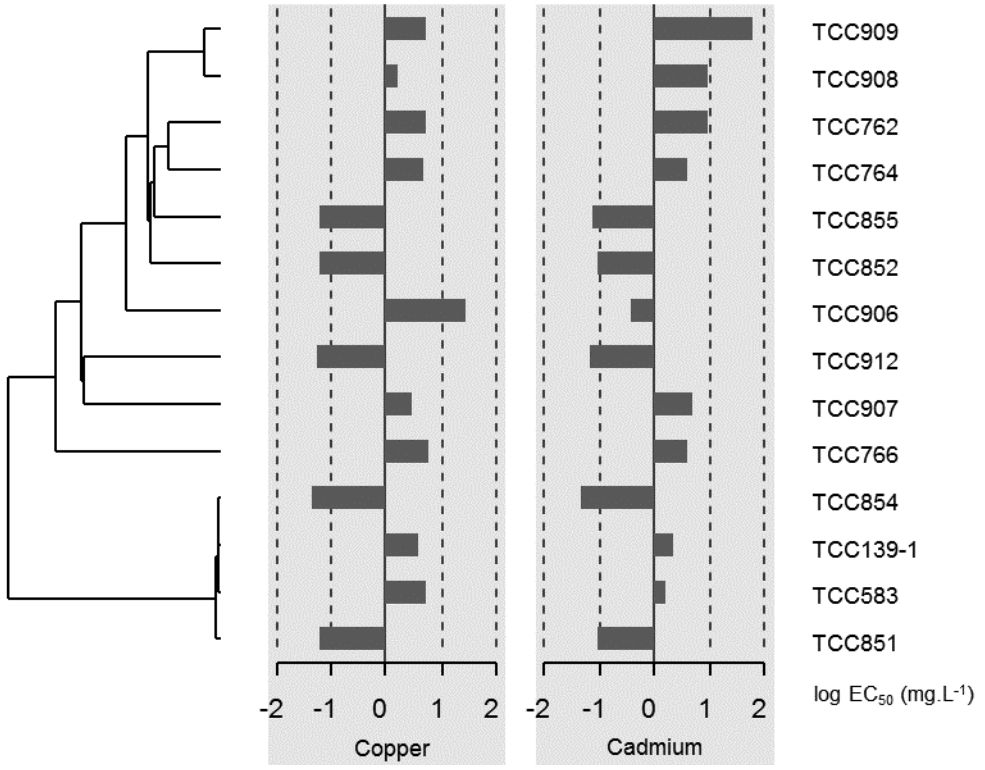

Figure 5 EC_{50} species scores of the 14 *Nitzschia. palea* strains mapped onto the phylogenetic tree. The size of the bar is proportional to the species log EC_{50}

2.3.5 Geographic influence

The effect of the sampling site origin on the pesticide sensitivity of the different strains of *Achnanthidium* and *Nitzschia palea*, measured by the Mantel's test (Table 8) The influence of the geographic distance in the phylogenetic distance was also tested, and was also of no statistical significance, even though the strains came from different countries.

No link was revealed between the strains' origin and their sensitivity to herbicides. On the other hand, for the metals tested, the origin played a significant role ($p < 0.01$) in the *N. palea*'s sensitivity to copper and cadmium.

Table 8 Results of the Mantel test for matrix correlation between phylogeny and geographic origin, and geographic origin and sensitivity of Nitzschia palea and Achnanthidium spp strains to herbicides. Statistically significant values ($p < 0.05$) are marked in bold

Tested factor	***Nitzschia palea* geographic origin**	***Achnanthidium* spp geographic origin**
Phylogenetic distance	0.062	0.305
Sensitivity to Pesticides	-0.004	-0.147
Sensitivity to Atrazine	-0.004	-0.140
Sensitivity to Diuron	0.040	-0.097
Sensitivity to Isoproturon	-0.078	-0.041
Sensitivity to Terbutryn	0.047	-0.135
Sensitivity to Metals	**0.801**	
Sensitivity to Cadmium	**0.661**	
Sensitivity to Copper	**0.769**	

2.4 Discussion

2.4.1 Phylogenetic tree

The maximum likelihood phylogenetic tree was well-supported in the terminal and mid-divergences of the tree, as similarly found by Rimet et al. (2011), with the exception of the nodes inside the Naviculales and *N. palea* clade. The *N. palea* clade also presented low support when analysed using the D1-D2 LSU marker, in a previous study (Trobajo et al., 2009). The internal nodes supporting the pennate diatoms presented a low support, unlike the full supported ones retrieved by Theriot et al. (2010), using the SSU rDNA, rbcL and the psbC markers.

The pennate diatoms used in this study did not group according to the division made by Round et al.(1990): the three araphids bioassayed clustered together with the *Achnanthidium* diatoms. Assumptions regarding the position of the Fragilariophyceae must be done with caution, considering the small representability of the family, despite the high support. In Medlin & Kaczmarska (2009) it was shown that although the raphids are a monophyletic group, the naviculoids evolved forming separate lineages (Medlin et al., 2000), which may be due to the low relatedness of some forms (Kociolek et al., 2015).

The 14 *Nitzschia palea* strains sequenced clustered together and were sister species with *N. pusilla*. Nonetheless, similarly to Rimet et al. (2011), some strains sampled in the same site formed clades with strains from different origins. This was especially true for the Portuguese and French strains. As for *A. minutissimum*, the only two strains from the same site clustered together.

Cryptic species, such as *N. palea* (Trobajo et al., 2009) and *A. minutissimum* (Wojtal et al., 2011), are morphologically undifferentiated but present differences at a genetic level (Vanelslander et al., 2009). Medlin et al., (1993) suggested a slower evolution of the 18S gene than of the morphological traits, the base of diatom identification. This may result in the separation of closely related species.

2.4.2 Toxicity bioassays

2.4.2.1 Herbicides

Diatoms' great species richness grants them sensitivity differences regarding herbicides (Roubeix et al., 2011). For this reason, understanding species autecology and also their individual sensitivities is of major importance for aquatic systems' health assessment (Kociolek, 2011).

Considering the EC_{50} values of the different species for each herbicide, we can order the herbicides from the least to the most toxic thus: atrazine < isoproturon < diuron < terbutryn. This ranking is somewhat dissimilar to the one obtained by Larras et al.(2012), which ranked atrazine< isoproturon< terbutryn < diuron. Furthermore(2012), the sensitivity differences between raphids and araphids were not observed: the sensitivity of the araphids was present in both the top 5 most and least sensitive strains for atrazine, isoproturon and terbutryn. Only the most sensitive strain *N. palea* (TCC855) presented a EC_{50} lower than the maximum allowable concentration of the environmental quality standards (MAC-EQS) established by the European Parliament and The Council (2013) of 1 μgL^{-1} for isoproturon. The remaining most sensitive strains exceeded the 2 μgL^{-1}, 1.8 μgL^{-1} and 0.34 μgL^{-1} MAC-EQS for atrazine, diuron and terbutryn.

Focusing on the least sensitive strains, whereas *N. palea* (TCC764) is polysaprobous (Kelly et al., 2005) and its low sensitivity is expected, *N. pusilla* (TCC894) and *L. ventricosa* (TCC892), oligo-β-mesosaprobic (Alfinito & Iberite, 2013), shouldn't tolerate the pesticides so well. As for the most sensitive, *U. acus* (TCC635) is an oligo-β-mesosaprobic (Alfinito & Iberite, 2013) and *A. minutissimum* (TCC688 and TCC676) is oligosaprobous (Alfinito & Iberite, 2013; Kelly et al., 2005), it's EC_{50} is consistent with those of species inhabiting waters with low pollution levels. In spite of its preference for polluted environments, two of the most sensitive strains to herbicides belonged to the *N. palea* species (TCC855 and TCC139-1). This high sensitivity to the herbicides may be due to decades of subculturing (TCC139-1) or sampling in uncontaminated sites. The absence of correlation goes against the findings of Larras et al. (2012), who linked species ecology given by the biotic indices and herbicide toxicity .

Two of the species chosen, *N. palea* and *A. minutissimum*, were bioassayed for several isolates. Since the other three Achnanthidium species (*A. catenatum, A. straubianum* and *A. pyrenaicum)* were part of the *A. minutissimum* complex

clade, their responses were also included in the statistical analysis of phylogenetic and geographic signals. As mentioned earlier, *A. minutissimum* is a species with a preference for oligotrophic waters, while *N. palea* thrives in highly polluted ones. When ranking the sensitivities, the *N. palea* strains tend to have a lower sensitivity and higher variation to the herbicides than the *Achnanthidium* spp. Nevertheless, the difference is only significant ($p < 0.05$) for the diuron treatment.

Typically, the dose-response curve can be modelled by a logistic regression. Some strains in this bioassay, however, presented a small stimulation at the lowest concentrations, an effect known as hormesis or greening effect (E. Calabrese & Baldwin, 2002). This greening effect, at low concentrations, is regularly induced by triazines (Gustavson et al., 2003) and phenylureas (Roubeix et al., 2011; Schmitt-Jansen & Altenburger, 2005). The *A. minutissimum* presented this effect in 54.5% strains tested for atrazine, diuron and terbutryn, and 45.5% when tested for isoproturon. *N. palea* presented this effect in 71.4%, 92.9%, 85.7% and 78.6% of the strains tested for atrazine, diuron, isoproturon and terbutryn, respectively. This effect may be a result from the intensified pigment production, to counteract the photosynthetic efficiency loss caused by the herbicide (Roubeix et al., 2011).

2.4.2.2 Metals

Copper was more toxic than cadmium, following the study of Emmanuel et al.(2008). Among the 14 strains, the cadmium EC_{50} ranged from 0.0061mgL^{-1} and 1.9325mgL^{-1}, values that comprise the 0.3mgL^{-1} reported by Heredia et al. (2012) and the 0.2mgL^{-1} reported by Branco et al. (2010). Regarding copper however, the mean EC_{50} value (0.05505) was far below the 1.5 reportedly tolerated (Patrick, 1977).

Copper in solution bind to the cell walls, reducing the availability of the dissolved cation (Franklin et al., 2002). Since algae require trace amounts of copper (Manahan & Smith, 1973), at low concentrations(0.001-0.15), this happening may lower its dose to a level where it can excerpt its micronutrient effect, thus explaining the observed hormesis observed in the first concentrations bioassayed for half of diatoms.Although this is true for copper, it does not apply to cadmium since its only biological role as a nutrient is in marine environments, while zinc deficit limits diatom growth (Lee et al.,

1995). In such circumstances, the cadmium molecule replaces the zinc one in the carbonic anhydrase (Lane et al., 2005).

The growth inhibition caused by copper is a result of the growth yield reduction, inhibition of the division and decrease of the photosynthetic pigment production (Masmoudi et al., 2013). Conversely, cadmium triggers a series of molecular, biochemical and cellular effects, from nutrient competition to xanthophyll cycle deceleration, which account for differences in individual sensitivities (Masmoudi et al., 2013). This may explain the 316-fold difference between the least and the most sensitive to cadmium but not the hormetic response observed in 64.3% strains tested (not shown). That response may result from increased enzymatic activity, sequestration and repairing mechanisms or a homeostatic response (Stebbing, 2003) of the cell.

2.4.3 Inter and intraspecific phylogenetic signal

In order to measure several species' trait values statistical dependence on their phylogenetic relationships, the phylogenetic signal is used (Revell et al., 2008). This signal is expected when analysing ecological variation (Kamilar & Cooper, 2013) therefore, its use has been suggested to predict pesticide sensitivity of not yet tested species (Guénard et al., 2014), reducing the need for laborious cell counting and expensive ecotoxicity bioassays.

However, this signal strongly depends on the phylogeny and data input (Losos, 2008), and the size of the sample will affect the uncertainty and variation of the phylogenetic signal index used (Münkemüller et al., 2012). In addition, trait variation along the phylogenetic tree (Guénard et al., 2011) and convergent evolution produce a positive signal, more related to adaptation than evolution (Carew et al., 2011; Losos, 2008).

Following the results of Larras, Keck et al. (2014), we tested the hypothesis of the presence of a phylogenetic signal at an interspecific level, but also at an intraspecific level. These species were chosen considering their importance in biomonitoring, derived from *N. palea* polluo-tolerance and *A. minutissimum* polluosensitivity (Kelly et al., 2005).

We observed a significant phylogenetic signal among all species tested for the herbicides, which is in line with what was expected from the literature (Larras,

Keck et al., 2014), only atrazine sensitivity (the least toxic herbicide) did not show a phylogenetic signal.

The case of the intraspecific variability, however, is more complex: a phylogenetic signal was found significant for *A. minutissimum* but not for *N. palea*. This may be a result of the *A. minutissimum* strains tested which were a collection of several morphological species (Wojtal et al., 2011), whereas *N. palea* is in fact a single monophyletic species (Rimet et al., 2014). Additionally, the sequence variability among *A. minutissimum* strains amounts to 4.9%, while *N. palea*'s variability remains at 1.4%. Considering that the strength of the signal is proportional to the number of base substitutions (Philippe et al., 2011), the absence of signal among the *N. palea* strains was expected. Furthermore, according to Ashton(2004), recent separations have a low phylogenetic signal.

The lability of the signal may be due to a random evolution of the phenotype, independently of the genotype (Kamilar & Cooper, 2013), resulting in differences among close relatives (Blomberg et al., 2003).

2.4.4 Geographic influence

In this study, the Mantel's test was used to test the assumption that genetic divergence of individuals is related to their geographical remoteness, and this could have also an impact of the sensitivity of *N. palea* and *A. minutissimum* strains.

With respect to unicellular organisms' distribution, two opposing schools of thought exist: the cosmopolitan one, supporting that communities are mainly under environmental control, and the other saying that communities exhibit biogeographic and macroecological patterns as macroorganisms, being under local and regional control (e.g. Soininen, 2012). Since the several strains of *N. palea* and *Achnanthidium* bioassayed had distinct geographical provenance, its weight in the diatoms sensitivity was measured.

2.4.4.1 Herbicides

According to Potapova & Hamilton (2007), *A. minutissimum* has six closely related groups, with distinct ecologies. In our study, the *Achnanthidium* group presented a phylogenetic signal for the herbicides bioassayed (excluding atrazine) but did not show any link between the geography effect on the sensitivity

or phylogenetic distance. For the *N. palea* strains, the sampling site did not have a significant impact on the sensitivity to the herbicides bioassayed or the populations' genetic diversity.

This absence of geographic influence doesn't agree with the limits imposed to populations' dispersion by geographic distance and the consequent positive relation found between geography and genetics (Casteleyn et al., 2009). The absence of geographic signal for the *N. palea* strains can be explained by the reduced number of strains we tested, compared to former studies showing the importance of geography (Rimet et al., 2014). For the *A. minutissimum* strains, it is probably due to the presence of more than one species (Wojtal et al., 2011) with different herbicides responses therefore hiding the geographic signal.

2.4.4.2 Metals

While the dimension of the *N. palea* dataset was identical for both herbicides and metals, the latter exhibit a link between diatom's source and their sensitivity. Given that no correlation was found that justifies local adaptation, alterations may be in gene expression rather than the gene itself (Morgan et al., 2007). Changes at the phenotype level have been previously described as an alternative to local adaptation (eg.: Matesanz et al., 2012; Sultan & Spencer, 2002).

Kawecki & Ebert (2004) suggested a heterogeneous environment favours plasticity over genetic adaptation, which is more advantageous than specific phenotypes for different medium composition (Rengefors et al., 2015). Taking this into account we may infer that, although the sampling sites differ in water composition, media's dissolved metals do not justify trait selection.

2.5 Conclusions

The 39 diatoms strains tested presented a phylogenetic signal at the interspecific level for Diuron, Isoproturon and Terbutryn but not Atrazine, the least toxic herbicide tested. At an intraspecific level, however, a significant phylogenetic signal was found only for one of the two cryptic species tested, *A. minutissimum*. Recent separation or being only one single species in fact may explain *Nitzschia palea*'s absence of signal. On the other hand, Achnanthidium minutissimum's phylogenetic signal may be due to the presence of several discrete but cryptic species.

3 Biochemical assays

3.1 Introduction

3.1.1 Effect of metals and herbicides on diatoms

Micropollutants, or emerging contaminants, are present in the aquatic ecosystems at low concentrations but pose, nonetheless, a toxicological threat to the environment (Schwarzenbach et al., 2006). In addition, numerous of the methods currently used in the treatment of water sources do not remove micropollutants efficiently (Bodzek, 2015).

Micropollutants can be distributed in two main categories: organic, such as pharmaceuticals, plant protection products, halo-organic and phenol compounds; and inorganic: metals and anions, such as chlorates and bromates. Some of these micropollutants are included in the 45 priority substance list of the Water Framework Directive (WFD, European Union 2000; European Union 2013). Despite that, atrazine, diuron, isoproturon and terbutryn are among the most commonly found herbicides in freshwater (Loos et al., 2009). Cadmium and copper are also of concern for aquatic organisms (Goudie, 2013). Still, research involving these micropollutants is uneven, once most existing ecotoxicological studies focus on the interaction of diatoms and metals, and only a small portion on pesticides (Rimet, 2012b). While belonging to different categories, both metals and herbicides are known to impair photosynthetic activity (Fig. 6), increasing the production of reactive oxygen species (ROS) (Kumar et al., 2014).

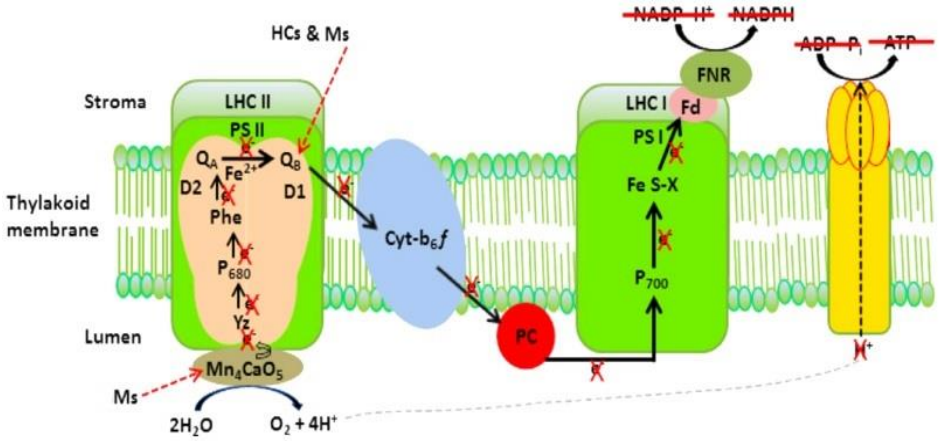

Figure 6 Schematic representation of the impact of metals (Ms) and herbicides (HCs) on the light reaction of photosynthesis in algae. Metals can bind to the Oxygen-Evolving Complex (Mn_4CaO_5), inhibiting electron transport through photosystem II (PSII) to the secondary electron acceptor quinone (Q_B). Quinone B can be directly inhibited by herbicides and metals, blocking the electron flow through cytochrome B6/F (Cyt-b_6/f) and plastocyanin (PC) until the primary donor (P700) of photosystem I (PSI). Electrons transferred through photosystem I are accepted by ferrodoxin (Fd) which, together with ferredoxin-NADP reductase (FNR), reduces NADP to NADPH. ATP synthase then generates ATP from ADP+P_i using the energy from the proton (H^+) gradient created during water split and proton translocation. [Adapted from Kumar et al. (2014)]

In addition to impact on photosynthesis, metals can also bind with biological molecules, due to their high affinity to oxygen, nitrogen and sulphur (Nieboer & Richardson, 1980), and promote oxidative stress, by disrupting the equilibrium between production and scavenging of ROS.

Pollution sensitivity in diatoms depends on the species considered. Differences in sensitivity are also used in order to evaluate water quality in guides, such as the specific pollution-sensitivity index (PSI, CEMAGREF 1982), regarding organic contamination. Diatoms' rapid response to chemical alterations to the surrounding media (Dixit et al., 1992), as well as their elevated diversity - estimated circa 100,000 taxa (reviewed in Mann & Vanormelingen, 2013)-, make them good monitors of aquatic systems.

3.1.2 Biochemical markers

Phenotypic traits may evolve independently of the genotype (Kamilar & Cooper, 2013), resulting in differences in sensitivity not visible at a genetic level. Adaptation to polluted environments can be a result of acclimatization (Marvá et al., 2010), physiological adaptation to the surrounding media's conditions. Several physiological responses to sub-lethal concentrations of chemicals have been described, such as biomass reduction photosynthetic impairment, activity stimulation of ROS-scavenging enzymes and molecules (Sabater et al., 2007). Among these responses, species' responses to oxidative stress have been used as exposure biomarkers to herbicides, metals and other pollutants (Branco et al., 2010; Lozano et al., 2014).

Oxidative stress is characterized as an imbalance between ROS production and the organism's ability to regulate them, possibly resulting in tissue damage (Betteridge, 2000). Despite ROS important role in signaling (reviewed in Mittler et al., 2011), if not regulated properly can interact with lipids, DNA, carbohydrates and proteins (Møller et al., 2007). In order to avoid the damage triggered by those interaction, microalgae engage several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), reductase (GR) and peroxidases (GPx); accompanied by non-enzymatic molecules, such as pigments and polysaccharides (reviewed in Cirulis et al., 2013).

Table 9 Reactive Oxygen Species (ROS) interactions and removal mechanisms [Adapted from Møller et al. (2007)]

ROS	Reacts with	Removal mechanisms	Inactivation product
Oxygen singlet ($^1O_2$)	Pollyunsaturated fatty acids (PUFAs) Guanine Tryptophane, Histidine, Tyrosine, Methionine, Cysteine	Carotenes	O_2
Superoxide ($O_2^{\bullet-}$)	Protein's iron-sulphur (Fe-S) centres	Superoxide dismutase (SOD)	H_2O_2
Hydrogen peroxide (H_2O_2)	Cysteine	Catalase (CAT) Glutathione peroxidases (GP*x*) Glutathione cycle	H_2O
Hydroxyl radical (HO•)	PUFAs DNA Carbohydrates Proteins	-	-

Quantifying ROS is challenging once their half-life is very short. Measuring antioxidant response or oxidative damage is a more straightforward way estimate the degree of stress diatoms are subject to, when expose to sub-lethal concentrations of a micropollutant. Diatoms are known to alter antioxidant response when exposed to metals (reviewed in Masmoudi et al., 2013) and herbicides (Lozano et al., 2014). This shifts, if correlated with micropollutant presence in the environment, can be used as a surrogate for strenuous diatom identification and counting. For instance, variations in chlorophyll a fluorescence due to pollutant exposition have the suitability to become a non-invasive way to monitor aquatic media (Kumar et al., 2014; Serôdio et al., 1997). Considering environmental concentrations tend to instigate more sub-lethal than lethal responses, evaluating the degree of stress the populations are subjected to, could help improve monitoring process and ecological management.

3.2 Material and Methods

3.2.1 Cell culture, counting and harvesting

Based on the standard tests, two *Nitzschia palea* strains, one tolerant and one sensitive, were selected for biochemical assays. The test concentrations chosen were based on the expected inhibition of 25%, 50% and 75% (Table 10). In order to produce the required biomass, the number of batch replicates was adjusted to each concentration tested, with a minimum of 3 replicates per condition. Erlenmeyers containing 50 mL of medium were grown under the same light, photoperiod and temperature conditions as the assays to determine the effective concentrations.

Table 10 Strains and concentrations tested, for the different chemicals

Chemical	Strain	C1 (mgL^{-1})	C2 (mgL^{-1})	C3 (mgL^{-1})
Copper (from $CuSO_4$)	TCC910	0.0004	0.004	0.04
	TCC912	0.040	0.080	0.160
Cadmium ($CdCl_2$)	TCC762	0.140	1.960	27.440
	TCC854	0.001	0.010	0.140
Atrazine	TCC852	0.250	0.630	1.560
	TCC907	0.040	0.100	0.250
Diuron	TCC908	0.035	0.120	0.430
	TCC907	0.003	0.010	0.035

At the end of the 96 hours, the cells were counted using the Utermöhl settling technique (Utermöhl, 1958), after homogenization in an ultrasonic bath. A drop of Lugol's solution was added to an aliquot, in order to kill, mark the starch and weight down the cells (Lund et al., 1958). The cells were then harvested by centrifugation at 10,000 xg speed, during 10 minutes, at 4ºC. The supernatant was discarded and the pellet was resuspended in medium, in order to obtain a concentration of $5x10^6$ cells mL^{-1}. Aliquots of $1x10^6$ cells mL^{-1} were made and harvested. The supernatant was discarded and the pellet was stored at -20 ºC until further use.

3.2.2 Pigment quantification

One aliquot was resuspended in 1mL of 90% acetone, on a 2.5mL Eppendorf tube covered with aluminium foil. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant kept for pigment quantification. Liposoluble pigments were quantified using spectrophotometry.

Sample's absorption was measured at five different wavelengths: 470nm, 581nm, 631nm, 664nm and 750nm. Chlorophyll *a* and chlorophylls *c* contents were calculated using the Jeffrey & Humphrey's formulas (1975):

$$\text{Chl } a = 11.47\text{E}_{664} - 0.40\text{E}_{630}$$
$$\text{Chl } c = 24.36\text{E}_{630} - 3.73\text{E}_{664}$$

Fucoxanthin was calculated using the Seely et al. (1972) formula:

$$\text{Carotenoids} = \frac{\text{E}_{470} - 1.239(\text{E}_{631} + \text{E}_{581} - 0.30\text{E}_{664}) - 0.275\text{E}_{664}}{141}$$

Cellular pigment content was expressed as ng.10^6 cells^{-1}.

3.2.3 Antioxidants

3.2.3.1 Reduced glutathione

Reduced glutathione was indirectly measured by quantifying the formation of 2-nitro-5-thiobenzoic acid (TNB) through 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) reaction with the thiol group of GSH (Rahman et al., 2006).

One aliquot was resuspended in 0.1% Triton X-100, 0.6% sulfosalicylic acid in 0.1M potassium phosphate, 5mM EDTA (pH= 7.5) extraction buffer. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant was stored at -20 ºC until use.

Eight standards (0, 3, 7.5, 15, 30, 45, 60, 75 nmol.mL^{-1}) were made by diluting a 200 µmol.mL^{-1} GSH stock solution in the potassium phosphate buffer, with the purpose of obtaining the calibration curve. To each well of a 96-well microplate 233 µL of reaction buffer (0.1M phosphate buffer (pH=7.5), 5mM EDTA), 33.3 µL of sample or reaction buffer (blank) and 20 µL of DTNB (1.7 mM) were added. Absorbance increase was followed every 30 seconds for 2 minutes, at 412 nm. Reduced glutathione content was then calculated using the calibration curve and results were expressed as nmol.10^6 cells^{-1}.

3.2.4 Activity of antioxidant enzymes

3.2.4.1 Superoxide dismutase

Superoxide dismutase activity was quantified using an indirect method that utilizes nitroblue tetrazolium (NBT) conversion to NBT-diformazan (formazan dye) via superoxide radical (adapted from Beauchamp & Fridovich, 1971).

Nitroblue tetrazolium was added to the reaction buffer (50 mM Tris-HCl (pH 8), 0,1 mM DTPA, 0,1 mM Hipoxantine) to make a 68.4 µM solution. Eleven standards (0, 0.05, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 30, 60 $U.mL^{-1}$) were made by diluting a 300 $U.mL^{-1}$ SOD stock solution in 50 mM Tris-HCL (pH= 8) solution, in order to obtain the calibration curve.

One aliquot was resuspended in 1% Triton X-100, 1mM EDTA, 1% PVP and 1mM DTT in 50mM potassium phosphate (pH= 7) extraction buffer. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant kept for quantification.

In a 96-well microplate 25 µl of sample (or 25 µl of standard +25 µl of extraction buffer D), 250 µl of reaction buffer+ NBT (or 225 µl for the standard) and 25 µl of xanthine oxidase (31.5 mU) were added. Plates were then incubated under agitation (50 rpm) for 20 minutes, after which the absorbance was read at 560nm. Activity was then calculated using the calibration curve and results were expressed as $U.10^6$ $cells^{-1}$.

3.2.4.2 Catalase

Catalase activity was indirectly measured through quantification of formaldehyde derived from enzymatic reaction with methanol, using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as a chromogen (Johansson & Borg, 1988).

Eight formaldehyde standards (0, 1, 2.5, 5, 15, 30, 45,60 µM) were made by dissolving 4.25 mM formaldehyde in reaction buffer (100 mM potassium phosphate, pH= 7), in order to obtain the calibration curb.

One aliquot was resuspended in 1% Triton X-100, 1mM EDTA, 1% PVP and 1mM DTT in 50mM potassium phosphate (pH= 7) extraction buffer. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10

minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant kept for quantification.

In a 96-well microplate were added 125 µL of reaction buffer, 37.5 µL methanol and 25 µL of sample or standard. To start the reaction, 25 µL of hydrogen peroxide was added to each well. Plates were then incubated for 20 minutes under agitation, after which 37.5 µL of potassium hydroxide (10 M) and 37.5 µL of Purpald (34.2 mM) were added to stop the reaction. Plates were incubated for 10 minutes with agitation, followed by the addition of 12.5 µL potassium periodate (65.2 mM) and additional 5 minutes of incubation The. absorbance was then read at 540 nm, activity was calculated and expressed as $nU.10^6$ $cells^{-1}$.

3.2.4.3 Glutathione peroxidase

Glutathione peroxidase activity was indirectly determined through NADPH consumption, which is used in GSSG reduction to GSH by GPx, after the enzyme reducing cumene hydroperoxide and producing GSSG (Paglia & Valentine, 1967).

One aliquot was resuspended in 1% Triton X-100, 1mM EDTA, 1% PVP and 1mM DTT in 50mM potassium phosphate (pH= 7) extraction buffer. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant kept for quantification.

To a reaction mix containing 15 µL of sample or blank, 154.5 µL of dilution buffer (50 mM Tris-HCl (pH 7.6), 5 mM EDTA), 60 µL of GSH (5mM), 45 µL pf cumene hydroperoxide (2mM) and 3 µL of glutathione reductase (25 $U.mL^{-1}$), 22.5 µL of NADPH (2 mM) was added to start the reaction, in a 96-well microplate.

Absorbance at 340 nm was read every 10 seconds for 5 minutes and the activity was calculated based on NADPH molar extinction coefficient (ε= 0.00622 μM^{-1} cm^{-1}) and expressed as $nU.10^6$ $cells^{-1}$.

3.2.4.4 Glutathione-S-transferases

Glutathione-S-transferases activity was measured by quantifying the increase of thioester resulting from conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974).

One aliquot was resuspended in 1% Triton X-100, 1mM EDTA, 1% PVP and 1mM DTT in 50mM potassium phosphate (pH= 7) extraction buffer. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant kept for quantification.

To each well of a 96-well microplate 100 µL of sample or phosphate buffer (blank) was added, followed by 200 µL of reaction mix, consisting of 165 µL 0.1 mM phosphate buffer (pH= 6.5), 30 µL of GSH (10 mM) and 5 µL of CDNB.

Absorbance at 340 nm was read every 10 seconds for 5 minutes and the activity was calculated based on CDNB molar extinction coefficient (ε= 9.6 mM^{-1} cm^{-1}) and expressed as $nU.10^6$ $cells^{-1}$.

3.2.5 Indicators of cellular damage

3.2.5.1 Protein content

Total protein content was directly assessed by quantifying the protein binding to Comomassie Brilliant Blue G-250, which changes the dye's maximum absorption wavelength, from 465 nm to 595 nm (Bradford, 1976).

One aliquot was resuspended in 1% Triton X-100, 1mM EDTA, 1% PVP and 1mM DTT in 50mM potassium phosphate (pH= 7) extraction buffer. Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant kept for quantification.

Ten standards (0, 0.005, 0.025, 0.05, 0.125, 0.25, 0.5, 1, 1.5, 2 $mg.mL^{-1}$) were made, by diluting Bovine Serum Albumin (BSA) in extraction buffer, to establish a calibration curve.

In a 96-well 5 µL of sample or standard and 250 µL of Bradford's reagent were added. Plates were incubated for 15 minutes under agitation, after which absorbance at 545 nm was read and protein determined based on the calibration curve and expressed as $mg.10^6$ $cells^{-1}$.

3.2.5.2 Lipid peroxidation

Lipid peroxidation was indirectly measured by quantifying fatty acids peroxidation products, which react with thiobarbituric acid (TBA), forming a coloured adduct (Ohkawa et al., 1979)

One aliquot was resuspended in 1mL of 20% trichloroacetic acid (TCA). Cells were then broken with the help of an ultrasonic probe, for 20 seconds. After 10 minutes centrifugation, at 4ºC and 10,000 xg speed, the pellet was discarded and the supernatant stored at -20 ºC.

In a 2.5 mL microtube were added 100 µL of sample or water (blank), 200 µL of TBA (0.5 % in 20 % TCA) and 300 µL of TCA. After puncturing the lid, microtubes were incubated at 96ºC for 25 minutes, and subsequently transferred to ice to cool. The concentration of LPO was calculated using MDA molar extinction coefficient (ε= 1.56x105$M^{-1}.cm^{-1}$) and expressed as nmol.10^6 $cells^{-1}$.

3.2.6 Statistical treatment

Biochemical parameters from each strain were submitted to hypothesis testing using the PERMANOVA routine (permutational multivariate analysis of variance) from PRIMERv6 (Anderson et al., 2008; Clarke & Gorley, 2006), following the calculation of Euclidean distance matrices for each strain. The Monte Carlo p-values in the PERMANOVA main tests were evaluated in terms of significance among different concentrations. Values lower than 0.05 were considered significantly different. Differences between concentrations were indicated by distinct letters. Also, the biochemical descriptors obtained from each chemical were normalized and submitted to the calculation of Euclidean distance matrices to perform Principal Coordinates Ordination (PCO, PRIMER v6).

3.3 Results

Several parameters were measured and analyzed as potential biomarkers for diuron toxicity. Parameters such as growth, protein content, lipid peroxidation levels and total glutathione content were quantified; antioxidant response of the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferases were determined and, finally, lipophilic pigment content was assessed through chlorophyll *a* chlorophyll *c* and carotenoids

3.3.1 Responses to Diuron

The sensitive strain's response to C3 (0.035 mg.L^{-1}) was not quantified due to a biomass loss, which averted the quantification.

3.3.1.1 Growth

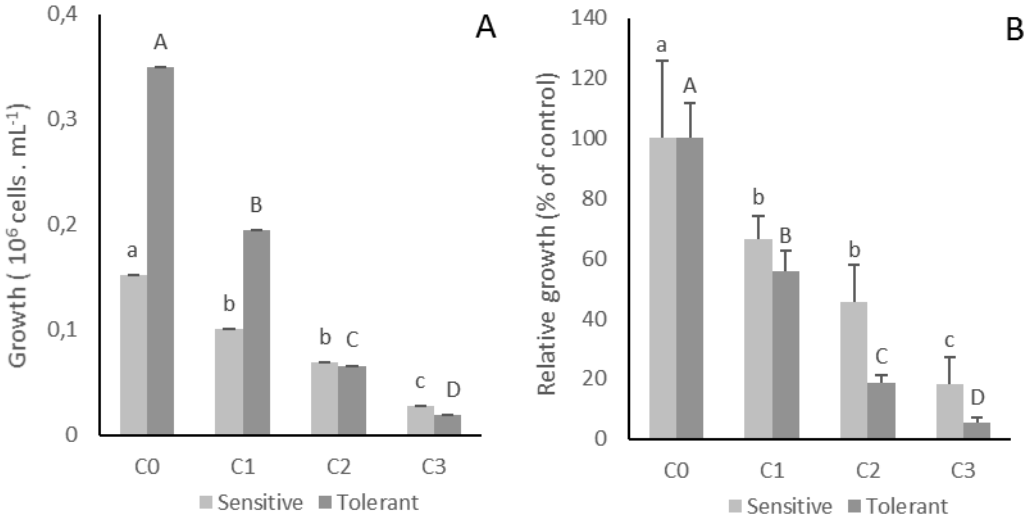

Figure 7 Growth (10^6 cells.ml^{-1}) (A) and relative growth (% of control) (B) of tolerant (TCC908) and sensitive (TCC907) *Nitzschia palea* strains exposed to diuron. For TCC907 diuron concentrations were C0: 0; C1: 0.003; C2: 0.01; C3: 0.035 mg.L^{-1} ; for TCC908 wereC0: 0; C1: 0.035; C2: 0.12; C3: 0.43 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

In the absence of diuron, the tolerant strain (TCC908) had a cell density 2.3 fold greater than the sensitive one. Diuron exerted an inhibitory effect on both strains (Fig. 7). The sensitive strain (TCC907) showed significant growth differences compared to the control, although no differences were found between C1 (0.003 mg L^{-1}) and C2 (0.01 mg L^{-1}). At C3 (0.035 mg L^{-1}) inhibition was about 82%. The tolerant strain had significant growth differences in all the concentrations tested, with a 95% growth impairment at C3 (0.43 mg L^{-1}). When exposed to the same concentration, the growth of the sensitive strain was almost twice inhibited compared to the tolerant strain.

3.3.1.2 Cellular damage

Proteins content

Protein content is higher for the tolerant than the sensitive strain in the absence of diuron (Fig. 8). The increase in protein content observed on the

sensitive strain was not significantly different from the control. The content variation on the tolerant strain was not significant either.

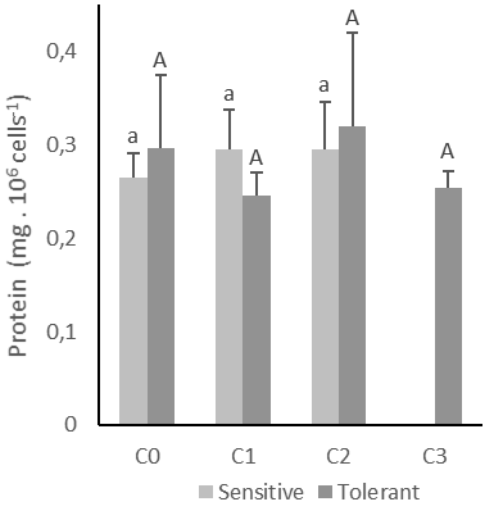

Figure 9: Cellular protein content (mg.10^6 $cells^{-1}$) of tolerant (TCC908) and sensitive (TCC907) *Nitzschia palea* strains exposed to diuron. For TCC907 diuron concentrations were C0: 0; C1: 0.003; C2: 0.01mg L^{-1}, for TCC908 were C0: 0; C1: 0.035; C2: 0.12; C3: 0.43 mg L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Lipid peroxidation levels

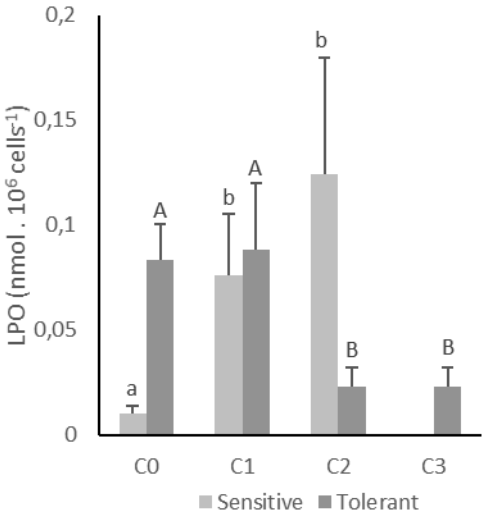

Figure 8 Lipid peroxidation (nmol.10^6 $cells^{-1}$) of tolerant (TCC908) and sensitive (TCC907) *Nitzschia palea* strains exposed to diuron. For TCC907 diuron concentrations were C0: 0; C1: 0.003; C2: 0.01mg.L^{-1} ; for TCC908 wereC0: 0; C1: 0.035; C2: 0.12; C3: 0.43 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Lipid peroxidation was eight times higher in the tolerant strain in the absence of diuron, (Fig. 9). Sensitive strain's lipid peroxidation increased significantly under diuron exposure. For the tolerant strain, exposition to diuron significantly decreased peroxidation levels at C2 (0.12 mg L^{-1}) and C3 (0.43 mg L^{-1}).

3.3.1.3 Antioxidants

Reduced glutathione content

Reduced glutathione contents were higher in the sensitive than in the tolerant strain (Fig. 10). The sensitive strain (TCC907) showed a significant increase in its total reduced glutathione content at C2 (0.01 mg.L^{-1}). As for the tolerant strain, reduced glutathione showed no significant alterations with diuron exposure.

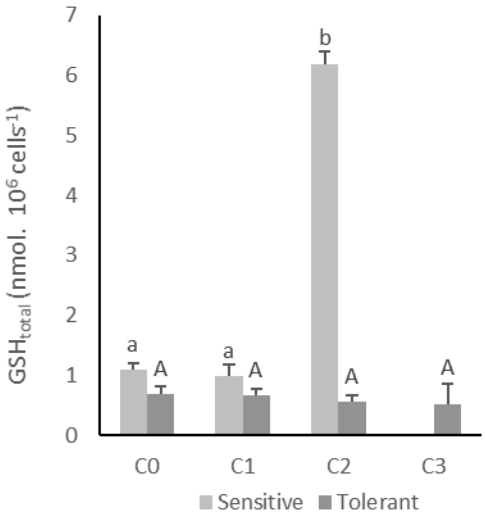

Figure 10 Reduced glutathione (nmol.10^6 $cells^{-1}$) of tolerant (TCC908) and sensitive (TCC907) *Nitzschia palea* strains exposed to diuron. For TCC907 diuron concentrations were C0: 0; C1: 0.003; C2: 0.01mg.L^{-1} ; for TCC908 wereC0: 0; C1: 0.035; C2: 0.12; C3: 0.43 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

3.3.1.4 Antioxidant enzymes

Antioxidant response was analysed by measuring the activity of four enzymes (figure 11), all involved in ROS detoxification.

Superoxide dismutase

SOD activity at control condition in the sensitive strain is higher than in the tolerant strain (Fig. 11-A). In the sensitive strain SOD activity increases with exposure, significantly differing from the control at C2 (0.01 mg.L^{-1}). The tolerant strain increases SOD activity under diuron exposure. The increase, however, is not significant.

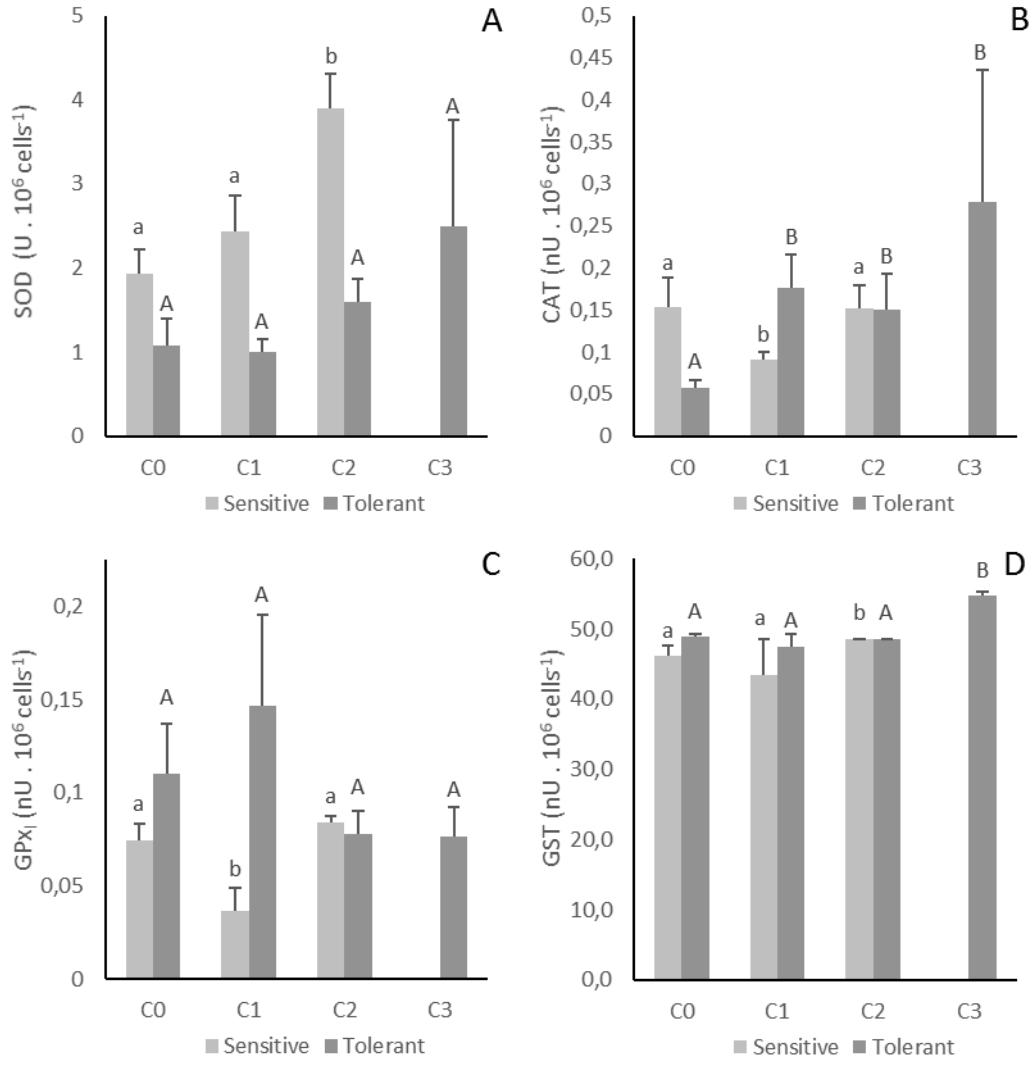

Figure 11 Antioxidant response: (A): superoxide dismutase (U.10^6 $cells^{-1}$); (B) catalase activity(nU.10^6 $cells^{-1}$); (C) glutathione peroxidase activity (nU.10^6 $cells^{-1}$); (D) glutathione S-transferases activity (nU.10^6 $cells^{-1}$) of tolerant (TCC908) and sensitive (TCC907) *N. palea* strains exposed to diuron. For TCC907 diuron concentrations were C0: 0; C1: 0.003; C2: 0.01mg.L^{-1} ; for TCC908 wereC0: 0; C1: 0.035; C2: 0.12; C3: 0.43 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences ($p \leq 0.05$) among concentrations for the sensitive and tolerant strains, respectively..

Catalase

At the control, sensitive strain's catalase activity is higher than the tolerant strain (Fig. 11-B). Sensitive strain presented an activity at C1 (0.003 $mg.L^{-1}$) compared to control. The tolerant strain significantly increases its catalase activity when exposed to diuron.

Glutathione peroxidase

In the absence of diuron, the tolerant strain has a higher glutathione peroxidase activity than the sensitive one (Fig. 11-C). Sensitive strain significantly decreased its enzymatic activity at C1 (0.003 $mg.L^{-1}$). The tolerant strain's activity doesn't differ from the control, presenting a non-significant activity increase at C1 (0.035 $mg.L^{-1}$).

Glutathione S-transferases activity

Glutathione S-transferases activity is slightly higher for the tolerant strain in the absence of diuron (Fig. 11-D). Sensitive strain shows a non-significant decrease at C1 (0.003 $mg.L^{-1}$) and a significant increase in GSTs activity at C2 (0.01 $mg.L^{-1}$). The tolerant strain exhibits a significant increase in activity at C3 (0.43 $mg.L^{-1}$).

3.3.1.5 Pigment content

In the absence of diuron, chlorophyll *a* content is higher in the sensitive than in the tolerant strain (Fig. 12-A) Sensitive strain significantly decreases chlorophyll *a* content at C1 (0.003 $mg.L^{-1}$) and C2 (0.01 $mg.L^{-1}$). The tolerant strain has its chlorophyll *a* content significantly decreased at C3 (0.43 $mg.L^{-1}$).

Chlorophyll *c* content is higher in the sensitive than in the tolerant strain, in the absence of diuron (Fig. 12-B). Sensitive strain's chlorophyll *c* content does not change significantly with exposure to diuron. In the tolerant strain, chlorophyll *c* content decreases non-significantly from the control.

Carotenoids content is higher in the tolerant strain in the absence of diuron, (Fig. 12-C). Sensitive strain carotenoid content increases significantly at C2 (0.01 $mg.L^{-1}$). Carotenoids in the tolerant strain do not present a significant variation under diuron exposure.

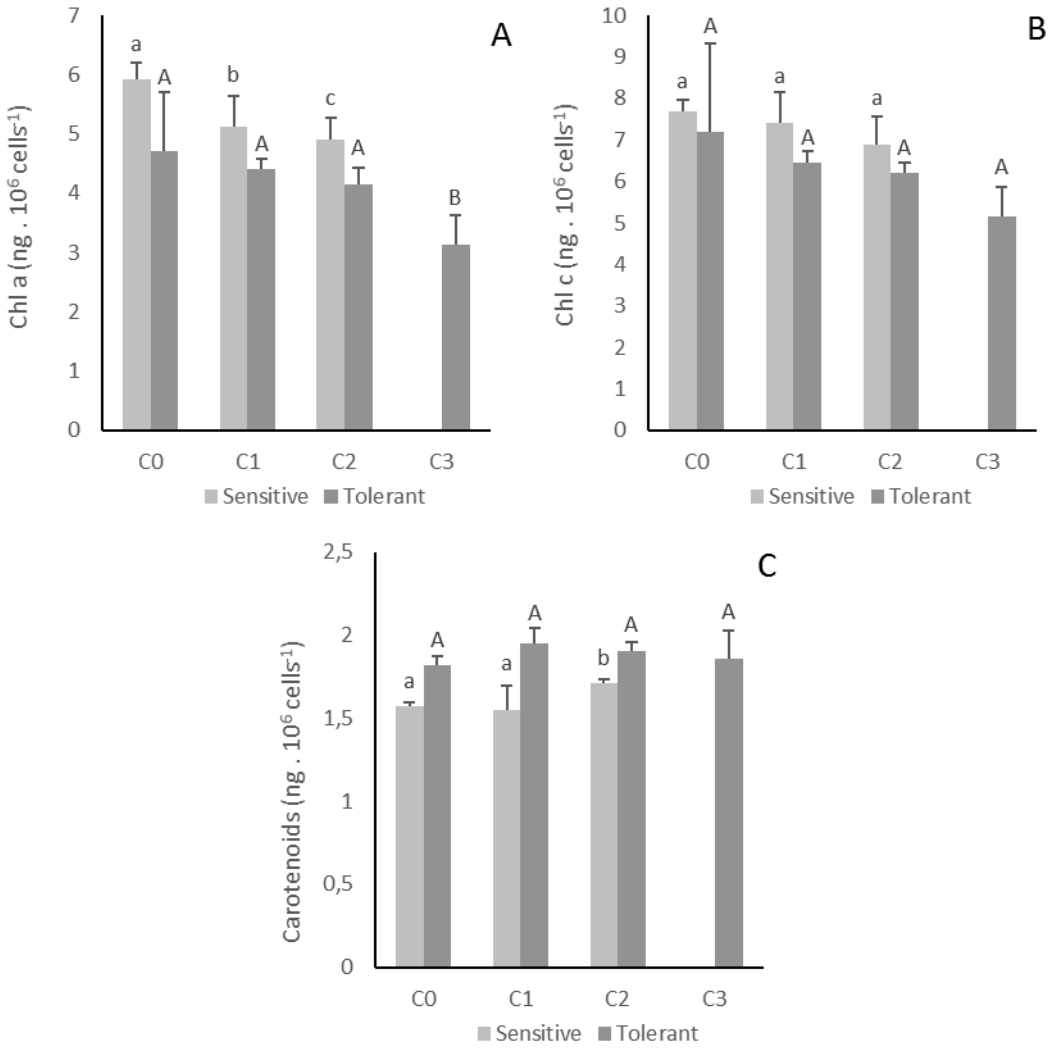

Figure 12 Pigment content: (A): chlorophyll a (ng.10^6 $cells^{-1}$); (B) chlorophyll b (ng.10^6 $cells^{-1}$); (C) Carotenoids (ng.10^6 $cells^{-1}$) of tolerant (TCC908) and sensitive (TCC907) *Nitzschia palea* strains exposed to diuron. For TCC907 diuron concentrations were C0: 0; C1: 0.003; C2: 0.01mg $Diu.L^{-1}$; for TCC908 wereC0: 0; C1: 0.035; C2: 0.12; C3: 0.43 mg $Diu.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences ($p \leq 0.05$) among concentrations for the sensitive and tolerant strains, respectively.

3.3.1.6 Multivariable overview of the biomarkers

The first two axes of the PCO explain about 71% of the total variation of the measured parameters for both strains (Fig. 13).

PCO1 is characterized by the positive weight of GSTs and CAT, and the negative load of SOD, Protein, Chl *a* and Chl *c*. Lipid peroxidation (LPO) and GSH contribute at a smaller scale. As for PCO2, it is characterized by the negative weight of Growth and carotenoids, and, to a minor extent, GP*x*.

PCO analysis shows the controls from both the sensitive and the tolerant strain in the same quadrant (-/-), which is characterized by elevated growth, protein and chlorophylls *a* and *c* content.

The response of the sensitive strain (TCC907) at the first and second concentrations tested (S1 and S2) was characterized by the increase of LPO, GSH and SOD activity.

Tolerant strain's (TCC908) response is distributed along the positive part of PCO1. The response of the first concentration tested, T1 is in the quadrant characterized by GP*x* and carotenoid content. The response to higher concentrations (T2 and T3) is characterized by CAT and GST activity.

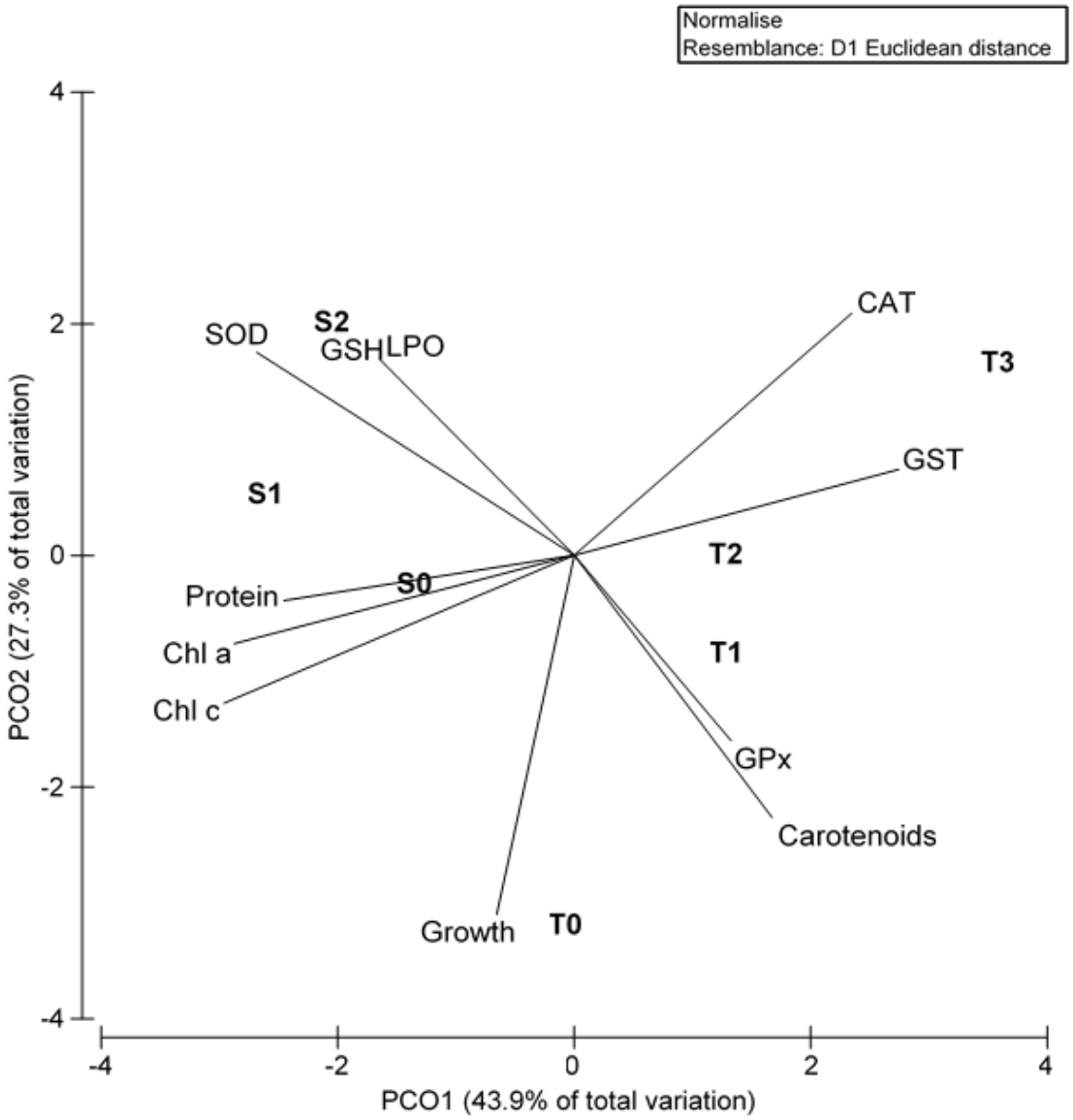

Figure 13 PCO multivariate analysis of diuron biomarkers by Primer 6, with a tolerant (T) and a sensitive (S) strain of *Nitzschia palea*

3.3.2 Responses to Atrazine

3.3.2.1 Growth

In the absence of atrazine, the tested strains presented differences in cell density, with the tolerant strain having 1.85 fold the density of the sensitive strain (Fig. 14-A). Atrazine exerted an inhibitory effect on both strains. The tolerant strain (TCC852) exhibited a significant growth impairment at all the concentrations tested. The sensitive strain also had significant growth inibitions, with a similar effect at C1 (0.04 mg L^{-1}) and C2 (0.1 mg L^{-1}). When exposed to the same concentraton, 0.25 mg L^{-1}(C1 for the tolerant and C3 for the sensitive strain), the tolerant strain was inhibited 56% while a 79% growth reduction was observed for the sensitive strain (Fig. 14-B).

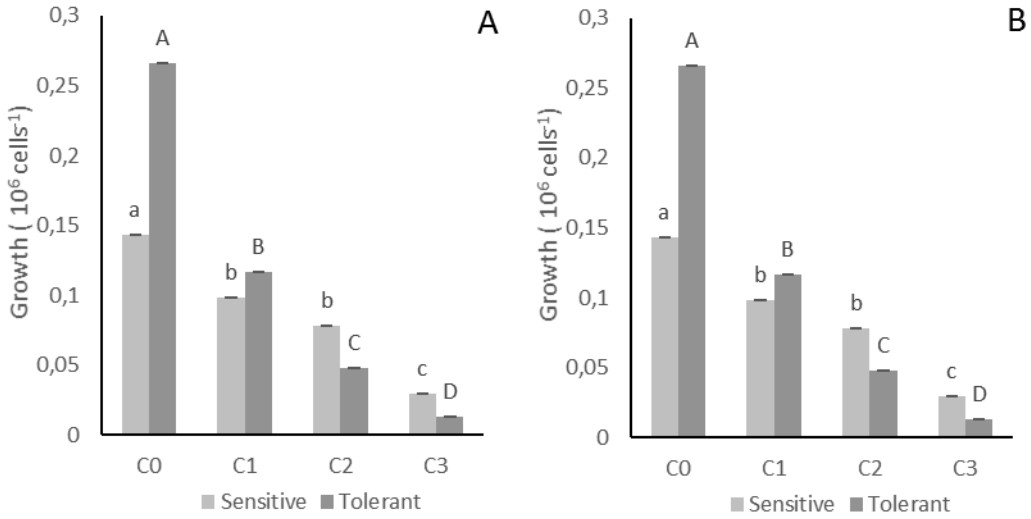

Figure 14 Growth (10^6 cells.ml^{-1}) (A) and relative growth (% of control) (B) of tolerant (TCC852) and sensitive (TCC907) *Nitzschia palea* strains exposed to Atrazine. For TCC907 atrazine concentrations were C0: 0; C1: 0.04; C2: 0.1; C3: 0.25 mg Atr.L^{-1} ; for TCC852 were C0: 0; C1: 0.25; C2: 0.63; C3: 1.56 mg Atr.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

3.3.2.2 Cellular damage

<u>Proteins content</u>

Protein content is higher for the sensitive strain in the absence of atrazine (Fig. 15). A significant protein content decrease was observed for the tolerant strain at C1 (0.25 mg.L^{-1}) and C2 (0.63 mg.L^{-1}). Sensitive strain's protein content

presented a decreasing trend, which was not significant. At the same concentration, 0.25 mg.L^{-1}, protein content in both strains is similar.

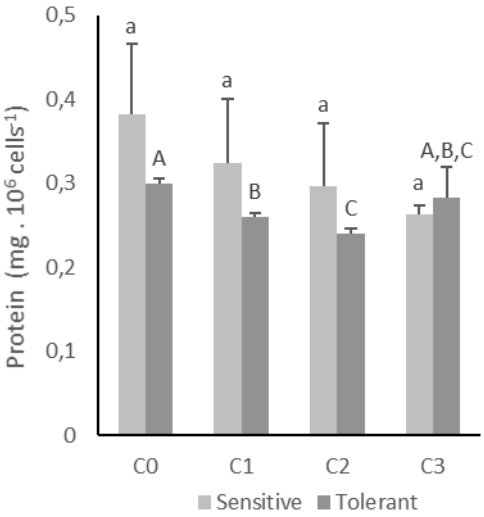

Figure 15 Cellular protein content (mg.10^{6}cells^{-1}) of tolerant (TCC852) and sensitive (TCC907) *Nitzschia palea* strains exposed to Atrazine. For TCC907 atrazine concentrations were C0: 0; C1: 0.04; C2: 0.1; C3: 0.25 mg Atr.L^{-1} ; for TCC852 were C0: 0; C1: 0.25; C2: 0.63; C3: 1.56 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Lipid peroxidation levels

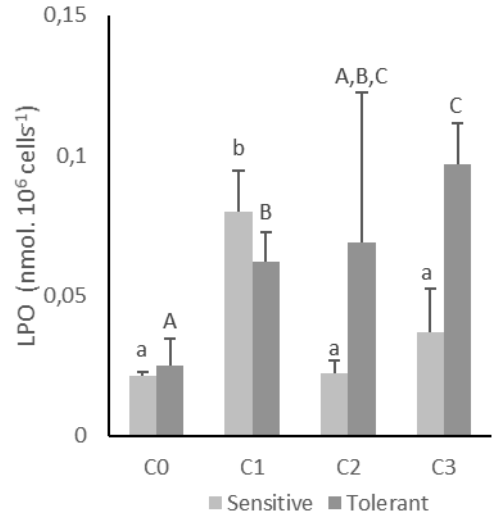

Figure 16 Lipid peroxidation (nmol.10^6 cells-1) of tolerant (TCC852) and sensitive (TCC907) Nitzschia palea strains exposed to Atrazine. For TCC907 atrazine concentrations were C0: 0; C1: 0.04; C2: 0.1; C3: 0.25 mg Atr.L-1 ; for TCC852 were C0: 0; C1: 0.25; C2: 0.63; C3: 1.56 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

In the absence of atrazine, both strains presented similar levels of lipid peroxidation (Fig. 16). Under atrazine exposition, the sensitive strain showed a LPO increase at C1 (0.04 mg.L^{-1}). Tolerant strain showed higher LPO levels when exposed to atrazine that were significant for C1 and C3. When exposed to the same concentration, LPO in the tolerant strain was higher than in the sensitive strain.

3.3.2.3 Antioxidants

Reduced glutathione content

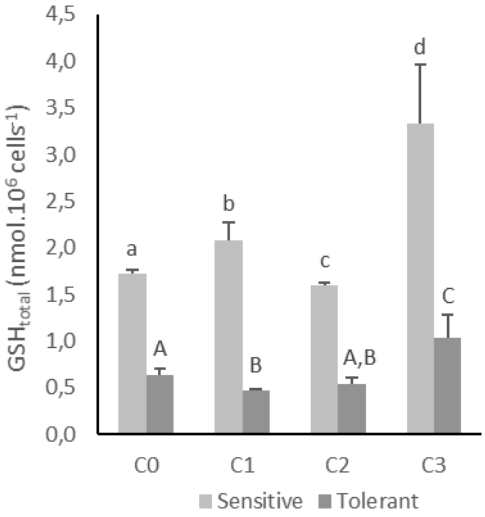

Figure 17 Reduced glutathione (nmol.10^6 cells-1) of tolerant (TCC852) and sensitive (TCC907) *Nitzschia palea* strains exposed to Atrazine. For TCC907 atrazine concentrations were C0: 0; C1: 0.04; C2: 0.1; C3: 0.25 mg Atr.L-1 ; for TCC852 were C0: 0; C1: 0.25; C2: 0.63; C3: 1.56 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Reduced glutathione in the sensitive strain is higher than in the tolerant strain at the control (Fig. 17). Sensitive strain shows a significant increase in GSH at C1 (0.04 mg.L^{-1}) and C3 (0.25 mg.L^{-1}), and a significant decrease at C2 (0.1 mg.L^{-1}). The tolerant strain presents a significant decrease in GSH content at C1 (0.25 mg.L^{-1}) and an increase at C3 (1.56 mg.L^{-1}). Exposed to the same atrazine concentration (0.25 mg.L^{-1}), the sensitive strain showed a significant increase in its glutathione content while the tolerant strain's decreased.

3.3.2.4 Antioxidant response

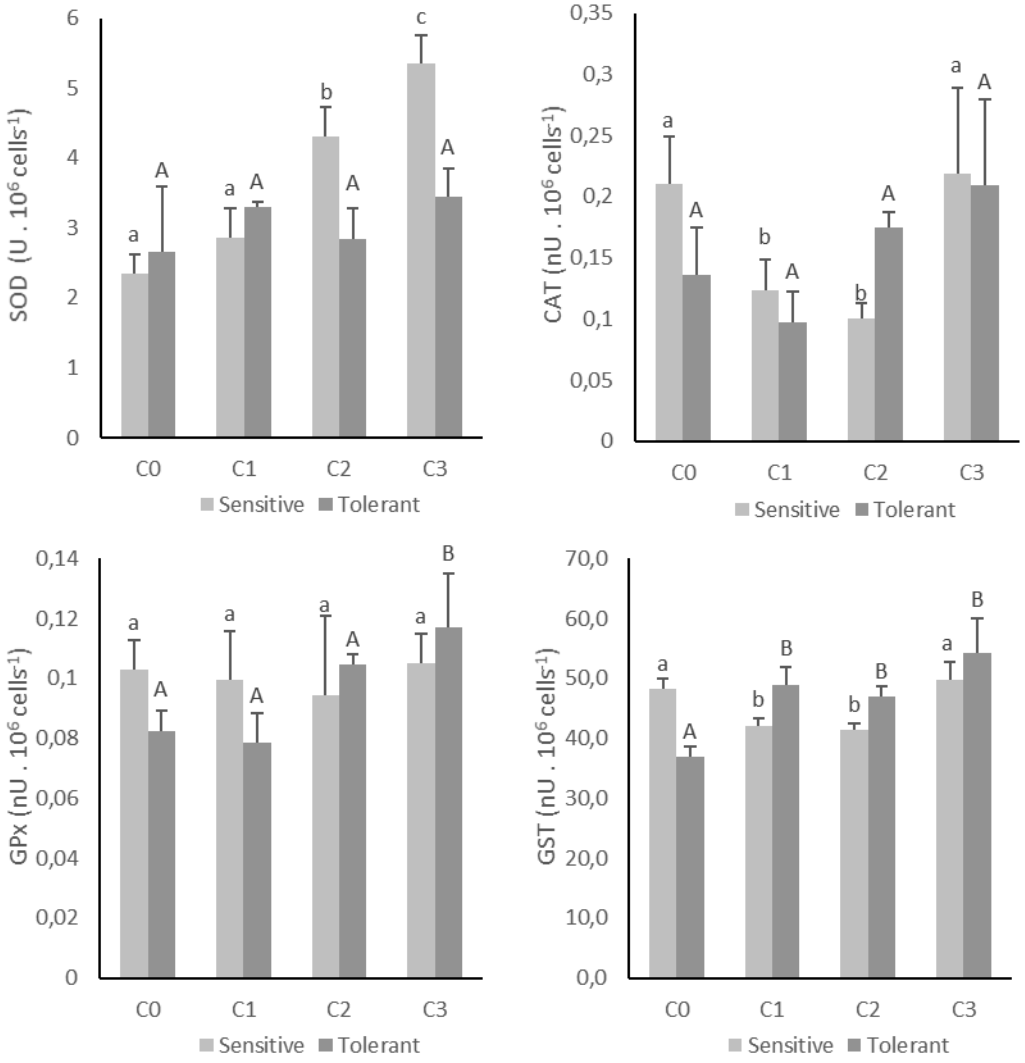

Figure 18 Antioxidant response: (A): superoxide dismutase (mU.10^6 cells^{-1}); (B) catalase activity(nU.10^6 cells^{-1}); (C) glutathione peroxidase activity (nU.10^6 cells^{-1}); (D) glutathione S-transferase activity (nU.10^6 cells^{-1}) of tolerant (TCC852) and sensitive (TCC907) *Nitzschia palea* strains exposed to Atrazine. For TCC907 atrazine concentrations were C0: 0; C1: 0.04; C2: 0.1; C3: 0.25 mg.L^{-1} ; for TCC852 were C0: 0; C1: 0.25; C2: 0.63; C3: 1.56 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Superoxide dismutase

Tolerant strain had a higher SOD activity in the absence of atrazine (Fig. 18-A) than the sensitive one. When exposed to the herbicide, the sensitive strain exhibited an activity increase, significant at C2 (0.1 mg.L^{-1}) and C3 (0.25 mg.L^{-1}). The tolerant strain displayed no alteration in enzymatic activity. At 0.25 mg.L^{-1} the sensitive strain's SOD activity was higher than the tolerant one's.

Catalase

At the control, sensitive strain's catalase activity is higher than the tolerant one's (Fig. 18-B). Sensitive strain presented an activity decrease regarding the control at C1 (0.04 $mg.L^{-1}$) and C2 (0.1 $mg.L^{-1}$). The tolerant strain presented no significant differences in CAT activity. For the same concentration, 0.25 $mg.L^{-1}$, enzymatic activity did not differ from the control for either strain.

Glutathione peroxidase

In the absence of atrazine, the sensitive strain has a higher glutathione peroxidase activity than the tolerant one (Fig. 18-C). The sensitive strain displays no changes in enzymatic activity with exposure to the herbicide. The tolerant strain increases activity at all concentrations tested, significantly at C3 (1.56 $mg.L^{-1}$). Sensitive strain's GPx activity is higher than the tolerant one when exposed to the same concentration.

Glutathione S-transferases activity

Glutathione S-transferases activity is higher for the sensitive strain in the absence of atrazine, C0 (Fig. 18-D). Sensitive strain shows a significant decrease in GST activity in C1 (0.25 $mg.L^{-1}$) and C2 (0.63 $mg.L^{-1}$). The tolerant strain, on the other hand, has a significant increase when exposed to atrazine. For the same concentration (0.25 $mg.L^{-1}$), GST activity is similar in both strains.

3.3.2.5 Pigment content

In the absence of atrazine, chlorophyll *a* content is higher in the sensitive strain than in the tolerant one (Fig. 19-A). Strains display opposit trends under exposure to atrazine. Sensitive strain increases its chlorophyll *a* content, significantly at C3 (0.25 $mg.L^{-1}$), and the tolerant strain significantly decreases chlorophyll *a* content under atrazine exposure.

Chlorophyll *c* content is higher in the sensitive strain than in the tolerant one, in the absence of atrazine (Fig. 19-B). Sensitive strain's chlorophyll *c* content, significantly decreases at C1 (0.04 $mg.L^{-1}$). In the tolerant strain, chlorophyll *c* content doesn't differ significantly from the control.

Sensitive strains' carotenoid content decreases with exposure to increasing levels of atrazine (Fig. 19-C). This increase is significant at C2 (0.1

mg.L^{-1}) and C3 (0.25 mg.L^{-1}). In the tolerant strain carotenoids increase significantly at the first concentration tested, C1 (0.25 mg.L^{-1}). Carotenoid content significantly increases at 0.14 mg.L^{-1} for the tolerant strain, coversly to the sensitive strain, where it decreases.

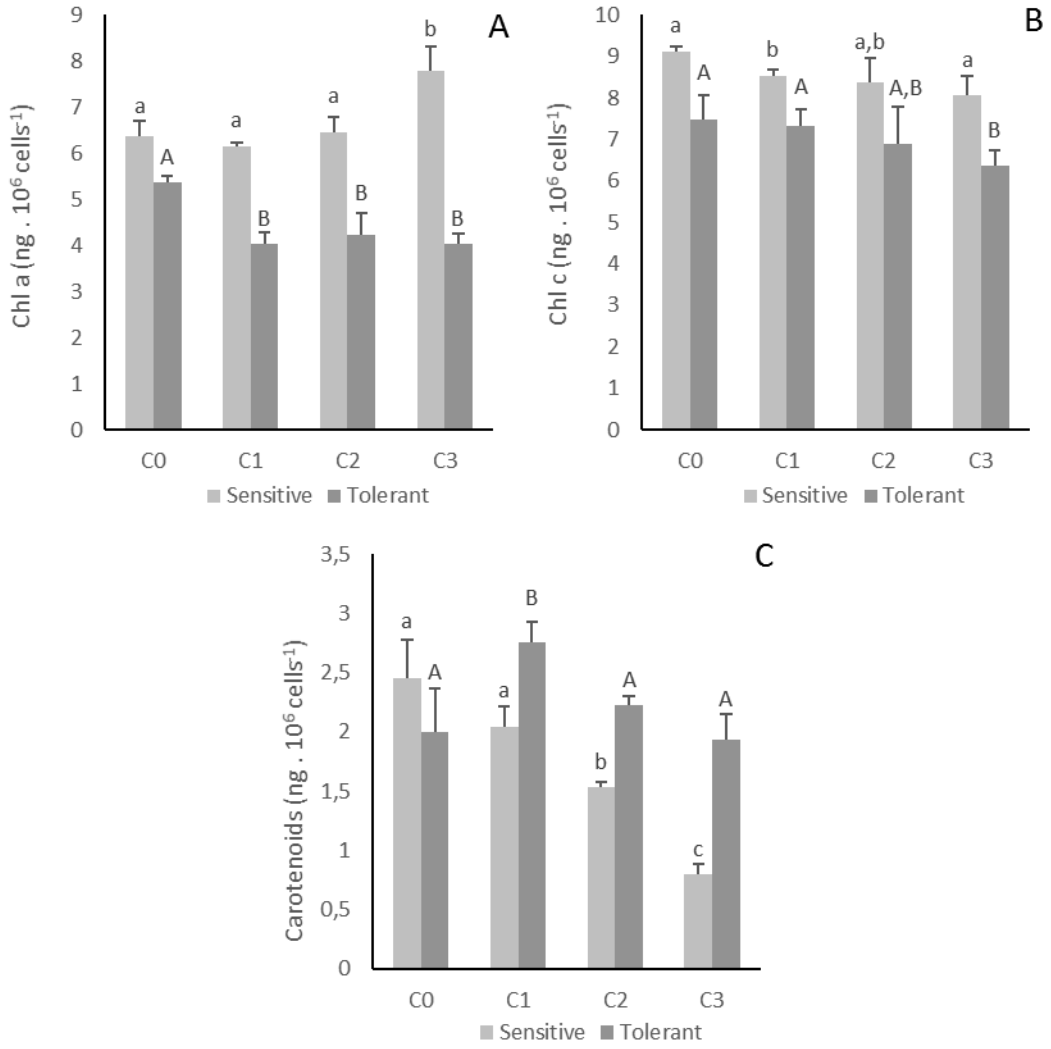

Figure 19 Pigment content: (A): chlorophyll a (ng.10^6 cells^{-1}); (B) chlorophyll c (ng.10^6 cells^{-1}); (C) Carotenoids (ng.10^6 cells^{-1}) of tolerant (TCC8522) and sensitive (TCC907) *Nitzschia palea* strains exposed to atrazine. For TCC907 atrazine concentrations were C0: 0; C1: 0.04; C2: 0.1; C3: 0.25 mg.L^{-1}; for TCC852 were C0: 0; C1: 0.25; C2: 0.63; C3: 1.56 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

3.3.2.6 Multivariable overview of the biomarkers

The first two axes of the PCO explain about 70% of the variation of the measured parameters for both strains (Fig. 20). The positive weight of carotenoids and the negative loads of CAT, GPx, SOD and GSH characterize

PCO1. As for PCO2, it is characterized by the negative weight of LPO, GST and the positive weight of Protein, growth and Chl *c*.

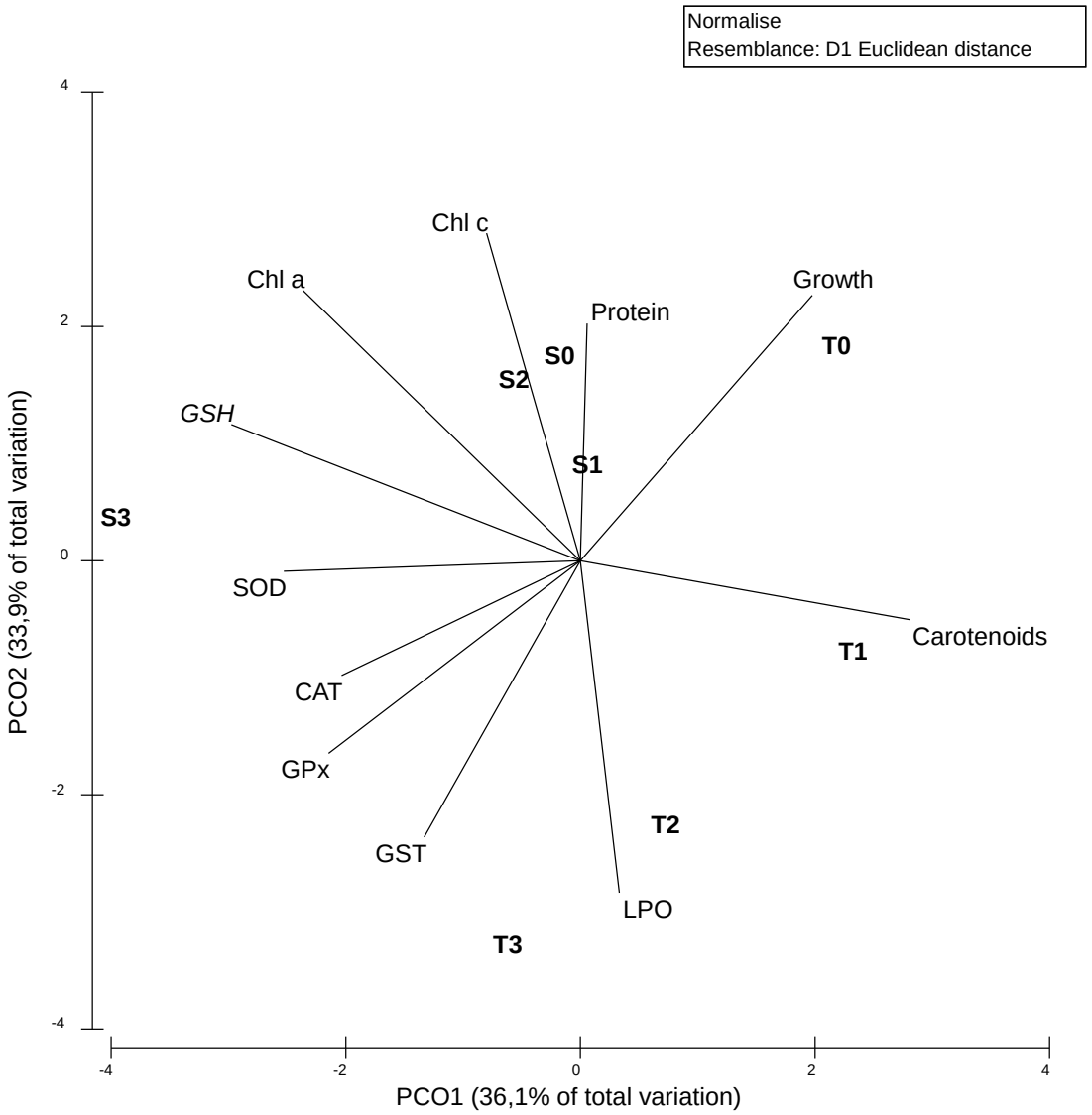

Figure 20 PCO multivariate analysis of atrazine biomarkers by Primer 6, with a tolerant (T) and a sensitive (S) strain of Nitzschia palea

PCO analysis shows the controls from both the sensitive and the tolerant strains, as well as the response under different concentrations of the sensitive strain, in the positive half of PCO2. Response to atrazine exposition, for the tolerant strain is in the negative half of PCO2.

The response of the sensitive strain to the first three exposures (S0, S1 and S2) is characterized by high protein and chlorophyll *c*, while high SOD and GSH, and low carotenoid levels characterize S3.

At T0, T1 and T2 conditions the tolerant strain is characterized by high growth, carotenoids and LPO levels, respectively. The response to the highest concentration is also characterized by the LPO, as well as elevated GSTs.

3.3.3 Responses to Copper

3.3.3.1 Growth

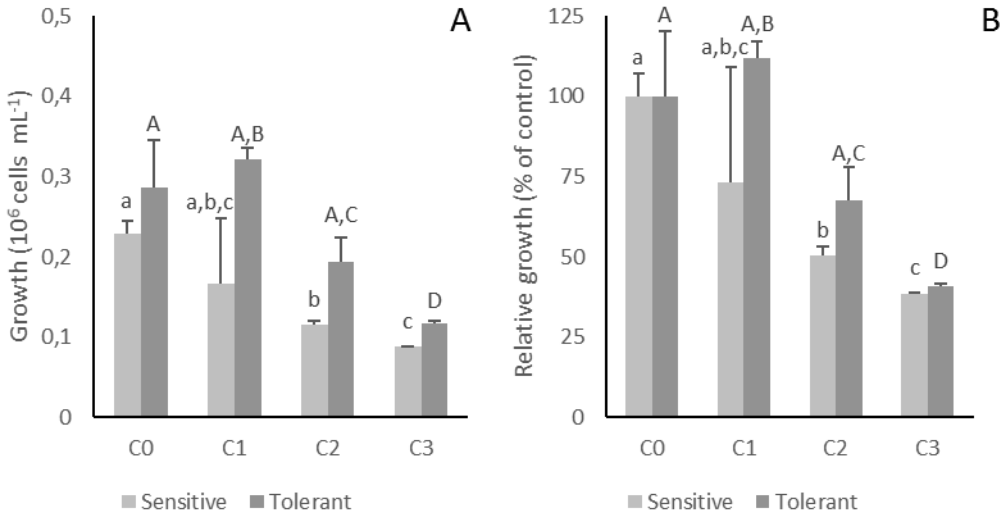

Figure 21 Growth (10^6 cells.ml^{-1}) (A) and relative growth (% of control) (B) of tolerant (TCC912) and sensitive (TCC910) *Nitzschia palea* strains exposed to copper. For TCC910 copper concentrations were C0: 0; C1: 0.0004; C2: 0.004; C3: 0.04 mg.L^{-1} ; for TCC912 wereC0: 0; C1: 0.04; C2: 0.08; C3: 0.16 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

In the absence of copper strains presented differences in cell density, with the tolerant strain having 1.25 fold the density of the sensitive strain (Fig. 21-A). Copper exerted an inhibitory effect on both strains. The tolerant strain exhibited a non-significant higher growth at C1 compared to control. At C2 no differences were observed, but a 51% growth inhibithion was observed at C3 (0.16 mg L^{-1}) compared to control (Fig 21-B). The sensitive strain had significant growth decreases at C2 (0.004 mg L^{-1}) and C3 (0.04 mg L^{-1}), 50% and 62% of control, respectively. At 0.04 mg L^{-1} the tolerant strain didn't present significant differences compared to control, while the sensitive strain's growth was inhibited.

3.3.3.2 Cellular damage

Proteins content

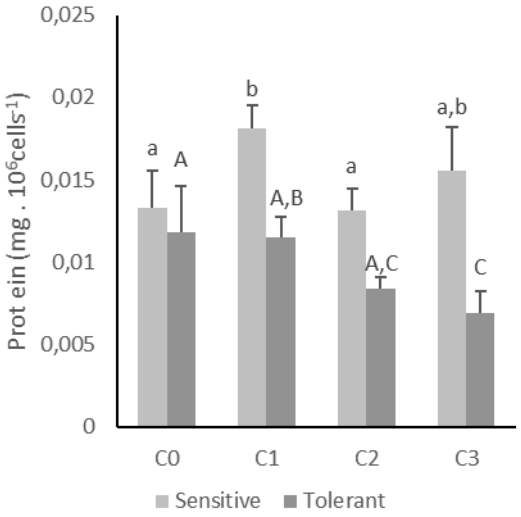

Figure 22 Cellular protein content (µg.10^6cells^{-1}) of tolerant (TCC912) and sensitive (TCC910) *Nitzschia palea* strains exposed to copper. For TCC910 copper concentrations were C0: 0; C1: 0.0004; C2: 0.004; C3: 0.04 mg.L^{-1} ; for TCC912 were C0: 0; C1: 0.04; C2: 0.08; C3: 0.16 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively..

Protein content is higher for the sensitive strain in the absence of copper (Fig. 22). A significant protein content increase was observed for the sensitive strain at C1 (0.0004 mg.L^{-1}), while no changes are observed at the other concentrations. Tolerant strain's protein content presented a decreasing trend, significant at C3 (0.16 mg.L^{-1}). At the same concentration (C1 for the tolerant and C3 for the sensitive strain) sensitive strain's protein content was higher than the tolerant one's.

Lipid peroxidation

In the absence of copper, both strains presented similar levels of lipid peroxidation (Fig. 23). No significant differences were observed, upon copper exposition, regarding the sensitive strain. Tolerant strain showed a significant LPO increase only at C1 (0.04 mg.L^{-1}). When exposed to the same concentration (0.04 mg.L^{-1}), tolerant strain had higher levels of LPO than the sensitive strain, which were close to control levels.

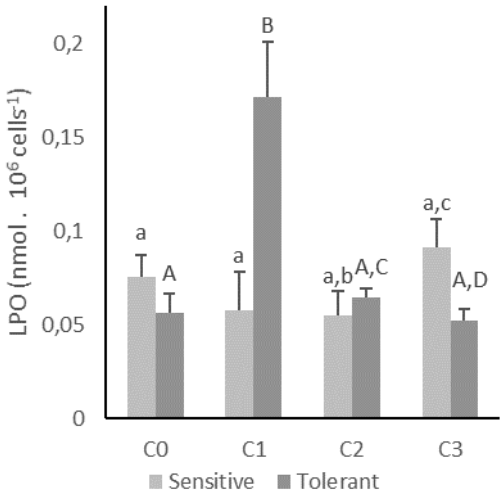

Figure 23 Lipid peroxidation (nmol.10^6 $cells^{-1}$) of tolerant (TCC912) and sensitive (TCC910) *Nitzschia palea* strains exposed to copper. For TCC910 copper concentrations were C0: 0; C1: 0.0004; C2: 0.004; C3: 0.04 $mg.L^{-1}$; for TCC912 were C0: 0; C1: 0.04; C2: 0.08; C3: 0.16 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

3.3.3.3 Antioxidants

Reduced glutathione

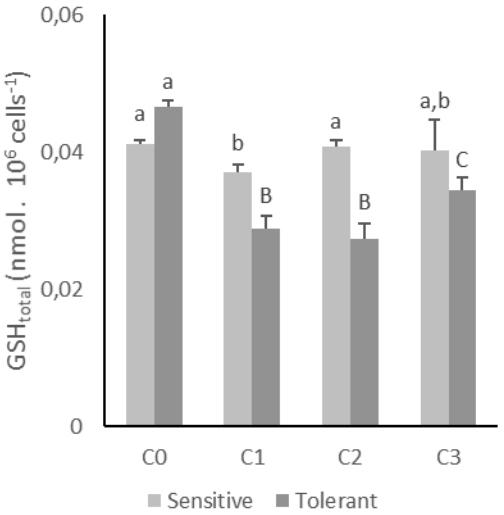

Figure 24 Total reduced glutathione (nmol.10^6 $cells^{-1}$) of of tolerant (TCC912) and sensitive (TCC910) *Nitzschia palea* strains exposed to copper. For TCC910 cadmium concentrations were C0: 0; C1: 0.0004; C2: 0.004; C3: 0.04 $mg.L^{-1}$; for TCC912 wereC0: 0; C1: 0.04; C2: 0.08; C3: 0.16 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Reduced glutathione content in the tolerant strain is higher than in the sensitive strain at the control (Fig. 24). Sensitive strain shows no significant

alterations with exposure. The tolerant strain presents a significant decrease in GSH. Exposed to the same copper concentration (0.04 $mg.L^{-1}$), the sensitive strain showed no changes in its glutathione content while the tolerant strain's decreased, having a lower content than the sensitive one.

3.3.3.4 Antioxidant enzymes

Superoxide dismutase

Sensitive strain had a higher SOD activity in the absence of copper (Fig. 25-A). when exposed to metal, neither the sensitive nor the tolerant strain

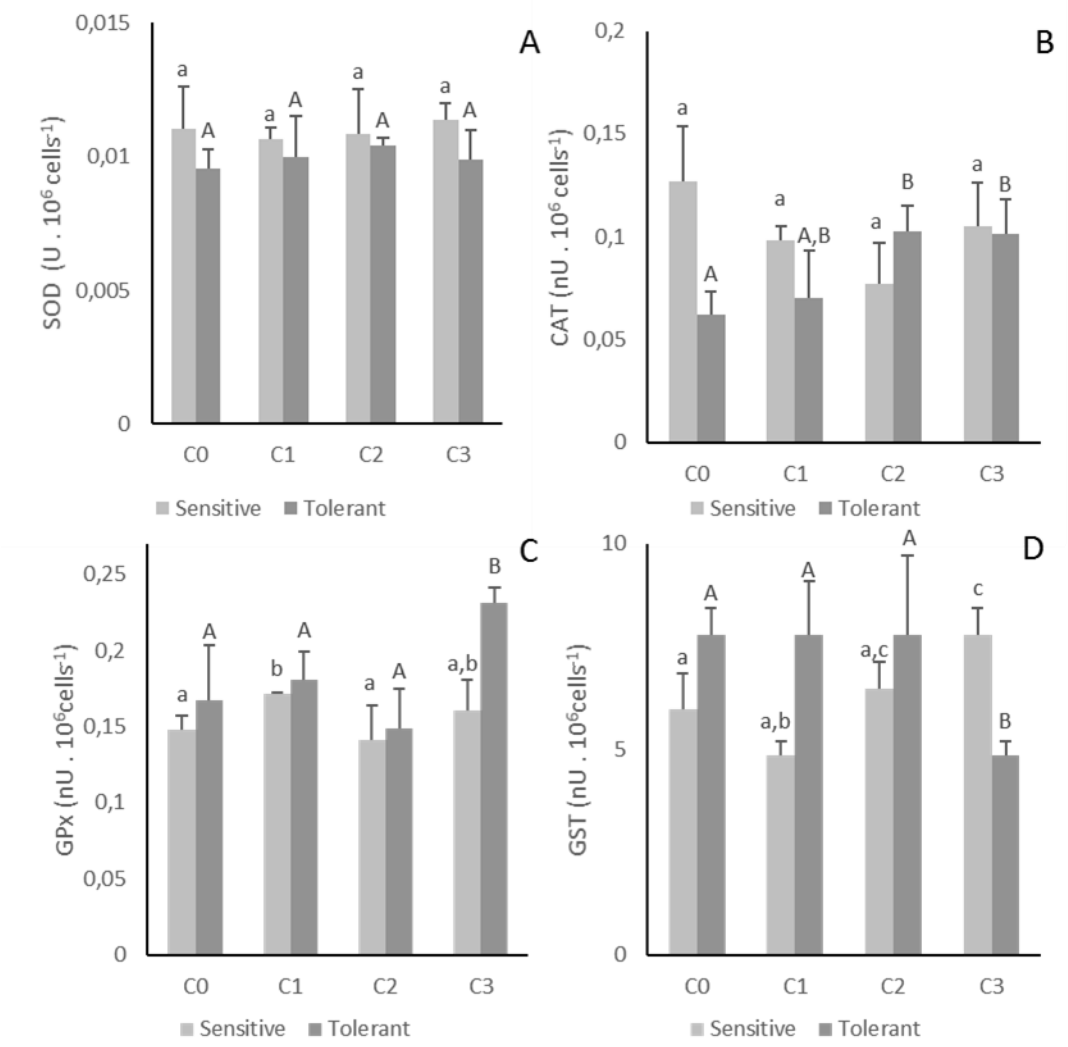

Figure 25 Antioxidant response: (A): superoxide dismutase ($U.10^6$ cells-1); (B) catalase activity ($nU.10^6$ $cells^{-1}$); (C) glutathione peroxidase activity ($nU.10^6$ $cells^{-1}$); (D) glutathione S-transferase activity ($nU.10^6$ $cells^{-1}$) of tolerant (TCC912) and sensitive (TCC910) *Nitzschia palea* strains exposed to copper. For TCC910 copper concentrations were C0: 0; C1: 0.0004; C2: 0.004; C3: 0.04 $mg.L^{-1}$; for TCC912 were C0: 0; C1: 0.04; C2: 0.08; C3: 0.16 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences ($p \leq 0.05$) among concentrations for the sensitive and tolerant strains, respectively.

displayed alterations in enzymatic activity. At 0.04 0.14 mg.L^{-1} the sensitive strain's SOD activity was higher than the tolerant one's.

Catalase

At the control, C0, sensitive strain's catalase activity is higher than the tolerant one's (Fig. 25-B). No significant changes in activity are observed for the sensitive strain. The tolerant strain as an activity increase when exposed to Cu, significant at C2 (0.08 mg.L^{-1}) and C3 (0.16 mg.L^{-1}). For the same concentration, 0.04 mg.L^{-1}, enzymatic activity doesn't differ from the control for both strains, being lower for the tolerant strain.

Glutathione peroxidase

In the absence of copper, the tolerant strain has a similar glutathione peroxidase activity to the sensitive one (Fig. 25-C). Tolerant strain significantly increased enzymatic activity at C3 (0.16 mg.L^{-1}). The sensitive strain only presents a significant activity increase at C1 (0.0004 mg.L^{-1}). When exposed to the same concentration, 0.04 mg.L^{-1}, strains do not differ significantly from the control.

Glutathione S-transferases

Glutathione S-transferases activity is higher for the tolerant strain in the absence of copper (Fig. 25-D). The sensitive strain showed a significant increase in GST activity in C3 (0.04 mg.L^{-1}) when compared to the control. For the tolerant strain, a significant decrease is observed at C3 (0.16 mg.L^{-1}). For the same concentration (0.04 mg.L^{-1}), GST activity significantly increases for the sensitive strain while it is similar to the control for the tolerant strain.

3.3.3.5 Pigment content

In the absence of copper, C0, chlorophyll *a* content is higher in the tolerant strain than in the sensitive one (Fig. 26-A) Sensitive strain significantly increases chlorophyll *a* content at all concentrations tested, having contents at C2 (0.004 mg.L^{-1}) and C3 (0.04 mg.L^{-1}) that are not significantly different. The tolerant strain significantly increases chlorophyll *a* content at C1 (0.0004 mg.L^{-1}) and C3 (0.04 mg.L^{-1}). When exposed to the same concentration, 0.04 mg.L^{-}1, both strains display a significant content increase compared to control.

Chlorophyll *c* content is higher in the tolerant strain than in the sensitive one, in the absence of copper, C0 (Fig. 26-B). Sensitive strain's chlorophyll *c* content augments with exposure, significantly at C2 (0.004 $mg.L^{-1}$) and C3 (0.04 $mg.L^{-1}$). In the tolerant strain, chlorophyll *c* content doesn't significantly differ from

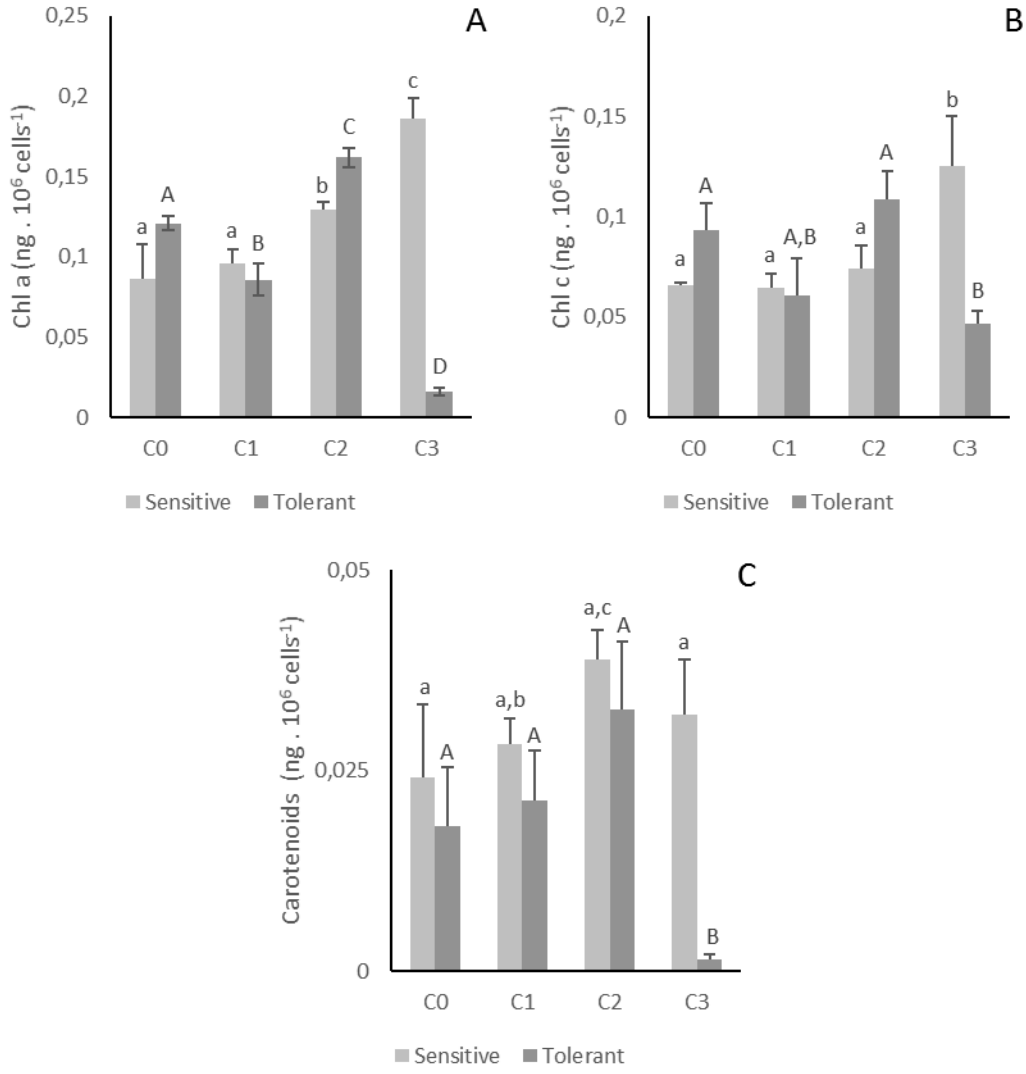

Figure 26 Pigment content: (A): chlorophyll a (ng.10^6 $cells^{-1}$); (B) chlorophyll b (ng.10^6 $cells^{-1}$); (C) carotenoids (ng.10^6 $cells^{-1}$) of tolerant (TCC912) and sensitive (TCC910) *Nitzschia palea* strains exposed to copper. For TCC910 copper concentrations were C0: 0; C1: 0.0004; C2: 0.004; C3: 0.04 $mg.L^{-1}$; for TCC912 were C0: 0; C1: 0.04; C2: 0.08; C3: 0.16 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Different letters (a-c) represent significant differences ($p \leq 0.05$) among concentrations

the control. At 0.04 $mg.L^{-1}$ the chlorophyll *c* content in the sensitive strain is similar to the content in the tolerant strain.

Carotenoids' content is higher in the tolerant strain in the absence of copper (Fig. 26-C). Sensitive strain carotenoids increase with exposure to Cu, with C2 (0.004 $mg.L^{-1}$) and C3 (0.04 $mg.L^{-1}$) having a similar content. Tolerant

strain's carotenoid content significantly increased at C1 (0.04 $mg.L^{-1}$) and C3 (0.16 $mg.L^{-1}$). Carotenoid content increases significantly from the control at 0.04 $mg.L^{-1}$ for both strains, being higher for the tolernat strain.

3.3.3.6 Multivariable overview of copper biomarkers

The first two axes of the PCO explain about 57% of the variation of the measured parameters for both strains (Fig. 27). PCO1 is characterized by the positive weight of GP*x*, and the negative load of carotenoids, SOD, Prot, GSH and Chl *c* to a minor extent. As for PCO2, it is characterized by the positive loads of GST, LPO, Growth and Chl a, and the negative weight of CAT.

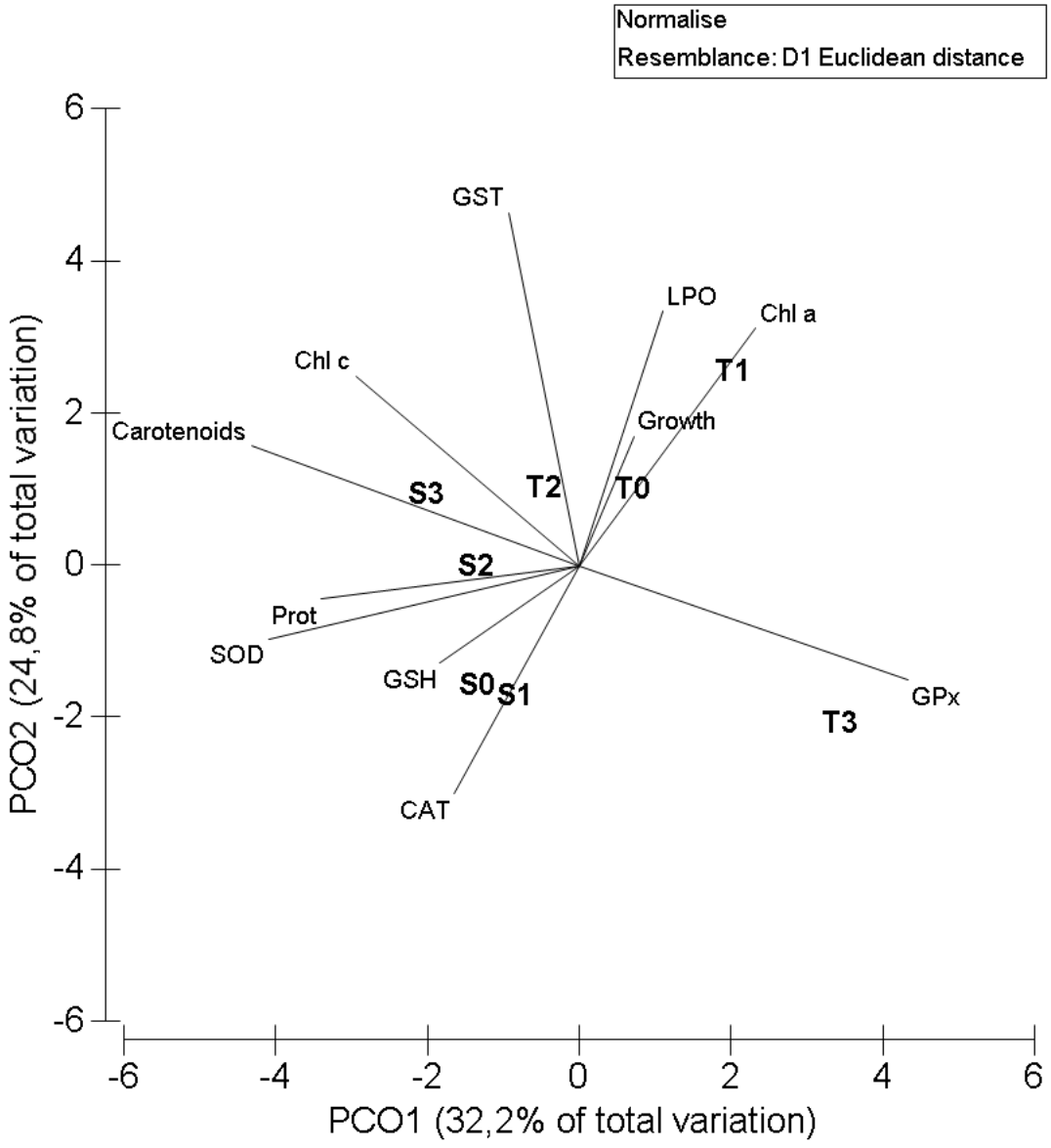

Figure 27 PCO multivariate analysis of copper biomarkers by Primer 6, with a tolerant and a sensitive strain of *Nitzschia palea*

PCO analysis shows a clear separation between both strains along a line that divides the positive quadrant at PCO1, with the tolerant strain in the positive side and the sensitive strain in the negative one.

The response of the sensitive strain (TCC910) is characterized by high Chl c and Carotenoids, which define C3 (0.04 $mg.L^{-1}$), Prot and SOD, which characterize C2 (0.004 $mg.L^{-1}$), GSH and CAT, which characterize the response from C0 and C1 (0.0004 $mg.L^{-1}$).

Tolerant strain's (TCC912) response is characterized by elevated LPO, Growth and Chl *a*, for C0 and C1 (0.04 $mg.L^{-1}$), GSTs for C2 (0.08 $mg.L^{-1}$) and GPx activity for C3 (0.16 $mg.L^{-1}$).

3.3.4 Responses to Cadmium

3.3.4.1 Growth

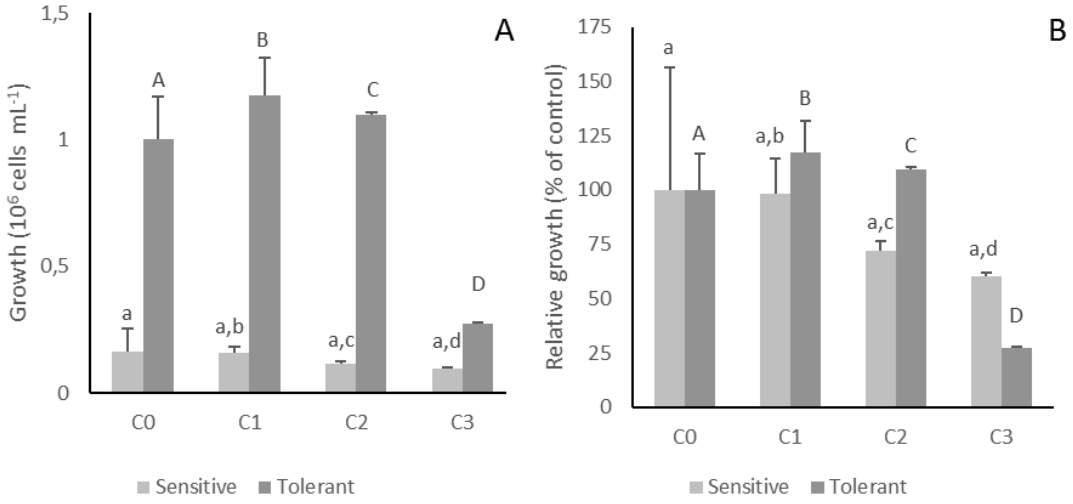

Figure 28 Growth (10^6 $cells.ml^{-1}$) (A) and relative growth (% of control) (B) of tolerant (TCC762) and sensitive (TCC854) *Nitzschia palea* strains exposed to cadmium. For TCC854 cadmium concentrations were C0: 0; C1: 0.001; C2: 0.01; C3: 0.14 $mg.L^{-1}$; for TCC762 were C0: 0; C1: 0.14; C2: 1.96; C3: 27.44 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively..

In the absence of cadmium, the tolerant strain had a higher cell density, 6.2 fold greater than the sensitive one. Cadmium exerted an inhibitory effect on both strains (Fig. 28). The sensitive strain (TCC854) did not show significant growth differences compared to the control, although at C3 (0.14 mg L^{-1}) its growth was inhibited in almost 70%. The tolerant strain (TCC762) had significant growth differences in all the concentrations tested. This strain exhibited a

hormetic effect at C1 (0.14 mg L^{-1}) and C2 (1.96 mg L^{-1}) and approximatly 73% inhibition at C3 (27.44 mg L^{-1}). At 0.14 mg L^{-1}, the tolerant strain grew more than the control, while the sensitive strain's growth was highly impaired

3.3.4.2 Cellular damage

Proteins content

Protein content is similar for both sensitive and tolerant strains in the absence of cadmium (Fig. 29). A non-significant protein increase was observed for the sensitive strain (TCC854) at C1, followed by decreases at higher concentrations, that weere not significantly different from control or C1. The tolerant strain (TCC762) didn't present significant alterations. At 0.14 mg.L^{-1},the protein content was similar for the sensitive and tolerant strains

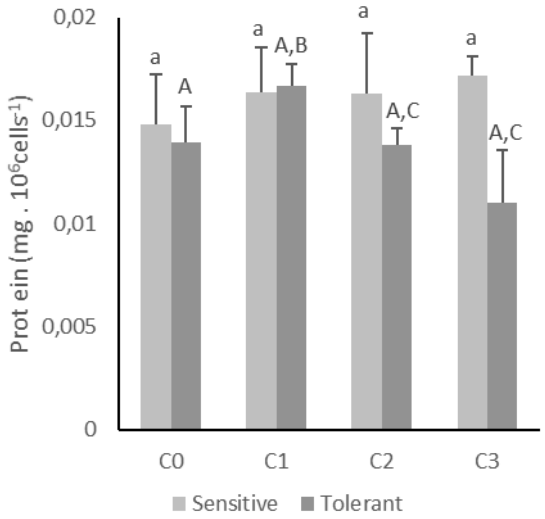

Figure 29 Cellular protein content (mg.10^6cells^{-1}) of tolerant (TCC762) and sensitive (TCC854) *Nitzschia palea* strains exposed to cadmium. For TCC854 cadmium concentrations were C0: 0; C1: 0.001; C2: 0.01; C3: 0.14 mg.L^{-1}; for TCC762 were C0: 0; C1: 0.14; C2: 1.96; C3: 27.44 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Lipid peroxidation

Lipid peroxidation is higher in the sensitive strain in the absence of cadmium, exposition to the metal increased lipid peroxidation in both strains (Fig. 31). For the sensitive strain, lipid peroxidation increased non-significantly to the concentrations exposed. For the tolerant strain, exposition to cadmium significantly rose peroxidation levels. At the same concentration, 0.14 mg L^{-1}, lipid peroxidation is higher in the sensitive strain.

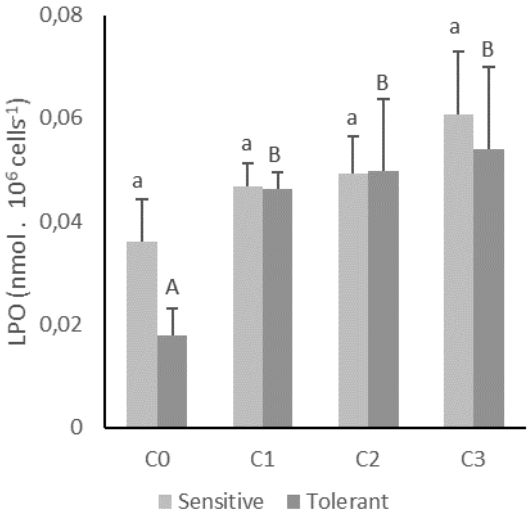

Figure 30 Lipid peroxidation (nmol.10^6 $cells^{-1}$) of tolerant (TCC762) and sensitive (TCC854) *Nitzschia palea* strains exposed to cadmium. For TCC854 cadmium concentrations were C0: 0; C1: 0.001; C2: 0.01; C3: 0.14 $mg.L^{-1}$; for TCC762 were C0: 0; C1: 0.14; C2: 1.96; C3: 27.44 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively..

3.3.4.3 Antioxidants

Reduced glutathione

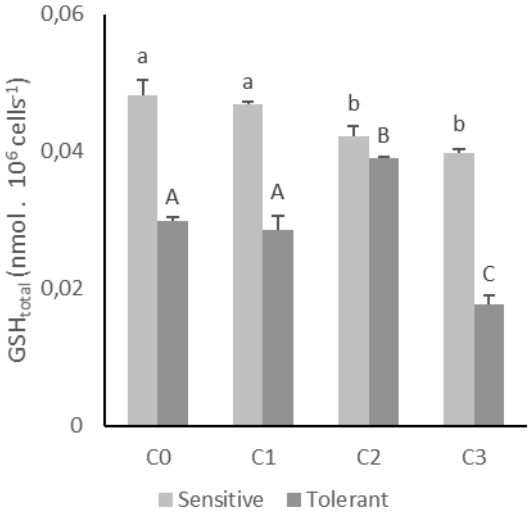

Figure 31 Total reduced glutathione (nmol.10^6 $cells^{-1}$) of tolerant (TCC762) and sensitive (TCC854) *Nitzschia palea* strains exposed to cadmium. For TCC854 cadmium concentrations were C0: 0; C1: 0.001; C2: 0.01; C3: 0.14 $mg.L^{-1}$; for TCC762 were C0: 0; C1: 0.14; C2: 1.96; C3: 27.44 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Different letters (a-c) represent significant differences (p≤0.05) among concentrations

.

The overall quantity of reduced glutathione is higher in the sensitive strain than in the tolerant one (Fig. 31). The sensitive strain (TCC854) showed a significant decrease in its reduced glutathione content at C2 (0.01 $mg.L^{-1}$) and C3 (0.14 $mg.L^{-1}$). As for the tolerant strain, reduced glutathione increases significantly at C2 (1.96 $mg.L^{-1}$) and decreases significantly at C3 (27.44 $mg.L^{-1}$), compared to control. At 0.14 $mg.L^{-1}$ the tolerant strain showed no changes in its glutathione content regarding the control, while the sensitive strain's decreased, but still higher than the tolerant levels'.

3.3.4.4 Antioxidant enzymes

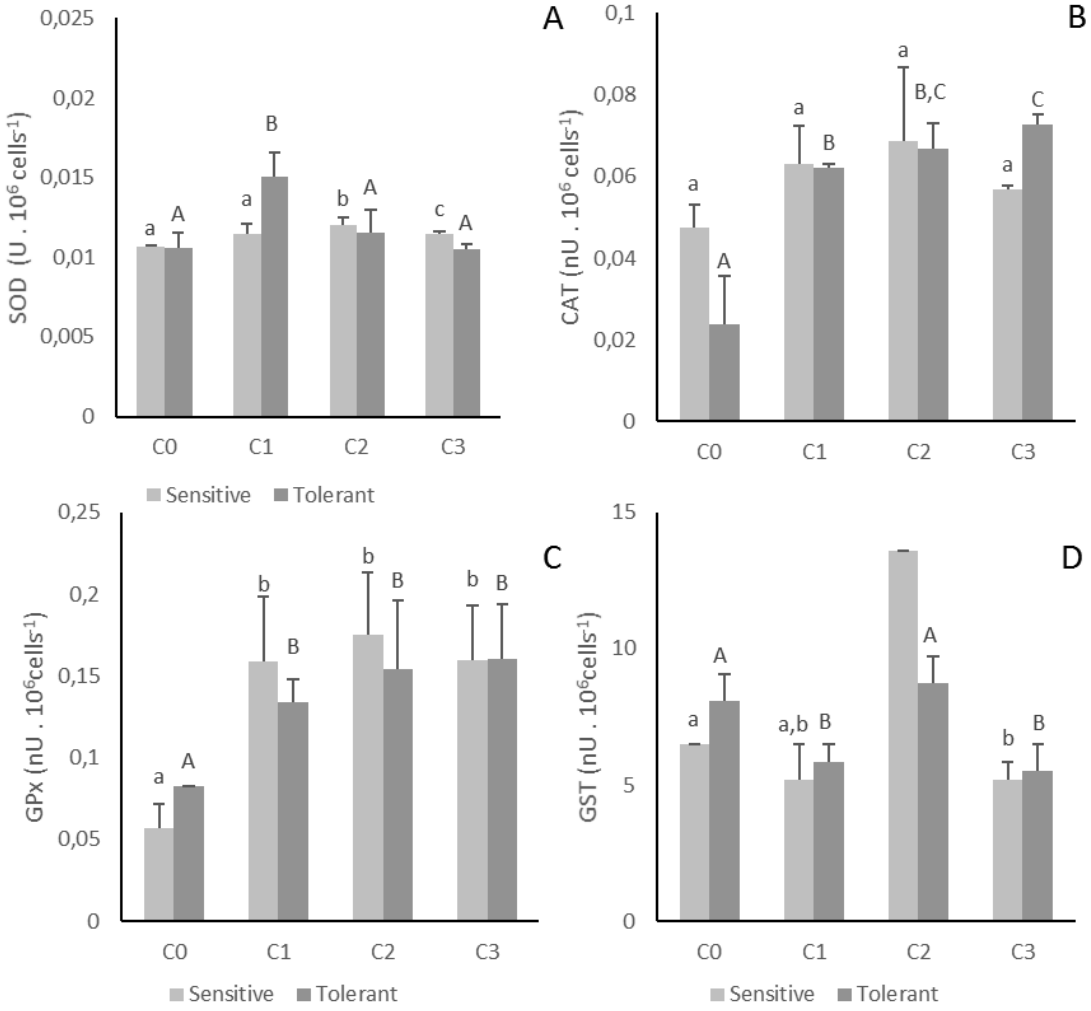

Figure 32 Antioxidant response: (A): superoxide dismutase ($U.10^6$ $cells^{-1}$); (B) catalase activity($nU.10^6$ $cells^{-1}$); (C) glutathione peroxidase activity ($nU.10^6$ $cells^{-1}$); (D) glutathione S-transferase activity ($nU.10^6$ $cells^{-1}$) of tolerant (TCC762) and sensitive (TCC854) *Nitzschia palea* strains exposed to cadmium. For TCC854 cadmium concentrations were C0: 0; C1: 0.001; C2: 0.01; C3: 0.14 $mg.L^{-1}$; for TCC762 were C0: 0; C1: 0.14; C2: 1.96; C3: 27.44 $mg.L^{-1}$). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences ($p \leq 0.05$) among concentrations for the sensitive and tolerant strains, respectively.

Superoxide dismutase

Both sensitive and tolerant strains have similar SOD activities when not exposed to Cd (Fig. 32-A). Sensitive strain's SOD activity increases slightly, differing from the control at C2 (0.01 $mg.L^{-1}$) and C3 (0.14 $mg.L^{-1}$). The tolerant strain significantly increases SOD activity at C1 (0.14 $mg.L^{-1}$), decreasing to control levels at hogher Cd concentrations. At 0.14 $mg.L^{-1}$, tolerant strain's SOD activity is higher than sensitive strain's.

Catalase

At the control, C0, sensitive strain's catalase activity is lower than the tolerant one's (Fig. 32-B). Both strains presented an activity increase compared to control. The increase is significant for the tolerant strain. For the same concentration, 0.14 $mg.L^{-1}$, enzymatic activity is slightly lower for the sensitive strain.

Glutathione peroxidase

In the absence of cadmium, the tolerant strain has a higher glutathione peroxidase activity than the sensitive one (Fig. 32-C). Both strains significantly increased their enzymatic activity with exposure. The sensitive strain presents a significant activity increase upon exposure. The tolerant strain only presented differences at C1 (0.14 $mg.L^{-1}$).and C3 (27.44 $mg.L^{-1}$) when compared to the control. Both strain's enzyme activity significantly increses, regarding the control, when exposed to the same concentration, 0.14 $mg.L^{-1}$.

Glutathione S-transferases

Glutathione S-transferases activity is higher for the tolerant strain in the absence of cadmium (Fig. 32-D). The sensitive strain presents a significant decrease in GSTs activity at C3 (0.14 $mg.L^{-1}$). The tolerant strain also displays an activity decrease at C3. For the same concentration (0.14 $mg.L^{-1}$), GST activity decreases, regarding the control, for both strains.

3.3.4.5 Pigment content

In the absence of cadmium, C0, chlorophyll *a* content is higher in the tolerant strain than in the sensitive one (Fig. 33-A) Sensitive strain significantly

increases chlorophyll *a* content at C2 (0.01 mg.L^{-1}) and C3 (0.14 mg.L^{-1}). The tolerant strain significantly increases chlorophyll *a* content at C2 (1.96 mg.L^{-1}) and decreases its content at C1 (0.14 mg.L^{-1}) and C3 (27.44 mg.L^{-1}). The significant decrease for the tolerant strain at 0.14 mg.L^{-1}contrasts with the increase observed in the sensitive strain.

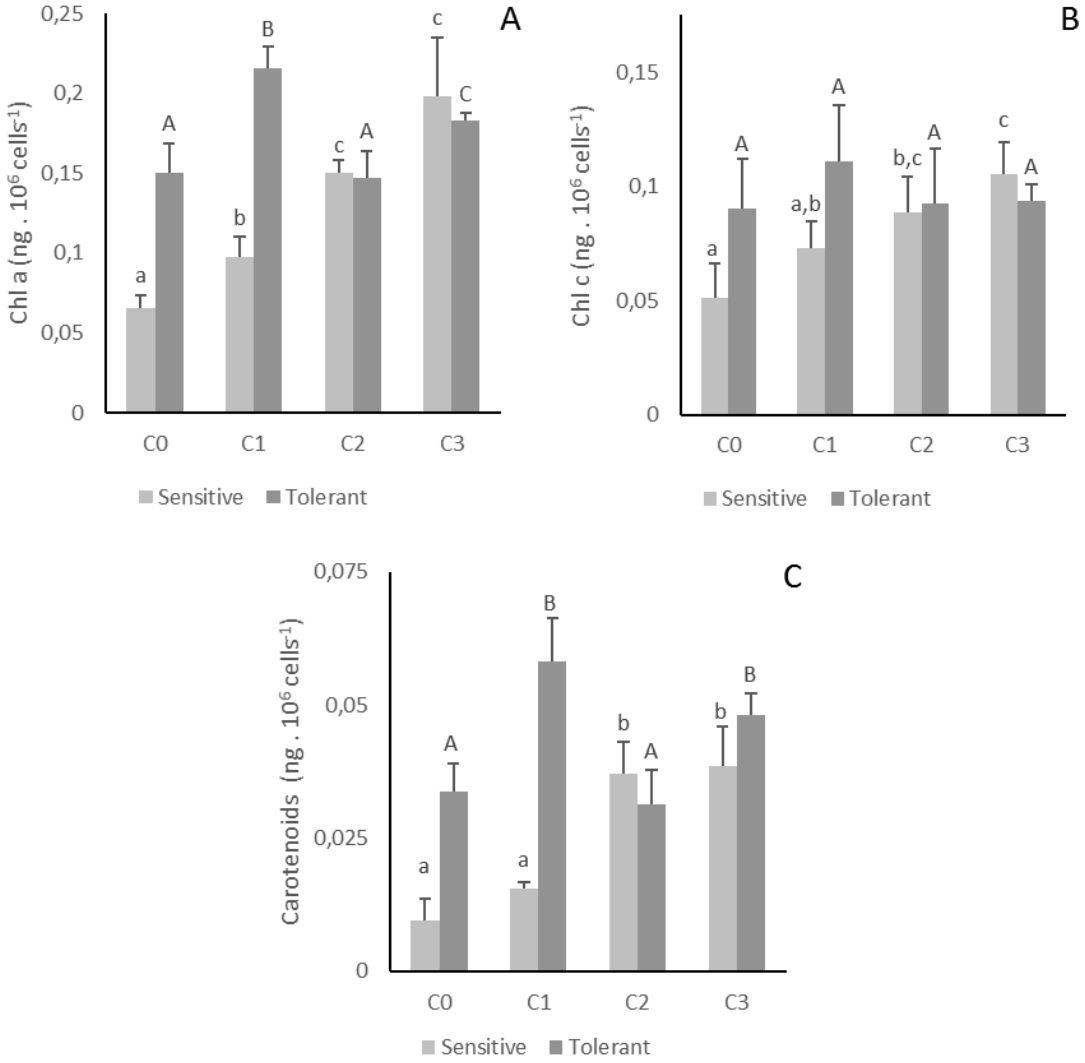

Figure 33 Pigment content: (A): chlorophyll *a* (ng.10^6 cells^{-1}); (B) chlorophyll *b* (ng.10^6 cells^{-1}); (C) Carotenoids (ng.10^6 cells^{-1}) of tolerant (TCC762) and sensitive (TCC854) *Nitzschia palea* strains exposed to cadmium. For TCC854 cadmium concentrations were C0: 0; C1: 0.001; C2: 0.01; C3: 0.14 mg.L^{-1} ; for TCC762 were C0: 0; C1: 0.14; C2: 1.96; C3: 27.44 mg.L^{-1}). Values represent the mean of the 3 replicates ± standard deviation. Lower and uppercase letters represent significant differences (p≤0.05) among concentrations for the sensitive and tolerant strains, respectively.

Chlorophyll *c* content is higher in the tolerant strain than in the sensitive one, in the absence of cadmium, C0 (Fig. 33-B). Sensitive strain's chlorophyll *c* content augments with exposure, significantly at C2 and at the last concentration, C3 (0.14 mg.L^{-1}). In the tolerant strain, chlorophyll *c* content doesn't significantly

differ from the control in the presence of the metal. At 0.14 mg.L^{-1} the chlorophyll *c* content in the sensitive strain is twice as high as the content in the tolerant strain.

Sensitive strain's carotenoids' content is higher than the tolerant one in the absence of cadmium, C0 (Fig. 33-C). Sensitive strains' carotenoid content increases with exposure. This increase is significant at C2 (0.01 mg.L^{-1}) and C3 (0.14 mg.L^{-1}). Carotenoids in the tolerant strain decrease significantly at the first C1 (0.14 mg.L^{-1})and the last concentration tested, C3 (27.44 mg.L^{-1}). Carotenoid content differs significantly from the control at 0.14 mg.L^{-1} for both strains.

3.3.4.6 Multivariable overview of cadmium biomarkers

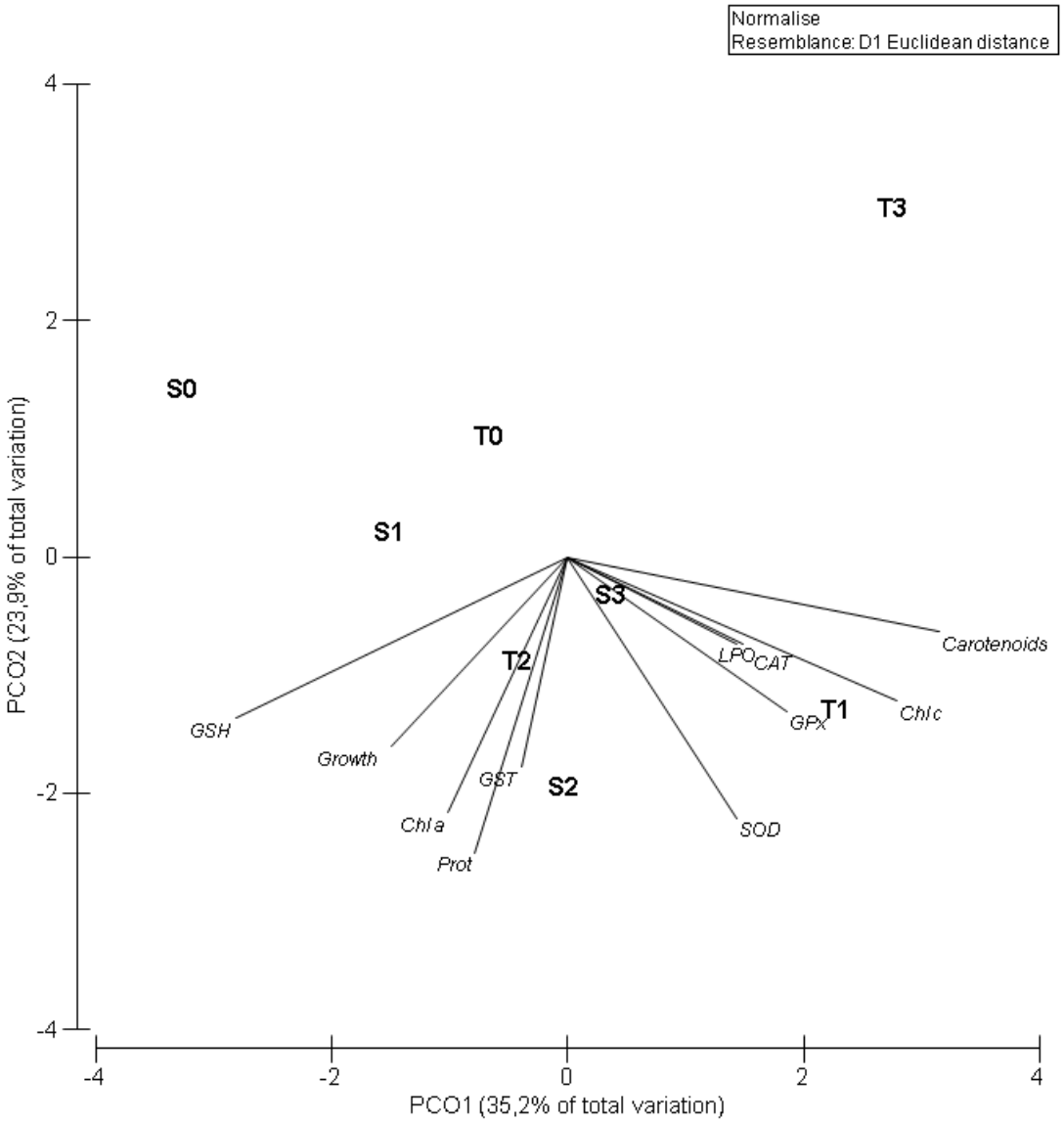

Figure 34 PCO multivariate analysis of cadmium biomarkers by Primer 6, with a tolerant and a sensitive strain of Nitzschia palea

The first two axes of the PCO explain about 59% of the variation of the measured parameters for both strains (Fig. 34). PCO1 is characterized by the positive weight of carotenoids, Chl *c*, GP*x*, CAT, LPO, and the negative load of GSH. As for PCO2, it is characterized by the negative weight of Prot, SOD, Chl *a*, GST and, to a minor extent, Growth.

PCO analysis shows the controls from both the sensitive and the tolerant strain in the same quadrant (-/+), along with response from the sensitive strain at C1 (0.001 $mg.L^{-1}$).

The response of the sensitive strain (TCC854) at C2 (0.01 $mg.L^{-1}$), C3 (0.14 $mg.L^{-1}$) and the tolerant strain at C1 (0.14 $mg.L^{-1}$) was characterized by the highest carotenoid and chlorophyll *c* content, antioxidant response (SOD, CAT and GP*x*), as well as lipid damage (LPO).

Tolerant strain's (TCC762) C2 (1.96 $mg.L^{-1}$) is characterized by chlorophyll *a*, reduced glutathione (GSH) and protein content, GST activity and growth. These parameters were low for the tolerant strain at C3 (27.44 $mg.L^{-1}$).

3.4 Discussion

In this study, a sensitive *Nitzschia palea* strain was compared to a tolerant one to assess if the herbicides, atrazine and diuron, and the metals, copper and cadmium, tolerance differences were supported at the biochemical level.

3.4.1 Herbicides

3.4.1.1 Diuron

In a study by Larras et al. (2013), *N. palea* was considered a tolerant species, with an EC_{50} of 1.6671 $mg.L^{-1}$. In this study, the EC_{50} were 0.0085 $mg.L^{-1}$ for the sensitive strain and 0.0481 mg L^{-1} for the tolerant strain, values in line with the ones obtained for *Fragilaria rumpens* (EC_{50}: 0,00889 $mg.L^{-1}$) and *Cyclotella meneghiniana* (EC_{50}: 0,049 mg L^{-1}), respectively (Larras et al., 2013).

Diuron is a PSII-inhibitor herbicide that binds to the D1 protein, hindering Hill reaction, leading to photosynthesis arrest and the production of ROS (reviewed in Kumar et al., 2014). Intriguingly, a 60% decrease in ROS content was observed for the diatom *Chaetoceros calcitrans* when exposed to 0.005 mg L^{-1} diuron, which the authors hypothesized to resulted from the scavenging properties of fucoxanthin, SOD and CAT (Dupraz et al., 2016). In this study, both

strains presented differences in those scavengers levels, even in the absence of the herbicide: while carotenoid content was higher for the tolerant strain, CAT and SOD activities were more elevated for the sensitive strain.

Although having higher SOD and CAT levels at control conditions, the sensitive strain didn't display an enzymatic boost of those or the other enzymes tested when exposed to the lower diuron concentration. In fact, the activity of both enzymes involved in H_2O_2 detoxification, CAT and GP*x* which are responsible for major detoxification under stress and finer tuning, respectively (Lozano et al., 2014), decreased. The inhibition of these enzymes, coupled with the electron flow disturbance resulted in an increase of LPO and oxidized chlorophylls (Kumar et al., 2014), which diminishes its photosynthetic ability.

Exposition to the second concentration appear to triggered the sensitive strain's defense mechanisms, since the enzymes previously inhibited (CAT and GP*x*) returned to the basal levels or were stimulated. In a preceding study (Teisseire & Vernet, 2000), diuron's ability to weakly induce the antioxidant system was already acknowledged. Activity increase of enzymes such as GSTs, involved in herbicide detoxification (Geoffroy et al., 2002) and SOD, part of the primary ROS defense response (Mittler, 2002) were observed. In addition to an increase in total GSH, substrate of GSTs, carotenoids, which quench both excited chlorophyll and ROS (Szymanska et al., 2012), also increased.

As for the tolerant strain, when exposed to diuron, an increase in CAT activity was observed for all concentrations tested. This results contrast with previous studies (Geoffroy et al., 2002; Keresztes et al., 2009). Despite the action of mechanisms not analyzed here (such as phytochelatins, polysaccharides, and polyphenols, Cirulis et al., 2013), CAT appears to be relevant in ROS reduction, perceptible by a significant reduction in the LPO levels. The activity of GSTs was only induced at the last concentration tested, as well as chlorophyll reduction. This is consistent with oxidative stress mitigation through photosynthesis decrease, conjugation of xenobiotics and LPO detoxification.

Both strains presented different strategies to handle the damage caused by diuron. Sensitive strain's mechanisms, SOD, GSTs and carotenoids, were only activated when exposed to a higher diuron concentration. This tardiness allowed the occurrence of membrane damage (LPO). As for the tolerant strain, CAT played a main role in the cell's defense as well as GSTs. In addition to increasing

these antioxidant enzymes' activity, the tolerant strain also decreased ROS production derived from photosynthesis by lowering the chl a content.

3.4.1.2 Atrazine

Nitzschia palea, alongside *Gomphonema parvulum* and *Achnanthidium minutissimum*, is considered tolerant to atrazine (Debenest et al., 2010). Atrazine is algistatic that, similarly to diuron, acts through inhibition of the oxygen evolution in photosynthesis, whose tolerance is linked to more than just plant's ability to metabolize it (Shimabukuro & Swanson, 1969). In this study, both strains had EC_{50} of the same order, 0.121 mgL^{-1} and 0.223 mgL^{-1}, similar to *Encyonema gracilis* - EC_{50}: 0.242 mgL^{-1} (Wood et al., 2016)-, but one order below the one obtained by Larras et al. (2012) for *N. palea* – EC_{50}: 1.539 mgL^{-1}.

The herbicide directly interferes with electron transfer in the chloroplast, but also causes damage to the chlorophylls through photodamage (Qian et al., 2014). This damage is observed for the tolerant strain, which displayed a significant chlorophyll a content decrease under all atrazine concentrations tested and chlorophyll c for the highest one; as well as for the sensitive strain, whose chlorophyll c content was reduced at the first concentration assayed. The tolerant strain tried to mitigate the damage by significantly increasing carotenoid content, a low weight molecule known to increase under low oxidative stress (Cirulis et al., 2013) and to be involved in short-term photoprotection in diatoms (Bertrand, 2010). The sensitive strain, in contrast, had its carotenoid content reduced at the highest concentrations tested, being less shielded from photodamage. A similar reduction was observed for *Phaeodactylum tricornutum* exposed to 0.200 $mg.L^{-1}$ and 0.500 $mg.L^{-1}$ of atrazine (Bai et al., 2015).

Atrazine can also impair protein synthesis and lead to lipid accumulation due to energy production deficit caused by the photosynthetic impairment (Weiner et al., 2007). Said impairment can lead to the formation of triplet state chlorophyll and Reactive Oxygen Species (ROS), which can then damage lipids and proteins (Qian et al., 2008; Rutherford & Krieger-Liszkay, 2001). This damage can be observed in both strains by the increase in lipid peroxidation, and by the decrease in protein content for the tolerant strain. This decrease in protein content may also relate to the D1 protein turnover (Mattoo et al., 1989), in which

damaged D1 protein is degraded and synthetized *de novo*. Given that D1 protein is part of the heterodimeric PSII reaction core, this process ensures photosystem function while the degradation rate does not surpass the repairing one, which is impaired by the herbicide.

The sensitive strain does not display significantly higher LPO levels when exposed to the higher concentrations, at which SOD activity rose. Superoxide dismutase, part of organisms first line of antioxidant defense (Mittler, 2002), may have decrease the cellular O_2^- pool. The resulting H_2O_2 is, at low concentrations, involved in cell signaling of processes such as growth, migration and apoptosis, while at higher concentrations it can react with intracellular copper or iron to form hydroxyl radicals which can damage DNA, lipids and proteins (Veal & Day, 2011). The activity of the main H_2O_2 detoxifying enzyme, catalase (Lozano et al., 2014), presented an activity decrease, which may be a result of an inefficiency of the activation and/or degradation by peroxisome's proteases (Hassan & Nemat Alla, 2005), that can lead to H_2O_2 accumulation. In addition, there is also a GSTs activity decrease, which may be due to the inhibition of some types of GSTs (Zhu et al., 2009). Total glutathione content increased, possibly related to reduced glutathione's potential as $^1O_2$, H_2O_2 and $OH^{\bullet}$ scavenger (reviewed in Gill & Tuteja, 2010). Given that neither main H_2O_2 detoxifying enzymes' activity (GP*x* and CAT), damage (LPO) or damage repairing mechanisms (GSTs) increase were observed, it is likely that H_2O_2 levels were only slightly elevated, conducing to apoptosis, rather than causing damage. The tolerant strain, on the other hand, managed atrazine driven stress by conjugating it, noticeable through the GSTs activity increase and GSH content. This defense mechanism did not prevent the strain from sustain damage in the membranes. According to Prade et al. (1998), conjugation plays an important role in plant tolerance to triazine herbicides. At the highest concentration, the activity of both GSTs and GP*x* increase, thus increasing the demand for GSH. GSH synthesis may be increased through feedback induction (Marrs, 1996), to counterweigh the atrazine excess.

When exposed to the same herbicide concentration, 0.25 $mg.L^{-1}$, each strain had different strategies to deal with damage. While the sensitive strain used SOD to deal with the excess ROS caused by herbicide exposure and probably engaged into apoptosis; the tolerant strain's reduced the amount of herbicide available, although sustaining some damage. At this concentration, while the

tolerant strain had its carotenoid content increased, it decreased in the sensitive strain. Although also having the GSTs-related mechanism enhanced, the tolerant strain suffered a higher damage than the sensitive one. This results follow the ones obtained by Szigeti & Lehoczki (2003), where atrazine-resistant *Conyza canadensis* showed alterations at lipid level and quenching ability, as well as higher photosensitivity and propensity to photo-inhibition.

Although the sensitivity of both strains remained in the same order, distinct biochemical responses when exposed to atrazine were observed. Sensitive strain's response consisted, mainly, in $O_2^{\bullet -}$ detoxification

3.4.2 Metals

3.4.2.1 Copper

Sensitivity differences among strains of several species to copper has been briefly reviewed in Masmoudi et al. (2013) where, in several papers analysed, copper EC_{50} for *Phaeodactylum tricornutum* had a range of two order of magnitude. In this study, *Nitzschia palea* sensitivity ranged from 0.005 $mg.L^{-1}$ to 0.134 $mg.L^{-1}$ for the sensitive and the tolerant strains, respectively. Similar results for the same species have been found by Takamura et al. (1989) (EC_{50}: 0.212 $mg.L^{-1}$) and Rai et al. (1981) (EC_{50}: 0.002 $mg.L^{-1}$).

Photosynthesis can be inhibited by copper (Macdowall, 1949) through mechanisms such as interaction with PSII donor side (Patsikka et al., 2001) or copper replacement of the magnesium in chlorophylls (Küpper et al., 2003). The pigment content boost observed at all concentrations tested, for both strains, may be seen as an attempt to compensate the photosynthetic impairment. This hypothesis is consonant with the ones from Lelong et al. (2012), where *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia delicatissima* increased the pigment content in response to a decline in photosynthetic yield. In addition to compensate for the photosynthetic efficiency loss, carotenoids are also one of the low molecular weight compounds synthetized in response to metal oxidative stress (Pinto et al., 2003).

The photosynthetic impairment caused by copper leads to the formation of reactive oxygen species (ROS). Copper competes with CAT for the degradation H_2O_2, however, this Fenton-type reaction leads to the formation of $^{\bullet}OH$, which will start the peroxidation chain (Sandmann & Boger, 1980). At the

lowest concentration, neither strain showed differences at CAT activity level. However, the sensitive strain had an increased GP*x* activity, which also detoxifies H_2O_2, and that may have avoided the increase in peroxidation levels. The tolerant strain, on the other hand, had a significant LPO increase, probably due to the maintenance of antioxidant enzymes at the basal level.

At the first concentration to which they were exposed, which did not cause significant inhibition to either strain, both exhibited a GSH decrease. Glutathione can be consumed in processes that include conjugation via GSTs catalysis and phytochelatins' production (Smith et al., 2014). Considering no alterations in GSTs activity, copper immobilization seems to be both strains' preferred defense mechanism. Similar GSH response was found in the marine diatom *Ceratoneis closterium* (Smith et al., 2014; Stauber & Florence, 1986). Furthermore, the elevated LPO levels observed in the tolerant strain imply existing damage in the cellular membrane, which may augment glutathione release to the medium (Tang et al., 2005). In the same study, Tang et al., (2005) found that concentrations of 0.0001 $mg.L^{-1}$ were enough to compromise membrane integrity.

Exposed to the second highest concentration, the sensitive strain displayed a growth inhibition, not compensated by the observed pigment increase. At the third concentration, the sensitive strain increased its pigment content and GSTs activity. The consumption of GSH by GSTs may lower the GSH:GSSG ratio, leading to inhibition of cellular division, due to interference in the formation of the mitotic spindle (Stauber & Florence, 1987), which halts mitosis.

On the other hand, the tolerant strain increased CAT activity which, paired with GSH consumption, managed to mitigate the potential damage caused by excess copper and allowing for non-significant growth decrease regarding the control. At the third concentration tested, the tolerant strain increased the carotenoid and chl *a*, boosted H_2O_2 detoxifying enzymes. Increasing CAT alone at the second concentration and CAT and GP*x* at the third one. These results follow the conclusion of Sofo et al., (2015) which stated that, due to its low affinity, CAT is activated only under stress circumstances.

When exposed to the same concentration, 0.04 $mg.L^{-1}$, both strains appear to have different strategies to cope with excess copper. On the one hand, the sensitive strain increased pigment production, in order to balance the

photosynthetic efficiency lost, and GSTs activity to reduce eventual oxidative stress. On the other hand, the tolerant strain also increased pigment content but also employed GSH-related mechanisms that, although protecting cell division, did not avoid lipid damage. In a similar study, Sáez et al. (2015) compared one sensitive and two tolerant strains of the brow algae *Ectocarpus siliculosus*, and identified different defense strategies in response to Cu-induced stress. In said study the sensitive strain decreased its chlorophyll *a* content, unlike in this one, and increased ascorbate peroxidase (AP*x*) levels, while the tolerant strains increased antioxidants content (among which GSH), AP*x* activity and protein content.

The two orders of magnitude for sensitivity difference observed between the sensitive and tolerant strain was noticeable at a biochemical level. At the control level, both strains had different values for the markers assessed but this trend was not maintained when both strains were exposed to the same copper concentration, with exception of growth, LPO, GST, GSH and chl *a*. The sensitive strain's stress response was restricted to pigment augmentation, in an attempt to restore the lost photosynthetic activity, and, at the highest concentration, GSTs activity increase in order to decrease metal's availability. In contrast, the tolerant strain increased the antioxidant response at the higher concentrations tested, in an effort to lessen the damage caused by the ROS generated by copper action.

3.4.2.2 Cadmium

Metals are known to induce oxidative stress and interact with molecules such as lipids, proteins and DNA (reviwed in Cirulis et al., 2013). Branco et al. (2010) studied alterations in biochemical markers in *N. palea* exposed to Cd stress. In that study, the EC_{50} calculated was 0.0276 $mg.L^{-1}$, one order below the EC_{50} estimated for the sensitive strain in the present work. Conversely, the value estimated for the tolerant strain (20.3975 mg L^{-1})was two orders higher than the sensitive strain (0.2489 mg L^{-1}) and three orders higher than the one obtained by Branco et al. (2010), being in line to the one obtained to the tolerant species *P. tricornutum*, -IC_{50}: 22.39 $mg.L^{-1}$ - (Torres et al., 1997). Sensitivity differences within a species can be minimal, or up to several orders of magnitude (Hammond et al., 2012; Santos et al., 2016).

At the lowest concentration of Cd to which the sensitive strain was exposed, the only parameter increasing significantly, compared to the control was GP*x* activity. This increase denotes potential stress or phytotoxicity caused by metals (Pinto et al., 2003). Indeed, at the second concentration tested, cells increased the activity of antioxidant enzymes (SOD, GST and GP*x*) as well as Chl *a* and carotenoids' content. A boost of antioxidant enzymes, as well as carotenoids is a common response to metal stress, contrasting with Chl *a* content, which tends to decrease in order to avoid further oxidative damage (Cirulis et al., 2013). Chlorophyll *a* can increase reactive oxygen species due to its role as electron donor in photosynthesis, but it is also a valuable antioxidant (Cirulis et al., 2013). At the highest concentration, there was an increase in photosynthetic pigments content, which, compensated with the increased activities of GP*x* and GSTs, may justify the absence of increase in LPO.

On the other hand, when exposed to cadmium, the tolerant strain experienced hormesis, which is growth stimulation upon exposure to a contaminant before the concentration instigates inhibition (Calabrese & Baldwin, 2002). Calabrese (1999) found evidence that this phenomenon was caused by an exaggerated attempt to balance a disturbance of contaminants in homeostasis. This disturbance may have been caused by damage to the membranes (LPO), which stimulated the antioxidant enzymes SOD, CAT and GP*x* at the lowest concentration tested, and CAT, GSH and Chl *a* at the second one. For the highest concentration tested, however, the disruption was not compensated, causing severe growth inhibition. The inhibition observed may be caused by the increase in LPO and reductions in GSH content, GSTs activity, Chl *a* and carotenoids. In this study, carotenoids had a higher decrease than Chl a, unlike the results obtained by Sivaci & Sivaci (2012). Cadmium is known to impair enzymes involved in chlorophylls' and carotenoids' biosynthesis (carotenoids: Bertrand, 2010; chlorophylls: Van Assche & Clijsters, 1990), which may explain the decrease in pigment content.

Comparing both strains when exposed to the same concentration ([Cd]: 0.14 mgL^{-1}), the tolerant strain had an increase in growth, as well as in the activity of the antioxidant enzymes SOD, CAT and GST when compared to the sensitive strain. Zhang et al (2009) found the same trend for SOD and CAT when studying hormesis in earthworms. On the other hand, the sensitive strain, which displayed

a non-significant growth inhibition compared to the control, had elevated LPO together with higher GP*x* activity, pigment and GSH contents. Since chlorophylls can increase oxidative stress through photosensitization (Pinto et al., 2003; Sharma et al., 2012), the contrast in LPO values can be explained by the differences observed at damage mitigation level (GSH and GP*x*). In both strains, protein content did not diverge or increase greatly, opposing the results of McLean & Williamson (1977), which observed anabolism-dependent Cd-intake.

Cadmium has a high pro-oxidant potential (Masmoudi et al., 2013). This status can be boosted by a reduction in GSH content (Pinto et al., 2003), such as the one observed in the sensitive strain. The tolerant strain, on the other hand, showed no alteration in GSH content at the first concentration assayed and an increase at the second one, concentrations that caused growth stimulation. At the highest concentration tested, which caused inhibition, GSH content decreased significantly, in agreement with the previous study (Pinto et al., 2003) but not with Branco et al. (2010) who observed an increase. The reduction of GSH content coupled with an increased GSTs activity in the sensitive strain suggests the formation of Cd-GSH complexes, given that metal-GSH complexes are easily excreted (Tang et al., 2005). Moreover, cadmium obstructs the electron flow in photosynthesis, namely at the water-splitting center (Mallick & Mohn, 2003), leading to $^1O_2$ formation (Pinto et al., 2003). At the lowest concentration, the tolerant strain increases SOD activity, unlike the sensitive one, in order to detoxify $^1O_2$. The partitioning process leads to the formation of H_2O_2, which is believed to be the main ROS in algal cells (Knauert & Knauer, 2008). Both strains increased GP*x* activity, a H_2O_2 detoxifying mechanism, which was paired with CAT increase in the tolerant strain. Superoxide dismutase activity was maintained or decreased, possibly due to excess H_2O_2 generated in other compartments which can inhibit its activity (Lee & Shin, 2003). The enzymes GP*x* and CAT are located in different cellular compartments: cytosol, chloroplasts and mitochondria, and peroxisomes, respectively (Racchi, 2013). Therefore, alterations in enzymatic activity can help estimate which organelles are under stress and where H_2O_2 is being detoxified.

The three orders of magnitude for sensitivity differences observed for the sensitive and tolerant strains were not reflected in all biochemical parameters studied. At the control level, both strains had different values for the markers

assessed and this trend was maintained when both strains were exposed to the same cadmium concentration. At the lower concentrations tested, the sensitive strain already presented a growth decrease while the tolerant strain had a growth stimulation consistent with activation of ROS-scavenging mechanisms.

3.5 Conclusions

Tolerant and sensitive strains presented, overall, different strategies to manage the damage caused by the herbicides and metal they were exposed to.

Regarding the herbicides, the responses varied although both shared the mode of action. For diuron, antioxidant response was weakly induced, leading to lipid peroxidation, while the tolerant strain detoxified H_2O_2 and reduced the production of photosynthetic ROS. For atrazine, the main action for the sensitive strain was $O_2^{\bullet -}$ detoxification, whereas the tolerant strain invested in conjugation pathways and Chl a reduction.

Considering the metals, with exception of the copper-sensitive strain, all others engaged in H_2O_2 detoxification. In a similar fashion, all excluding the cadmium-tolerant strain boosted their pigment content. Although surviving under higher concentrations of cadmium, the tolerant strain sustained more damage than the sensitive one.

4 General conclusions

Diatoms' distribution and ecology have long been subject of discussion, boosted by the discovery of new diatom species, as well as (pseudo)cryptic species, which may present a completely new set of traits. Molecular methods gave an important contribution in the taxonomic improvement and endemic species definition.

Diatom populations, however, are not as uniform as previously thought. This may raise some apprehension in the monitoring field, based on reference species. Many species' ecological preferences are unknown or may vary, influencing said species' sensitivity. Since a populations' sensitivity may not represent the species' average sensitivity to a chemical, current approaches, considering a species to have homogeneous behavior towards a pollutant, may under or overestimate the ecological status of an aquatic ecosystem.

Considering *N. palea*, no link was found between phylogeny and sensitivity therefore, it was expected that herbicide and metal tolerance would be connected to the diatoms' ability to manage stress caused by those chemicals. However, none of the parameters evaluated stud out as a potential biomarker of herbicide or metal contamination, with exception of GP*x* for the cadmium treatment.

These results demand caution when analyzing data regarding different strains from a species. Since they employ distinctive biochemical pathways to deal with metal and herbicide toxicity, sensitivity differences of several orders of magnitude can be observed. Therefore, intraspecific variability must be accounted for either performing toxicity assays or using a species as biomonitors of environment health status.

5 References

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