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Marisa Alexandra Marques de Freitas

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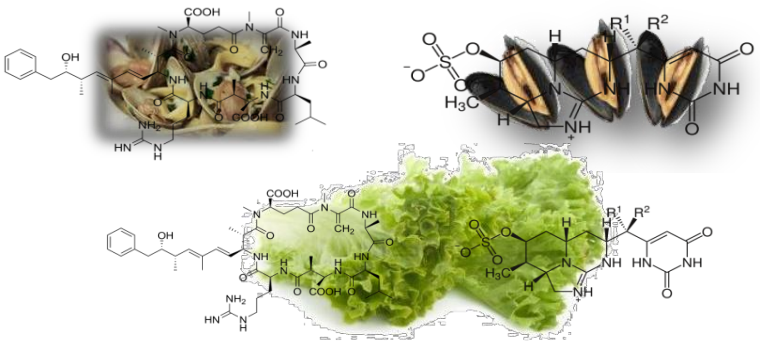

Effects of interaction, availability and bioaccessibility of microcystin-LR and cylindrospermopsin in terrestrial and aquatic species

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Abstract

The occurrence and proliferation of toxic cyanobacterial blooms are an emergent environmental concern worldwide. Microcystin-LR (MC-LR), mainly produced by *Microcystis aeruginosa*, is the most documented and studied cyanotoxin. Cylindrospermopsin (CYN) has been recognized of increased concern due to the invasive nature of its main producer, *Cylindrospermopsis raciborskii*. Recent studies support the hypothesis that MC-LR and CYN exert harmful effects on crop plants. Lettuce is an important commercial leafy vegetable that supplies important nutrients for human diet. Thus, it is of particular interest to know its sensitivity to environmentally relevant concentrations of cyanotoxins, including mixtures. Proteomic technologies seem to be suitable for the identification of early stress responses, which are not perceptible by traditional endpoints. Proteomics may also provide the identification of allergenic proteins, which may be of particular interest for human health risk assessment. However, human health problems due to MC-LR and CYN are most likely associated to the chronic exposure by contaminated drinking water and food. Previous studies have shown that edible aquatic organisms, especially bivalves, can accumulate high levels of these cyanotoxins without lethal effects. MC-LR and CYN are water-soluble and stable at a wide range of temperatures and pHs, thus the knowledge of the influence of storage and cooking practices as well as human digestion on MC-LR and CYN concentration in food is required to achieve a more accurate risk assessment. This thesis aimed to: (1) assess biochemical and physiological effects of MC-LR, CYN and MC-LR/CYN mixture in lettuce, using conventional endpoints and a proteomic-based approach; (2) assess the changes on MC-LR and CYN concentration in edible bivalves after applying different storage and cooking practices, and (3) assess the MC-LR and CYN bioaccessibility.

Lettuce plants (*Lactuca sativa* L.) were exposed to concentrations of 1, 10 and 100 µg/L of MC-LR, CYN and MC-LR/CYN mixture for five and ten days, and the effects were assessed by the parameters fresh weight, activity of antioxidant enzymes and mineral content in edible parts. The lettuce leaves were also studied by a comparative proteomics approach. To assess the changes on MC-LR and CYN concentration in bivalves after common food storage and processing practices, clams (*C. fluminea*) fed MC-LR-producing *M. aeruginosa* and mussels (*M. galloprovincialis*) fed CYN-producing *C. raciborskii* were refrigerated, frozen, boiled, steamed and subjected to microwave

radiation over different periods of time and then analyzed by LC-MS/MS. Bioaccessibility of MC-LR and CYN were assessed in uncooked clams and in uncooked and steamed mussels, respectively.

Overall, an increase in root growth was obtained, however, leaf-fresh weight was significantly reduced in plants exposed to 100 µg/L. The GST activity was significantly increased in roots, contrary to GPx activity, which decreased in roots and leaves. In general, the mineral content in lettuce leaves decreased with MC-LR and increased with CYN, and apparently these effects are time and concentration-dependent. The effects of the MC-LR/CYN mixture were almost always similar to the single cyanotoxins. Some of these physiological and biochemical effects were further elucidated by the proteomics analysis, and at proteome level, the effects of the mixture were clearly stronger than those of CYN alone. The biological functions of the proteins that were most represented in both experiments were related to photosynthesis and carbon metabolism and stress/defense response. Such variations could have altered the rates of mineral uptake by lettuce plants and also conferred putative tolerance of lettuce plants to CYN.

The recovery of free MC-LR in clams increased with freezing storage and with cooking for short periods of time; specifically with the microwave radiation treatment for 0.5 and 1 min and boiling treatment for 5 and 15 min. The bioaccessibility of MC-LR after proteolytic digestion was reduced to 83%, potentially because of MC-LR degradation by pancreatic enzymes. In mussels stored frozen a significantly higher recovery of CYN was obtained. The cooking treatments did not produce significant differences in CYN concentration in mussel matrices (flesh), however, CYN was found in the cooking water, suggesting that heat processing can be used to reduce the availability of CYN in this food item. The *in vitro* digestion with salivary and gastrointestinal juices considerably decreased the CYN availability in uncooked and steamed mussels, highlighting the importance in integrating the bioaccessibility in the human health risk assessment.

In conclusion, these findings provide new insights into the biochemical and physiological mechanisms of the lettuce response to MC-LR and CYN (inclusive in mixture) and may contribute to the understanding of potential mechanisms that may confer tolerance to CYN. This study also provides an enhancement of knowledge on the MC-LR and CYN concentration available in food after employing techniques

commonly used for their preservation and processing, which might be of particular interest for the definition of critical control limits, considering the HACCP approach as a promising tool for risk management. Our results also suggest that risk assessment based on MC-LR and CYN concentration in raw products might not be representative of true human exposure.

Keywords: Cylindrospermopsin, bioaccessibility, bivalves, *Lactuca sativa*, Microcystin-LR, mixture.

Resumo

A ocorrência e a proliferação de florescências de cianobactérias tóxicas são um problema ambiental emergente a nível mundial. Microcistina-LR (MC-LR), produzida principalmente por *Microcystis aeruginosa*, é a cianotoxina mais estudada e documentada. A cianotoxina cilindrospermopsina (CYN) tem sido alvo de crescente preocupação devido à natureza invasiva da principal cianobatéria que a produz, *Cylindrospermopsis raciborskii*. Estudos recentes suportam a hipótese de que a MC-LR e a CYN podem exercer efeitos tóxicos em plantas agrícolas. A alface é um vegetal de grande importancia a nível comercial, o qual fornece nutrientes essenciais para a dieta humana. É portanto, de particular interesse conhecer a sua sensibilidade a concentrações ambientalmente relevantes de cianotoxinas, inclusive quando em mistura. A abordagem proteómica parece ser adequada para a identificação de respostas ao stress que não são perceptíveis pelos *endpoints* tradicionais. O estudo do proteoma pode também permitir a identificação de proteínas alergénicas, que podem ter particular interesse em termos de avaliação de risco para a saúde humana. No entanto, as implicações para a saúde humana devido às cianotoxinas, MC-LR e CYN, são mais susceptíveis de ocorrer pela exposição crónica através da ingestão de água e alimentos contaminados. Estudos prévios demonstraram que os organismos aquáticos, especialmente bivalves, podem acumular níveis elevados de cianotoxinas sem que ocorram efeitos letais. Por outro lado, a MC-LR e a CYN são solúveis em água e estáveis a uma ampla gama de temperaturas e pHs. Portanto, a compreensão da influência dos métodos de armazenamento e processamento de alimentos, assim como da digestão humana sobre a concentração de MC-LR e CYN nos alimentos é de grande relevância para alcançar uma avaliação de risco mais precisa. Os objetivos desta tese foram: (1) avaliar os efeitos bioquímicos e fisiológicos provocados pela MC-LR, CYN e pela sua mistura em alface, através de *endpoints* convencionais e de uma abordagem proteómica; (2) avaliar o efeito de diferentes práticas de armazenamento e de processamento de alimentos sobre a concentração de MC-LR e CYN em bivalves edíveis, e (3) conhecer a biaccessibilidade de MC-LR e CYN.

As plantas de alface (*Lactuca sativa* L.) foram expostas às concentrações de 1, 10 e 100 µg/L de MC-LR, CYN e da mistura de MC-LR/CYN durante cinco e dez dias e os efeitos foram avaliados pelos parâmetros peso fresco, atividade de enzimas antioxidantes e conteúdo mineral na parte edível das plantas. As folhas de alface

foram também estudadas utilizando uma abordagem proteómica comparativa. Para avaliar os efeitos das práticas de armazenamento e procesamento dos alimentos na concentração de MC-LR e CYN disponível em bivalves, ameijoas (*C. fluminea*) e mexilhões (*M. galloprovincilais*) após serem alimentados com as cianobactérias produtoras de MC-LR e CYN, *M. aeruginosa* e *C. raciborskii*, respetivamente, foram refrigerados, congelados, cozidos, cozidos a vapor e submetidos a radiação microondas durante diferentes períodos de tempo e de seguida analisados por LC-MS/MS. A bioaccessibilidade da MC-LR e CYN foi estudada em ameijoas cruas e em mexilhões crus e cozidos a vapor, respetivamente.

Na generalidade, obteve-se um aumento do crescimento das raízes, no entanto, o peso fresco das folhas foi significativamente reduzido nas plantas expostas a 100 µg/L. A atividade da GST foi significativamente aumentada nas raízes, ao contrário da atividade da GPx, que diminuiu nas raízes e nas folhas. A concentração de minerais diminuiu nas folhas das plantas de alface expostas a MC-LR, contrariamente às plantas expostas a CYN, onde o teor de minerais foi significativamente aumentado, e aparentemente estes efeitos foram dependentes do tempo e da concentração de exposição. Os efeitos da mistura de MC-LR/CYN foram semelhantes aos da exposição às cianotoxinas individualmente. Alguns destes efeitos fisiológicos e bioquímicos foram elucidados através da análise peoteómica, e a este nível, os efeitos da mistura foram claramente mais potentes do que na exposição isolada a CYN. As funções biológicas das proteínas que foram mais representadas em ambos os ensaios estavam relacionadas com a fotossíntese e o metabolismo de carbono assim como com a resposta ao stress/mecanismos de defesa. Estas variações poderiam ter alterado a taxa de absorção de minerais pelas plantas de alface e também conferido uma potencial tolerância destas plantas à CYN.

A recuperação da MC-LR livre nas ameijoas aumentou devido à armazenagem sob congelação e ao cozimento por curtos períodos de tempo; especificamente, nos tratamentos com radiação microondas durante 0,5 e 1 min e na cozedura durante 5 e 15 min. A biodisponibilidade da MC-LR após a digestão proteolítica foi reduzida para 83%, possivelmente devido à degradação da MC-LR pelas enzimas pancreáticas. O armazenamento dos mexilhões sob congelação permitiu também uma maior recuperação da CYN. Os tratamentos de cozedura não produziram diferenças significativas na concentração da CYN na matriz do mexilhão (tecidos), no entanto, a toxina foi encontrada na água de cozedura, o que sugere que o processamento pode ser utilizado para reduzir a sua disponibilidade neste organismo edível. A digestão *in*

vitro com os sucos salivares e gastrointestinais diminuiu consideravelmente a disponibilidade da CYN nos mexilhões crus e cozidos a vapor, o que destaca a importância da integração da bioacessibilidade na avaliação de risco para a saúde humana.

Em conclusão, estes resultados permitem uma compreensão mais abrangente dos mecanismos bioquímicos e fisiológicos de resposta das plantas de alface à MC-LR e à CYN (isoladas e em simultâneo), e podem contribuir para um maior entendimento do mecanismo que parece conferir tolerância à CYN. Este estudo também potencia o conhecimento sobre a concentração da MC-LR e CYN disponível em bivalves após o uso de práticas comumente aplicadas na sua conservação e processamento, podendo estes resultados ser utilizados para definição de limites críticos de controlo, considerando a abordagem HACCP como uma ferramenta promissora para a gestão de riscos para saúde humana. Estes resultados também sugerem que a avaliação de riscos com base na concentração de MC-LR e CYN em produtos crus pode não ser representativa da real exposição humana.

Palavras-chave: Cilindrospermopsina, bioacessibilidade, bivalves, *Lactuca sativa*, Microcistina-LR, mistura.

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Chapter 4

Effects of storage, processing and proteolytic digestion on the microcystin-LR concentration in edible clams.

List of Abbreviations

2-DE	Two-dimensional electrophoresis
AAS	Atomic Absorption Spectroscopy
ACN	Acetonitrile
APX	Ascorbate peroxidase
BSA	Bovine serum albumin
BW	Body Weight
CAT	Catalase
CHAPS	3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate
CYP450	Cytochrome P450
CYN	Cylindrosdpermopsin
ESI	Electrospray
EST	Expressed sequence tag
FA	Formic acid
FAS	Fatty acid synthesis
FAAS	Atomic absorption spectrometer
FAO	Food and Agriculture Organization of the United Nations
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-S-transferase
HACCP	Hazard Analysis Critical Control Points
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HSP	Heat shock protein
IARC	International Agency for Research on Cancer
IEF	Isoelectric focusing
ICP–MS	Inductively coupled plasma – mass spectrometry
I.P.	Intraperitoneal
IPG	Immobilized pH gradient
LC-MS/MS	Liquid Chromatography Coupled to Tandem Mass Spectrometry
LD_{50}	Median Lethal Dose
LEA	Embryogenesis abundant protein
LOD	Limit of detection
LOQ	Limit of quantification

MALDI-TOF/TOF-MS	Matrix-assisted laser desorption/ionization time of flight-mass spectrometry
Mdha	N-methyl-dehydroalanine
MeOH	Methanol
MC-LR	Microcystin-LR
MCs	Microcystins
MRM	Multiple reaction monitoring mode
MW	Microwave
$NaHCO_3$	Sodium Bicarbonate
NOAEL	No Observed Adverse Effect Levels
OATPs	Organic Anion Transporting Polypeptides
PAL	Phenylalanine ammonia lyase
PCA	Principal component analysis
PDA	Photoelectric diode array
PP	Protein phosphatases
PPIase	Peptidyl-prolyl cis-trans isomerase
PPO	Polyphenoloxidase
POD	Peroxidase
PR	Pathogenesis-related
PRK	Phosphoribulokinase
PS	Photosystem
ROS	Reactive oxygen species
RuBisCO	Ribulose bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SB	Solubilization buffer
SBPase	Sedoheptulose-1,7-bisphosphatase
SD	Standard deviation
SOD	Superoxide dismutase
SPE	Solid-Phase Extraction
TCA	Tricarboxylic acid
TDI	Tolerable daily intake
TFA	Trifluoroacetic acid
WHO	World Health Organization
WW	Wet weight

Chapter 1

Introduction

1. Introduction

1.1. General introduction

Cyanobacteria, commonly designated as 'blue-green algae', are a group of unicellular and multicellular photosynthetic prokaryotes that occur worldwide in freshwater, brackish and coastal marine ecosystems (Sivonen and Jones, 1999). Blooms of cyanobacteria can be potentiated by a combination of several environmental factors, such as nutrient availability, water temperature, light intensity, salinity and water stagnation (Merel et al., 2013). The frequency and intensity of cyanobacteria blooms, including toxin-producing taxa, have become increasingly in the last decades because of eutrophication of surface waters mainly due to anthropogenic sources (Paerl and Paul, 2012). In addition, warmer temperatures and low river flows associated to global climate change seem to profit the occurrence and distribution of highly toxic cyanobacteria (Elliott, 2012; O'Neil et al., 2012; Paerl and Paul, 2012). Warming can promote cyanobacterial expansion because as prokaryotes, their growth rates are optimized at relatively high temperatures, supporting a competitive advantage under nutrient-enriched conditions in comparison to eukaryotic phytoplankton (Paerl and Paul, 2012). The blooms of cyanobacteria represent an emerging human and environmental concern because of some species produce toxins (cyanotoxins) that can affect a large number of organisms, such as zooplankton, mollusks, crustaceans, fish, birds, mammals and plants.

Microcystins (MCs) are the highest widespread group of cyanotoxins, being the microcystin-LR (MC-LR) the most common variant. Nevertheless, the increasing occurrence of blooms of cylindrospermopsin-producing cyanobacteria inclusive in temperate areas, suggests that cylindrospermopsin (CYN) may be regarded as an emergent human and ecological threat worldwide.

The effects of MC-LR and CYN in agriculture have been a field of increasing interest, since recent studies have suggested phytotoxic effects of these cyanotoxins on terrestrial plants (Corbel et al., 2014). The use of water for irrigation from sources containing toxic cyanobacterial blooms can present harmful effects on growth and development of plants, and potential risks to human health due to the hypothetical accumulation of cyanotoxins in edible parts (Corbel et al., 2014). Furthermore, in aquatic ecosystems, it is common to find several cyanobacteria species; thus, the

existence of mixtures of cyanotoxins is expected and it was already reported for MC-LR and CYN (Brient et al., 2008). The exposure of crop plants to a mixture of MC-LR and CYN may lead to potential additive, synergistic or antagonistic effects. Nevertheless, only few studies have proven adverse effects of single cyanotoxins on plants at environmentally relevant concentrations (Gehringer et al., 2003; Pflugmacher et al., 2007; Pichardo and Pflugmacher et al., 2011), leading to the hypothesis that plants have appropriate protective mechanisms to tolerate cyanotoxins.

On the other hand, it can be questioned if the traditional endpoints used to assess toxic effects (e.g., growth, photosynthetic rate and the activity of antioxidant enzymes and nonenzymatic substances) exhibit enough sensitivity to evaluate understated biochemical alterations. Proteomics is a field of growing interest in the agricultural sector because it has contributed to a better understanding of the specific biological functions of the proteins involved in plant responses to environmental stresses, and may enable the discovery of proteins underlying stress tolerance (Afroz et al., 2011; Kosová et al., 2011; Abreu et al., 2013). Nevertheless, some secreted proteins with defensive or protective functions on stress factors are recognized to also have allergenic potential (Abreu et al., 2013). From the health risk point of view, proteomics data associated with allergen identification may provide promising insights into the protein composition, quality, and safety of edible plants exposed to environmentally relevant concentrations of cyanotoxins. Moreover, tolerant plants can accumulate high levels of cyanotoxins, which can be considered a great risk to public health.

Organisms in direct contact with toxic cyanobacterial blooms, such as aquatic species, are more prone to accumulate cyanotoxins, and several studies have reported the bioaccumulation of MC-LR and CYN in common aquatic vertebrates and invertebrates, including zooplankton, mollusks, crustaceans and fish (Ibelings and Chorus, 2007). Among them, bivalves (clams and mussels), as sessile species and filter-feeding organisms, can be important vehicles of MC-LR and CYN to both animals and humans. The oral route is by far the most representative of human exposure to cyanotoxins. Thus, the pattern and frequency of exposed populations through contaminated-food consumption is required to a more accurate health risk assessment. Human exposure assessment has been based on the total concentration of MC-LR and CYN in raw edible organisms (Ibelings and Chorus,

2007). Nevertheless, the risks associated to the consumption of contaminated food may change if the consumers use storage and processing practices that alter the concentration of cyanotoxins. Furthermore, to exert toxic effects, the cyanotoxins have to be released from food matrix to be absorbed by intestinal epithelium. Thus, the study of the bioaccessibility of cyanotoxins is of major interest for a more accurate human health risk assessment due to the consumption of contaminated food, once bioaccessibility represents the maximum bioavailability of any contaminant (Versantvoort et al., 2005).

1.2. Microcystin-LR

The most widespread and studied cyanotoxins are the cyclic heptapeptide hepatotoxins MCs (MW 900–1200). Among the several structural variants of MCs, MC-leucine and arginine (MC-LR) (Fig. 1) is highlighted due to its toxicity and dominance in cyanobacterial blooms. *Microcystis aeruginosa* is the most common bloom former and the main producer of MC-LR. However, this cyanotoxin can be also produced by *Anabaena, Oscillatoria, Planktothrix, Nostoc* and *Anabaenopsis* (Sivonen and Jones, 1999).

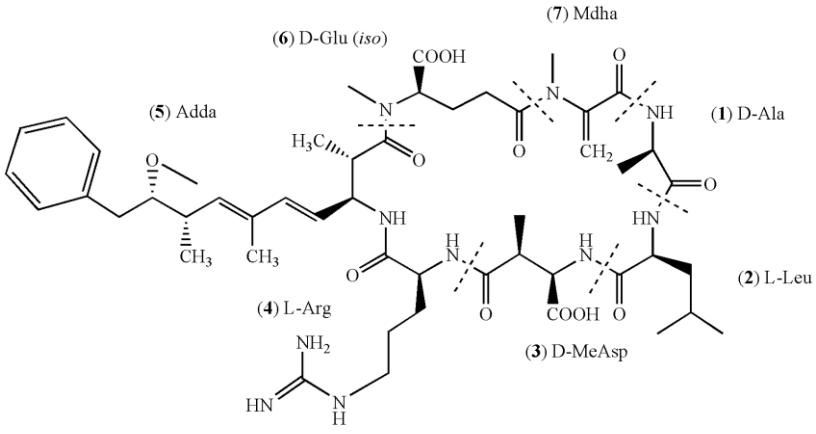

Fig. 1. The chemical structure of the heptapeptide MC-LR, where *D*-Ala is *D*-alanine (**1**), *L*-Leu is *L*-leucine (**2**), *D*-Me-Asp is *D*-erythro-*β*-methylaspartic acid (**3**), *L*-Arg is *L*-arginine (**4**), Adda is the unusual amino acid (2s,3s,8s,9s)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoicacid (**5**), *D*-Glu is *D*-glutamic acid (**6**), and Mdha is N-methyl-dehydroalanine (**7**).

The main mechanism of MC-LR toxicity in both animals and higher plants is the irreversible inhibition of serine/threonine protein phosphatases (PP) (PP1 and PP2A) (MacKintosh et al., 1990). The mechanism involved in PP inhibition by MC-LR consists in a rapid and reversible binding, followed by a slower covalent binding (occurs over several hours) between the N-methyl-dehydroalanine (Mdha) residue of toxin and cysteine-273 of the catalytic subunit of PP1 (cysteine-266 of PP2A) (Craig et al., 1996; MacKintosh et al., 1995).

1.3. Cylindrospermopsin

The tricyclic alkaloid CYN (MW 415) has been recognized of increased concern due to the progressive widespread distribution of its main producer, *Cylindrospermopsis raciborskii* (Ohtani et al., 1992), including into temperate zones (Kinnear, 2010; Poniedziałek et al., 2012) (Fig. 2).

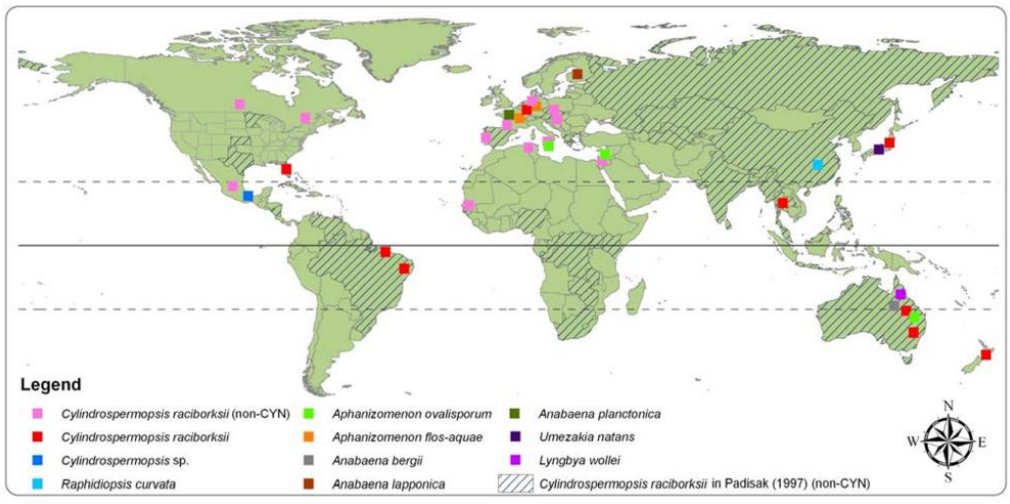

Fig. 2. Global distribution of blooms of cyanobacteria known as CYN-producers. Note: 'non-CYN' denote a fluorescence from which toxin production was not confirmed or studied (Kinnear, 2010).

Furthermore, other cyanobacterial species such as *Umezakia natans* (Terao et al., 1994), *Aphanizomenon ovalisporum* (Banker et al., 1997), *Raphidiopsis curvata* (Li et al., 2001), *Lyngbya wollei* (Seifert et al., 2007), *Anabaena bergii*, *Aphanizomenon flos-aquae* (Preussel et al., 2006), and *Anabaena lapponica* (Spoof et al., 2006) have been reported as CYN producers (Fig. 3). So far, two structural congeners of CYN, 7-epi-CYN and deoxy-CYN (Norris et al. 1999; Banker et al., 2000; Seifert et al., 2007), have also been identified (Fig. 3).

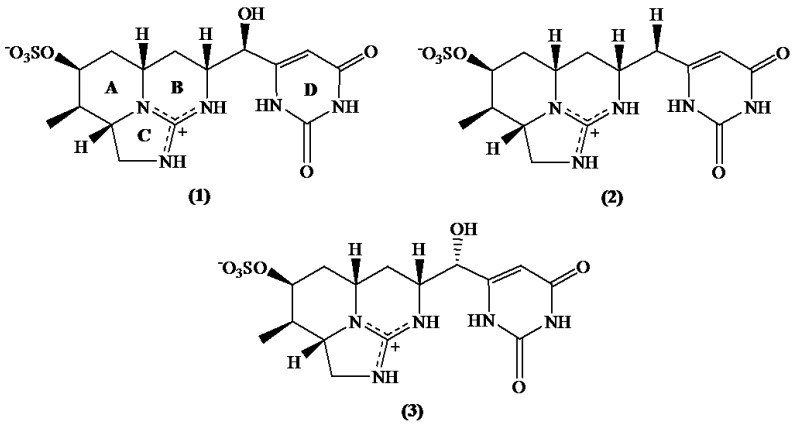

Fig. 3. The molecular structure of cylindrospermopsin **(1)** and its analogs 7-deoxy-cylindrospermopsin **(2)** and 7-epicylindrospermopsin **(3)**.

Although the molecular mechanism of CYN toxicity has not yet been established, it is known that it inhibits eukaryotic protein synthesis with similar intensity in plant and mammalian cell extracts (Terao et al., 1994; Runnegar et al., 2002; Froscio et al., 2008). Moreover, CYN was reported to inhibits the glutathione (GSH) synthesis (Runnegar et al., 1995) and the metabolic activation of CYN by cytochrome P450 (CYP450) seems to contribute to its high toxicity (Runnegar et al., 1995; Froscio et al., 2003).

1.4. Effects of MC-LR and CYN on plants

The majority of MC-LR- and CYN-related research have been focused on mammalian toxicity; however, plants can also be affected by these cyanotoxins through several molecular pathways (Babica et al., 2006; Corbel et al., 2014). Cyanotoxins can be released from toxic cyanobacterial cells into water due to their natural metabolism (e.g., CYN) (Chiswell et al., 1999; Rücker et al., 2007) or following a cellular lyse during cell senescence or through water treatment processes such as algaecide application (e.g., MC-LR) (Babica et al., 2006). The concentration of MCs in surface waters vary from 4-50 µg/L up to 6500 µg/L, however, the higher concentrations would be found in blooms and scums and correspond to intracellular plus dissolved cyanotoxin (Corbel et al., 2014). Although the studies reporting the concentrations of CYN in the environment are scarce, the concentration of total extracellular CYN in water seem to vary from undetectable values up to 126 µg/L (Corbel et al., 2014). The phytotoxic effects of MC-LR and CYN on higher plants were firstly focused on aquatic macrophytes and floating plants that are naturally exposed to cyanotoxins (Pflugmacher et al., 1999; Pflugmacher, 2002; Pietsch et al., 2001; Mitrovic et al., 2005; Saqrane et al., 2007; Kinnear et al., 2008). MCs are very stable and may persist in aquatic systems for weeks after being released from the cells (Sivonen and Jones, 1999). Also, CYN can persist in the water because its photodegradation is very low under natural conditions (Wörmer et al., 2010). Therefore, the use of contaminated surface waters for agricultural irrigation may also allows that these cyanotoxins enter into terrestrial ecosystems, leading to potential risks for crop production and quality. MC-LR, by acting as PP1 and PP2A inhibitors and inducers of ROS production, could be involved in several physiological and molecular processes in higher terrestrial plants (Corbel et al., 2014). Indeed, numerous studies have reported that MC-LR produces several perturbatory effects on plant physiology and metabolism.

As is shown in Table 1, the growth, photosynthesis, antioxidant system and mineral content of several aquatic and terrestrial plant species can be affected due to MC-LR exposure.

Table 1. General effects of the MC-LR on different species of aquatic and terrestrial plants.

Physiological response	Plant species	Endpoint	Effect	Concentration of exposure (µg/L)	Crude extract	Purified toxin	Reference
Growth	*Oryza sativa*	Fresh weight	○	0.26; 13; 78	X	-	Azevedo et al., 2014
	Brassica napus; Oryza sativa	Fresh and dry weight; height	↓	24; **120**; **600**; **3,000**	X	-	Chen et al., 2004
	Medicago sativa	Fresh weight; stem length	↓	2,220; **11,120**; **22,240**	X	-	El Khalloufi et al., 2011
	Lycopersicon esculentum	Stem length	↓	**2,220**; **11,120**; **22,240**	X	-	El Khalloufi et al., 2012
	Lepidium sativum	Fresh weight; length	↓	1; **10**	X	X	Gehringer et al., 2003
	Brassica oleracea; Sinapis alba	Stem length	○	1; 10	-	X	Järvenpää et al., 2007
	Vicia faba	Length, dry weight, number of nodes and leaves	↓	**50**; **100**	X	-	Lahrouni et al., 2013
	Solanum tuberosum	Fresh weight; shoot length	↓	1; 5; **10**; **50**; **100**; **500**; **1,000**; **2,500**; **5,000**	-	X	McElhiney et al., 2001
	Lemma minor	Fresh and dry weight; frond number	↓	1,500; 3,000; **10,000**; **20,000**	-	X	Mitrovic et al., 2005
	Wolffia arrhiza	Frond number	↓	1,500; 3,000; 6,000; 10,000; **15,000**	-	X	Mitrovic et al., 2005
	Ceratophyllum demesum	Fresh weight	↓	**0.1**; **0.5**; **1**; **5**	-	X	Pflugmacher, 2002
	Oryza sativa	Fresh weight	○	50	X	-	Prieto et al., 2011
	Oryza sativa	Fresh weight of root	↓	500; 1,000; **2,000**; **4,000**	-	X	Chen et al., 2013
	Lemna gibba	Fronds number	↓	75; **150**; **220**; **300**	X	-	Saqrane et al., 2007
	Triticum durum; Zea mays; Pisum sativum; Lens esculenta	Fresh and dry weight	↓	**500**; **1,050**; **2,100**; **4,200**	X	-	Saqrane et al., 2009
Photosynthesis	*Oryza sativa*	Fv/Fm fluorescence	○	0.26; 13; 78	X		Azevedo et al., 2014
	Medicago sativa	Fv/Fm fluorescence	↓	2,220; **11,120**; **22,240**	X	-	El Khalloufi et al., 2011
	Lycopersicon esculentum	Fv/Fm fluorescence	↓	2,220; 11,120; **22,240**	X	-	El Khalloufi et al., 2012
	Brassica oleracea; Sinapis alba	Fv/Fm fluorescence; Chlorophyll (a+b)	○	1; 10	-	X	Järvenpää et al., 2007
	Vicia faba	Fv/Fm fluorescence; Chlorophyll (a+b)	↓	50; **100**	X	-	Lahrouni et al., 2013

	Solanum tuberosum	Chlorophyll (a+b)	↓	1; 5; **10**; **50**; **100**; **500**; **1,000**; **2,500**; **5,000**	-	X	McElhiney, et al., 2001
	Ceratophyllum demesum	Fv/Fm fluorescence	↓	0.1; **0.5**; **1**; **5**	-	X	Pflugmacher, 2002
	Ceratophyllum demesum	Oxygen production	○	0.25	-	X	Pietsch et al., 2001
	Ceratophyllum demesum	Fv/Fm fluorescence	↓	0.25	X	-	Pietsch et al., 2001
	Lemna gibba	Chlorophyll (a+b)	↓	**75**; **150**; **220**; **300**	X	-	Saqrane et al., 2007
	Triticum durum; Pisum sativum	Fv/Fm fluorescence/ Chlorophyll (a+b)	↓/○	500; 1,050; 2,100; 4,200	X	-	Saqrane et al., 2009
	Zea mays; Lens esculenta	Fv/Fm fluorescence/ Chlorophyll (a+b)	↓/↓	500; 1,050; **2,100**; **4,200**	X	-	Saqrane et al., 2009
Oxidative stress	*Oryza sativa*	GST; GPx	○	0.26; 13; 78	X		Azevedo et al., 2014
	Oryza sativa	SOD; POD	↑/ ○	**24**; **120**; 600; 3,000	X	-	Chen et al., 2004
	Brassica napus	SOD/ POD	↓/ ↑	24; 120; **600**; **3,000**	X	-	Chen et al., 2004
	Medicago sativa	POD	↑	2,220; **11,120**; **22,240**	X	-	El Khalloufi et al., 2011
	Lycopersicon esculentum	POD	↑	2,220; **11,120**; **22,240**	X	-	El Khalloufi et al., 2012
	Lepidium sativum	GST; GPx	↑	1; 10	X	X	Gehringer et al., 2003
	Vicia faba	POD; CAT	↑	50 ; **100**	X	-	Lahrouni et al., 2013
	Lemma minor	POD	↑	1,500; 3,000; **10,000**; **20,000**	-	X	Mitrovic et al., 2005
	Ceratophyllum demesum	sGST	↑	0.5	-	X	Pflugmacher et al., 1999
	Phragmites australis	GST	↑	0.5	-	X	Pflugmacher et al., 2001
	Spinacia oleracea	GST; GSH; GR; SOD; POD; CAT	↑	0.5	X	-	Pflugmacher et al., 2007
	Ceratophyllum demesum	sGST/ POD	○/↑	0.25	-	X	Pietsch et al., 2001
	Ceratophyllum demesum	sGST/ POD	↓/ ↑	0.25	X	-	Pietsch et al., 2001
	Oryza sativa	GST/ GPx	↑/ ○	50	X	-	Prieto et al., 2011
	Lemna gibba	POD	○	75; 150; 220; 300	X	-	Saqrane et al., 2007
	Lepidium sativum	GST, GPx; GR	↑	0.5	X	X	Stüven and Pflugmacher, 2007
Mineral content	*Lycopersicon esculentum*	Na; K and Ca	↑	**2,220**; **11,120**; **22,240**	X		El Khalloufi et al., 2012
	Vicia faba	Na/ K; Ca and	↑/ ↓	50; **100**	X	-	Lahrouni et

		N					al., 2013
	Triticum durum; Zea mays; Pisum sativum; Lens esculenta	Ca; Na; K; P; N	↑	500; 1,050; 2,100; 4,200	X	-	Saqrane et al., 2009
Non-enzymatic and enzymatic antioxidant content	*Medicago sativa*	Phenols content	↑	2,220; **11,120**; **22,240**	X	-	El Khalloufi et al., 2011
	Lycopersicon esculentum	Phenols content	↑	2,220; **11,120**; **22,240**	X	-	El Khalloufi et al., 2012
	Vicia faba	PPO; PAL; Phenols content	↑	50; **100**	X	-	Lahrouni et al., 2013
	Medicago sativa	α and β Tocopherols	↑	0.05; 0.5; 5	X	X	Peuthert and Pflugmacher, 2010
	Spinacia oleracea	Ascorbate; α Tocopherol	↑	0.5	X	-	Pflugmacher et al., 2007
	Lemna gibba	Phenols content	↑	**75**; **150**; **220**; **300**	X	-	Saqrane et al., 2007
	Lepidium sativum	α and β Tocopherols	↑	0.5	X	X	Stüven and Pflugmacher, 2007

↑ Increased in comparison to control group; ↓ Decreased in comparison to control group; ○ There were no effects in comparison to control group; - Not measured; Concentration values highlighted in bold indicate more pronounced effects.

Although the reported biochemical and physiological effects may vary depending on the (1) use of purified toxins or crude extracts, (2) plant species, (3) stage of plant development, (4) time of exposure, and (5) range of concentrations studied, overall, the growth and photosynthesis are inhibited and the antioxidant system, either enzymatic and non-enzymatic, is induced. Nevertheless, an aspect that is important to point out is the ecological relevance of the most of these studies, since the concentrations of MCs that actually produce effects appear to be non-environmentally realistic, being 10 to 1000-fold higher than those usually found in the ecosystems. Furthermore, most of these studies were carried out with plants in early stage of development and it could be hypothesized that mature plants could have more complex defense strategies to tolerate the stress promoted by cyanotoxins, at least if they are exposed to ecologically relevant concentrations.

The toxic effects of CYN on plants are far less studied. However, on the contrary of MC-LR, CYN may often be found at higher levels in dissolved form than within cyanobacterial cells (Chiswell et al., 1999; Rücker et al., 2007). Thus, the transdermal absorption of CYN may be a relevant route of plant exposure. Indeed,

implications of this cyanotoxin were already observed in vegetable cells and the few studies that were performed indicate that, at low concentrations, the exposure to CYN results in an induction of plant growth, inhibition of photosynthesis and increase in oxidative stress (Table 2).

Table 2. General effects of the CYN on different species of aquatic and terrestrial plants.

Physiological response	Plant species	Endpoint	Effect	Concentration of exposure (µg/L)	Crude extract	Purified toxin	Reference
Growth	*Hydrilla verticillata*	Biomass of roots	↑	25; 50; 100; 200; **400**	X	-	Kinnear et al., 2008
	Brassica juncea/ Brassica oleracea	Fresh weight of leaves	○/ ○	18.2; 35.5	X	X	Kittler et al., 2012
	Oryza sativa	Roots/ leaves fresh and dry weight	↑/ ○	2.5	X	-	Prieto et al., 2011
	Sinapis alba	Dry weight	↓	2,500; 5,000; 10,000; **20,000**; **40,000**; **80,000**; **160,000**	-	X	Vasas et al., 2002
Photosynthesis	*Hydrilla verticillata*	Chlorophyll (a+b)	↓/ ○	25; 50; 100; 200; 400	X	-	Kinnear et al., 2008
Oxidative stress	*Oryza sativa*	GST; GPx	↑	2.5	X	-	Prieto et al., 2011

↑ Increased in comparison to control group; ↓ Decreased in comparison to control group; ○ There were no effects in comparison to control group; - Not measured; Concentration values highlighted in bold indicate more pronounced effects.

In aquatic ecosystems, the existence of mixtures of cyanotoxins is highly expected, however, the toxicological experiments are predominantly carried out on individual cyanotoxins. The impact of cyanotoxin mixtures, especially the more prevalent, is a matter of high priority. Simultaneous exposure to MC-LR and CYN may lead to changes in the response capability of crop plants, triggering potential synergistic, additive or antagonistic effects. However, the studies regarding to effects of interaction of the MC-LR/CYN mixture are very scarce. According to our knowledge, only Prieto et al. (2011) have studied the interaction effects of MC-LR and CYN in plants. The authors suggested a synergistic effect on the oxidative stress response (GST activity) of rice plants when exposed to cyanobacterial extracts containing ecological relevant concentrations of both CYN (0.13 µg/L) and MC-LR (50 µg/L) (Table 3).

Table 3. General effects of the MC-LR/CYN on different species of aquatic and terrestrial plants.

Physiological response	Plant species	Endpoint	Effect	Concentration of MC-LR and CYN exposure (µg/L)	Crude extract	Purified toxin	Reference
Growth	*Oryza sativa*	Roots/ leaves fresh and dry weight	○	50 and 0.13, respectively	X	-	Prieto et al., 2011
Oxidative stress	*Oryza sativa*	GST/ GPx	↑/ ○	50 and 0.13, respectively	X	-	Prieto et al., 2011

↑ Increased in comparison to control group; ↓ Decreased in comparison to control group; ○ There were no effects in comparison to control group; - Not measured.

Since there is a great lack of studies assessing the effects of MC-LR/CYN mixtures in aquatic and terrestrial species, it is of particular relevance to cover this topic, especially because of its increasing tendency of occurrence (Brient et al., 2008).

On the other hand, the ability of MC-LR and CYN to accumulate in edible tissues of terrestrial plants has been described (Gutiérrez-Praena et al., 2013), leading to potential health hazards for animals and humans (Corbel et al., 2014). Although the knowledge concerning the uptake of cyanotoxins by agricultural crops is particularly scarce, it could be hypothesized that the tolerant plants are more susceptible to accumulate them.

Overall, the exposure of plants to ecologically relevant concentrations of these cyanotoxins, even in mixture, seems to produce no significant effects, leading to the hypothesis that plants have developed appropriate protective or compensatory homeostatic mechanisms to tolerate cyanotoxins. However, it can be questioned if the traditional endpoints used to assess toxicity (e.g., growth, photosynthetic rate, antioxidative enzymes and nonenzymatic substances) exhibit enough sensitivity to evaluate understated biochemical alterations. Recently, Azevedo et al. (2014) reported the lack of sensitivity of the conventional parameters for the analysis of the toxicity of *M. aeruginosa* extract on rice (*Oryza sativa*) plants (MC-LR concentrations of 0.26–78 µg/L); however, significant alterations were observed through proteomic analyses. Proteomics is a promising tool in the agricultural sector because it can contributes to a better understanding of the specific functions of the proteins involved in plant responses to environmental stresses (Afroz et al., 2011; Kosová et al., 2011; Abreu et al., 2013).

1.4.1. Proteomics applied to agricultural sector to assess the effects of cyanotoxins

Proteomics is an emergent research tool that offers several advantages over the standard enzymatic and biochemical assays. By the investigation of protein dynamics and variations in plant metabolism in response to an environmental stimulus, proteomic technologies may enable the potential identification of protein biomarkers of stress response and the discovery of proteins involved in stress tolerance (Afroz et al., 2011; Gutiérrez-Praena et al., 2014; Kosová et al., 2011). Recent advances in accomplishment of genome sequencing of crop plants (e.g., rice) and the development/improvement of analytical methods for protein characterization makes the proteomics analysis appropriate for the agricultural sector. Proteomic studies are extensively applied to genetically modified plants, in which new proteins are incorporated into food crops, for instance to promote resistance to pests, pesticides and other stressors. Proteomic studies investigating the effects of CYN and MC-LR have been performed on bivalves, including mixtures with other environmental pollutants (e.g., herbicides) (Martins et al., 2009; Puerto et al., 2011; Malécot et al., 2013). However, so far only two studies were developed using a proteomic approach to investigate the effects of cyanotoxins (MC-LR) in crop plants. Concisely, Azevedo et al. (2014) studied the biochemical responses of rice (*Oryza sativa*) seedlings exposed to low concentrations of MC-LR; and Gutiérrez-Praena et al. (2014) studied the effects of MC-LR in the leaf proteome of tomato (*Lycopersicon esculentum*). In these two studies, the combination of two-dimensional electrophoresis (2DE) and matrix-assisted laser desorption/ionization time of flight-tandem mass spectrometry (MALDI-TOF/TOF MS), allowed the identification of the function of several proteins that complement the current understanding of the mode of action of MC-LR in those plants. Thus, these techniques could be applied for other commercially important species, such as lettuce (*Lactuca sativa*), a leafy vegetable worldwide used for human consumption.

From the health risk point of view, proteomics may provide also new insights on safety and quality of edible plants exposed to environmentally relevant concentrations of cyanotoxins due to the potential identification of allergenic proteins secreted as a defensive or protective mechanism, such as pathogenesis-related (PR) proteins (Abreu et al., 2013).

1.5. Human exposure to MC-LR and CYN

The human health risks associated to the exposure to cyanotoxins are of growing concern due to the progressive occurrence and expansion of harmful cyanobacterial blooms. Humans are potentially exposed to cyanotoxins by three major routes: (1) ingestion of edible organisms that accumulate cyanotoxins, and cyanobacteria-based dietary supplements; (2) inhalation/ingestion and dermal contact by recreational activities and (3) ingestion of contaminated drinking water (Fig. 4) (Merel et al., 2013).

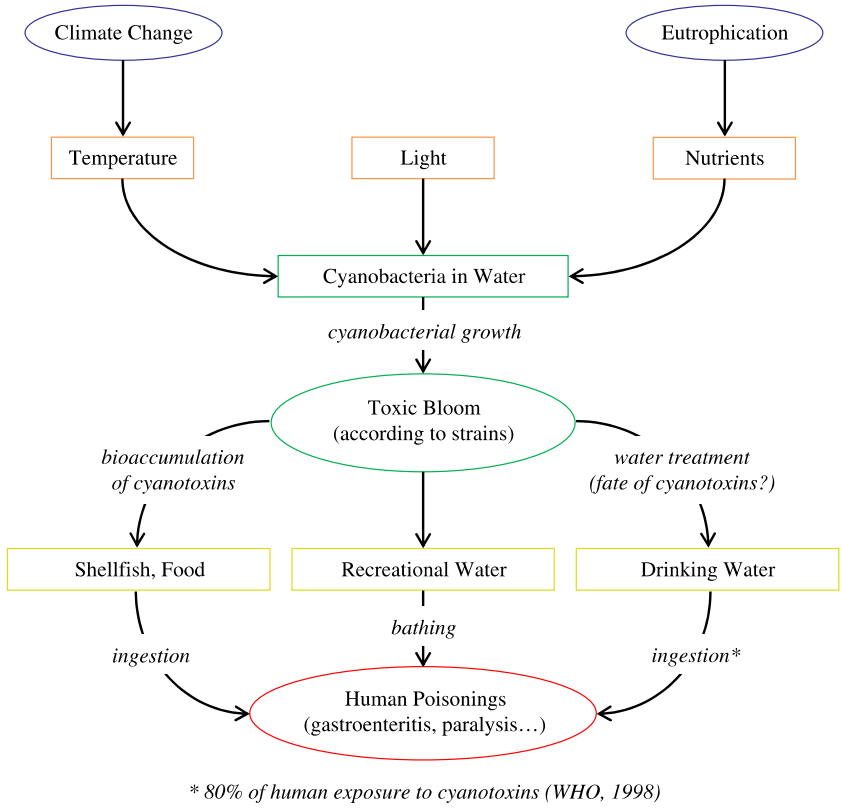

Fig.4. The main factors that influence the expansion of toxic cyanobacterial blooms and the routes of human exposure to cyanotoxins (Merel et al., 2013).

Although drinking water is considered to be the major route of chronic human exposure to cyanotoxins, the consumption of edible organisms that directly graze toxic cyanobacteria (e.g., clams, oysters and mussels, among others) should be similarly considered. Several studies have reported the occurrence of MC-LR and

CYN in the tissues of edible aquatic organisms (e.g., fish, bivalves) (Gutiérrez-Praena et al., 2013; Kinnear, 2010; Ibelings and Chorus, 2007), reaching levels at which human consumption should be avoided (Chen and Xie, 2005; Ibelings and Chorus, 2007). Bivalves, as filter-feeding organisms, have shown to accumulate high concentrations of MCs and CYN in both laboratory and field conditions (Gutiérrez-Praena et al., 2013; Kinnear, 2010), making them potential vehicles of these cyanotoxins to higher trophic levels (Saker et al., 2004; Vasconcelos et al., 2007). Considering the human health effects, cyanotoxins can be classified according to their target organs, as follows: (1) neurotoxins (nervous system), (2) hepatotoxins (liver), (3) cytotoxins (several organs: liver, kidneys, adrenal glands, small intestine), and (4) dermatotoxins (causing skin irritation). In mammals, the acute exposure to the hepatotoxin MC-LR produces a cascade of events (cytoskeleton alterations, lipid peroxidation, oxidative stress, apoptosis) leading to cell necrosis, intrahepatic hemorrhage and death (Funari and Testai, 2008). Although human acute intoxication is rare, unfortunately, it already occurred in 1996 at the Brazilian dialysis center of Caruaru, which caused the death of 60 patients due to the use of contaminated water for hemodialysis (Pouria et al., 1998). Nevertheless, human health problems due to MC-LR are most likely associated with chronic exposure, where phosphatases inhibition induces cellular proliferation and hepatic hypertrophy (Funari and Testai, 2008). Epidemiological studies in China support the association of chronic exposure to MCs from contaminated drinking water with primary liver and colorectal cancer (Ueno et al., 1996; Zhou et al., 2002). On the basis of data on tumor promoting mechanisms, the International Agency for Research on Cancer (IARC) classified MC-LR as ‘‘possibly carcinogenic to humans’’ (group 2B) (Grosse et al., 2006).

Regarding to the cytotoxin CYN, the most famous case of human intoxication occurred in 1979 in Australia, where 149 people supplied with drinking water from a reservoir with CYN-producing *C. raciborskii* suffered a hepatoenteritis-like illness (Griffiths & Saker, 2003). Although a lack of epidemiological studies regarding to chronic exposure to CYN, it has been suggested that tumors are generated in mice by oral exposure to the toxin (Falconer & Humpage, 2001; Falconer and Humpage, 2006).

Based on the potential for human health risks, the World Health Organization (WHO) proposed a provisional upper limit in drinking water of 1 µg/L for MC-LR and a tolerable daily intake (TDI) of 0.04 µg/kg of body weight (WHO, 1998). Likewise, a guideline for maximum concentration of 1 µg/L was proposed in drinking water for

CYN as well as a provisional TDI of 0.03 µg/kg body weight (Humpage and Falconer, 2003) (Table 4).

Table 4. The TDI of MC-LR and CYN derived from the respective no observed adverse effect level (NOAEL). The LD_{50} of MC-LR and CYN by intraperitoneal and oral routes. Adapted from Funari and Testai (2008).

Cyanotoxin	TDI (µg/Kg/d)	NOAEL (µg/Kg/d)	i.p. LD_{50} (µg/Kg bw)	Oral LD_{50} (µg/Kg bw)
MC-LR	0.04*	40 (Mice; 13 weeks; gavage)	50-1,200	5,000
CYN	0.03**	30 (Mice; 11 weeks; gavage)	2,100 (24h); 200 (6 days)	4,400-6,900 (2-6 days)

* WHO, 1998.

** Humpage and Falconer, 2003.

The median lethal dose (LD_{50}) of MC-LR by the oral route is considerably lower (30- to 100-fold) than by intraperitoneal (i.p.) injection. The lower acute toxicity in mice by the oral route is likely due to toxicokinetic differences: MC-LR require uptake via the bile acid transport system (Organic Anion Transporting Polypeptides (OATPs: human) (Fischer et al., 2005) for the absorption through the gastrointestinal (GI) mucosa and this is bypassed by i.p. injection, in which MC-LR is directly available for internalization into hepatocytes (Funari and Testai, 2008). Although the information of CYN is very limited in comparison to those focused on MC-LR, similarly, the intestinal absorption of CYN is potentially dependent on a transporter (Chong et al., 2002), and also the acute toxicity via the oral route is lower than i.p. route. Therefore, determining the right scenario of exposure to MC-LR and CYN is of crucial importance for a representative human health risk assessment due to the consumption of cyanotoxin-contaminated food.

1.5.1. Factors affecting human exposure to MC-LR and CYN by the consumption of contaminated edible aquatic organisms

The severity degree of MC-LR and CYN induced toxicity depends on the levels and duration of exposure, determined by the balance between the absorption, detoxification and excretion**.** In addition, another factor of uncertainty in assessing human exposure derives from the fact that it is not clear whether the levels of MC-LR and CYN measured in raw edible aquatic organisms correspond to the total bioavailable amount. Some external factors such as food storage, preparation, processing and the human digestion itself can change the MC-LR and CYN availability and thus the risk of human exposure.

1.5.1.1. Food storage and processing

Food is generally consumed after being stored and processed. The processing of food products generally implies the transformation of the perishable raw commodity to value added product that has larger shelf life (Kaushik et al., 2009). The common food storage (e.g., refrigeration, freezing) and processing practices (e.g., boiling, frying, microwave cooking, steaming, canning) at domestic and industrial level could lead to significant variations of MC-LR and CYN availability in edible organisms. Domingo (2011) has studied the influence of cooking processes on the concentrations of toxic metals and various organic environmental pollutants in food and found that the main changes in contaminant levels depend basically on the food item and the cooking conditions, as follows: (1) cooking method, (2) time, (3) temperature, and (4) medium of cooking. Kaushik et al. (2009) reviewed the effects of food processing in pesticide dissipation and found that in most cases processing leads to large reductions in pesticide residue levels. MC-LR and CYN are water-soluble and stable at high temperatures (Chiswell et al., 1999; Sivonen and Jones, 1999), thus studies are necessary to know whether storage and cooking practices can be suitable of reducing the content of these cyanotoxins in food. Very few studies have been developed in the investigation of the effects of food storage and processing practices in the availability of cyanotoxins in edible organisms (Guzmán-Guillén et al., 2011; Morais et al., 2008; Zhang et al., 2010), and these studies concern only to MC-LR (Table 5).

Table 5. Effects of food storage and processing practices on the MC-LR availability in tissues of edible aquatic organisms.

Conditions	Without refrigeration		Refrigeration			Freezing			Edible aquatic organism	Reference
	6 h	48 h	24 h	48 h	72 h	48 h	1 week	1 month	Mussels (*Mytilus galloprovincialis*)	Morais et al., 2008
Storage	↓	↓	↓	↓	↓	↓	↓	↓		
	Boiling		**Microwaving**			**Toxin in cooking water**			**Edible aquatic organism**	**Reference**
	5 min	30 min	1 min	5 min					Mussels *(Mytilus galloprovincialis)*	Morais et al., 2008
	○	○	↓	↓			-			
	2 min	2 min Continuously	1 min	5 min					Tilapia (*Oreochromis niloticus*)	Guzmán-Guillén et al., 2011
Cooking	↓	↓	○	↓			X			
	5 min								Bighead carp (*Aristichthys nobilis*)	Zhang et al., 2010
	↑		-				X			

↑ Increased in comparison to control group; ↓ Decreased in comparison to control group; ○ There were no effects in comparison to control group; X The toxin was found in cooking water; - Not measured.

Overall, storage and microwaving methods seem to reduce the availability of MC-LR in edible aquatic organisms, while the results regarding to boiling treatment are controversial. The detection of MCs in the boiling water suggests also the potential of this process for removal of cyanotoxins from edible tissues.

Regarding to CYN, as reviewed by Gutiérrez-Praena et al. (2013) and according to our knowledge, so far, no data have been published on the effects of storage and processing in its availability in edible organisms.

1.5.1.2. Bioaccessibility

Food, once ingested, is subjected to the physical and chemical conditions of the mouth, stomach and small intestine, which may change the bioavailability of several contaminants. The oral bioavailability of a compound is a result of three processes:

(1) release of the compound from food matrix into digestive juices (i.e., bioaccessibility), (2) transport across the intestinal epithelium into the *vena Portae* (intestinal transport) and (3) degradation of the compound in the liver (and intestine) (metabolism) (Versantvoort et al., 2005). In more detail, bioaccessibility is defined as the fraction of the contaminant that is released from the food matrix by the action of digestive enzymes and is then available for absorption by the intestinal mucosa (Versantvoort et al., 2005). The bioaccessibility indicates the maximal oral bioavalability of any contaminant (Versantvoort et al., 2005) (Fig. 5).

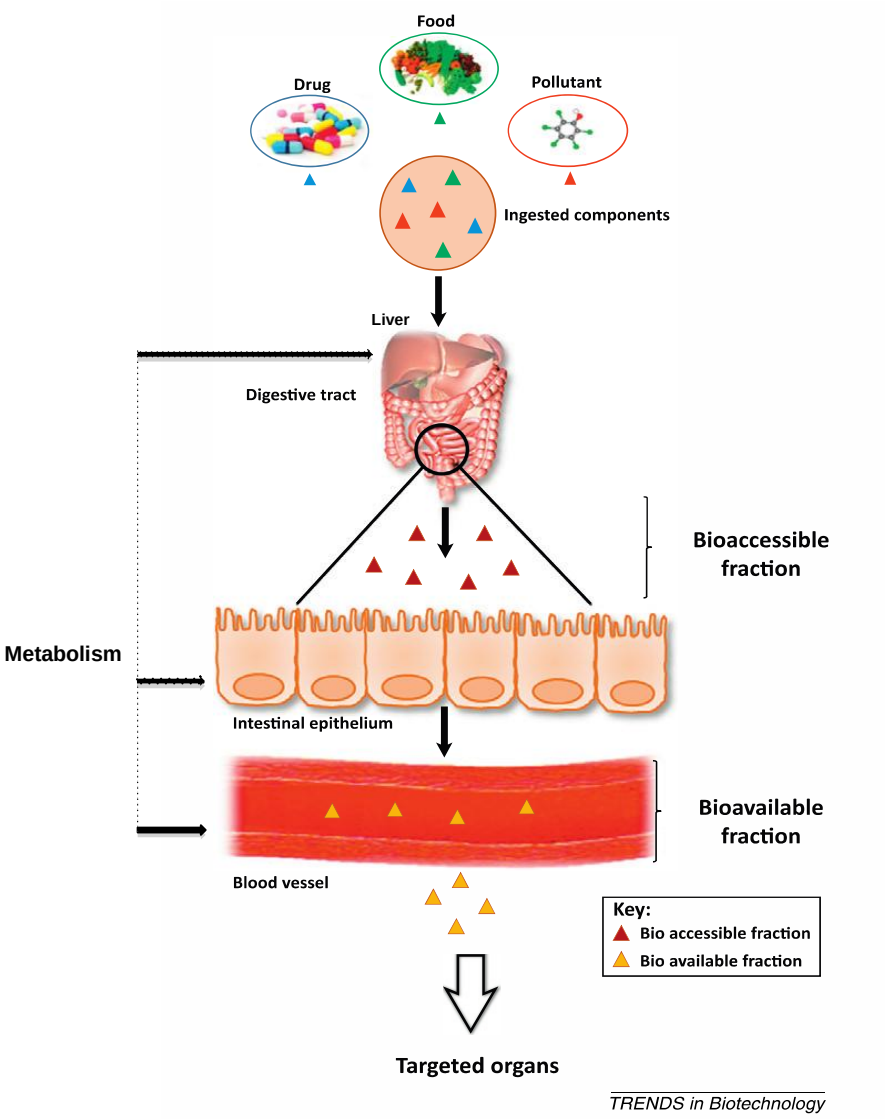

Fig. 5. Schematic representation of the difference between bioaccessibility and bioavailability of food contaminants (Adapted from Guerra et al., 2012).

There are several *in vitro* methods that simulate the human digestion process, most of them comprising three phases: mouth, stomach and small intestine. Thus, these models enable the investigation of the bioaccessibility of a compound during the transit in the gastrointestinal tract (Versantvoort et al., 2005; Maulvault et al., 2011; Guerra et al., 2012). The bioaccessibility of a contaminant can be influenced by several factors, as follows: (1) the type of food matrix, (2) cooking preparation, (3) digestion model, (4) initial concentration of the contaminat, and (5) the chemical properties of the contaminant (Hur et al., 2009; Maulvault et al., 2011; Xing et al., 2008). The determination of the bioaccessibility of MC-LR and CYN is of great relevance, once it will provide data for a more accurate human health risk assessment due to consumption of contaminated food. The current health risk assessment generally considers the concentration of these cyanotoxins in the raw edible organisms.

There were only few reports on the digestibility of MCs with gastric and intestinal juices, but these studies were carried out with toxin in its free form in solution (without food matrix) (Table 6).

Table 6. Digestion of MC-LR (in solution) by proteolytic enzymes of gastric and intestinal juices.

Pepsin	Trypsin	Chymotrypsin	Reference
+	-	-	Moreno et al., 2004
-	-	-	Smith et al., 2010
NA	+	+	Kankaanpää et al., 2005

+ Digested; - non-digested; NA, not analyzed.

Although the contradictory results of these studies, probably due to the differences in the experimental designs and/or analysts and analysis equipment, they give an indication of the effects occurring in MCs (heptapeptides) when they are alternatively exposed to gastric or intestinal digestive conditions. However, it is important to notice that if the toxin is ingested within food, this may be not straightforward because of the matrix effects. According to our knowledge there are no studies on the bioaccessibility of MC-LR and CYN. The study of bioaccessibility of these cyanotoxins in edible bivalves can provide relevant information for risk assessment, supporting potential improvements in the management measures.

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Chapter 2

Structure of the thesis and objectives

2.1. Structure of the thesis

This thesis comprises six chapters consisting in a general introduction (Chapter 1), which describes the state-of-the-art of the field of this work, followed by the description of the objectives (Chapter 2).

The Chapter 3 comprises two manuscripts already submitted to international peer-reviewed journals (ISI) that explore the effects of interaction of MC-LR and CYN on lettuce, an important edible vegetable. The first manuscript describe the effects these cyanotoxins on the traditional endpoints, such as growth, activity of the antioxidative enzymes and mineral content. The second manuscript describes the effects of CYN and MC-LR/CYN mixture at the proteome level, in an attempt to identify early stress responses and affected pathways, which are not perceptible by traditional endpoints, as well as to identify effects which may have implications on lettuce nutritional quality and safety, such as the presence of allergenic proteins.

The Chapter 4 comprises one scientific paper and one manuscript already submitted to an international peer-reviewed journal (ISI) that explore the availability and bioaccessibility of MC-LR and CYN in bivalves, which are an important human food resource and also susceptible to accumulate high levels of these cyanotoxins. In this chapter the effects of common practices of food storage and processing, as well as the human digestion were considered to assess the availability and the bioaccessibility of MC-LR and CYN in edible bivalves.

In Chapter 5 is presented the overall discussion of the results, the conclusions and future perspectives for research.

Finally, the Chapter 6 includes supporting information of the second manuscript presented in this thesis: Lettuce (*Lactuca sativa* L.) leaf-proteome profiles after exposure to cylindrospermopsin and to microcystin-LR/cylindrospermopsin mixture: a concentration-dependent response.

These four experimental works were published or submitted for publication in international peer-reviewed journals (ISI), as is indicated in the respective chapters. The structure of the manuscripts and the scientific paper was maintained according to the journal guidelines in which they were published or submitted, including the reference style.

Overall, the scope of this thesis was to study the effects of these two emergent cyanotoxins in an important crop plant, *Lactuca sativa*; and to explore the effects of common practices of food storage and processing, as well as human digestion on the concentration of MC-LR and CYN available in two edible bivalves, in order to improve human health and environmental risk assessment associated to the cyanobacterial toxins as a threat to food safety and security. The structure of this thesis follows a logical progression of the topics addressed that are briefly shown in Fig. 1.

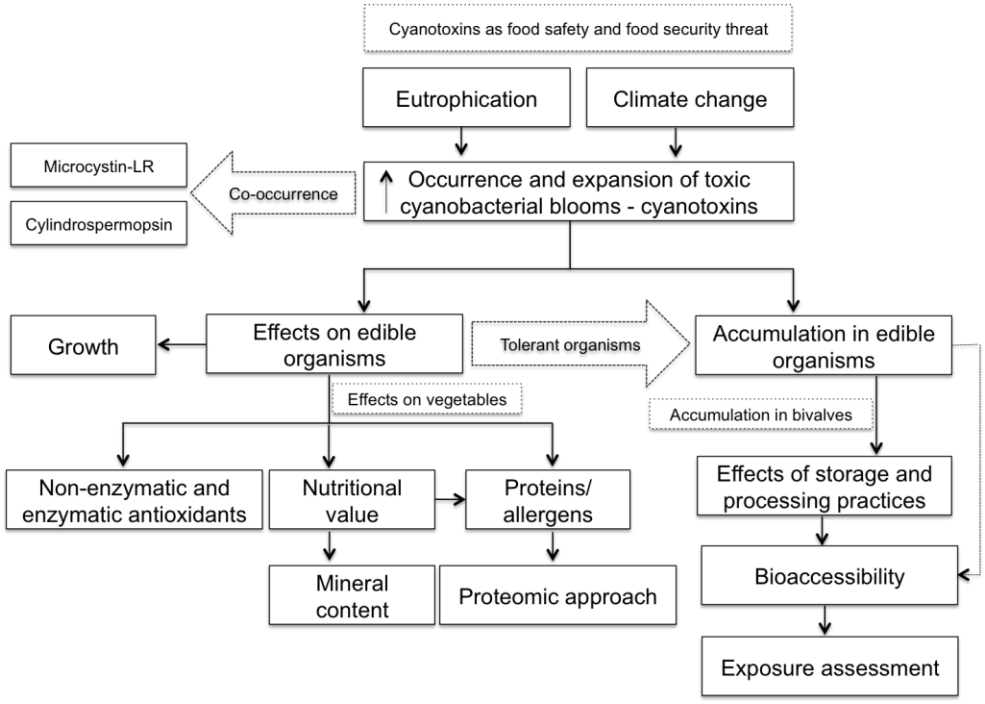

Fig. 1. Scheme of the logical progression of the topics included in this thesis.

2.2. Objectives

This thesis aimed to enhance the knowledge on the effects of MC-LR, CYN and the mixture of the two cyanotoxins in lettuce plants; and the effects of the common food storage and processing practices as well as human digestion on the availability and bioaccessibility, respectively, of MC-LR and CYN in edible bivalves. Specific aims were as follows:

1 - To assess the effects of environmentally relevant concentrations (1, 10 and 100 µg/L) of MC-LR, CYN and a mixture of MC-LR and CYN on growth (fresh weight), antioxidant defense system (GST and GPx activities), and mineral content of lettuce plants (*Lactuca sativa* L.).

2 - To use a 2-DE proteomic approach and MALDI-TOF/TOF MS to investigate the leaf-proteome profiles of lettuce (*Lactuca sativa* L.) plants exposed to environmentally relevant concentrations (1, 10, and 100 µg/l) of a CYN and MC-LR/CYN mixture.

3 - To assess the changes on MC-LR concentration in edible clams after applying common food storage and processing practices as well as to determine MC-LR bioaccessibility after proteolytic digestion.

4 - To assess the changes on CYN concentration in edible mussels with storage and processing as well as to assess the bioaccessibility of CYN in raw and processed (steamed) mussels.

Chapter 3

Effects of interaction of microcystin-LR and cylindrospermopsin in lettuce

This chapter has been adapted from the following manuscripts:

Marisa Freitas, Joana Azevedo, Edgar Pinto, Joana Neves, Alexandre Campos, Vitor Vasconcelos. Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/ cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (*Lactuca sativa* L.). Submitted to the Journal Ecotoxicology and Environmental Safety.

Marisa Freitas, Alexandre Campos, Joana Azevedo, Aldo Barreiro, Sébastien Planchon, Jenny Renaut, Vitor Vasconcelos. Lettuce (*Lactuca sativa* L.) leaf-proteome profiles after exposure to cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture: a concentration-dependent response. Accepted for publication in the Journal Phytochemistry.

Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (*Lactuca sativa* L.)

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Abstract

Toxic cyanobacterial blooms are documented worldwide as an emerging environmental concern. Recent studies support the hypothesis that microcystin-LR (MC-LR) and cylindrospermopsin (CYN) produce toxic effects in crop plants. Lettuce (*Lactuca sativa* L.) is an important commercial leafy vegetable that supplies essential elements for human nutrition; thus, the study of its sensitivity to MC-LR, CYN and a MC-LR/CYN mixture is of major relevance. This study aimed to assess the effects of environmentally relevant concentrations (1, 10 and 100 µg/L) of MC-LR, CYN and a MC-LR/CYN mixture on growth, antioxidant defense system and mineral content in lettuce plants.

In almost all treatments, an increase in root fresh weight was obtained; however, the fresh weight of leaves was significantly decreased in plants exposed to 100 µg/L concentrations of each toxin and the toxin mixture. Overall, GST activity was significantly increased in roots, contrary to GPx activity, which decreased in roots and leaves. The mineral content in lettuce leaves changed due to its exposure to cyanotoxins; in general, the mineral content decreased with MC-LR and increased with CYN, and apparently these effects are time and concentration-dependent. The effects of the MC-LR/CYN mixture were almost always similar to the single cyanotoxins, although MC-LR seems to be more toxic than CYN. Our results suggest that lettuce plants in non-early stages of development are able to cope with lower concentrations of MC-LR, CYN and the MC-LR/CYN mixture; however, higher concentrations (100 µg/L) can affect both lettuce yield and nutritional quality.

Keywords: microcystin-LR, cylindrospermopsin, GST, GPx, minerals, *Lactuca sativa*

Abbreviations: *AAS, atomic absorption spectroscopy; APX, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; CYN, cylindrospermopsin; GSH, glutathione; GPx, glutathione peroxidase; GST, glutathione-S-transferase; ICP–MS, inductively coupled plasma – mass spectrometry; MeOH, methanol; MC-LR, microcystin-LR; MCs, microcystins; PDA, photoelectric diode array; PP, protein phosphatases; PPO, polyphenoloxidase; POD, peroxidase; ROS, reactive oxygen species; SPE, solid phase extraction; SOD, superoxide dismutase; TFA, trifluoroacetic acid.*

1. Introduction

Toxic cyanobacterial blooms have become increasingly widespread in aquatic ecosystems, potentially as a consequence of eutrophication and climate change (Elliott, 2012; O'Neil et al., 2012). Among cyanobacteria, *Microcystis* is recognized as the most common bloom forming genus, and microcystin-LR (MC-LR), primarily produced by *Microcystis aeruginosa*, is the predominant variant of microcystins (MCs). Nevertheless, the tricyclic alkaloid cylindrospermopsin (CYN) has been recognized to

be of increased concern due to the invasive nature of its main producer, *Cylindrospermopsis raciborskii* (Kinnear, 2010; Poniedziałek et al., 2012). The use of irrigation water containing toxic cyanobacterial blooms can be hazardous to the agricultural sector because several studies have reported that cyanotoxins negatively impact the yield, quality and safety of crop plants. The primary mechanism of the toxicity of MC-LR in both animals and higher plants is well recognized and consists of the irreversible inhibition of serine/threonine protein phosphatases 1 and 2A (PP; PP1 and PP2A) by covalent binding (Mackintosh et al., 1990). Potentially associated with this mechanism, several studies have shown that MCs, including MC-LR, inhibit germination, decrease plant growth and crop yield and alter chlorophyll content and photosynthesis (Chen et al., 2004; El Khalloufi et al., 2011; Gehringer et al., 2003; McElhiney et al., 2001; Mitrovic et al., 2005; Pflugmacher, 2002; Pflugmacher et al., 2006; Pflugmacher et al., 2007; Pietsch et al., 2001; Saqrane et al., 2009). The induction of oxidative stress by the production of reactive oxygen species (ROS) seems to be another important biochemical mechanism of MC-LR toxicity in plants. Several studies have been performed on the oxidative stress generated in plants due to MC exposure, and changes in the antioxidant mechanisms (enzymatic and non-enzymatic components) have been reported (Gehringer et al., 2003; El Khalloufi et al., 2011; 2012; 2013; Lahrouni et al., 2013; Pereira et al., 2009; Pflugmacher et al., 1999; Pflugmacher et al., 2001; Pflugmacher, 2004; Pflugmacher et al., 2006; Pflugmacher et al., 2007; Saqrane et al., 2009; Stüven and Pflugmacher, 2007). Among the antioxidant enzymes, glutathione-S-transferase (GST) has been successfully employed to assess the oxidative stress promoted by MC-LR in plants. This strategy was developed because the described pathway of MC-LR detoxification is by its conjugation with tripeptide glutathione (GSH), catalyzed via GST (Pflugmacher et al., 1998; 2001). Nevertheless, Gehringer et al. (2003) and Stüven and Pflugmacher (2007) have obtained a significant increase in glutathione peroxidase (GPx) activity in seedlings of *Lepidium sativum* exposed to MC-LR either purified or from extracts, suggesting that GPx may play an important role to mitigate the negative effects of ROS generated by MC-LR in plants. However, if the antioxidant mechanisms are not efficient to scavenge the enhanced amount of ROS promoted by cyanotoxins, extensive cellular damage can occur, which may lead to potential negative effects on plant nutrient uptake and translocation. Minerals are essential to plant growth and development; they are intrinsic components in their structure and normal metabolism and function (Taiz and Zeiger, 2002). Interestingly, Saqrane et al. (2009) have reported that the exposure of *Triticum durum*, *Zea mays*, *Pisum sativum* and *Lens esculenta* plants to MC-containing extracts resulted in changes in the mineral content in roots in a concentration-dependent

manner. More recently, El Khalloufi et al. (2012) and Lahrouni et al. (2013) have also demonstrated that cyanobacterial bloom extracts containing MCs induced changes in mineral assimilation and content in tomato (*Lycopersicon esculentum*) and faba bean (*Vicia faba*).

Although the effects of CYN in plants have been studied to a much lesser extent than MC-LR, this toxin is expected to become increasingly recurrent and thus enhancing the knowledge of its impact on crop plants is of critical importance. So far, the molecular mechanism of toxicity of CYN has not yet been established; however, CYN is known to inhibit protein synthesis with similar intensities in plant and mammalian cell extracts (Froscio et al., 2008). The few studies that have arisen regarding the effects of CYN on plants indicate that CYN results in the induction of oxidative stress (Prieto et al., 2011), the reduction of pollen germination (Metcalf et al., 2004) and the inhibition of growth (Beyer et al., 2009; Vasas et al., 2002).

In the majority of the studies performed on the effects of cyanotoxins in plants, the concentrations of the cyanotoxins used did not take into account their ecological relevance. Furthermore, in the aquatic environment, the simultaneous occurrence of different cyanotoxins can be highly expectable; inclusively, the co-occurrence of MC-LR and CYN has already been reported (Brient et al., 2008). In laboratory studies, synergistic effects have been suggested on the oxidative stress response (GST activity) of rice plants (*Oryza sativa*) exposed to cyanobacterial extracts containing CYN (0.13 µg/L) and MC-LR (50 µg/L) (Prieto et al., 2011). Thus, a study of the effects of the mixture of these two prevalent cyanotoxins (MC-LR and CYN) at environmentally relevant concentrations is of major significance to predict the potential impact of their interaction in crop plants.

Lettuce (*Lactuca sativa* L.) is a leafy vegetable widely used for human consumption due to its extensive production, convenience and nutritional value. Among other nutrients, lettuce provides an important source of minerals for the human diet (Pinto et al., 2014). The inhibition of lettuce root growth has been demonstrated after exposure to extracts of *Microcystis aeruginosa* containing MCs in a concentration ranging from 5.9 to 56.4 µg/L (Pereira et al., 2009). However, this study was performed with plants in early stages of development, and mature plants could be hypothesized to have more complex defense strategies to cope with stress promoted by cyanotoxins.

The aim of this study was to assess the effects of environmentally relevant concentrations (1, 10 and 100 µg/L) of MC-LR, CYN and a mixture of MC-LR and CYN on growth, antioxidant defense systems and mineral content in lettuce plants (*Lactuca*

sativa L.) in non-early stages of development.

2. Materials and Methods

2.1. Cyanobacterial culture and toxin purification and quantification

2.1.1. Culture of *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*

M. aeruginosa (LEGE 91094) and *C. raciborskii* (LEGE 97047) were grown to exponential phase in Z8 medium (Kotai, 1972) (6-L flasks) under fluorescent light with a light/dark cycle of 14/10 h and a temperature of 25 ± 1°C. The cultured cells were gathered by centrifugation (20 min, 4°C, 4495 *g*), frozen at -80°C and then freeze-dried. As CYN is highly hydrophilic, the culture medium of *C. raciborskii* was also freeze-dried. The lyophilized material was stored at room temperature in the dark until toxin extraction and purification. In this study, purified toxins were chosen for the experiments to find the specific effects of the MC-LR/CYN mixture, avoiding interferences of other potentially toxic metabolites (e.g., lipopolysaccharides) deriving from cyanobacterial crude extracts.

2.1.2. MC-LR extraction, purification and quantification by HPLC-PDA

MC-LR was extracted from *M. aeruginosa* cells according to Ramanan et al. (2000), with some modifications. Briefly, the lyophilized *M. aeruginosa* biomass was extracted with 75% (v/v) methanol (MeOH) (Fisher Scientific, UK) by continuous stirring for 20 min at room temperature. The sample was then ultrasonicated five times on ice at 60 Hz for 1 min (Vibra-Cell 50-sonics & Material Inc. Danbury, CT, USA). The homogenate was centrifuged at 10,000 *g* for 15 min, and the resulting supernatant was collected and stored at 4°C. The pellet was re-extracted with an equal volume of solvent, and the pooled supernatants were subjected to solid phase extraction (SPE) with a Water Sep-Pak® Vac 6-ml C18 cartridge preconditioned with 100% MeOH and distilled water at a flow rate of 1 mL/min. The loaded column was washed with 20% MeOH, and the MC-LR was then eluted using 80% MeOH. The MC-LR fraction was evaporated by rotary evaporation at 35°C to remove the entire MeOH portion. The concentrated MC-LR was

thereafter purified and quantified by a Waters Alliance e2695 HPLC system coupled with a photoelectric diode array (PDA) 2998. The MC-LR semi-preparative assay was performed using a reversed-phase column (Phenomenex Luna RP-18 (250 mm × 10 mm, 10 µm) maintained at 35°C. The gradient elution was performed with MeOH and water, both acidified with 0.1% trifluoroacetic acid (TFA), with a flow rate of 2.5 mL/min. The injection volume was 500 µL. The peak purity and percentage of purified MC-LR were calculated at 214 and 238 nm. The fraction with purified MC-LR was then evaporated with nitrogen air for one day until all of the solvent was removed. Then, the residue was resuspended in distilled water. The chromatographic purity of MC-LR was 97%. The purified fractions of MC-LR were then quantified in the same HPLC system on a Merck Lichrospher RP-18 endcapped column (250 mm x 4.6 mm, 5 µm) equipped with a guard column (4 × 4 mm, 5 µm), both maintained at 45°C. The PDA range was 210-400 nm with a fixed wavelength of 238 nm. The linear gradient elution consisted of (A) MeOH + 0.1% TFA and (B) H_2O + 0.1% TFA (55% A at 0 min, 65% A at 5 min, 80% A at 10 min, 100% A at 15 min, and 55% A at 15.1 and 20 min) with a flow rate of 0.9 mL/min. The injected volume was 20 µL. The MC-LR was identified by a comparison of its spectra and retention time with that of the MC-LR standard (batch number 018K1209, 10.025 µg/mL in MeOH, 98% purity, Cyano Biotech GmbH, Berlin, Germany). The system was calibrated using a set of seven dilutions of the MC-LR standard (0.5 to 20 µg/mL) in 50% MeOH. Each vial was injected in duplicate, and every HPLC runs series of 10 samples included a blank and two different standard concentrations. The Empower 2 Chromatography Data Software was used for calculating and reporting the peak information. The retention time of the MC-LR peak was 10.44 min (data from method validation not published).

2.1.3. CYN extraction, purification and quantification by HPLC-PDA

CYN was extracted from *C. raciborskii* cells and culture medium following a modified version of the method described by Welker et al. (2002). Briefly, the freeze-dried material (0.7 g) was first sonicated in a bath for 15 min in 5 mL of 0.1% (v/v) TFA (spectrophotometric grade) and then subjected to five cycles of ultrasonication with a Vibra-Cell at 60 Hz for 1 min. The homogenate was stirred for 1 h at room temperature and centrifuged (20,000 *g*, 4°C, 20 min). The supernatant was collected and the pellet was subjected to a second extraction. The supernatants were then pooled and stored at -20°C. CYN was thereafter purified in the same HPLC-PDA system on a semi-

preparative Gemini C18 column (250 mm x 10 mm, 5 µm) from Phenomenex that was maintained at 40°C. The isocratic elution utilized a 5% MeOH solution containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 2.5 mL/min and an injection volume of 1000 µL. Working solutions of CYN (0.08-5.0 µg/mL) were prepared in water. Standard CYN was supplied by Alexis (San Diego, CA, USA). The purified CYN fractions were then quantified in an HPLC system on an Atlantis® HILIC phase column (250 mm x 4.6 mm, 5 µm) from Waters maintained at 40°C. The PDA range was 210-400 nm with a fixed wavelength of 262 nm. The isocratic elution was also a 5% MeOH solution containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 0.9 mL/min and an injection volume of 10 µL. The system was calibrated using a set of seven dilutions of the CYN standard (25, 20, 10, 5, 2, 1 and 0.5 µg/mL) in ultrapure water. Each vial was injected in duplicate, and every HPLC run consisting of a series of 10 samples included a blank and two different standard concentrations. The chromatographic purity of CYN was 98%. The Empower 2 Chromatography Data Software was used for the calculation and reporting the peak information. The retention time of the CYN peak was 7.35 min (data from method validation not published).

2.2. Plant material and exposure to MC-LR, CYN and MC-LR/CYN mixture

The lettuce plants (*Lactuca sativa L.* var. 'Susybel') were purchased in a commercial soil substrate at four to five weeks' maturity. The roots were carefully washed with tap water until complete soil removal, and 20 lettuce plants were then transferred to the holes of plastic boards (PVC), which were placed on black glass trays (35 x 25 x 5 cm deep). The roots were completely immersed in 3 L culture medium (adapted from Jensen and Malter (1995), (table 1)), pH 6.5, which was continuously aerated.

Table 1. Composition (constituents and concentration) of the culture medium used to expose lettuce plants to MC-LR, CYN and a MC-LR/CYN mixture in a closed hydroponic system. Adapted from Jensen and Malter (1995).

Chemical compound	Principal element	Concentration
Macronutrients		**(g/L)**
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Mg	0.5
Monopotassium phosphate (KH_2PO_4)	K, P	0.27
Potassium nitrate (KNO_3)	K, N	0.2
Potassium sulfate (K_2SO_4)	K	0.1
Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$)	N, Ca	0.5
Iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Fe	0.025
Ethylenediaminetetraacetic acid (EDTA)		0.03
Micronutrients		**(mg/L)**
Boric acid (H_3BO_3)	B	4.17
Manganous chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Mn	3.75
Cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	Cu	0.21
Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	Mo	0.14
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Zn	0.66

The lettuce plants were acclimated for one week with fluorescent white light (light/dark cycle of 14/10 h) and a temperature of 21 ± 1°C. After acclimation, the culture medium was renewed and the purified toxins were diluted to the ecologically relevant concentrations of 1, 10, and 100 µg/L, in which the lettuce plants were exposed for five days. After this time, 10 lettuce plants were harvested and the remnant ones were exposed again to the same conditions for five days further. Three independent trials were performed (exposure to MC-LR, CYN and a MC-LR/CYN mixture) in triplicate in a total of 12 trays for each assay (control group and the three concentrations of exposure). To monitor the stability of the cyanotoxins, samples of 500 µL were taken every day, during five days, and the analysis confirmed that MC-LR and CYN were stable throughout the experiments (data not shown).

2.3. Determination of plant growth

At the end of five days of exposure, 10 lettuce plants were randomly harvested and the roots and leaf tissues were separated. The fresh weight (fw) was determined, and tissues were then stored at -80°C for further analysis. This procedure was repeated for the ten-remainder lettuce plants exposed for 10 days. Plant growth was expressed as the mean fresh weight (fw) ± standard deviation (SD) of n =10.

2.4. Enzyme activity measurement

The measurements of soluble (cytosolic) GST and GPx activities were performed in the roots and leaves of two lettuce plants. Tissues were ground in liquid nitrogen to a powder with a pestle and mortar and then homogenized in phosphate buffer, (100 mM) pH 6.5, in a ratio of 1.5 g/1 mL (tissue/buffer). The homogenates were centrifuged at 4495 *g* for 20 min at 4°C, and the supernatants were recovered. Aliquots were stored at –80°C until further analysis. Protein content was determined according to the method of Bradford (1976), in which bovine serum albumin (BSA) was used as a standard. The sGST activity was determined according to the method of Habig et al. (1974), whereas GPx activity was determined according to the method of Lawrence and Burk (1976). GST and GPx activities were performed with 0.1 mg protein/mL, and the enzymatic activities were calculated according to Azevedo et al. (2014) and expressed in nkat/mg protein.

2.5. Determination of mineral content in lettuce leaves

Leaf tissues of three lettuce plants from the MC-LR, CYN and MC-LR/CYN experiments were analyzed regarding to their content of calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), sodium (Na), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu) and molybdenum (Mo). The digestion and analysis of lettuce samples were performed according to Pinto et al. (2014). Freeze-dried leaves of lettuce plants were digested in an MLS 1200 Mega high performance microwave digestion (Milestone, Sorisole, Italy) unit. Samples were weighed into the PTFE vessels and 5 mL of HNO_3 (65% w/w, *Trace*SELECT® Ultra) and 2 mL of 30% H_2O_2 (30% v/v, *Trace*SELECT® Fluka) were added to each vessel. Afterwards, the mixture was submitted to the following microwave heating programs (power/time): 250 W/1 min, 0 W/2 min, 250 W/5 min, 400 W/5 min and 600 W/5 min. Sample solutions were then analyzed by a 3100 flame atomic absorption spectroscopy (AAS) instrument (Perkin Elmer, Überlingen, Germany) and by inductively coupled plasma – mass spectrometry (ICP-MS) (VG Elemental PlasmaQuad 3, Winsford, UK) for total metal content. For AAS analysis, multi-element calibration standards were prepared from 1000 mg/L single-element standard solutions (Sigma, MO) of Ca, Mg, K and Na. For ICP-MS analysis, calibration standards were prepared from AccuStandard® (New Haven, CT) 10 µg/mL multi-element ICP-MS standard solution (ICP-MS-200.8-CAL1-1). All solutions were prepared using ultrapure water (>18.2 MΩ cm at 25°C) obtained with a

Milli-Q (Millipore, Billerica, MA) water purification system.

The ICP–MS instrument was equipped with a glass concentric nebulizer (Meinhard® Type A), a water-cooled glass spray chamber with impact beads, a standard quartz torch and nickel skimmer and sampling cones. For sample introduction, a Minipuls 3 (Gilson, Villiers le Bel, France) peristaltic pump was used. Argon of 99.9% purity (Alphagaz 2™, supplied by Air Liquide, Maia, Portugal) was used as the plasma source. The ICP-MS analysis was performed under the following conditions: argon flow rate (13 L/min); auxiliary argon flow rate (0.7 L/min); nebulizer flow rate (0.8 L/min); RF power (1350 W); scan regions dwell time (200 ms); and detection mode (pulse counting). The elemental isotopes (m/z ratios) ^{55}Mn, ^{56}Fe, ^{65}Cu, ^{66}Zn and ^{95}Mo were monitored for analytical determination and ^{45}Sc, ^{89}Y and ^{115}In were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and stability using ^{115}In as the target isotope. P was determined according to Murphy and Riley (1962). Results were expressed on a dry weight (dw) basis.

2.6. Statistical analysis

The statistical analysis of lettuce growth and enzymatic activity was performed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The homogeneity of variance was checked with the Levene test, and when it was not observed, data were transformed to achieve this assumption. Statistical analysis of mineral content was conducted using the Mann-Whitney U test (*IBM® SPSS® Statistics version 21.0 for Mac OS X*). The significance level was set at $P < 0.05$.

3. Results and Discussion

3.1. Effects of MC-LR, CYN and MC-LR/CYN mixture on lettuce growth

Overall, at morphological levels, the treatments applied in this study did not produce perceptible deleterious effects in lettuce plants (e.g., chlorosis or necrosis). The effects of MC-LR, CYN and MC-LR/CYN mixture on lettuce growth were studied by comparing the mean fresh weight of the control and treated plants (Fig. 1).

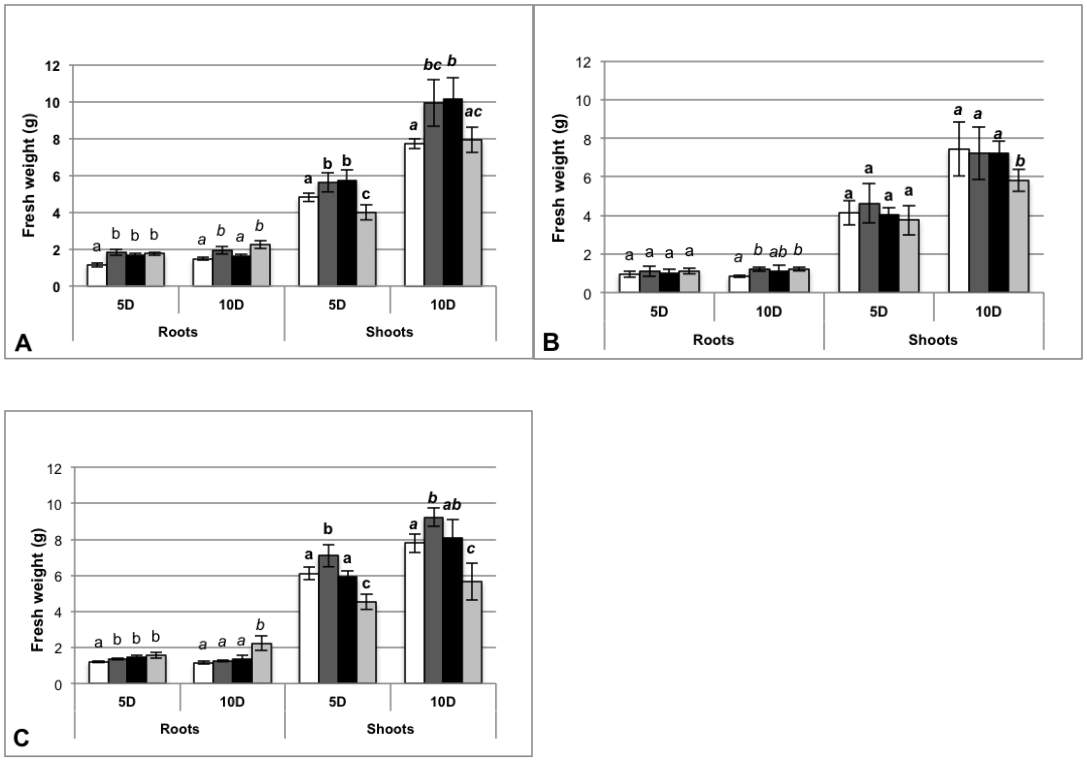


Fig. 1. The fresh weight of lettuce plants (roots and leaves) after being exposed 5 and 10 days (5D and 10D, respectively) to MC-LR (A), CYN (B) and MC-LR/CYN (C). Control: white bars; 1 µg/L: dark gray bars; 10 µg/L: black bars; 100 µg/L: light gray bars. Values are expressed as the mean ± SD (n=10). Different letters (a, b and c) indicate significant differences ($p < 0.05$).

The exposure of lettuce plants to 1, 10 and 100 µg/L of MC-LR led to a significant increase in root fresh weight ($P < 0.05$). Interestingly, the fresh weight of lettuce leaves was also significantly increased ($P < 0.05$), after exposure to low concentrations (1 and 10 µg/L) of MC-LR. In contrast, the highest concentration of MC-LR (100 µg/L) produced a significant decrease ($P < 0.05$) in fresh weight of lettuce leaves after five

days of exposure; however, this effect attenuated after 10 days of exposure. Unlike our results, lettuce root growth has been inhibited by extracts of *M. aeruginosa* containing MCs in concentrations ranging from 5.9 to 56.4 µg/L, following five days of exposure (Pereira et al., 2009). Furthermore, several studies have reported the growth inhibition of roots and leaves of plants as result of MC-LR exposure (Chen et al., 2004; El Khalloufi et al., 2011; Gehringer et al., 2003; McElhiney et al., 2001; Mitrovic et al., 2005; Pflugmacher, 2002; Pflugmacher et al., 2006; Pflugmacher et al., 2007; Saqrane et al., 2009). However, as evidenced by these studies, various factors can influence the toxicological effects. These include plant species, stage of plant development, the use of purified toxins or crude extracts, time of exposure and the range of concentrations studied. In most of these studies, the toxic effects of MC-LR were produced in plants in early stages of development and/or by high concentrations, approximately 100- to 1,000-fold higher than those used in this study. The contradictory results obtained by Pereira et al. (2009) could be explained by the differences in the stage of development of lettuce plants, the parameter used to assess the growth (root length) and the use of crude extracts instead of purified toxin. Interestingly, in the latter study, the strain of *M. aeruginosa* that produced the most pronounced root growth inhibition contained the lowest concentration of MCs. This suggests that at ecologically relevant concentrations, other components of the extracts of *M. aeruginosa* may induce inhibitory effects on lettuce root growth, but these findings may not be a result of MC-LR by itself.

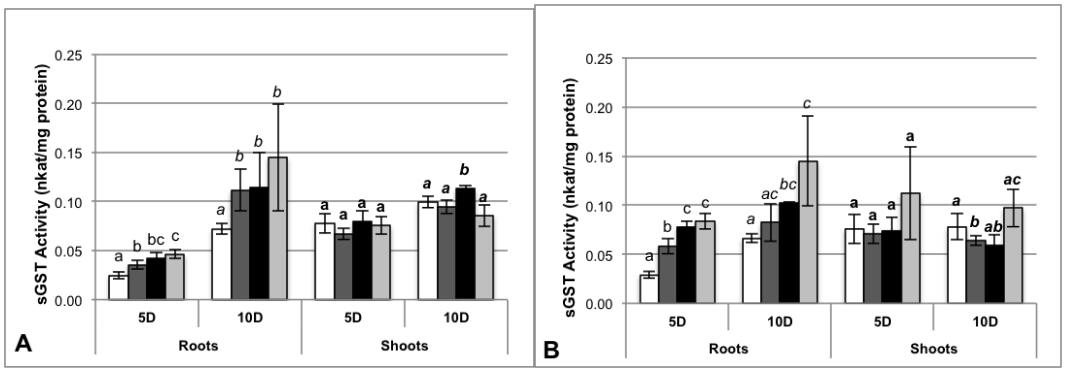
The effects of time of exposure were more evident in the CYN trial. The fresh weight of lettuce plants was only affected after 10 days of exposure, whereas the growth of roots was stimulated in all concentrations of exposure, and the leaf fresh weight decreased at 100 µg/L ($P < 0.05$) of CYN. Similar results in leaf fresh weight of rice plants exposed nine days to extracts of *A. ovalisporum* containing 2.5 µg/L of CYN have been reported (Prieto et al., 2011). *Hydrilla verticillata* has been reported to exhibit in increase in root growth following 14 days of exposure to extracts of *C. raciborskii* containing 400 µg/L of CYN (Kinnear et al., 2008). According to Kinnear et al. (2008), several benefits could result from the increased root production, such as the potential production of exudates, allelochemicals or phytochelatins, which might scavenge CYN, preventing its uptake by plant cells. In all of the experiments in our study, we observed a gradual abundance of exudates in the culture medium that were proportional to the toxin concentration (data not shown). Although whether these compounds are effective to protect lettuce plants to cyanotoxins is not known, their potential contribution should

be studied further. However, the increased metabolic activity in roots required for growth, production of exudates and potential detoxification could be hypothesized to compromise the leaf water content and the growth of the lettuce plants exposed to 100 µg/L concentrations.

The exposure of lettuce plants to a MC-LR/CYN mixture promoted an increase in root growth in all concentrations after five days of exposure and at the highest concentration (100 µg/L) after 10 days of exposure. The fresh weight of leaves significantly increased with 1 µg/L and decreased with 100 µg/L of MC-LR/CYN after both five and 10 days of exposure ($P < 0.05$). Apparently, the MC-LR/CYN mixture produces similar effects of the single toxins, not enabling the assumption of additive, synergistic or antagonistic effects on the lettuce fresh weight. Prieto et al. (2011) have studied the effects of *A. ovalisporum* and *M. aeruginosa* cell extracts containing CYN and MCs, respectively, on rice plants and neither extracts nor its mixture produce significant changes in fresh and dry weight of roots and leaves after 48 h of exposure. In general, lettuce plants seem to be susceptible of homeostatic compensation with low concentrations of MC-LR, CYN and MC-LR/CYN (e.g., 1 µg/L and 10 µg/L); however, high concentrations (e.g., 100 µg/L) may cause adverse effects on leaf yield.

3.2. Effects of MC-LR, CYN and MC-LR/CYN mixture on oxidative stress response

The phase II detoxification enzymes GST and GPx have been widely used as indicators of oxidative stress promoted by cyanotoxins in several target plant species. In this study, the effects of MC-LR, CYN and a MC-LR/CYN mixture on the GST and GPx activities in roots and leaves of lettuce plants were also assessed (Fig. 2).



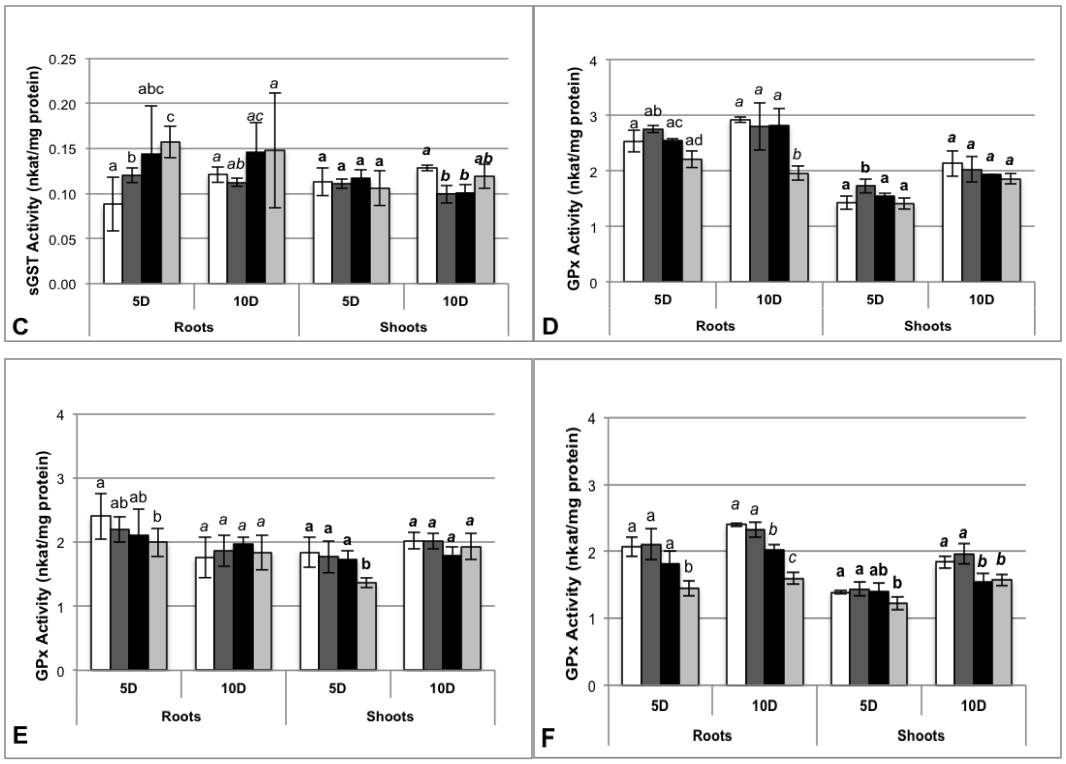


Fig. 2. GST (A, B and C) and GPx (D, E and F) activities in lettuce plants (roots and leaves) after being exposed 5 and 10 days to MC-LR, CYN and MC-LR/CYN, respectively. Control: white bars; 1 µg/L: dark gray bars; 10 µg/L: black bars; 100 µg/L: light gray bars. Values are expressed as the mean ± SD (n=3). Different letters (a, b, c and d) means significant differences ($p < 0.05$).

The GST activity was significantly increased ($P < 0.05$) in the roots of plants exposed to MC-LR and CYN, and this increase seems to be time- and concentration- dependent. Likewise, the MC-LR/CYN mixture promoted a significant increase ($P < 0.05$) of GST activity in roots after five days of exposure, and the high activity obtained (≈ 0.15 nkat/mg) when compared to MC-LR or CYN alone led us to suppose that synergistic effects may have occurred. After 10 days of exposure to the MC-LR/CYN mixture, the GST levels in roots remained high, although no significant differences in comparison to control groups were found. The GST activity in leaves was similar to the corresponding control groups and in some cases was found to be even lower. These results suggest that roots were more affected than leaves by the exposure to cyanotoxins, most likely due to the direct contact with the cyanotoxins and higher uptake/accumulation. However, this differentiated response could be related to a replacement in leaves by other antioxidant components of the defense system (enzymatic and/or non-enzymatic). Chloroplasts, where photosynthesis takes place, are the major cellular compartments of ROS production because of their photoactive nature (Gill and Tuteja, 2010). Lettuce leaves are rich in antioxidant compounds (e.g., carotenoids and

phenolics) that play an important role to protect the photosynthetic apparatus against oxidative stress. Inclusively, the non-enzymatic antioxidants, such as phenolic compounds and α- and β-tocopherol, have been reported to be enhanced in plants after their exposure to MCs (El Khalloufi et al., 2012; Lahrouni et al., 2013; Stüven and Pflugmacher, 2007). This raises attention to the impact of cyanotoxins in the nutritional value of lettuce leaves because the content of antioxidants can be enhanced as a physiological mechanism of response.

According to our results, GST activity in the roots of lettuce plants showed that it was a good marker of stress induced by MC-LR, CYN and its mixture. Similarly, Prieto et al. (2011) have reported that the exposure of rice plants to a mixture of *A. ovalisporum* and *M. aeruginosa* cell extracts containing CYN and MC-LR, respectively, resulted in a significant increase in GST activity in roots, also suggesting that a synergistic effect of both extracts may exist; however, no changes in GPx activity have been observed in rice plants after 48 h of exposure.

In this study, the GPx activity was significantly decreased (P <0.05) in the roots of lettuce plants exposed for 10 days to 100 µg/L of MC-LR, whereas it was significantly increased (P <0.05) in leaves of plants exposed to 1 µg/L for five days. GPx activity decreased in both roots and leaves of lettuce plants exposed to 100 µg/L of CYN for five days. A similar pattern was found for the MC-LR/CYN trial, and in this experiment, the GPx activity significantly decreased (P <0.05) in roots and leaves exposed for 10 days to 10 and 100 µg/L of the mixture. Although the GPx activity has been successfully used to assess the effects of MC-LR in *Lepidium sativum* (Gehringer et al., 2003; Stüven and Pflugmacher 2007), GPx may not be the best biomarker of these cyanotoxins in lettuce plants. The activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) increased in *M. sativa* seedlings after exposure to ecologically relevant concentrations of MCs (Pflugmacher et al., 2006). Recently, El Khalloufi et al. (2013) also reported that peroxidase (POD), polyphenoloxidase (PPO) and CAT activities were significantly increased in leaves, roots and nodules of *M. sativa* exposed to cyanobacterial extract containing MCs. The enzyme GPx scavenges peroxides, especially hydrogen peroxide. However, CAT and APX can also convert hydrogen peroxide to water (Gill and Tuteja, 2010); thus, these enzymes should be further studied as potential indicators of oxidative stress generated in lettuce plants due to the exposure to cyanotoxins.

3.3. Effects of MC-LR, CYN and MC-LR/CYN mixture on mineral content in lettuce leaves

The impact of cyanotoxins on mineral content in crop plants has been barely studied. In this work we explored the effects of ecologically relevant concentrations of MC-LR, CYN and a MC-LR/CYN mixture on the mineral content in lettuce leaves, the edible portion of this plant. The basal levels of the minerals analyzed in lettuce leaves in each experiment at the end of five and 10 days (control groups) are shown in table 2.

Table 2. Mineral content in the lettuce leaves of the control groups from the three experiments at the end of 5 and 10 days (5D and 10D, respectively). Values are expressed as the mean ± SD (n=3).

Mineral concentration		Control group of MC-LR		Control group of CYN		Control group of MC-LR/CYN	
		5D	10D	5D	10D	5D	10D
(mg/Kg DW)	Ca	694.1 ± 1.2	721.7 ± 6.8	599.6 ± 34.8	524.9 ± 59.7	595.1 ± 42.3	532.1 ± 79.0
	Mg	236.1 ± 3.2	227.9 ± 11.1	207.4 ± 3.1	202.8 ± 26.5	238.21 ± 14.4	193.16 ± 31.6
	K	4257.4 ± 40.6	4868.9 ± 420.6	4500.4 ± 278.1	5626.6 ± 113.5	4795.1 ± 323.1	4535.7 ± 163.5
	P	447.4 ± 4.4	343.5 ± 20.6	544.2 ± 15.3	548.9 ± 20.9	494.2 ± 18.7	457.5 ± 17.2
	Na	55.3 ± 3.3	100.4 ± 13.2	115.7 ± 10.5	64.5 ± 2.4	184.1 ± 6.8	80.9 ± 2.4
	Mn	28.4 ± 2.3	39.5 ± 3.5	26.3 ± 1.6	14.1 ± 1.9	16.8 ± 2.2	13.6 ± 0.2
	Fe	9.4 ± 1.2	7.4 ± 0.4	8.6 ± 1.2	3.2 ± 0.4	3.0 ± 0.7	1.97 ± 0.6
	Zn	8.6 ± 0.8	7.9 ± 0.2	5.3 ± 0.3	2.0 ± 0.1	2.7 ± 0.3	2.1 ± 0.1
(µg/Kg DW)	Cu	1439.4 ± 114.7	1190.5 ± 65.5	1112.1 ± 89.2	447.4 ± 35.6	624.3 ± 40.8	413 ± 57.2
	Mo	275.6 ± 6.1	264.9 ± 12.4	267.9 ± 11.0	89.5 ± 7.9	73.9 ± 9.9	61.3 ± 2.0

Overall, the exposure of lettuce plants to MC-LR, CYN and a MC-LR/CYN mixture resulted in significant changes in mineral contents in their leaves. The ratios of the mean concentration of minerals in lettuce leaves from treated groups *vs* control groups are shown in table 3. The exposure of lettuce plants to MC-LR produced, in general, a decrease in the mineral content in leaves, and the effects were more pronounced at the highest time and concentration of exposure. Significant differences ($P < 0.05$) were observed for the macronutrients Ca, Mg, K and P after five days of exposure and for all macro and micronutrients (except for Na) after 10 days of exposure. Minerals, after being absorbed by the roots, are translocated to various parts of the plant where they are used in numerous biological functions (Taiz and Zeiger, 2002). In this study, in general, the antioxidant response to stress promoted by MC-LR was more pronounced in roots than in leaves. Thus, the oxidative stress and cellular damage potentiated in roots may have considerably affected the uptake and/or translocation of nutrients and water to the edible tissues of lettuce plants.

Table 3. Ratio of the mineral content in lettuce leaves exposed for 5 and 10 days (5D and 10D, respectively) to MC-LR, CYN and a MC-LR/CYN mixture. Values express the ratio between the mean concentrations obtained in each condition by the mean concentration obtained in the respective control group. Values are expressed as the mean ± SD (n=3). Values in bold represent the concentrations that exceeded the screening value (control group, Table 2).

Mineral	Condition	MC-LR		CYN		MC-LR/CYN	
		5D	10D	5D	10D	5D	10D
Ca	1 µg/L	**1.14 ± 0.16**	**1.03 ± 0.03**	**1.14 ± 0.10**	**1.03 ± 0.08**	0.94 ± 0.09	**1.19 ± 0.06**
	10 µg/L	**1.00 ± 0.09**	0.92 ± 0.06*	**1.28 ± 0.08***	**1.08 ± 0.07**	0.91 ± 0.05	**1.14 ± 0.04**
	100µg/L	0.84 ± 0.12*	0.83 ± 0.03*	**1.25 ± 0.02***	**1.10 ± 0.03**	**1.06 ± 0.11**	0.68 ± 0.02*
Mg	1 µg/L	0.93 ± 0.13	0.98 ± 0.01	**1.18 ± 0.13**	0.99 ± 0.05	0.95 ± 0.07	**1.25 ± 0.06***
	10 µg/L	0.87 ± 0.09*	0.90 ± 0.04*	**1.27 ± 0.09***	**1.06 ± 0.09**	0.84 ± 0.06	**1.17 ± 0.11**
	100µg/L	0.83 ± 0.05*	0.82 ± 0.08*	**1.23 ± 0.04***	**1.04 ± 0.02**	**1.14 ± 0.11**	**1.04 ± 0.15**
K	1 µg/L	**1.16 ± 0.07***	0.97 ± 0.01	**1.14 ± 0.06**	0.96 ± 0.06	0.91 ± 0.01	0.95 ± 0.04
	10 µg/L	**1.10 ± 0.07**	0.99 ± 0.02	**1.16 ± 0.10**	0.93 ± 0.02	0.94 ± 0.03	**1.01 ± 0.11**
	100µg/L	0.94 ± 0.07*	0.81 ± 0.06*	**1.10 ± 0.13**	0.95 ± 0.05	0.79 ± 0.07*	0.64 ± 0.11*
P	1 µg/L	0.95 ± 0.03*	**1.18 ± 0.06**	**1.18 ± 0.04***	0.67 ± 0.01*	**1.03 ± 0.07**	**1.05 ± 0.04**
	10 µg/L	0.85 ± 0.05*	0.94 ± 0.12	**1.23 ± 0.02***	0.69 ± 0.03*	0.97 ± 0.03	**1.07 ± 0.09**
	100µg/L	0.86 ± 0.07*	0.74 ± 0.11*	**1.17 ± 0.01***	0.60 ± 0.01*	**1.16 ± 0.09***	0.91 ± 0.07
Na	1 µg/L	**1.82 ± 0.01***	0.93 ± 0.01	0.85 ± 0.07	0.97 ± 0.13	0.74 ± 0.04*	**2.01 ± 0.11***
	10 µg/L	**2.29 ± 0.40***	0.82 ± 0.12	**1.34 ± 0.04***	**1.18 ± 0.16***	0.81 ± 0.04	**1.75 ± 0.17***
	100µg/L	**1.14 ± 0.35**	**1.11 ± 0.06**	**1.31 ± 0.04***	**1.21 ± 0.17***	**1.16 ± 0.001**	**1.19 ± 0.04**
Mn	1 µg/L	**1.42 ± 0.20***	0.95 ± 0.04	**1.41 ± 0.21***	**1.77 ± 0.33**	0.82 ± 0.12	**1.04 ± 0.12**
	10 µg/L	**1.16 ± 0.07***	0.84 ± 0.09	**1.78 ± 0.02***	**2.77 ± 0.41***	0.71 ± 0.02	**1.20 ± 0.15**
	100µg/L	**1.00 ± 0.02**	0.63 ± 0.04*	**1.71 ± 0.01***	**3.68 ± 0.29***	0.98 ± 0.14	0.54 ± 0.02*
Fe	1 µg/L	**1.19 ± 0.13***	**1.10 ± 0.02**	**1.15 ± 0.12**	**2.08 ± 0.08***	**1.44 ± 0.01**	**2.02 ± 0.08***
	10 µg/L	0.94 ± 0.10	0.66 ± 0.03*	**1.41 ± 0.11**	**3.20 ± 0.98***	0.74 ± 0.19	**1.94 ± 0.17***
	100µg/L	0.89 ± 0.03	0.68 ± 0.07*	**1.14 ± 0.11**	**2.05 ± 0.25***	**1.23 ± 0.21**	**1.35 ± 0.03**
Zn	1 µg/L	0.99 ± 0.10	0.98 ± 0.003	**1.13 ± 0.06**	**2.10 ± 0.09***	0.73 ± 0.07*	**1.18 ± 0.12**
	10 µg/L	**1.03 ± 0.02**	0.81 ± 0.07	**1.11 ± 0.07**	**2.26 ± 0.17***	0.84 ± 0.10	**1.00 ± 0.10**
	100µg/L	0.67 ± 0.06*	0.63 ± 0.03*	**1.13 ± 0.07**	**2.14 ± 0.03***	**1.05 ± 0.03**	0.66 ± 0.07*
Cu	1 µg/L	0.94 ± 0.08	**1.07 ± 0.02**	**1.51 ± 0.02***	**1.79 ± 0.02***	**1.03 ± 0.03**	**1.41 ± 0.14***
	10 µg/L	0.94 ± 0.02	0.88 ± 0.08	**1.12 ± 0.13**	**2.01 ± 0.04***	**1.02 ± 0.06**	**1.75 ± 0.03***
	100µg/L	0.87 ± 0.08*	0.82 ± 0.05*	**1.10 ± 0.01**	**1.96 ± 0.07***	**1.22 ± 0.11**	**1.10 ± 0.04***
Mo	1 µg/L	**1.48 ± 0.06***	**1.01 ± 0.10**	**1.03 ± 0.07**	**1.88 ± 0.11***	**1.51 ± 0.06***	0.91 ± 0.05
	10 µg/L	**1.38 ± 0.03**	0.89 ± 0.08	**1.01 ± 0.07**	**2.24 ± 0.17***	0.78 ± 0.04	**1.53 ± 0.02***
	100µg/L	0.80 ± 0.02	0.73 ± 0.05*	0.94 ± 0.01	**1.84 ± 0.06***	**1.37 ± 0.10**	0.37 ± 0.05*

The mineral content in the roots of *T. durum*, *Z. mays*, *P. sativum* and *L. esculenta* (Ca, Na, K, P and N), as well as *L. esculentum* (Ca, Na, K), increased in a concentration-dependent manner after 30 days of exposure to *M. aeruginosa* extract containing MCs (500-4,200 µg/L; 2,220-22,240 µg/L, respectively) (El Khalloufi et al., 2012; Saqrane et al., 2009). However, similar to our results and the range of concentrations used in this study, the K and Ca content in the shoots of *V. faba* have been recently reported to

decrease, whereas Na increased after two months of exposure to *M. aeruginosa* extract containing 50 and 100 µg/L of MCs (Lahrouni et al., 2013).

Contrary to the MC-LR, the exposure of lettuce plants to CYN produced an enhancement in leaf mineral content at almost all concentrations after five days of exposure. Moreover, after 10 days of exposure to CYN, the content of the minerals Mn, Fe, Zn, Cu and Mo in the leaves was significantly increased at all concentrations of exposure ($P < 0.05$). In the CYN experiment only P was significantly decreased at all concentrations after 10 days of exposure ($P < 0.05$). However, generally, crop plants need small amounts of micronutrients; thus the excessive increase of these elements in the leaves of lettuce plants exposed to CYN for 10 days could be an indication of some physiological disorders, the minerals can be hypothesized to have been used as a defense mechanism. Exploring the mechanisms underlying the enhancement of the uptake of mineral nutrients after five days of exposure to CYN is important. The lettuce plants were able to retain almost all of the minerals (especially the macronutrients) in a concentration higher than the corresponding control value, and this can be an indication of the potential tolerance of lettuce plants to CYN. However, studies on the effects of cyanotoxins in crop plants have been primarily focused on yield losses; yet, the physiological stress promoted by cyanotoxins seems to alter the chemical composition of plants and can therefore change its nutritional quality. The ability of crop plants to cope with abiotic stress and also maximize its nutritional quality is of major relevance for food security. The leaves of lettuce plants exposed to CYN demonstrate the ability to retain a higher content of minerals; however other quality parameters should be assessed, such as antioxidants, proteins, non-structural carbohydrates, lipids and sensory quality traits, which are particularly prone to be changed due to exposure to abiotic stress (Wang and Frei, 2011).

The mixture of MC-LR/CYN resulted in a general decrease of nutrient content after five days of exposure. However, this tendency was reverted with the increase in time of exposure, and after 10 days only the 100 µg/L concentration resulted in lower concentrations of the minerals in comparison to the correspondent control group. Once again, apparently, the MC-LR/CYN mixture did not produce additive, synergistic or antagonistic effects on mineral content in lettuce leaves.

Plants require Ca, Mg, K and P in relatively high amounts, and these nutrients are essential to ensure its life cycle. Although each mineral contributes to several metabolic reactions, some general functions in plant metabolism could be affected due to the

disturbance of mineral uptake and translocation promoted by MC-LR, CYN and its mixture in lettuce plants. Mg supports other functions related to respiration and the synthesis of DNA and RNA and is also a central atom of the chlorophyll molecule; thus, it plays an important role in the light-dependent reactions of photosynthesis. Additionally, both K and P are crucial for respiration and photosynthesis. Mn also plays an important role in the structure of photosynthetic proteins and enzymes, as well as ATP synthesis, as it activates several enzymes involved in tricarboxylic acid metabolism. Likewise, Zn is required for many enzymes and also for chlorophyll biosynthesis (Taiz and Zeiger, 2002). The decline of these minerals in lettuce leaves as was found after MC-LR exposure, could compromise the chlorophyll content and photosynthetic rates; thus, the chronic exposure of lettuce to MC-LR could result in loss of productivity. Indeed, the photosynthesis impairment by MC-LR is well recognized and has already been reported by several authors (El Khalloufi et al., 2011; McElhiney et al., 2001; Pietsch et al., 2001; Pflugmacher, 2002; Saqrane et al., 2009). Although other biological functions could be affected by mineral imbalances, including antioxidant activity and cell growth (for example, Fe and Cu are associated with enzymes involved in redox reactions; and Ca and Na are involved in the synthesis of new cell walls, cell division and cell expansion (Taiz and Zeiger, 2002)), photosynthesis, which plays an important role as energy source, and thus crop yield, could be one of the most affected processes by mineral imbalance due to exposure to MC-LR and CYN.

4. Conclusions

In the present study, the effects of ecologically relevant concentrations of MC-LR, CYN and a MC-LR/CYN mixture were investigated regarding to growth, oxidative stress and mineral content in lettuce plants. This is of particular relevance, facing a tendency towards an increasing occurrence of toxic cyanobacterial blooms and the challenge of the intensification of agricultural productivity. Our results suggest that lettuce plants are able to cope with lower concentrations (1 and 10 µg/L) of MC-LR, CYN and MC-LR/CYN mixture by ensuring the maintenance and even increasing the fresh weight and mineral content and controlling oxidative stress, as was indicated by the significant increase of the GST activity in roots. Furthermore, the enhancement of the mineral

content in the leaves of plants exposed to CYN for five days may provide an indication of the potential tolerance of lettuce plants to this cyanotoxin. However, the concentration and the time of exposure are preponderant factors for the toxic effects of these cyanotoxins in lettuce plants. The exposure of lettuce plants to 100 µg/L of cyanotoxins resulted in a significant decrease in fresh weight of leaves and its mineral content, especially for MC-LR and the MC-LR/CYN mixture, highlighting the potential implications of these toxins for lettuce yield and nutritional quality. Furthermore, the potential tolerance of lettuce plants to CYN raises the possibility of its accumulation in edible tissues, and this issue should be further studied to avoid risks in human health.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Lettuce (*Lactuca sativa* L.) leaf-proteome profiles after exposure to cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture: a concentration-dependent response

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Abstract

The intensification of agricultural productivity is an important challenge worldwide. However, environmental stressors can provide challenges to this intensification. The progressive occurrence of the cyanotoxins CYN and MC-LR as a potential consequence of eutrophication and climate change is of increasing concern in the agricultural sector because it has been reported that these cyanotoxins exert harmful effects in crop plants. A proteomic-based approach has been shown to be a suitable and powerful tool for the detection and identification of the early responses of organisms exposed to cyanotoxins. The aim of this study was to compare the leaf-proteome profiles of lettuce plants exposed to environmentally relevant concentrations of CYN and a MC-LR/CYN mixture. Lettuce plants were exposed to 1, 10, and 100 µg/l

CYN and a MC-LR/CYN mixture for five days. The proteins of lettuce leaves were separated by two-dimensional electrophoresis (2-DE), and those that were differentially abundant were then identified by matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF/TOF MS). Although the lettuce genome has not been sequenced, a high rate of differentially abundant proteins was identified (>73%). The biological functions of the proteins that were most represented in both experiments were photosynthesis and carbon metabolism and stress/defense response. Proteins involved in protein synthesis and signal transduction were also highly observed in the MC-LR/CYN experiment. Although distinct protein abundance patterns were observed in both experiments, the effects appear to be concentration-dependent, and the effects of the mixture were clearly stronger than those of CYN alone. The obtained results highlight the putative tolerance of lettuce to CYN at concentrations up to 100 µg/l. Furthermore, the combination of CYN with MC-LR at low concentrations (1 µg/l) stimulated a significant increase in the fresh weight (fr. wt) of lettuce leaves and at the proteomic level resulted in the increase in abundance of a high number of proteins. In contrast, many proteins exhibited a decrease in abundance or were absent in the gels of the simultaneous exposure to 10 and 100 µg/l MC-LR/CYN. In the latter, also a significant decrease in the fr. wt of lettuce leaves was obtained. These findings provide new insights into the molecular mechanisms of the lettuce response to CYN and MC-LR/CYN and may contribute to the identification of potential protein markers of exposure and novel proteins that may confer tolerance to CYN and MC-LR/CYN, although these need to be functionally characterized and validated. Furthermore, because lettuce is an important crop worldwide, this study may improve our understanding of the potential impact of environmental stress conditions on its quality traits (e.g., presence of allergenic proteins).

Keywords: *Lactuca sativa*, Proteomics, Cylindrospermopsin, Microcystin-LR, Mixture

Abbreviations: *2-DE, two-dimensional electrophoresis; APX, ascorbate peroxidase; CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate; CYN, cylindrospermopsin; EST, expressed sequence tag; FAS, fatty acid synthesis; GSH, glutathione; GST, glutathione-S-transferase; HPLC, High-performance liquid chromatography; HSP, heat shock protein; IPG, immobilized pH gradient; IEF, isoelectric focusing; LEA, embryogenesis abundant protein; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ionization time of flight-mass spectrometry; MC-LR, microcystin-LR; MeOH, methanol; PCA, principal component analysis; PDA,*

photoelectric diode array; PP, protein phosphatases; PPIase, peptidyl-prolyl cis-trans isomerase; PR, pathogenesis-related; PRK, phosphoribulokinase; PS, photosystem; ROS, reactive oxygen species; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate carboxylase/oxygenase; SB, solubilization buffer; SBPase, sedoheptulose-1,7-bisphosphatase; SD, standard deviation; SOD, superoxide dismutase; TCA, tricarboxylic acid; TFA, trifluoroacetic acid.

1. Introduction

The progressive occurrence and global expansion of harmful cyanobacteria blooms have been forecasted as consequences of eutrophication and climate change (Elliott, 2012; O'Neil et al., 2012). Among freshwater cyanobacteria, *Microcystis aeruginosa* is the most common bloom former (O'Neil et al., 2012). However, the invasive species *Cylindrospermopsis raciborskii* has shown a substantial widespread distribution, including into temperate zones (Kinnear, 2010; Poniedziałek et al., 2012). The use of irrigation water from sources that contain toxic cyanobacterial blooms of *C. raciborskii* and *M. aeruginosa* may pose a threat on the agricultural sector because their cyanotoxins (the cytotoxic cylindrospermopsin (CYN) and the hepatotoxic microcystin-LR (MC-LR), respectively) appear to generate phytotoxic effects on crop plants. CYN is a tricyclic alkaloid, and although the molecular mechanism of its toxicity has not yet been established, it is known that CYN inhibits eukaryotic protein synthesis with similar intensity in plant and mammalian cell extracts (Froscio et al., 2008). Although studies on the toxic effects of CYN on plants are scarce, the few studies that have analyzed CYN indicate that it results in the reduction of pollen germination (Metcalf et al., 2004), inhibition of plant growth (Vasas et al., 2002), induction of abnormal mitosis, alteration of microtubule organization, inhibition of root and shoot elongation (Beyer et al., 2009), and increase in oxidative stress (Prieto et al., 2011). MC-LR, the most studied structural variant of microcystins, is a cyclic heptapeptide that irreversibly inhibits, by covalent binding, serine/threonine protein phosphatases (PP; PP1 and PP2A), and this is the main mechanism of its toxicity in both animals and higher plants (Mackintosh et al., 1990). The induction of oxidative stress by the production of reactive oxygen species (ROS) appears to be another important biochemical mechanism of MC-LR

toxicity that may cause serious oxidative damage to DNA (Pflugmacher, 2004; Stüven and Pflugmacher, 2007). The toxic effects of MC-LR on plants have also been characterized. It has been reported that MC-LR results in the inhibition of germination (Pflugmacher et al., 2006; Pflugmacher et al., 2007; El Khalloufi et al., 2011), inhibition of growth and development (McElhiney et al., 2001; Pflugmacher, 2002; Gehringer et al., 2003; Chen et al., 2004; Mitrovic et al., 2005; Pflugmacher et al., 2006; Pflugmacher et al., 2007; El Khalloufi et al., 2011), alteration of microtubule organization (Máthé et al., 2009), induction of changes in photosynthesis (Pietsch et al., 2001; Pflugmacher, 2002; El Khalloufi et al., 2011), induction of changes in chlorophyll content (McElhiney et al., 2001; Pflugmacher, 2002), and induction of changes in antioxidative response parameters (Pflugmacher et al., 1999; Pflugmacher et al., 2001; Gehringer et al., 2003; Pflugmacher, 2004; Pflugmacher et al., 2006; Stüven and Pflugmacher, 2007; Pflugmacher et al., 2007; Saqrane et al., 2009; El Khalloufi et al., 2011). Nevertheless, the reported biochemical and physiological effects vary depending on the (1) use of purified toxins or crude extracts, (2) the plant species, (3) the stage of plant development, (4) the time of exposure, and (5) the range of concentrations studied. Therefore, it is important to note the ecological relevancy of these studies because few have confirmed the effects at environmentally relevant concentrations (Gehringer et al., 2003; Pichardo and Pflugmacher et al., 2011). The concentrations required to exhibit effects in a wider range of species appear to be non-environmentally realistic because these are 10–1000-fold higher than those usually found in ecosystems (McElhiney et al., 2001; Mitrovic et al., 2005; Crush et al., 2008; Beyer et al., 2009; Saqrane et al., 2009). It has been reported that exposure concentrations of pure CYN below 100 µg/l appear to have no significant harmful effects on a wide range of species (e.g., a floating macrophytes and green algae) (Kinnear, 2010), leading to the hypothesis that plants have developed appropriate protective mechanisms to tolerate CYN. Otherwise, it can be questioned whether the traditional endpoints used to assess toxicity exhibit sufficient sensitivity to evaluate understated biochemical alterations. Recently, Azevedo et al. (2014) reported the lack of sensitivity of the conventional parameters for the analysis of the toxicity of *M. aeruginosa* extract on rice (*Oryza sativa*) plants (MC-LR concentrations of 0.26–78 µg/l); however, significant alterations were observed through proteomic analyses. The inhibition of protein synthesis by CYN and the inhibition of PP1/PP2A activities by MC-LR appear to interfere with a wide range of molecular processes in plants (Máthé et al., 2013). Although the conventional biochemical biomarkers of stress induced by CYN and MC-LR (antioxidative enzymes and nonenzymatic substances) appear to be suitable, because proteins are the main targets of these cyanotoxins, it is particularly

important to investigate how these operate in plant systems at the protein level. Proteomics is a field of growing interest in the agricultural sector because it has contributed to a better understanding of the specific functions of the proteins involved in plant responses to environmental stresses (Afroz et al., 2011; Kosová et al., 2011; Abreu et al., 2013). A proteomic approach may enable the identification of protein biomarkers of the plant stress response and the discovery of the biological processes underlying stress tolerance, which may be used to enhance agricultural productivity (Kosová et al., 2011; Abreu et al., 2013). Moreover, some secreted proteins with defensive or protective functions on stress factors are recognized to also have allergenic potential (Abreu et al., 2013). From the health risk point of view, proteomics data associated with allergen identification may provide new insights into the protein composition, quality, and safety of edible plants exposed to environmentally relevant concentrations of cyanotoxins. Nevertheless, in aquatic ecosystems, single species of cyanobacteria are almost never found; hence, the existence of mixtures of cyanotoxins in the water column is likely and it was already reported for MC-LR and CYN (Brient et al., 2008). Simultaneous exposure to a mixture of CYN and MC-LR may lead to changes in the response capability of crop plants, triggering potential synergistic or antagonistic effects. Recently, Prieto et al. (2011) suggested a synergistic effect on the oxidative stress response of rice plants due to its exposure to cyanobacterial extracts containing low concentrations of both CYN (0.13 µg/l) and MC-LR (50 µg/l). Proteomics studies investigating the effects of CYN and MC-LR have been performed on bivalves, including mixtures with other environmental pollutants (e.g., herbicides) (Martins et al., 2009; Puerto et al., 2011; Malécot et al., 2013). Proteomics studies on the plant response to abiotic stress factors are mainly related to cold, heat, drought, waterlogging, salinity, ozone treatment, hypoxia/anoxia, herbicide treatments, inadequate or excessive light conditions, deficient or excessive mineral nutrition, enhanced concentrations of heavy metals, radioactivity, and mechanical wounding, as was reviewed by Kosová et al. (2011). As above mentioned, more recently, Azevedo et al. (2014) successfully applied a proteomic approach to assess the early physiological and biochemical responses of rice seedlings to environmentally relevant concentrations of MC-LR.

This work aimed to use a 2-DE proteomic approach and MALDI-TOF/TOF MS to investigate the leaf-proteome profiles of lettuce (*Lactuca sativa* L.) plants exposed to environmentally relevant concentrations (1, 10, and 100 µg/l) of a CYN and MC-LR/CYN mixture.

2. Materials and Methods

2.1. General Experimental Procedures

The lettuce plants (*Lactuca sativa L.* var. 'Susybel') were exposed to environmentally relevant concentrations (1, 10, and 100 µg/l) of a CYN and MC-LR/CYN mixture in a hydroponic system. The CYN and MC-LR were purified and quantified by High-performance liquid chromatography (HPLC) and then diluted in the culture medium to the desired concentrations of exposure. All of the HPLC solvents were of high-purity chromatography grade and were filtered (Pall GH Polypro 47 mm, 0.2 µm) and degassed with an ultrasound bath. After exposure, to investigate the leaf-proteome profiles, the proteins of lettuce leaves were separated by 2-DE, and those that were differentially abundant were then identified by Matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF/TOF MS).

2.2. Cyanobacterial culture and toxin purification and quantification

2.2.1. Culture of *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*

M. aeruginosa (LEGE 91094) and *C. raciborskii* (LEGE 97047) were grown to exponential phase in Z8 medium (Kotai, 1972) (6-l flasks) under fluorescent light with a light/dark cycle of 14/10 h and a temperature of 25 ± 1ºC. The cultured cells were gathered by centrifugation (20 min, 4ºC, 4495 *g*), frozen at -80ºC, and then freeze-dried. Due to the high hydrophilicity of CYN, the culture medium from *C. raciborskii* was also frozen at -80ºC and freeze-dried. The lyophilized material was stored at room temperature in the dark until toxin extraction and purification. In this study, purified toxins were chosen for the experiments to address the specific effects of CYN and MC-LR/CYN on the lettuce leaf-proteome and to avoid interference from other potentially toxic metabolites (e.g., lipopolysaccharides) because cyanobacterial crude extracts appear to induce more pronounced effects than purified toxins at equivalent concentrations (Pietsch et al., 2001).

2.2.2. CYN extraction, purification, and quantification by HPLC-PDA

CYN was extracted from *C. raciborskii* cells and culture medium following a modified version of the method described by Welker et al. (2002). Briefly, the freeze-dried cells and medium (0.7 g) were first sonicated in a bath for 15 min in 5 ml of 0.1%, v/v trifluoroacetic acid (TFA) of spectrophotometric grade and then subjected to five cycles ultrasonication with Vibra-Cell at 60 Hz for 1 min. The homogenate was stirred for 1 h at room temperature and centrifuged (20,000 *g*, 4ºC, 20 min), and the supernatant was collected. The cell pellet was subjected to a second extraction. The supernatants were pooled and stored at -20ºC. CYN was thereafter purified using a Waters Alliance e2695 HPLC system coupled with a photoelectric diode array (PDA) 2998 on a semi-preparative Gemini C18 column (250 mm x 10 mm i.d., 5 µm) from Phenomenex that was maintained at 40ºC. The isocratic elution utilized a 5% methanol (MeOH) solution containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 2.5 ml/min and an injection volume of 1000 µl. Working solutions of CYN (0.08-5.0 µg/ml) were prepared in water. Standard CYN was supplied by Alexis (San Diego, CA, USA). The purified CYN fractions were then quantified in an HPLC system on an Atlantis® HILIC phase column (250 mm x 4.6 mm i.d., 5 µm) from Waters maintained at 40ºC. The PDA range was 210-400 nm with a fixed wavelength of 262 nm. The isocratic elution was also a 5% MeOH solution containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 0.9 ml/min and an injection volume of 10 µl. The system was calibrated using a set of seven dilutions of the CYN standard (25, 20, 10, 5, 2, 1, and 0.5 µg/ml) in ultrapure water. Each vial was injected in duplicate, and every HPLC run series of ten samples included a blank and two different standard concentrations. The chromatographic purity of CYN was of 98%. The Empower 2 Chromatography Data Software was used for the calculation and reporting the peak information. The retention time of the CYN peak was 7.35 min (data from method validation not published).

2.2.3. MC-LR extraction, purification, and quantification by HPLC-PDA

MC-LR was extracted from *M. aeruginosa* cells according to Ramanan et al. (2000) with some modifications. Briefly, the lyophilized *M. aeruginosa* biomass was extracted with 75% (v/v) MeOH (Fisher Scientific, UK) by continuous stirring for 20 min at room

temperature. The sample was then ultrasonicated five times on ice at 60 Hz for 1 min (Vibra-Cell 50-sonics & Material Inc. Danbury, CT, USA). The homogenate was centrifuged at 10,000 *g* for 15 min to remove the cell debris. The resulting supernatant was then collected and stored at 4ºC. The pellet was re-extracted with an equal volume of solvent, and the pooled supernatants were subjected at a flow rate of at 1 ml/min to SPE with a Water Sep-Pak® Vac 6-ml C18 cartridge preconditioned with 100% MeOH and distilled water. The loaded column was washed with 20% (v/v) MeOH, and the MC-LR was then eluted using 80% (v/v) MeOH. The MC-LR fraction was evaporated by rotary evaporation at 35ºC to remove the entire MeOH portion. The concentrated MC-LR extract was thereafter purified and quantified by HPLC-PDA. The MC-LR semi-preparative assay was performed using a reversed-phase column (Phenomenex Luna RP-18 (250 mm × 10 mm, 10 µm) maintained at 35ºC. The gradient elution was performed with MeOH and water, both of which were acidified with 0.1% TFA, with a flow rate of 2.5 ml/min. The injection volume was 500 µl. The peak purity and percentage of purified MC-LR were calculated at 214 nm and 238 nm. The fraction with purified MC-LR was then evaporated with nitrogen air for 1 day until all of the solvent was removed. The residue was resuspended in distilled water to the desired concentration. The chromatographic purity of MC-LR was of 97%. The purified fractions of MC-LR were then quantified in the same HPLC system on a Merck Lichrospher RP-18 endcapped column (250 mm x 4.6 mm i.d., 5 µm) equipped with a guard column (4 × 4 mm, 5 µm), both of which were maintained at 45ºC. The PDA range was 210-400 nm with a fixed wavelength of 238 nm. The linear gradient elution consisted of (A) MeOH + 0.1% TFA and (B) H_2O + 0.1% TFA (55% A and 45% B at 0 min, 65% A and 35% B at 5 min, 80% A and 20% B at 10 min, 100% A at 15 min, and 55% A and 45% B at 15.1 and 20 min) with a flow rate of 0.9 ml/min. The injected volume was 20 µl. The MC-LR was identified by a comparison of its spectra and retention time with that of the MC-LR standard (batch nº 018K1209, 10.025 µg/ml in MeOH, 98% purity, Cyano Biotech GmbH, Berlin, Germany). The system was calibrated using a set of seven dilutions of the MC-LR standard (0.5 to 20 µg/ml) in 50% MeOH. Each vial was injected in duplicate, and every HPLC run series of ten samples included a blank and two different standard concentrations. The Empower 2 Chromatography Data Software was used for calculating and reporting the peak information. The retention time of the MC-LR peak was 10.44 min (data from method validation not published).

2.3. Plant exposure to CYN and MC-LR/CYN mixture

Lettuce is an important leafy vegetable worldwide that contains substantial amounts of health-promoting phytochemicals (including polyphenols, carotenoids, and vitamin C). Lettuce has been shown to be an excellent experimental system for the assessment of the effects of MC-LR (Crush et al., 2008; Pereira et al., 2009), and although its genome has not been sequenced, lettuce has been the object of genomics and proteomics studies (Choi et al., 2008; Cho et al., 2009). The lettuce plants used in the experiments were purchased at four to five weeks' maturity after sowing in a commercial soil substrate. The roots were carefully washed with tap water until complete soil removal was achieved, and twenty lettuce plants were then transferred to the holes of plastic boards (PVC), which were placed on black glass trays (35 x 25 x 5 cm deep) in a hydroponic system that was continuously aerated. The roots were completely immersed in 3 l of culture medium (Jensen and Malter, 1995), pH 6.5. The lettuce plants were acclimated for 1 week with fluorescent white light (light/dark cycle of 14/10 h) and a temperature of 21 ± 1ºC. After the acclimation period, the culture medium was renewed, and the plants were exposed to CYN and the MC-LR/CYN mixture at ecologically relevant concentrations of 1, 10, and 100 µg/l for five days. After the exposure time, the plants were harvested. The roots and leaf tissues were separated, weighed (fr. wt) and stored at -80ºC for proteomic analysis. Three biological replicates for each experimental group (control (C), C1, C10, and C100) were prepared in a total of 12 trays.

2.4. Proteomics analysis

A proteomics analysis of the lettuce roots was attempted; however, likely due to salt interference, a slow progression of the first-dimensional isoelectric focusing (IEF) and the heat produced on the IPG gel strips may the analysis impracticable.

2.4.1. Protein extraction from lettuce leaves

The leaf tissues from one lettuce plant exposed for five days to the above-mentioned concentrations of CYN and MC-LR/CYN were ground in liquid nitrogen to a powder with a pestle and mortar. The protein extraction was performed immediately with

acetone containing 10% trichloroacetic acid and 0.07% β-mercaptoethanol for 1 h at -20ºC. After centrifugation at 4495 *g* and 0ºC for 45 min, the pellet was washed with acetone containing 0.07% β-mercaptoethanol for 1 h at -20ºC and centrifuged at 4495 *g* and 0ºC for 50 min. The pellet was dried with nitrogen gas, and the proteins were then solubilized in solubilization buffer (SB) composed of urea (7 M), thiourea (2 M), 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS) (4%, w/v), dithiothreitol (65 mM), ampholytes, pH 4–7 (0.8%, v/v), and polyvinylpolypyrrolidone (0.025 g/mL) for 1 h. The homogenate was centrifuged at 16,000 *g* and 4ºC for 20 min. The supernatant was collected, and the proteins were quantified according to the method described by Bradford (1976) using bovine serum albumin as the standard. The protein samples were stored at -80ºC until further analysis.

2.4.2 IEF and 2-DE

IEF and 2-DE were performed according to Puerto et al. (2011). Briefly, 300 µl of SB with 400 µg of proteins were loaded in 17-cm pH 4–7 IPG gel strips (Bio-Rad, Hercules, CA, USA). The proteins were separated by IEF in a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) using the following program: 16 h at 50 V (strip rehydration); step 1, 15 min at 250 V; step 2, linear voltage increase to 10,000 V over 3 h; step 3, linear increase from 10,000 V to 90,000 V/h; and step 4: 500 V/h. After rehydration, wet paper wicks were placed between the immobilized pH gradient (IPG) gel strip and electrode to remove the excess salts from the samples. The IPG gel strips were frozen at -20ºC prior to 2-DE. The IPG gel strips were equilibrated with 10 mg/ml dithiothreitol in buffer containing urea (6 M), SDS (2%, w/v), glycerol (30%, v/v), and Tris (50 mM), pH 8.8, for 15 min and then with 25 mg/ml iodoacetamide in the same buffer for 15 min. The equilibrated IPG gel strips were placed on top of 12% (w/v) acrylamide SDS-PAGE slab gels (20 cm×20 cm) and sealed with 0.5% agarose. The proteins were separated by SDS-PAGE in a Protean Xi Cell (Bio-Rad, Hercules, CA, USA) at 18 mA per gel for 30 min and then at 24 mA per gel until the dye reached the bottom of the gel. One 2-DE gel was run for each experimental replicate for a total of 24 gels. The gels were stained overnight at room temperature with Colloidal Coomassie Blue as described by Neuhoff et al. (1988).

2.4.3. Gel image acquisition and protein abundance variation analysis

The image acquisition and analysis of the protein abundance from the 2-DE gels were performed as described previously by Puerto et al. (2011). Briefly, the gel images were acquired using a calibrated scanner (GS-800, Bio-Rad, Hercules, CA, USA), and the protein spots were detected automatically with the PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA, USA). The spot intensities were normalized based on the total density in the gel image, and manual spot corrections, including re-matching, were also made using the software. The protein spots were considered differentially abundant when the intensity levels exhibited at least a two-fold difference that was statistically significant at a level of $p<0.05$ (univariate approach, *t*-test).

2.4.4. Protein identification by MALDI-TOF-TOF MS and peptide mass fingerprinting

A selection list of the spots of interest was generated based on the differentially abundant protein spots ($p<0.05$) between the C group and the C1, C10, and C100 groups of the CYN and MC-LR/CYN exposure experiments. The spots were excised from the gels and automatically digested with trypsin and spotted on MALDI targets as previously described by Printz et al. (2013). The identification of proteins was performed through mass spectrometry (MALDI-TOF/TOF). For each spot 1 MS spectrum was acquired and the 10 highest peaks selected for MS/MS analysis, excluding known contaminants and trypsin autocleavage products. All of the searches were conducted allowing for a peptide mass tolerance of 100 ppm and a fragment mass tolerance of 0.5 Da within NCBInr Viridiplantae (Green Plants) (downloaded 2013.01.18; 1,162,105 sequences) and EST_Lettuce databases (downloaded on 2013.04.19; 1,763,496 sequences; 357736570 residues) using MASCOT (Matrix Science, www.matrixscience.com, London, UK). Variable modifications [Dioxidation (W), Oxidation (HW), Oxidation (M), Trp -> Kynurenine (W)] and fixed modifications [Carbamidomethyl (C)] have been allowed for the database search. In general, a protein was considered identified when the protein scores were greater than 73 and were significant ($p<0.05$). When a protein or peptide was identified based on an EST sequence or a protein with a trivial name, the protein sequence was used in a Blast

alignment. Proteins were considered as being significantly identified when two individual non-identical peptides surpassed the threshold for identification or when one peptide resulted in a protein e-value of < 0.005. All identifications were manually validated and extra data was acquired when insignificant identifications were obtained. The manual validations were done as previously described by Printz et al. (2013). For some spots, manual sequence determinations were performed and the sequences found used for cross-species identification with FASTS and MSBlast.

2.5. Statistical analysis

The statistical analysis of the fr. wt of the lettuce plants (n=10) was conducted using the Mann-Whitney U test (p<0.05) (*IBM® SPSS® Statistics version 21.0 for Mac OS X*). The remaining statistical analyses and plots were performed using *R software 2.15.1* (*R Core Team*, 2012). A multivariate analysis using PCA was performed after the data were pre-processed through missing value imputation (*sequential KNN* method, *R Environment*) and logarithm transformation. Significant differences between the groups in each PCA dataset were tested by ANOSIM test (p<0.05). The *heatmap.2* function in the *gplots package* was used to generate the heat map. The clustering method that was used with this function was the default method, which consists of hierarchical clustering using the complete linkage method operated with a matrix of dissimilarities calculated as *Euclidean* distances.

3. Results and Discussion

In the present study, well-defined differences were observed in the leaf-proteome profiles of lettuce plants exposed for five days to ecologically relevant concentrations (1, 10 and 100 µg/l) of CYN and MC-LR/CYN. The proteomics approach was found to be a suitable tool that was sufficiently sensitive to recognize changes in the plant physiological responses that were not perceptible at the morphological level. Overall, the treatments applied in this study did not affect lettuce plants at the morphological level, with the exception of the leaf fr. wt of plants exposed to 1 µg/l and 100 µg/l MC-LR/CYN, which was significantly higher and lower than that of the control group (p<0.05), respectively (Fig. 1).

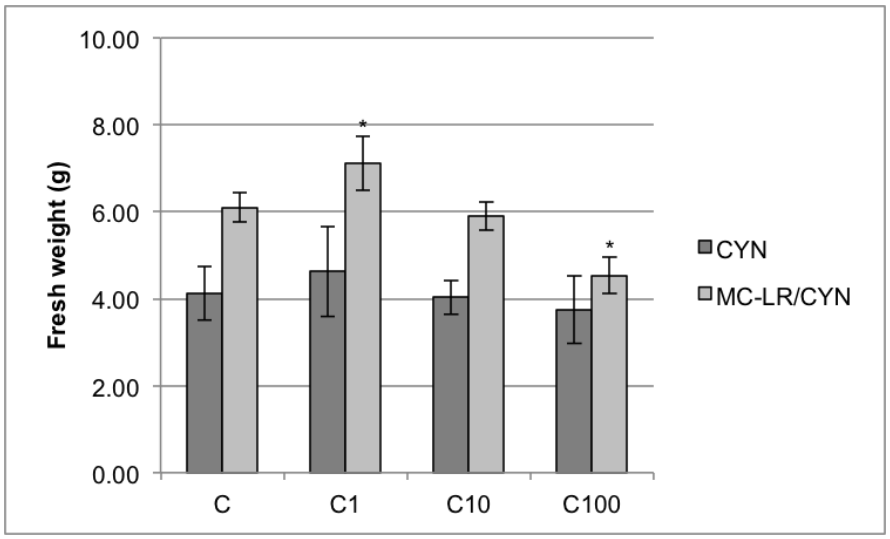

Fig. 1. Fr. wt (g) of leaves of lettuce plants exposed to CYN and MC-LR/CYN. The values are expressed as the means ± SD (n=10). The asterisk (*) indicates significant differences (*P*<0.05) between the control and exposed groups.

Although there is scarce information on the lettuce genome, a high rate of proteins was successfully identified by the combination of 2-DE, MALDI-TOF/TOF MS, and lettuce expressed sequence tag (EST) databases (NCBI). A functional characterization of the differentially abundant leaf-lettuce proteins was performed to better understand the physiological response of this important crop plant to CYN and MC-LR/CYN exposure.

3.1. Differential leaf-proteome profiles related to CYN and MC-LR/CYN exposure

The analysis of the leaf-proteome profiles of lettuce plants exposed to 1 (C1), 10 (C10), and 100 (C100) µg/l CYN and MC-LR/CYN revealed a total of 68 and 286 protein spots with significant abundance variations, respectively (p<0.05, spot intensity variation of at least two-fold). Specifically, the individual comparisons were made between the control group (C) and the groups with different exposure concentrations (C1, C10, and C100), as follows: C/C1, C/C10, and C/C100 (Figs. 2A and 2B).

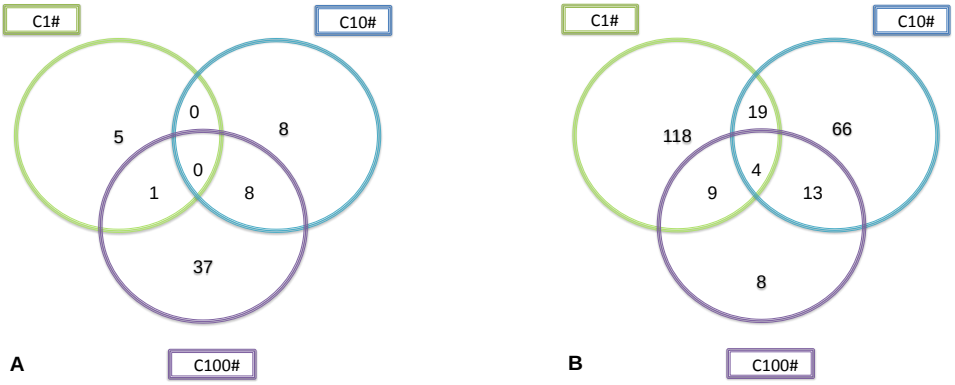

Fig. 2. Venn diagram of the protein spots that exhibit differential abundance on leaf-lettuce plants exposed to CYN (**A**) and MC-LR/CYN (**B**) compared with the control group. The intersections also show the number of common proteins spots between different groups. C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l.

Although some differentially abundant protein spots were common in the groups exposed to different concentrations, each group (C1, C10, and C100) exhibited a specific response pattern. The multivariate principal component analysis (PCA) (Figs. 3 and 4) allowed an accurate classification of the different experimental groups (p<0.05, ANOSIM test) (Supporting information Figs. 2 and 3).

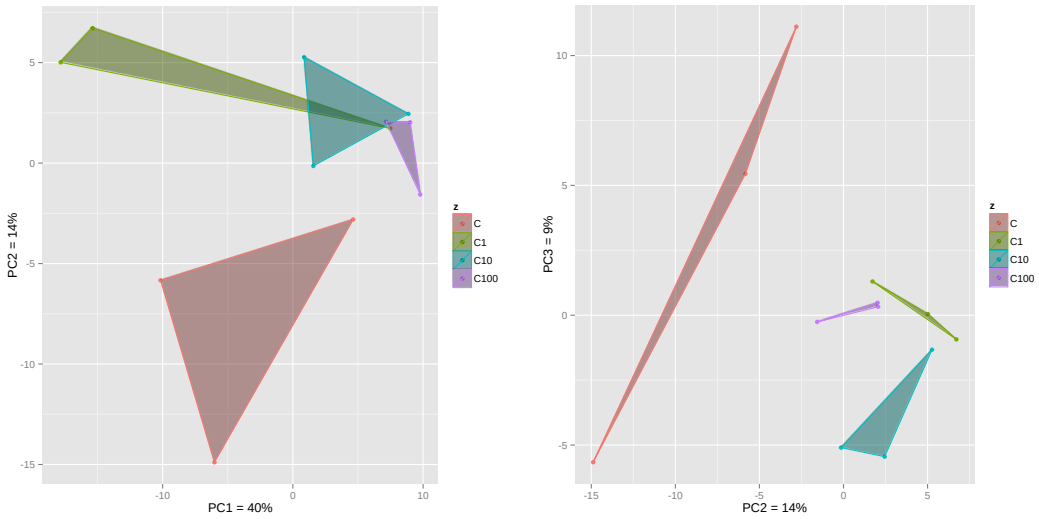

Fig. 3. PCA diagrams representing the first and second components and the second and third components of the differential protein abundance (spot intensity) on the 2-DE gel of leaf-lettuce plants exposed to CYN. C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l.

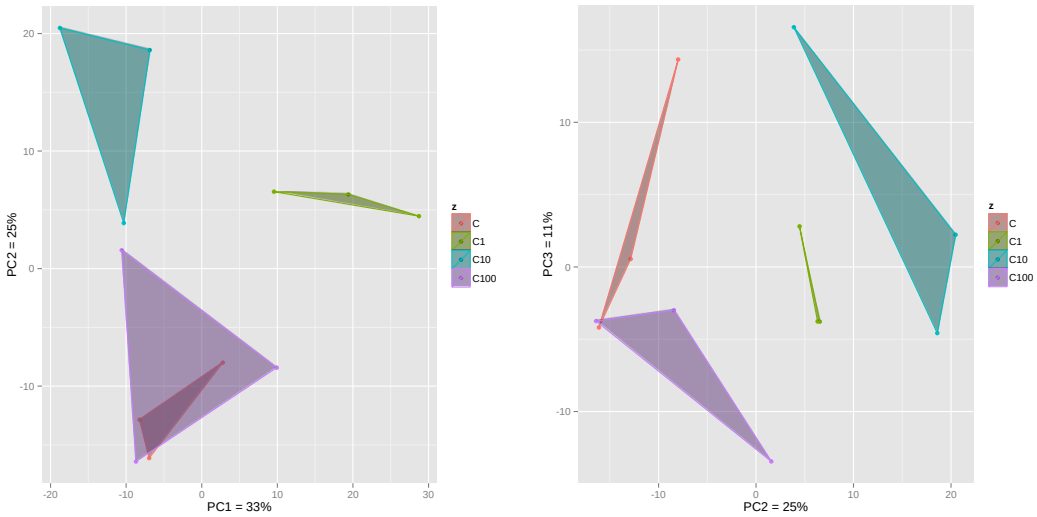

Fig. 4. PCA diagrams representing the first and second components and the second and third components of the differential protein abundance (spot intensity) on the 2-DE gel of leaf-lettuce plants exposed to MC-LR/CYN. C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l.

In the CYN exposure experiment, the first component explains 40% of the variation and separates groups C and C1 from groups C10 and C100. The second and third components explain 14% and 9% of the proteome variation, respectively, and these separate group C from groups C1, C10, and C100. In the MC-LR/CYN exposure experiment, the experimental groups were also well separated by the first three components. PC1 explains 33% of the proteome variation and separates groups C, C10, and C100 from group C1. Groups C10 and C100 are in the same component. PC2 explains 25% of the variation and separates groups C and C100 from groups C1 and C10, whereas the third component, which explains 11% of the variation, clearly separates group C from group C100.

The number of protein spots with significant abundance variation appears to be concentration-dependent in the CYN exposure experiment (Table 1).

Table 1. Quantitative description of the differentially abundant protein spots of the lettuce leaf-proteome profile obtained after treatment with ecologically relevant concentrations of CYN and the MC-LR/CYN mixture.

Exposure conditions	CYN			MC-LR/CYN		
	C1	C10	C100	C1	C10	C100
Spots with increased relative protein abundance	0	11	39	138	26	7
Spots with decreased relative protein abundance	5	1	0	1	11	5
Protein spots absent in C	1	4	7	7	4	5
Protein spots absent in C1	-	-	-	4	-	-
Protein spots absent in C10	-	-	-	-	61	-
Protein spots absent in C100	-	-	-	-	-	17
Differentially abundant protein spots (identified proteins)	6 (6)	16 (12)	46 (38)	150 (127)	102(74)	34 (25)

(C: control; C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l)

A low number of significantly different protein spots (6) were found in group C1, and almost all of these proteins decreased in abundance (5) compared with that observed in group C. In contrast, exposure to 10 and 100 µg/l CYN resulted in 11 and 39 differentially protein spots that increased in abundance and four and seven protein spots that were only present in the gels of these groups, respectively (Table 1). The number of differentially abundant protein spots in the MC-LR/CYN exposure experiment also appears to be concentration-dependent but following a reverse trend, i.e., a high number of proteins increased in abundance in group C1 (150), and 61 and 17 protein spots were absent in the gels of groups C10 and C100, respectively (Table 1).

For a broader visualization of these extensive results, individual heat maps were generated for the CYN and MC-LR/CYN exposure experiments. Associated with each heat map, two hierarchical clusterings were performed to display the similarities between the experimental groups exposed to CYN and MC-LR/CYN and to determine the distribution pattern of the differentially abundant proteins (Figs. 5 and 6).

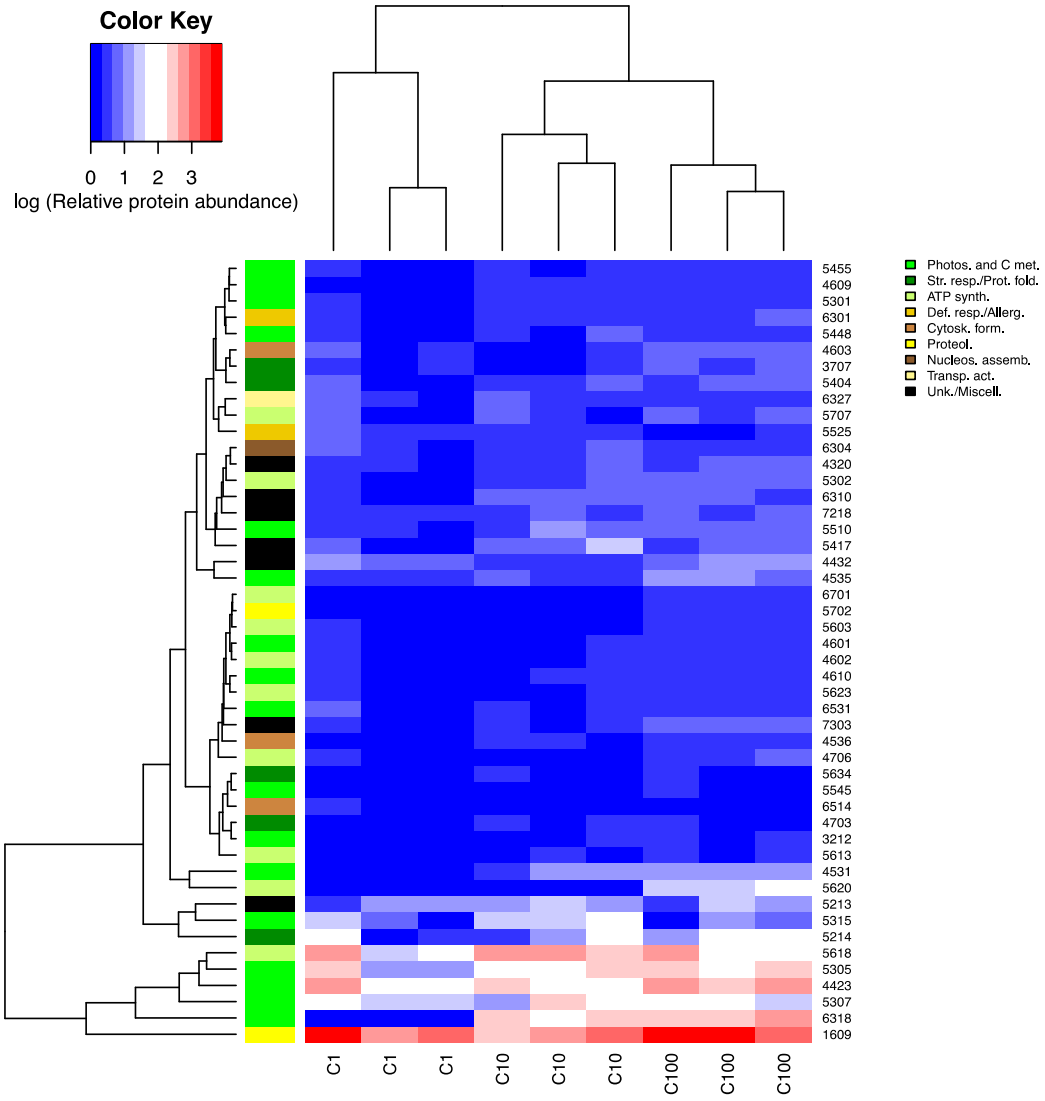

Fig. 5. Heat map of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to CYN. The values shown were normalized and standardized using the control group as the reference. Two hierarchical clusterings were made to display the similarities of the tested concentrations and the distribution pattern of the differentially abundant proteins. The functional categorization of the identified proteins is shown on the left side of the heat map. Photos. and C met.: photosynthesis and carbon metabolism; Str. resp./Prot. fold.: stress response/protein folding; ATP synth.: ATP synthesis; Def. resp./Allerg.: defense response/allergens; Cytosk. Form.: cytoskeleton formation; Proteol.: proteolysis; Nucleos. assemb.: nucleosome assembly; Transp. Act.: transport activity; Unk./Miscell.: unknown/miscellaneous.

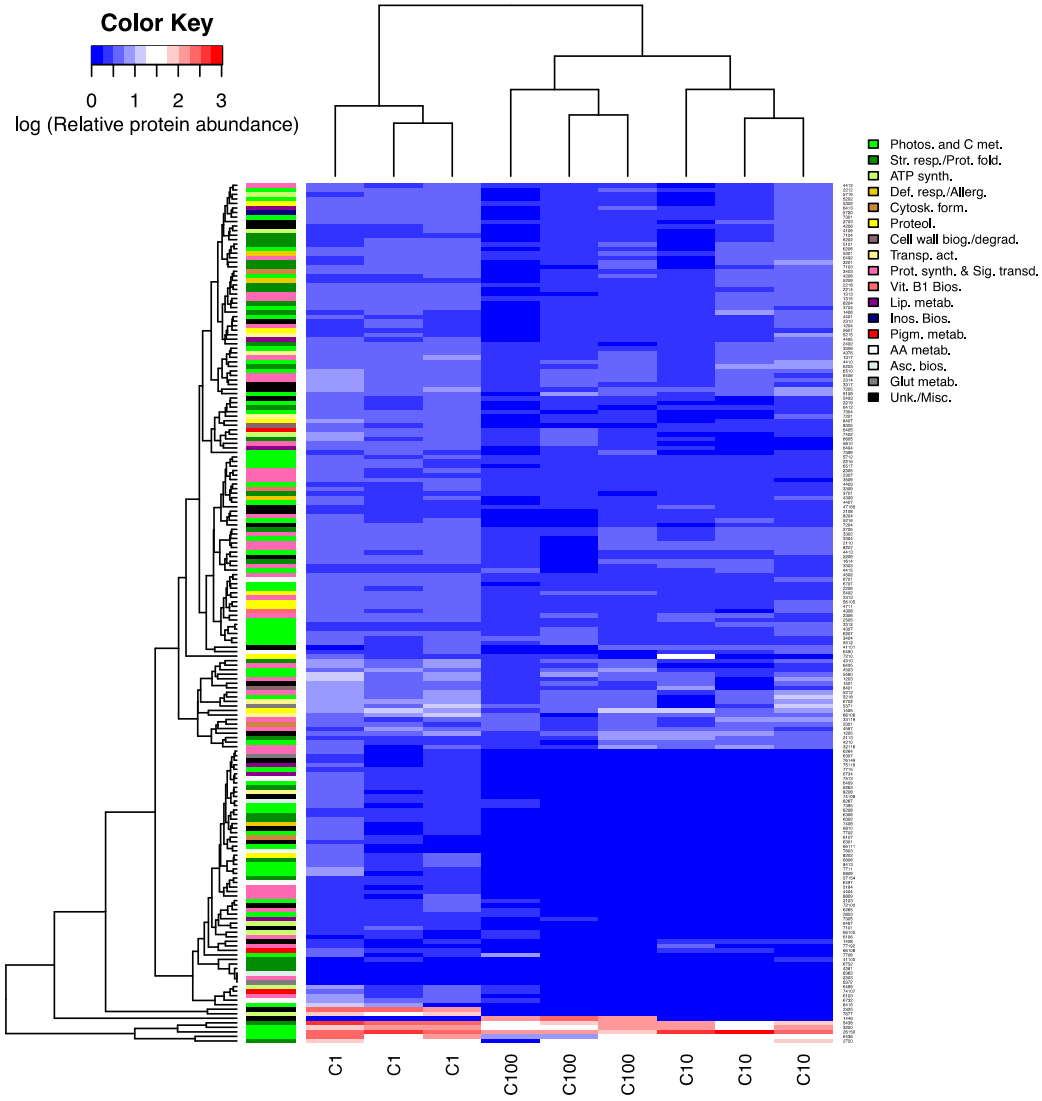

Fig. 6. Heat map of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to MC-LR/CYN. The values shown were normalized and standardized using the control group as the reference. Two hierarchical clusterings were made to display the similarities of the tested concentrations and the distribution pattern of the differentially abundant proteins. The functional categorization of identified proteins is shown on the left side of heat map. Photos. and C met.: photosynthesis and carbon metabolism; Str. resp./Prot. Fold.: stress response/protein folding; ATP synth.: ATP synthesis; Def. resp./Allerg.: defense response/Allergens; Cytosk. Form.: cytoskeleton formation; Proteol.: proteolysis; Cell wall biog./degrad.: Cell wall biogenesis/degradation; Transp. Act.: transport activity; Prot. Synth. & Sig. transd.: protein synthesis and signal transduction; Vit. B1 Bios.: vitamin B1 biosynthesis; Lip. metab.: lipid metabolism; Inos. Bios.: inositol biosynthesis; Pigm. metab.: pigment metabolism; AA. metab.: amino acid metabolism; Asc. bios.: ascorbic acid biosynthesis; Glut metab.: glutathione metabolism; Unk./Miscell.: unknown/miscellaneous.

In both the CYN and MC-LR/CYN exposure experiments, the clusters above the plots show two major groups (C1 and C10/C100). These clusters are corroborated by PCA (first component in the CYN experiment and first and second components in the MC-LR/CYN experiment) and suggest that the concentration of 1 µg/l in both experiments produced distinctive effects relative to the concentrations of 10 and 100 µg/l. The clustering of proteins by their abundance variation also generated two major groups. The first small group encloses the proteins that displayed major differences in abundance comparatively to group C (absence of spots in the gels or high-fold variation). In the CYN experiment, the color gradient shows a common pattern of the proteins that decreased in abundance in group C1 (dark blue/grey pattern). Group C100 is adjacent to group C10 and is clustered away from group C1 because all of the protein spots increased in abundance (light blue/grey pattern). With respect to the MC-LR/CYN experiment, the trend is reversed, and the majority of proteins identified in group C1 exhibited an increase in abundance (light blue/grey pattern), whereas the proteins in groups C10 and C100 decreased in abundance or were absent in the gels (dark blue/grey pattern).

3.2. Identification and functional classification of differentially abundant leaf-proteins

Six (100%), 12 (75%), and 38 (83%) proteins from the C1, C10, and C100 groups in the CYN exposure experiment, respectively, and 127 (85%), 74 (73%), and 25 (74%) proteins from the C1, C10, and C100 groups of the MC-LR/CYN exposure experiment, respectively, were successfully identified (Table 1). In both experiments, there were multiple spots corresponding to the same protein, such as ATP synthase CF1 β-subunit (spots 4706, 5603, and 5707) in the CYN experiment and chloroplastic-like thiamine thiazole synthase chloroplastic-like (spots 3309 and 4308) in the MC-LR/CYN experiment. Furthermore, some of the identified proteins exhibited an unexpected molecular mass relative to its position in the 2-DE gels (Supporting information Figs. 4 and 8). Similar results have been reported in other proteomics studies, and this variability may be related to the identification of proteins from other plant species, the identification of multiple isoforms of the same protein, and post-translational modifications (Sheoran et al., 2007; Pinheiro et al., 2013).

The identified proteins were classified based on their putative designated functions, which were mainly gathered from Gene Ontology (UniProt/Swiss-Prot) (details of the identification and changes in abundance are presented in the supplementary data; Tables 1 and 2). The identified proteins from the CYN and MC-LR/CYN experiments were classified into 10 (Fig. 7) and 18 functional categories (Fig. 8), respectively.

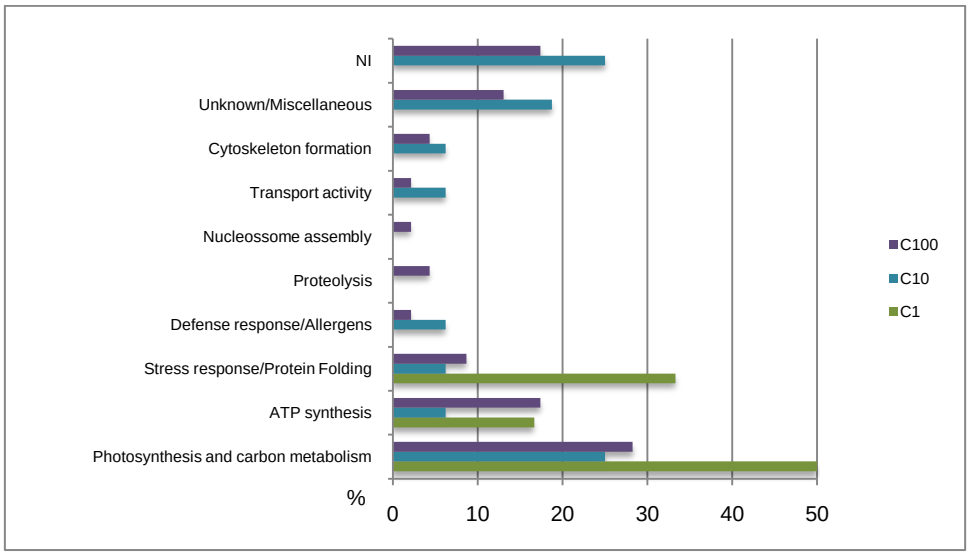

Fig. 7. Functional categorization of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to CYN. C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l.

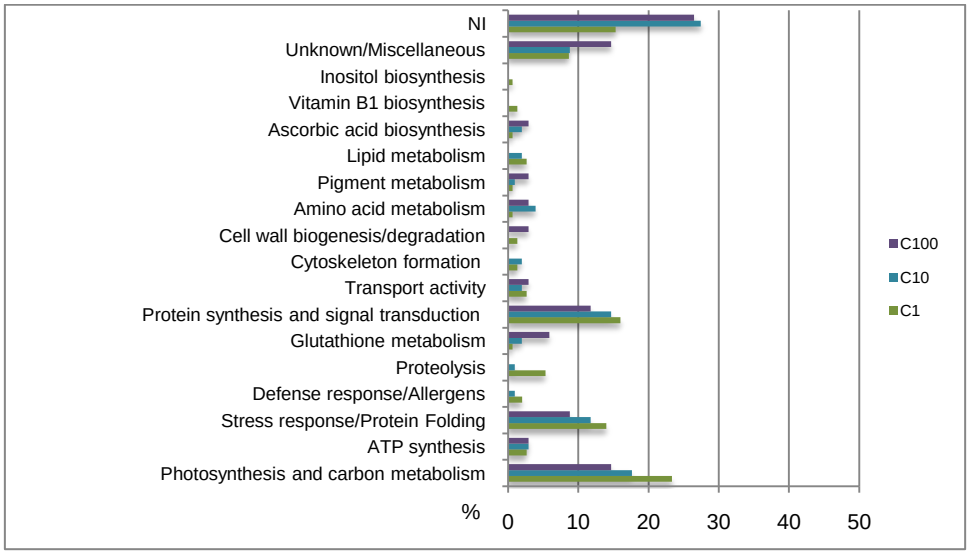

Fig. 8. Functional categorization of the proteins identified from the differentially abundant protein spots of leaf-lettuce plants exposed to MC-LR/CYN. C1: 1 µg/l; C10: 10 µg/l; and C100: 100 µg/l.

A large number of identified proteins in both experiments with all exposure concentrations are mostly involved in photosynthesis and carbon metabolism, ATP synthesis, and stress/defense response and protein folding (Figs. 7 and 8). Proteins related to protein synthesis and signal transduction were also well represented in the MC-LR/CYN experiment. To summarize, groups of more comprehensive functions were created to discuss the potential effects of environmentally relevant concentrations of CYN and MC-LR/CYN on leaf-lettuce plants.

3.2.1. Energy-related metabolism (photosynthesis/carbon metabolism; ATP synthesis)

The impact of the cyanotoxins on photosynthesis is of major interest because plant productivity and hence crop yield depend strongly of the efficiency of this process.

In the C1 group of the CYN experiment, although only six proteins showed differential abundance, three (50%) were related to energy production, and these decreased in abundance (plastocyanin, ATP synthase CF1 β subunit, and NADP-dependent malate dehydrogenase (pyruvate metabolism)). In contrast, energy production appears to be enhanced in the leaves of the plants in groups C10 and C100, the latter of which presented the highest number of proteins involved in this process. The abundance of chlorophyll a-b-binding proteins, which gather and transfer light energy to photosynthetic reaction centers, was increased; in particular, chlorophyll a-b-binding protein 8 exhibited a 50.7-fold increase in abundance in the C10 group. Oxygen-evolving enhancer proteins, which are responsible for water oxidation in photosystem II (PSII), are also increased in abundance in groups C10 and C100; and the same results were obtained with quinone oxidoreductase-like protein At1g23740, ATP synthase CF1 α (C10 and C100) and β subunits (C100), and ATPase epsilon chain (C100). In addition to proteins associated with primary photosynthesis reactions (light reactions), proteins involved in the Calvin cycle (carbon fixation reactions) exhibit an increase in abundance in group C100: ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RuBP activase), ribulose bisphosphate carboxylase/oxygenase activase 1 (RuBisCO activase 1), phosphoribulokinase (PRK), and sedoheptulose-1,7-bisphosphatase (SBPase).

The effects of MC-LR/CYN were much more complex and appear to have been

stronger in groups C10 and C100, the gels of which showed several absent proteins. The combined MC-LR/CYN exposure affected not only photosynthesis but also cellular respiration. Interestingly, in group C1, several proteins involved in energy production are increased in abundance. In detail, these proteins are associated with the light reactions of photosynthesis (chlorophyll a-b-binding proteins, oxygen-evolving enhancer proteins, chloroplast PsbO4 precursor, cytochromeb_6/f heme-binding protein 2-like, ATP synthase α subunit, PSI reaction center subunit II, PSII stability/assembly factor HCF136, quinone oxidoreductase-like protein At1g23740 chloroplastic-like, and ferredoxin-NADP reductase), photorespiration (gamma carbonic anhydrase-like 2), the Calvin cycle (RuBP activase and large subunit, RuBisCO activase 1, and PRK), glycolysis and pentose phosphate pathway (β-xylosidase/α-L-arabinofuranosidase 2-like, fructan 1-exohydrolase IIa, triosephosphate isomerase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, phosphoglycerate kinase 3, ribose-5-phosphate transaldolase), tricarboxylic acid (TCA) cycle (mitochondrial dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 2), and oxidative and photo phosphorylation (mitochondrial ATP synthase subunit delta' and chloroplastic soluble inorganic pyrophosphatase 1). In contrast, few proteins related to photosynthesis and cellular respiration were increased in abundance in groups C10 and C100 (chloroplast light-harvesting chlorophyll a/b-binding protein, RuBP activase (C100), pyruvate dehydrogenase E1 component subunit β-like, transaldolase-like, and chloroplastic soluble inorganic pyrophosphatase 1). Furthermore, there were proteins that were absent in the gels or exhibited decreases in abundance in groups C10 and C100, and these proteins are associated with the light reactions of photosynthesis (oxygen-evolving enhancer protein 1, ATP synthase α subunit, ATP synthase gamma chain, and chloroplastic-like isoform 1) and the Calvin cycle (RuBP activase, transketolase, putative), which is dependent on the ATP and NADPH generated by the light reactions to assimilate CO_2. Additionally, proteins involved in glycolysis (pyrophosphate--fructose 6-phosphate 1-phosphotransferase β subunit, and glyceraldehyde-3-phosphate dehydrogenase (C100)) and the TCA cycle (putative cytosolic NADP-malic enzyme, α isopropylmalate synthase, succinate dehydrogenase, isocitrate dehydrogenase (NAD+) (C100), and malate dehydrogenase (C100)) were absent in the gels or presented decreases in abundance in groups C10 and C100. Several studies have proposed that photosynthesis-related proteins are differentially regulated under abiotic stress. In general, photosynthesis is impaired in sensitive plants and enhanced in tolerant plants (Kosová et al., 2011; Abreu et al., 2013). Nothing definite is known regarding how CYN interferes in plant photosynthesis. However, negative effects of MC-LR in the

photosynthesis activity of plants have been reported (Abe et al., 1996; Pflugmacher, 2002; El Khalloufi et al., 2011). Our results show that MC-LR/CYN concentrations of 10 and 100 µg/l may produce harmful effects on the energy production of lettuce leaves. Nevertheless, the highest number of proteins that decreased in abundance was found in group C10, which may suggest that the effects are produced through different mechanisms. The roots exposed to 100 µg/l MC-LR/CYN had a high amount of exudates (data not shown). It could be hypothesized that this accumulation could prevent the uptake of the toxins, thereby mitigating their negative effects, but can also impair the uptake of water and nutrients, which can be related to the significant reduction in the fr. wt of these lettuce leaves (p<0.05) (Fig. 1). In contrast, the fr. wt of the lettuce leaves of group C1 was significantly increased (p<0.05), likely due to the enhancement of photosynthesis and carbon metabolism. As mentioned above, no differences were found in the fr. wt of lettuce leaves in the CYN experiment (Fig. 1); thus, it is likely that the increase in abundance of proteins related to photosynthesis and carbon metabolism in group C100 may contribute to the maintenance of cellular homeostasis and an active adaptation to the stress promoted by CYN. Plant productivity and hence crop yield depend strongly on the prevailing photosynthetic rates. The Calvin cycle is autocatalytic and can thus be enhanced by increases in the concentrations of its biochemical intermediates (Taiz and Zeiger, 2002). Some of the identified proteins belonging to the Calvin cycle, such as SBPase (which exhibits a 11.8-fold increase in abundance in group C100 of the CYN exposure experiment), have been reported to be effective for the improvement of abiotic stress tolerance (Abreu et al., 2013) and have also been studied to determine whether their use may increase crop production (Lefebvre et al., 2005). Lefebvre et al. (2005) reported that an increase in SBPase activity in tobacco plants (*Nicotiana tabacum*) leads to improvements in the photosynthesis rate, levels of sucrose and starch, leaf area, and biomass, which may be associated with an increase in the RuBP regenerative capacity. Because photosynthesis and carbon metabolism seem to have been the main biological processes affected by CYN and MC-LR/CYN, it is hypothesized that some of these identified proteins can be considered potential biomarkers of these cyanotoxins.

3.2.2. Stress and defense response (stress response/protein folding; glutathione metabolism; proteolysis; defense response/allergens)

In the stress/defense response, plants need to regulate a variety of processes that

require energy. In fact, the profile of abundance of proteins involved in the stress/defense response followed the same pattern observed in the analysis of energy-related proteins: group C100 of the CYN exposure experiment and group C1 of the MC-LR/CYN exposure experiment stand out due to their high number of proteins that exhibited an increase in abundance. Although it is well recognized that ROS are generated during normal plant metabolism (e.g., in chloroplasts), it has also been stated that the cyanotoxins CYN and MC-LR induce oxidative stress in plants (Pflugmacher et al., 2006; Pflugmacher et al., 2007; Stüven and Pflugmacher, 2007; Saqrane et al., 2009; Prieto et al., 2011). A protein involved in glutathione (GSH) metabolism (S-formylglutathione hydrolase), specifically in GSH synthesis, was only present in the C group of the MC-LR/CYN experiment. However, protein IN2-1 homolog B-like (glutathione-S-transferase (GST) superfamily) increased in abundance in group C100 (3.3-fold). Runnegar et al. (1995) showed that CYN inhibits GSH synthesis in cultured rat hepatocytes. It is interesting to note that in this study this effect was not observed in lettuce leaves exposed to CYN even at a concentration of 100 µg/l. Nevertheless, it is well recognized that MC-LR is detoxified by conjugation with GSH via GST (Pflugmacher et al., 1998). It can be hypothesized that the simultaneous exposure to MC-LR/CYN may lead to a reduction of GSH pool in cells due to MC-LR detoxification (via GST), which may have resulted in a higher requirement of GSH biosynthesis. Because CYN is an inhibitor of GSH synthesis, it can impair the capacity of plants to detoxify MC-LR. On the other hand, the weakened response of lettuce plants to MC-LR may contribute to oxidative stress and may enhance its toxic effects in a concentration-dependent manner, leading to an inefficient response of plants to CYN and consequently the inhibition of a higher number of proteins in the groups exposed to higher concentrations of the toxin. In contrast, at low concentrations, lettuce plants appear to be able to cope with oxidative stress by inducing the production of other antioxidant enzymes. Chloroplastic Cu/Zn superoxide dismutase (SOD), ascorbate peroxidase (APX), other peroxidases, and ferritin, which is reported to be responsive to stress, were identified in leaf-lettuce plants exposed to 1 µg/l MC-LR/CYN (chloroplastic 2-Cys peroxiredoxin BAS1, peroxiredoxin 2, thioredoxin-dependent peroxidase, oxidoreductase, chloroplastic SOD [Cu-Zn], aldo-keto reductase 2-like, and chloroplastic peroxiredoxin-2E (also in the group C10)). The coordinated action of these enzymes prevents the oxidative damage of cells generated by ROS. SOD catalyzes the dismutation of superoxide radical to hydrogen peroxide and oxygen. The resulting hydrogen peroxide is reduced by APX and other peroxidases to yield water (Pflugmacher et al., 2006). However, if the detoxification enzymes produced are not sufficient, proteins damaged by ROS may accumulate in plant cells under stress

conditions. Some of the strategies developed by plants to overcome this accumulation are to refold misfolded proteins using helper proteins, such as chaperones, and to remove these by protease activity. Heat shock protein (HSP) 70, which is one of the most important HSPs involved in the plant response to abiotic stresses (Abreu et al., 2013), was decreased in abundance in the C1 group of the CYN exposure experiment. However, in group C100 of the CYN exposure experiment, putative thioredoxin-dependent peroxidase and RuBisCO large subunit-binding protein subunit α, which has chaperone and refolding activity, exhibited increases in abundance. In addition, the chloroplastic protein peptidyl-prolyl cis-trans isomerase (PPIase) FKBP16-3, which is a folding catalyst, exhibited a 107-fold increase in abundance in group C100 (CYN exposure). With respect to the MC-LR/CYN experiment, several proteins involved in redox homeostasis and some proteins with chaperone functions (chloroplastic PPIase CYP38, RuBisCO subunit binding-protein α subunit precursor, 60-kDa chaperonin α subunit, chloroplastic 20-kDa chaperonin, protein disulfide isomerase-like 2-3-like, calreticulin (C10), protein disulfide isomerase (C10), and HSP 90 (C10)) were increased in abundance in groups C1 and C10 (exhibited less fold-variation in group C10). The late embryogenesis abundant protein (LEA), Lea14-A, is well recognized for its role in stress tolerance (Abreu et al., 2013); however, this protein was absent in the gels of groups C1 and C10 of the MC-LR/CYN exposure experiment. Additionally, some proteins involved in redox homeostasis and some proteins with chaperone activity were either decreased in abundance or absent in the gels of groups C10 and C100 of the MC-LR/CYN exposure experiment (chloroplastic chaperone protein ClpC (C10), glutaredoxin S16 (C10), thylakoid-bound ascorbate peroxidase (C10), thioredoxin reductase 2-like (C10), PITH domain-containing protein At3g04780 (C100), and chloroplastic chaperone protein ClpB3). The latter protein was also decreased in abundance in the C1 group. Almost all of the chaperones and peroxidases are chloroplastic, which suggests that photosynthetic complexes are the most affected by CYN and MC-LR/CYN exposure. In addition to detoxification and chaperone proteins, an increase in the abundance of proteases and proteasomes was observed in group C1 of the MC-LR/CYN exposure experiment. Probable 26S proteasome non-ATPase regulatory subunit 7, which belongs to the proteolytic complex that regulates cytosolic protein turnover by the ubiquitin pathway (Taiz and Zeiger, 2002) and plays an essential role in removing regulatory proteins and abnormal polypeptides in plants during stress, exhibited a 4-fold increase in abundance. The putative zinc-dependent protease was increased in abundance in group C100 of the CYN exposure experiment.

Proteins related to the bacterium defense response also presented changes in abundance in the CYN and MC-LR/CYN experiments. Eugenol synthase 1, which catalyzes the synthesis of the phenylpropene eugenol, a defense compound with antimicrobial properties, exhibited an increase in abundance in group C10 of the CYN exposure experiment and in group C1 of the MC-LR/CYN exposure experiment. In the latter experiment, harpin binding protein 1 also presented increases in abundance. The increase in abundance of pathogenesis-related (PR) proteins may be promising for the improvement of the response to abiotic stress; however, it may also constitute a threat to food safety because most of these proteins have allergenic potential. Thaumatin-like protein-like, which appears to have allergenic properties (Palacín et al., 2010), was increased in abundance in group C100 of the CYN exposure experiment (2.7-fold) and in group C1 of the MC-LR/CYN exposure experiment (2.2-fold).

3.2.3. Protein synthesis and signal transduction (transcription, RNA processing and translocation, and translation)

Several proteins involved in protein synthesis and signal transduction were found in almost all of the groups of the MC-LR/CYN experiment. The C1 group presented a high number of these proteins that were increased in abundance. Although to a lower extent, groups C10 and C100 also showed proteins associated with: mRNA processing (chloroplast putative ribonucleoprotein (C1, C10, and C100), chloroplast 31-kDa ribonucleoprotein (C1 and C100)), transcription factors containing DNA-binding motifs and regulators of transcription (transcription factor Pur-alpha 1-like, U2 small nuclear ribonucleoprotein A, zinc finger protein, proliferating cell nuclear antigen, minor allergen Alt *a*, nascent polypeptide-associated complex subunit alpha-like), and mRNA transport and translation (eukaryotic translation initiation factor 3 subunit D-like, elongation factor 1-beta, eukaryotic translation initiation factor 3 subunit F-like, eukaryotic translation initiation factor 3 subunit J-like (C10), chloroplast 30S ribosomal protein S1 (C1 and C10), 60S acidic ribosomal protein P0, and chloroplast 50S ribosomal protein L12 (C10)) that presented increases in abundance. The mRNA processing and translational apparatus may indicate a rapid protein synthesis, which could be related to the increase and maintenance of the fr. wt of lettuce leaves in groups C1 and C10, respectively. Nevertheless, some proteins associated with RNA recognition motifs and regulators of ribonuclease activity (poly(A)-binding protein (C10), and regulator of ribonuclease activity A (C10)) and translation (eukaryotic translation initiation factor 5A

(C10), 40S ribosomal protein (C10), eukaryotic translation initiation factor 3 subunit K-like (C10), and elongation factor 2 (C100)) decreased in abundance or were absent in the gels of groups C10 and C100. In this study, the changes in abundance of proteins related to protein synthesis suggest that this activity may be of particular importance in the lettuce response to MC-LR/CYN. The little that is known regarding how CYN inhibits protein synthesis in plants is that the soluble proteins associated with the eukaryotic translation system appear to be the target of the toxin (Froscio et al., 2008). Additionally, this toxin appears to interfere with the elongation step, which indicates that elongation factors may also be a target (Froscio et al., 2008). In fact, in group C100 of the MC-LR/CYN experiment, elongation factor 2 was the unique protein that exhibited a decrease in abundance. The potential decrease of energy-related enzymes (e.g., Calvin cycle enzymes), likely as a result of protein synthesis inhibition promoted by CYN, may lead to reduced synthesis of carbohydrates, which are essential for the support of cell division and elongation (Taiz and Zeiger, 2002). This decrease may also have contributed to the reduction in the fr. wt of lettuce leaves; however, this hypothesis should be further studied. Exposure to CYN results only in an increased abundance of histone H4 in the C100 group. This protein is a core component of the nucleosome and plays a central role in transcription regulation, DNA repair, DNA replication, and chromosomal stability. It could be hypothesized that CYN at a concentration up to 100 µg/l is not sufficient to cause cessation of protein synthesis; however, in combination with MC-LR, the inhibition of PP1 and PP2A and the induction of oxidative stress promoted by MC-LR may have enhanced the protein synthesis inhibition produced by CYN. Furthermore, phosphorylation and dephosphorylation play important roles in signal transduction, and in the MC-LR/CYN exposure experiment, proteins involved in signal transduction pathways (14-3-3-like protein D-like (C1), 14-3-3 protein 1-like, and 14-3-3 protein (C10)) present increases in abundance in groups C1 and C10. The plant 14-3-3 isoforms regulate a diverse range of proteins, including kinases, transcription factors, structural proteins, ion channels, and pathogen defense-related proteins (Denison et al., 2011). The differential accumulation of these proteins appears to be implicated in the response to abiotic stress (Abreu et al., 2013).

3.2.4. Transport activity

Proteins and lipoproteins involved in the transport of macromolecules, small molecules, and ions, mainly in the chloroplast, exhibited changes in abundance in both

experiments. In groups C10 and C100 of the CYN exposure experiment, temperature-induced lipocalin exhibited 4.4-and 2.5-fold increases in abundance, respectively. This protein as well as chloroplast processing peptidase-like, protein TIC 62 chloroplastic-like, and apolipoprotein d, also increased in abundance in group C1 of the MC-LR/CYN exposure experiment. However, in groups C10 and C100, chloroplastic protein TIC 62 was decreased in abundance, and GTP-binding nuclear protein Ran1A was absent in the gels of the C10 group.

3.2.5. Structural activity (cytoskeleton formation; cell wall biogenesis/degradation)

It is well recognized that MC-LR alters the cytoskeletal structure of animal cells (Toivola and Eriksson, 1999). Phosphorylation/dephosphorylation and the synthesis of certain regulatory proteins are key mechanisms in cytoskeletal regulation (Máthé et al., 2013). Similarly, CYN induces the reorganization of the cytoskeleton in animal cells (CHO K1 cells) (Fessard and Bernard, 2003). In this study, exposure to CYN promoted a decrease in the abundance of actin in the C10 group. In contrast, plastid-dividing ring protein, which is related to microtubule-based process and protein polymerization, increased in abundance in group C100 of the CYN exposure experiment and in group C1 of the MC-LR/CYN exposure experiment. Additionally, fibrillin, a glycoprotein that contributes to the structural integrity and assembly within or outside cells, increased in abundance in groups C1 and C10 of the MC-LR/CYN exposure experiment. UDP-arabinopyranose mutase 1, which is involved in the biosynthesis of the cell wall, exhibited an increase in abundance in group C1 of the MC-LR/CYN exposure experiment. However, xyloglucan endotransglucosylase/hydrolase, an enzyme involved in wall assembly, decreased in abundance in groups C1 and C100 of the MC-LR/CYN exposure experiment. Exposure to these cyanotoxins appears to lead to the reorganization of cytoskeletal components in lettuce leaves. Furthermore, it could be hypothesized that proteins involved in cytoskeleton assembly and cell wall biosynthesis played a role in the increase, maintenance, and decrease of the observed fr. wt of lettuce leaves.

2.2.6. Other metabolisms (amino acid metabolism; pigment metabolism; lipid metabolism; ascorbic acid biosynthesis; vitamin B1 biosynthesis; inositol biosynthesis; hormone regulator)

Overall, proteins involved in the metabolism of amino acids, pigments, lipids, ascorbic acid, vitamin B1, inositol, and hormone regulation exhibited increases in abundance in group C1 and decreases in abundance in groups C10 and C100 of the MC-LR/CYN exposure experiment. Ketol-acid reductoisomerase, which promotes the formation of amino acids containing a branched carbon skeleton, increased in abundance in the C1 group. In contrast, other proteins that have been identified to be involved in amino acid metabolism, such as acetohydroxyacid synthase 1, diaminopimelate decarboxylase 2 chloroplastic isoform 1, vitamin-b12 independent methionine synthase 5-methyltetrahydropteroyltriglutamate-homocysteine, and putative thiosulfate sulfurtransferase (C100) decreased in abundance or were absent in groups C10 and C100. Chloroplastic coproporphyrinogen-III oxidase, which is involved in porphyrin and chlorophyll metabolism and biosynthesis, increased in abundance in the C1 group, and chloroplastic uroporphyrinogen decarboxylase was absent in the C10 group. The polyphenol oxidase precursor, which is involved in the pigment biosynthetic process, was absent in group C100. A diverse family of lipases and esterases (GDSL esterase/lipase At5g45670, GDSL esterase/lipase LTL1-like, and bifunctional epoxide hydrolase 2-like) and a key enzyme of the type II fatty acid synthesis (FAS) system (enoyl-ACP reductase 1) presented increases in abundance in the C1 group. However, the protein 2-hydroxyacyl-CoA lyase-like, which plays a key role in redox signaling and lipid homeostasis, and the protein acetyl-CoA C-acetyltransferase, which is involved in the beta oxidation pathway of fatty acid degradation, were absent in the C10 group. Phosphomannomutase, which is involved in ascorbic acid biosynthesis, was absent in all of the groups of the MC-LR/CYN experiment. Chloroplastic-like thiamine thiazole synthase and L-myo-inositol-1-phosphate synthase, which are involved in vitamin B1 and inositol biosynthesis, respectively, increased in abundance in the C1 group. Auxin-binding protein ABP20-like, a probable receptor for the plant growth-promoting hormone auxin, exhibited an increase in abundance in groups C1 and C10 (to a higher extent in group C1), whereas abscisic acid receptor PYR1-like, a plant hormone associated with signal transduction, was absent in group C10. The simultaneous exposure to low concentrations of MC-LR and CYN (1 µg/l) appears to stimulate the synthesis of different constituents in lettuce leaves that are concomitantly involved in

plant growth and development and also in the response to stress, such as hormones, amino acids, lipids, important membrane components, and vitamins. Exposure to environmental stress induces several physiological changes in plants that can alter the chemical composition and thus the quality of crops (Wang and Frei, 2011). Dependent on numerous factors, such as the time of stress and the crop species, the plant stress response appears to result in increasing the concentrations of some constituents, such as proteins and antioxidants (Wang and Frei, 2011). Depending on the concentration of MC-LR/CYN exposure, positive changes in the nutritional quality of lettuce leaves may be attained. However, this issue should be further studied through the quantification of the respective constituents in plants exposed to these cyanotoxins.

3.2.7. Unknown/miscellaneous

Proteins with no well-defined function (several functions or unknown function) were identified as unknown/miscellaneous.

4. Conclusions

In this study, we applied a differential-expression proteomics approach to understand the mechanisms underlying the response of leaf-lettuce plants to environmentally relevant concentrations of CYN and MC-LR/CYN. The abundance of proteins was affected in a concentration-dependent manner by the simultaneous activation of several metabolic pathways, which are mainly related to photosynthesis, response/defense to stress, and protein synthesis and transduction. The activation of these pathways appears to confer tolerance to lettuce plants against CYN at concentrations up to 100 µg/l. The simultaneous exposure to MC-LR and CYN resulted in a ‘dualistic response’, and exposure to a concentration of 1 µg/l promoted an increase in the abundance of proteins associated with general biological processes and a significant increase in the fr. wt of leaves. In contrast, the concentrations of 10 and 100 µg/l appeared to be deleterious to lettuce because a high number of proteins associated with general biological processes exhibited decreases in abundance or were absent in the gels. The latter concentration also promoted a reduction in the fr. wt of the leaves. This effect is of major concern because the occurrence of mixtures of cyanotoxins is expected to become increasingly recurrent. This study also provides

new insights into potential protein markers of exposure to cyanotoxins and of novel proteins that may confer tolerance to CYN and MC-LR/CYN, although these need to be functionally characterized and validated. Furthermore, the proteomics analysis was found to be suitable for the discovery of some traits associated with the quality and safety of edible tissues of lettuce exposed to environmentally relevant concentrations of CYN and MC-LR/CYN, such as the presence of allergenic proteins.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Chapter 4

Availability and bioaccessibility of microcystin-LR and cylindrospermopsin in bivalves

This chapter has been adapted from the following scientific paper and manuscript:

Marisa Freitas, Joana Azevedo, António Paulo Carvalho, Alexandre Campos, Vitor Vasconcelos. 2014. Effects of storage, processing and proteolytic digestion on the microcystin-LR concentration in edible clams. Food and Chemical Toxicology 66 (2014) 217–223.

Marisa Freitas, Joana Azevedo, António Paulo Carvalho, Vera Mendes, Bruno Manadas, Alexandre Campos, Vitor Vasconcelos. Bioaccessibility and changes on cylindrospermopsin concentration in edible mussels over storage and processing time. Submitted to the Journal Food and Chemical Toxicology.

Effects of storage, processing and proteolytic digestion on the microcystin-LR concentration in edible clams

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Abstract

Accumulation of microcystin-LR (MC-LR) in edible aquatic organisms, particularly in bivalves, is widely documented. In this study, the effects of food storage and processing conditions on the free MC-LR concentration in clams (*Corbicula fluminea*) fed MC-LR-producing *Microcystis aeruginosa* (1×10^5 cell/mL) for four days, and the bioaccessibility of MC-LR after *in vitro* proteolytic digestion were investigated. The concentration of free MC-LR in clams decreased sequentially over the time with unrefrigerated and refrigerated storage and increased with freezing storage. Overall, cooking for short periods of time resulted in a significantly higher concentration ($P < 0.05$) of free MC-LR in clams, specifically microwave (MW) radiation treatment for 0.5 (57.5%) and 1 min (59%) and boiling treatment for 5 (163.4%) and 15 min (213.4%). The bioaccessibility of MC-LR after proteolytic digestion was reduced to 83%, potentially because of MC-LR degradation by pancreatic enzymes. Our results suggest that risk assessment based on direct comparison between MC-LR concentrations determined in raw food products and the tolerable daily intake (TDI) value set for the MC-LR might not be representative of true human exposure.

Keywords: Bioaccessibility; Clams; Food processing; Food storage; Microcystin-LR; Proteolytic digestion

Abbreviations: *ESI, Electrospray; GST, glutathione-S-transferase; HACCP, Hazard Analysis Critical Control Points; HCl, Hydrochloric Acid; IARC, International Agency for Research on Cancer; LC-MS, Liquid Chromatography-Mass Spectrometry; MCs, Microcystins; MC-LR, Microcystin-LR; Mdha, N-methyl-dehydroalanine; MeOH, Methanol; MRM, Multiple Reaction Monitoring mode; MW, Microwave;* $NaHCO_3$*, Sodium Bicarbonate; OATPs, Organic Anion Transporting Polypeptides; PDA, Photoelectric Diode Array; PP, Protein Phosphatases; SD, Standard Deviation; SPE, Solid-Phase Extraction; TDI, Tolerable Daily Intake; WHO, World Health Organization; WW, Wet Weight.*

1. Introduction

The occurrence of cyanobacterial blooms in freshwater is well recognized and documented. The main concerns for the environment and human health due to cyanobacterial blooms are the potential presence of high amounts of cyanotoxins in the water. Globally, the most studied cyanotoxins are microcystins (MCs), which are mainly produced by *Microcystis* but also by *Anabaena, Oscillatoria, Planktothrix, Nostoc,* and *Anabaenopsis* (Sivonen and Jones, 1999). Several structural variants of MCs have been identified. MC-LR (Fig. 1) is highlighted due to its toxicity and dominance in cyanobacterial blooms.

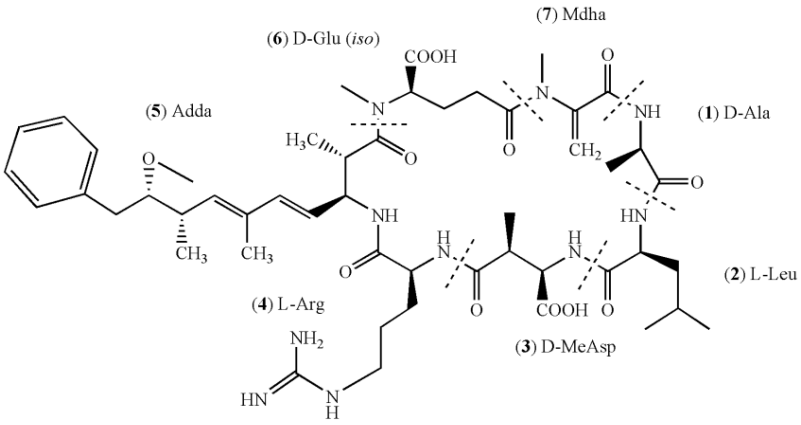

Fig.1. The chemical structure of the heptapeptide MC-LR, where *D*-Ala is *D*-alanine (**1**), *L*-Leu is *L*-leucine (**2**), *D*-Me-

Asp is *D*-erythro-*β*-methylaspartic acid (**3**), *L*-Arg is *L*-arginine (**4**), Adda is the unusual amino acid (2s,3s,8s,9s)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoicacid (**5**), *D*-Glu is *D*-glutamic acid (**6**), and Mdha is N-methyl-dehydroalanine (**7**).

The main mechanism of MC-LR toxicity is the irreversible inhibition of serine/threonine protein phosphatases (PP) (PP1 and PP2A) (MacKintosh et al., 1990). The following two-step mechanism is involved in PP inhibition by MCs: (1) a rapid and reversible binding, followed by (2) a slower covalent binding (occurs over several hours) between the N-methyl-dehydroalanine (Mdha) residue of toxin and cysteine-273 of the catalytic subunit of PP1 (cysteine-266 of PP2A) (Craig et al., 1996; MacKintosh et al., 1995). MC-LR is preferentially taken up by hepatocytes due to active transport by the bile acid carrier transport system, a member of the family of Organic Anion Transporting Polypeptides (OATPs: human) (Fischer et al., 2005), with the liver as the main target. The formation of stable complexes between PP1/PP2A and MC-LR has been suggested to be critical for liver tumor promotion. Epidemiological studies in China may support this suggestion through the association of chronic exposure to MCs from contaminated drinking water with primary liver and colorectal cancer (Ueno et al., 1996; Zhou et al., 2002). Furthermore, the International Agency for Research on Cancer (IARC) classified MC-LR as ‘‘possibly carcinogenic to humans’’ (group 2B) (Grosse et al., 2006). Human health problems due to MC-LR are most likely associated with chronic exposure. Although the major source of long-term human exposure to MCs seems to be drinking water, exposure through contaminated food must be further studied. Based on the potential for human health risks, the World Health Organization (WHO) established a provisional TDI of 40 ng/kg body weight for MC-LR. There are several reports of MCs accumulation in edible aquatic organisms (Amorim and Vasconcelos, 1999; Vasconcelos, 1995; Chen and Xie, 2005; Ibelings and Chorus, 2007). Bivalves (mussels and clams), which are filter-feeding organisms, may collect large amounts of toxic cyanobacterial cells. Bivalves seem to be insensitive to cyanotoxins, and, although most of them can detoxify MCs via the GST (glutathione-S-transferase) metabolic pathway (Pflugmacher et al., 1998; Vasconcelos et al., 2007), several studies have shown that MCs are stored in their organs (Vasconcelos, 1995; Amorim and Vasconcelos, 1999; Chen and Xie, 2005; Ibelings and Chorus, 2007). Furthermore, these organisms are usually eaten whole, which may enhance human exposure to MCs. Estimated daily intake of MCs was studied in four edible aquatic organisms, including clams, which seem to be unsafe for human consumption, once

TDI proposed by the WHO was exceeded several times (Chen and Xie, 2005). Currently, most of the analytical methods used for MC-LR extraction from food matrices use organic solvents such as methanol (MeOH), which do not enable complete MC-LR (dissolved, non- and covalently bound) extraction (Williams et al., 1997). MeOH only retrieves dissolved MCs or those in non-covalent bonds (free), while covalently bound MCs are supposedly not available when food is consumed (Ibelings and Chorus, 2007; Smith et al., 2010). Therefore, it is accepted that only free MCs are relevant to human exposure estimation and risk assessment by contaminated food consumption. Nevertheless, the estimation of exposure to MCs as food contaminants has been based on the direct comparison of the concentration determined from studied organisms (raw food) with the TDI value (Chen and Xie, 2005; Ibelings and Chorus, 2007), assuming that the available concentration of MCs in raw and in ready-to-eat food products is similar. The risk assessment of human exposure to MCs through food must include detailed knowledge of the variation of the free MC content in the different steps of food storage (e.g., refrigeration, freezing) and processing (e.g., boiling, frying, microwaving), because food is generally consumed after such processing. Data on the effects of storage and processing practices on MC availability in food have been reported (Morais et al., 2008; Zhang et al., 2010; Guzmán-Guillén et al., 2011). For instance, Zhang et al. (2010) found that the mean concentration of MCs in bighead carp muscle was significantly increased after boiling. These findings suggest that the MC concentration in some contaminated food-stuffs may have been underestimated. Furthermore, once ingested, the food is subjected to the physical and chemical conditions of the stomach and small intestine, which may change the MC bioavailability. Bioaccessibility is one of the main factors limiting bioavailability. Bioaccessibility is defined as the fraction of the contaminant that is released from the food matrix by the action of digestive enzymes and is then available for absorption by the intestinal mucosa (Cabañero et al., 2004; Versantvoort et al., 2005). Bioaccessibility has been studied for several food chemical contaminants, such as mycotoxins (Versantvoort et al., 2005), mercury (Cabañero et al., 2004) and polychlorinated biphenyls (Xing et al., 2008). To our knowledge, there are no studies that estimate MC-LR bioaccessibility after *in vitro* proteolytic digestion. This study aims (1) to assess changes in the MC-LR concentration after common practices of food storage and processing as well as (2) the determination of MC-LR bioaccessibility after proteolytic digestion to create a more suitable estimation of human exposure to MC-LR through consumption of contaminated food.

2. Material and methods

2.1. Reagents and chemicals

The mammalian enzymes pepsin (P7000), trypsin (T0303) and chymotrypsin (C4129) were purchased from Sigma-Aldrich (Spain). Aqueous solutions of hydrochloric acid (HCl) (37%) (Sigma, USA), phosphate buffer and sodium bicarbonate ($NaHCO_3$) (Sigma, USA) were prepared with ultrapure water supplied by a Millipore water purification system (0.0054 µS/cm) (MilliQ water). Acetic acid was purchased from Sigma (USA). The MeOH used for MC-LR extraction was analytical grade (Fisher Scientific, UK). All solvents used in LC-MS analysis were high-purity chromatography grade (LiChrosolv, Merck). Reagents used in the Z8 medium were analytical grade, and formic acid was LC-MS grade (Fisher Scientific, USA). MC-LR was used as the reference standard (lot nº SZBB069X, 95% purity, Sigma-Aldrich).

2.2. Biological material – cyanobacterial culture and clams

The exposure experiment was carried out with MC-LR-producing cells of *Microcystis aeruginosa* (LEGE 91094). Cyanobacteria were cultured to the exponential phase in Z8 medium (Kotai, 1972) (6 L flasks) under fluorescent light (light/dark cycle of 14/10h) and a temperature of 25 ± 1°C. *M. aeruginosa* LEGE 91094, produces MC-LR (95%) and low amounts of MC-LA and [D-Asp^3]-MC-LR (Vasconcelos, 1995). Specimens of Asian clams (*Corbicula fluminea*) (Müller, 1774), ranging from 25 to 30 mm in size, were collected in the estuary of River Minho (Valença, North Portugal). No MCs were detected in water where *C. fluminea* was collected (data not shown). The organisms were acclimated for one month prior to the experiment in 40 L aquaria with dechlorinated tap water. During this period, the organisms were fed twice a week with *Chlorella vulgaris* ($1x10^5$ cell/mL). The water was renewed weekly.

2.3. Exposure and experiment

2.3.1. Clam intoxication

Clams were intoxicated with whole cyanobacterial cells at the ecologically relevant

concentration of $1x10^5$ cell/mL (≈1.45 µg/L of MC-LR) for four days in static conditions. During the exposure experiment, the physical and chemical conditions of the water were as follows: temperature 16.5 ± 0.8°C, dissolved oxygen 9.6 ± 0.3 mg/L and pH 8.6 ± 0.1.

2.3.2. Simulation of thermal food storage and processing

After four days of exposure, clams were removed from shells and weighed. For each treatment, 10 g of clams, (wet weight (ww)), were submitted to the common practices of food storage and processing shown in Table 1.

Table 1. Detailed information of the food storage and processing conditions applied in experiment.

Conditions	**Storage process**			**Cooking process**	
	25°C	4°C	- 20°C	Microwave (550W)	100°C
Time	12h; 24h; 48h	24h; 48h; 72h	48h; 1 week; 1 month	0.5; 1; 3; 5 min	5; 15; 30 min

The experimental control group was not stored or processed and was immediately analyzed after exposure. Data from the control group was used for comparison (graphical and statistical analysis) with data from the storage and processing conditions applied. The boiling time began after the water reached to 100°C. The ratio of water: clams in the boiling method was 1 mL of MilliQ water: 1 g of clams. Clams and the water in which they were boiled were analyzed altogether. Treatments were performed in triplicate. A negative control with non-intoxicated clams (organisms fed with green algae *C. vulgaris* at the same concentration) was also assessed.

2.3.3. *In vitro* proteolytic digestion

Based on the assumption that gastrointestinal enzymes, which digest carbohydrates and lipids, have a negligible effect on the level of MC mobilization from a food matrix, only proteolytic digestion was assessed. The *in vitro* model used to simulate proteolytic mammalian digestion focused on two stages, physical processing in the mouth and chemical processing in the stomach and small intestine (duodenum) (Fig. 2).

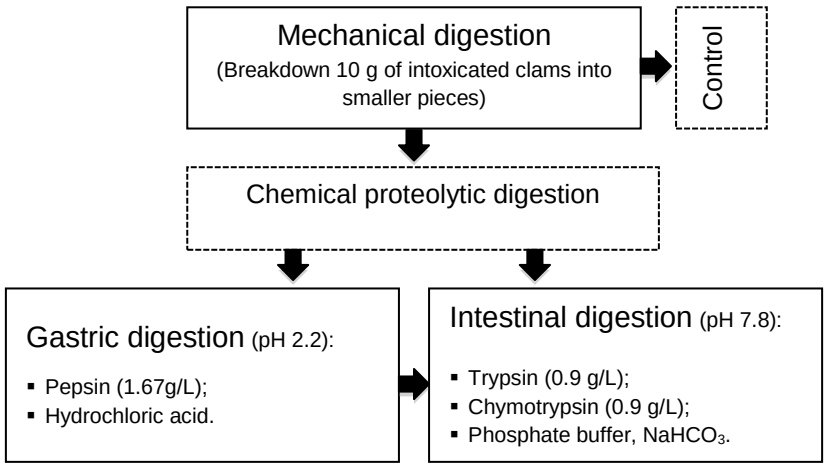

Fig. 2. Schematic representation of *in vitro* proteolytic digestion experiment.

For the latter, two treatments were performed, one simulating gastric digestion (pepsin in an acid environment) and the other simulating complete gastrointestinal digestion (pepsin in an acid environment followed by trypsin and chymotrypsin in an alkaline environment). The procedure was carried out according to Bauer et al. (2003) with some modifications. Briefly, intoxicated organisms (10 g) were mashed (Heidolph, Silent Crusher M, Germany) for 5 to 10 min and incubated in a shaking water bath (GFL 1083, GFL, Germany) for 1 h at 37°C in artificial gastric juice consisting of 85 mL pepsin solution (1.67 g/L in 0.1 M HCl). The pepsin activity was stopped by the addition of 22.5 mL sodium bicarbonate (0.39 M). Samples were incubated in a shaking water bath for 2 h at 37°C in artificial intestinal juice consisting of a mixture of 100 mL trypsin solution (0.9 g/L in 0.16 M phosphate buffer) and 100 mL chymotrypsin solution (0.9 g/L in 0.16 M phosphate buffer). The trypsin and chymotrypsin activities were stopped by the addition of 16 mL of HCl (1 M): acetic acid solution (1 M) in a ratio of 5:2. The experimental control used intoxicated organisms without chemical digestion. A negative control with non-intoxicated clams subjected to both chemical treatments was also performed to negate any interference from the matrix in LC-MS analysis (data not shown). All treatments and controls were run in triplicate.

2.4. MC-LR determination

2.4.1. MC-LR extraction and clean-up

To avoid interference in the final concentration of the toxin through sample freezing/thawing, free MC-LR was immediately extracted after each treatment, according to the method of Amorim and Vasconcelos (1999), with some modifications. Briefly, all samples were extracted in 50% MeOH at room temperature, then submitted to ultrasonication (50 MHz) (Vibra-Cell 50, Sonics & Materials Inc. Danbury, CT, USA) in an ice bath for 10 min and then centrifuged (Thermo Scientific - Legend^{TM} T/RT QUIKset^{TM}, Germany) (20 min, 4°C, 4495 *g*). The pellet was re-extracted with fresh 50% MeOH overnight. Samples were centrifuged again, and both supernatants were pooled together. Extracts from samples were then concentrated and cleaned in SPE (solid-phase extraction) cartridges (10g/60mL C-18-E, Strata® Phenomenex, USA), which were preconditioned with 100% MeOH, followed by MilliQwater and 20% MeOH. After loading the sample, the SPE cartridge was washed with 20% MeOH, and target compounds were eluted in 80% MeOH. The supernatant was evaporated to dryness under vacuum in a rotary evaporator (RotoquímicaBüch, Switzerland), and the residue was resuspended in 1 mL 50% MeOH. Samples were frozen at -80°C until later LC-MS analysis.

2.4.2. LC-MS analysis

The LC-MS system was a LCQ Fleet ion trap MS^{n} (ThermoScientific, USA) with an electrospray (ESI) interface, including a Surveyor LC pump, a Surveyor autosampler and a Surveyor photoelectric diode array (PDA) detector. Separation was achieved on a C18 Hypersil Gold column (100×4.6mm I.D., 5µm, ThermoScientific, USA) kept at 25°C with a flow rate of 0.8 mL/min. The injected volume was 25 µL. A gradient elution was used with mobile phase A, MeOH, and B, water, both acidified with 0.1% formic acid. Mobile phase A was linearly increased from 55 to 90% in 12 min, then increased to 100% in 0.5 min and held for 2.5 min, and finally brought back to 55% and held for 10 min until the next injection. The retention time of MC-LR was 5.8 min. The mass spectrometer was operated in a multiple-reaction monitoring mode (MRM) with a collision energy of 35 eV. The capillary voltage and tube lens were maintained at 22 and 120 V, respectively. Nitrogen was used as the sheath and auxiliary gas. Helium was used as the collision gas in the ion trap. The sheath gas flow rate was set at 80

(arb units), and the capillary temperature was held at 350°C. Samples were analyzed using the mass-to-charge ratio (*m/z*) transition of 995>599 at 23 V collision energy. The MC-LR transition was monitored over one microscan.

2.5. Statistical analysis

Data from the experiments were analyzed using sample means comparison by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (SPSS® version 18.0 for Windows). Statistical analysis was performed between three individual groups: 1) samples of the control group and samples of the stored clams; 2) samples of the control group and samples of the cooked clams; 3) samples of the control group and samples subjected to gastric and gastrointestinal digestion.

The significance level was set at $P<0.05$. Results are expressed in ng/g (ww) as the mean ± standard deviation (SD) for three replicates of each treatment. Bioaccessibility (%) after *in vitro* proteolytic digestion was calculated as follows: [(MC-LR extracted from the fraction after proteolytic digestion) ÷ (MC-LR extracted from the food matrix before proteolytic digestion)] × 100.

3. Results and Discussion

The ecologically relevant concentration of toxic *M. aeruginosa* used in the experiment did not result in mortality in *C. fluminea*. The Asian clam *C. fluminea* is an important invasive species in aquatic ecosystems, because it is supposedly a substantial food source for higher trophic levels and therefore a vehicle of MC-LR. The concentration of free MC-LR quantified in *C. fluminea* (whole organism) after four days of exposure to MC-LR-producing *M. aeruginosa* ($1x10^5$ cell/mL) was between 26.17 and 100.33 ng/g.

3.1. Effects of storage processes on the free MC-LR concentration

Understanding the effects of thermal storage on the available concentration of MC-LR in clams is of particular relevance because this practice is extensively applied to extend the shelf life and maintain the quality of the shellfish. Fig. 3 shows the changes in the free MC-LR concentration after clams are stored under unrefrigerated (25°C), refrigerated (4°C) and freezing (–20°C) conditions.

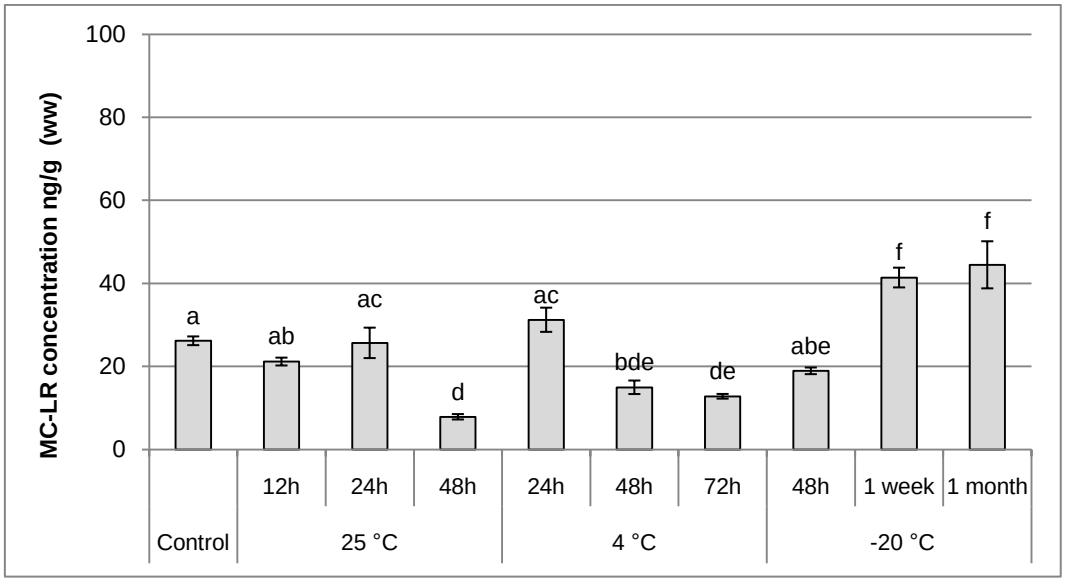

Fig. 3. The MC-LR concentration (ng/g) in intoxicated *C. fluminea* after different thermal storage conditions. Values are expressed as the mean ± SD (n=3). Different letters (a, b, c, d, e, and f) indicate significant differences (P<0.05). Columns that share the same letter are not significantly different.

The concentration of the free MC-LR in clams stored at unrefrigerated and refrigerated conditions decreased over the storage time and was significantly lower after 48 h at 25°C (69.9%, P<0.05) and after 48 h and 72 h at 4°C (42.8% and 51.1%, respectively, P<0.05), than the control group. Clams stored frozen (-20°C), had a significantly higher concentration of free MC-LR from 48 h to one week and one month (58.2% and 69.9%, respectively, P<0.05). The pattern of decrease in the free MC-LR concentration in clams over time in both the unrefrigerated and refrigerated storage conditions can be related to the state of deterioration of fresh clams. Shellfish is extremely perishable, needing to be chilled immediately after removal from shells to delay autolytic and microbial spoilage. It is likely that, as the storage time increases, cell breakage by

autolytic reactions may have occurred, allowing the interaction of MC-LR and internal cellular constituents, including thiols. The conjugation of MC-LR to glutathione, catalyzed by GSTs, is considered the major detoxification pathway of MCs (Pflugmacher et al., 1998 Vasconcelos et al., 2007), and this binding makes the MC-LR undetectable by MeOH extraction. Temperature variation (storage at 25ºC or 4ºC) can alter the affinity conformation for glutathione binding and especially GST activity (the physiological temperature for fish enzymes is 28ºC) (Pflugmacher et al., 1998), which may be related to the lowest concentration of free MC-LR being detected in clams stored at 25ºC for 48h. However, to confirm this hypothesis, further studies should be performed in an attempt to identify and quantify the metabolite corresponding to the glutathione conjugate of MC-LR (Ion *m/z* 1302.8 $[M + H]^+$) (Pflugmacher et al., 1998). Furthermore, the hypothesis that some MC-LR could have been removed by bacterial action of natural flora and contaminant microorganisms should not be discarded. Although the microbial flora of mollusks may vary due to many factors, namely the water quality, some genera of bacteria have been recognized as ubiquitous, such as *Pseudomonas* spp. and lactic acid bacteria (Manousaridis et al., 2005), whose ability to degrade and to remove MC-LR from aqueous solutions has been shown (Takenaka and Watanabe 1997; Nybom et al., 2007; Surono et al., 2008).

Storage by freezing is more effective in spoilage control, so autolytic reactions and microbial growth are unlikely to occur. Furthermore, freezing may cause considerable protein denaturation with corresponding changes in protein structure, which increase over time. Thus, it may also be hypothesized that MC-LR is more efficiently extractable and available after freezing storage for one week and one month. To our knowledge, only Morais et al. (2008) has studied the effects of unrefrigerated, refrigerated and freezing storage on changes in the available MCs in an edible organism (*Mytilus galloprovincialis*). The authors found a significantly lower (P<0.05) concentration of available MCs than in control group in all conditions, although, in contrast to our results, there were no significant differences between different periods of storage.

In any case, as clams were exposed to whole cyanobacterial cells, is likely that some cells have been accumulated in its digestive tract (Amorim and Vasconcelos, 1999; Vasconcelos, 1995). It is important to point out that if the clams were submitted to depuration after exposure, free MC-LR concentration after storage could be quite different due to the emptiness of the digestive tract.

3.2. Effects of cooking process on the free MC-LR concentration

In this study, we explored the effects of two cooking methods (microwaving and boiling for different periods of time) on the free MC-LR concentration in clams (Fig. 4).

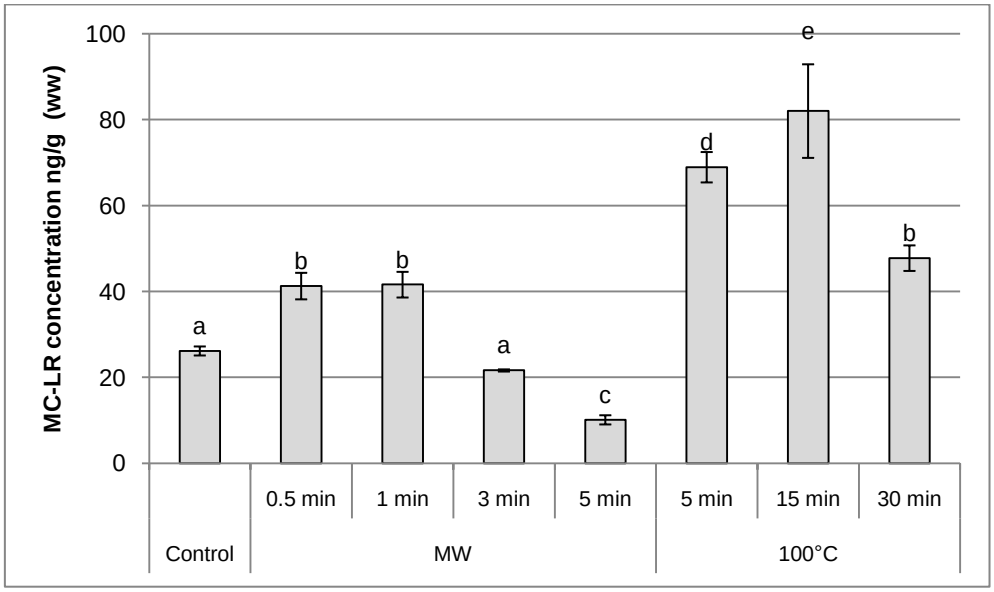

Fig. 4. The MC-LR concentration (ng/g) in intoxicated *C. fluminea* after different cooking conditions. Values are expressed as the mean ± SD (n=3). Different letters (a, b, c, d, and e) indicate significant differences (P< 0.05). Columns that share the same letter are not significantly different.

The clams exposed to MW radiation for 0.5 and 1 min and boiled for 5 and 15 min revealed a significantly higher concentration of free MC-LR (57.5%, 59%,163.4% and 213.4% respectively, P<0.05) compared with the control group. Overall, our results suggest that in short periods of cooking, MC-LR is released more easily from the food matrix and is therefore more available in cooked clams than in raw clams. Furthermore, the boiling method seems to be more effective in such release, most likely due to the MCs solubility in water. MeOH extraction may also not be sufficient to extract all free MC-LR from food matrices. The simultaneous application of different extraction methods (microwaving/MeOH and boiling/MeOH) seems to be more effective and representative of the MC-LR available in food. However, it is important to note that, as is normal in cooking procedures, in this study the evaporation of water was not prevented, so higher levels of free MC-LR may have been obtained by the reduced water content in the clams (e.g., MW treatment for 0.5 and 1 min), once the concentration presented was given by 10 g (ww) of clams. Nevertheless, in clams

exposed to longer periods of MW radiation (3 and 5 min), where major water evaporation occurred, there was a decrease in the free MC-LR concentration (16.8% and 61.3%, respectively), compared with the control group. Similarly, in clams boiled for 30 min, although the free MC-LR concentration had been significantly higher than uncooked clams (82.5%, $P<0.05$), it was significantly reduced in comparison to clams boiled for 5 and 15 min. These results also suggest that the cooking time is critical for the stability of MC-LR. So far, few studies have tested changes in the MC-LR concentration in the matrices of raw and cooked edible aquatic organisms. Morais et al. (2008) reported a significant decrease in the MCs concentration of toxic *M. aeruginosa*-fed mussels (*M. galloprovincialis*) after MW radiation treatment for 1 and 5 min. Similarly, Guzmán-Guillén et al. (2011) found a decrease of 36% in the MC-LR concentration of fish muscle (Tilapia, *Oreochromis niloticus*) spiked with a solution of pure MC-LR, MC-YR and MC-RR, after treatment with MW radiation by 5 min, although after 1 min, the MC-LR concentration remained stable relative to the control group. Our results are in line with previous studies in which a significant decrease of the free MC-LR concentration occurred after 5 min of MW treatment. MW radiation for 5 min may allow that the temperature of the clams to cause the evaporation of almost all the water, leading to a high dehydration state and consequent matrix modification by protein degradation. This process might have made MC-LR inaccessible for extraction or promoted its degradation. However, the reason for the decrease in the free MC-LR concentration in food matrices after longer periods of cooking is not fully understood and should be further studied, because previous studies have shown that the stability of pure MC-LR was not affected by extraction using a domestic microwave oven (650 W) for 9 min (Metcalf and Codd, 2000), or by boiling several hours (Van Apeldoorn et al., 2007). Unfortunately, although MW radiation for 5 min seems to lead to lower levels of MC-LR concentration in clams, the dehydration and the physical damage caused by this treatment renders its organoleptic properties unappealing for human consumption.

Regarding to boiling treatments, in our study, we analyzed boiled clams together with the water in which they were boiled, so the free MC-LR concentration determined is the combination of the contents of both matrices. The results of previous studies in this field are not in agreement. Zhang et al. (2010) generated intraperitoneal intoxication of MC-RR and MC-LR in bighead carp (*Aristichthys nobilis*) and found a significant increase in MCs concentration in boiled muscle (5 min) compared with lyophilized muscle ($P<0.01$). Furthermore, Zhang et al. (2010) found a substantial amount of MCs in the water in which the muscle fish was boiled. However, Morais et al. (2008) boiled *M. galloprovincialis* fed with toxic *M. aeruginosa* for 5 and 30 min and did not find any

differences between the control and boiling treatments. Guzmán-Guillén et al. (2011) boiled *Tilapia* muscle spiked with a solution of MC-LR, MC-YR and MC-RR continuously for 2 min and found a decrease in the MC-LR concentration (59.3%), although the boiling water also had a substantial amount of MCs. In fact, MC-LR transference to boiling water may constitute a risk to human health if the water and animal tissue are consumed. However, this process could be applied to reduce the free MC-LR concentration in food if the boiling water is discarded. Ibelings and Chorus (2007) suggest that in industrially processed "seafood", the removal of parts in which the cyanotoxins can accumulate (e.g., viscera and liver of fishes or guts and hepatopancreas of macrofauna) prior to processing could be effective in avoiding the hazard of MCs. However, this is impracticable in mussels. Considering the HACCP (Hazard Analysis Critical Control Points) approach set by the *Codex Alimentarius Commission*, our results present a new insight to set critical limits for food hypothetically contaminated with MC-LR, in which the boiling process is a critical control point. Control of the cooking time and the rejection of cooking water may be suitable procedures to reduce MC-LR to an acceptable level in food. Our results also suggest that direct comparison between the MC-LR concentration determined in raw edible aquatic organisms and the TDI set for MC-LR may be erroneous and may underestimate the risk of exposure by contaminated food consumption.

3.3. Bioaccessibility of MC-LR after proteolytic digestion

Because the ingestion of contaminated food is one of the most important routes for chronic exposure to MC-LR, it is of major importance to estimate the true exposure after food proteolytic digestion. The changes in the free MC-LR concentration in intoxicated clams after simulating gastric and gastrointestinal proteolytic digestion are shown in Fig. 5.

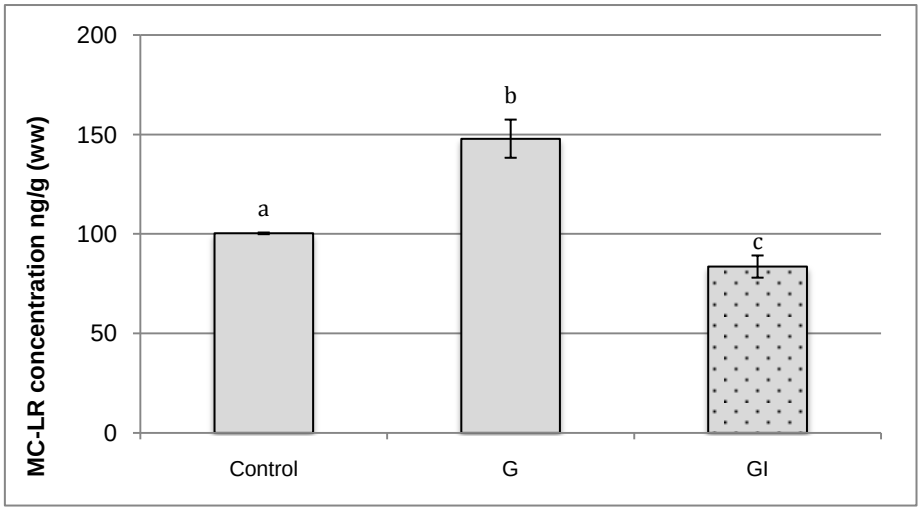

Fig. 5. The MC-LR concentration (ng/g) after *in vitro* proteolytic gastric (G) and gastrointestinal (GI) digestion. The column filled with black points represents the bioaccessibility. Values are expressed as the mean ± SD (n=3). Different letters (a, b, and c) indicate significant differences (P<0.05). Columns that share the same letter are not significantly different.

The simulation of gastric digestion resulted in a significant increase (47.3%, P<0.05) of the free MC-LR concentration, suggesting that pepsin and the acidic conditions found in the stomach may allow easier release of MC-LR from the food matrix. The denaturation of proteins with low pH may increase their digestibility, enhancing MC-LR extraction. The results of previous studies on the degradation of MCs by digestive enzymes are not in agreement. Moreno et al. (2004) observed that MC-LR was degraded either by the action of pepsin or the acidic environment found in the stomach, while Smith et al. (2010) found no digestion of MCs under gastric conditions. In the present study, even if some degradation occurred at this stage, the overall result was an increase in free MC-LR concentration, indicating that the major impact of gastric conditions was the enhancement of MC-LR availability. When gastric digestion was followed by intestinal digestion, the concentration of free toxin significantly decreases (P<0.05) to levels below the control group, which seems to suggest the degradation of free MC-LR by the pancreatic enzymes trypsin and chymotrypsin. It is likely that, with sequential digestion, a prolonged incubation of MC-LR with proteases could make it more susceptible to hydrolysis; however, this issue should be further studied. Controversial results have also been reported regarding the effect of pancreatic proteases on MC-LR, such as resistance to degradation by pancreatin (an enzyme mix), trypsin and chymotrypsin (Moreno et al., 2004; Smith et al., 2010) in opposition to

significant digestion by trypsin and chymotrypsin (Kankaanpää et al., 2005). It should be emphasized that in all these studies, MC was used in its free form in solution and alternatively exposed to gastric or intestinal digestive conditions. The results from these studies should be therefore considered carefully when evaluating the effect of digestion on MC toxicity because they do not take into consideration the bonds between the toxin and molecules (such as PP, glutathione) from food items or the effect of the previous gastric digestion on the intestinal digestion of MCs. In the present study, MC-LR-contaminated food was sequentially subjected to gastric and intestinal conditions to simulate complete gastrointestinal digestion, therefore providing the first, much more realistic, insight on the fate of MC-LR after entering the human digestive tract. The sequential proteolytic digestion allowed the calculation of the bioaccessibility of MC-LR, which was 83.1%. Bioaccessibility of less than 100% suggests that the true exposure to the contaminant is lower than expected, i.e., when raw food is analyzed (Versantvoort et al., 2005). It has been recognized that the toxicity of MCs by oral route is approximately 100-fold lower than by the intraperitoneal route. According to our findings, a decrease in MC-LR bioaccessibility and thus bioavailability after proteolytic digestion may be an important factor. However, it is surely not the only process that can decrease the toxicity. Indeed, several factors may contribute, including the following: MC-LR uptake dependent on the bile acid transport system; biotransformation; excretion; degradation by intestinal microflora; and potential interaction with different nutrients, minerals and antioxidants in food. However, this may be not considered enough to evidence for the assurance of food safety. As shown previously, frozen and undercooked clams generally have a higher available concentration of MC-LR. Furthermore, heat treatment usually increases the digestibility of the proteins, so it is imperative to study the influence of different cooking treatments on the bioaccessibility of MC-LR. Finally, because there may be specific compounds (e.g., MC-LR bound to a small residue of peptide) derived from the digestion of the MC-LR-PP, that have half the toxicity of the parent toxin (Smith et al., 2010), they should also be considered in the evaluation of the total toxicity of MCs after the proteolytic digestion of food.

4. Conclusions

In conclusion, this study provides an enhancement of knowledge on the MC-LR concentration available in food after employing techniques commonly used for their preservation and processing. This might be of particular interest in setting acceptable

thresholds in the industry, because the TDI can be exceeded in undercooked food. Treatments applied in this study could also be considered for the definition of critical control limits, considering the HACCP approach as a promising tool for risk management. Furthermore, the combination of these methods (freezing, boiling, and microwaving) with MeOH extraction may provide a more accurate determination of MC-LR concentration available in food. The bioaccessibility of MC-LR after proteolytic digestion is significantly reduced likely due to degradation by the pancreatic enzymes, trypsin and chymotrypsin. However, many other properties of proteolytic digestion should be further studied. To generate a more representative determination of bioaccessibility in ready-to-eat food products, future research should combine food storage and processing methods with *in vitro* enzymatic digestion.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Bioaccessibility and changes in the cylindrospermopsin concentration in edible mussels with storage and processing time

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Abstract

The cyanobacterial alkaloid cylindrospermopsin (CYN) is recognized as being of increased concern due to the global expansion of its main producer, *Cylindrospermopsis raciborskii*. Previous studies have shown that aquatic organisms, especially bivalves, can accumulate high levels of CYN without lethal effect. Based on the potential for human health risks, a provisional tolerable daily intake of 0.03 µg/kg body weight has been recommended. However, the human exposure assessment has been based on the CYN concentration in raw food items. Thus, this study aimed to assess the changes in CYN concentration in edible mussels with storage and processing time as well as CYN bioaccessibility. Mussels (*Mytilus galloprovincialis*) fed CYN-producing *C. raciborskii* were subjected to storage and processing treatments and were then analyzed by LC-MS/MS. Mussels stored frozen allowed a significantly higher recovery of CYN (52.5% in 48 h and 57.7% in 1 week, $p < 0.05$). The cooking treatments did not produce significant differences in the CYN concentration in the

mussel matrices (flesh); however, CYN was found in the cooking water, suggesting that heat processing can be used to reduce the availability of CYN in this food item. The *in vitro* digestion with salivary, gastric and duodenal juices considerably decreased the CYN availability in uncooked and steamed mussels. The digestion of purified CYN (100 ng/mL) confirmed its removal by salivary and gastrointestinal juices, highlighting the importance of integrating the bioaccessibility of CYN into the human health risk assessment.

Keywords: Bioaccessibility, cylindrospermopsin, food storage, food processing, *in vitro* digestion, *Mytilus galloprovincialis*.

Abbreviations: *ACN, acetonitrile; CYN, cylindrospermopsin; CYP450, cytochrome P450; FA, formic acid; FAO, Food and Agriculture Organization of the United Nations; GSH, glutathione; HACCP, Hazard Analysis Critical Control Points; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; SD, standard deviation; TDI, tolerable daily intake; ww, wet weight.*

1. Introduction

The occurrence and progressive proliferation of harmful cyanobacteria in freshwater, estuarine and marine ecosystems have been recognized as a potential consequence of eutrophication and climate change (O'Neil et al., 2012). During the few last decades, the expansion of *Cylindrospermopsis raciborskii* (a planktonic freshwater cyanobacterium) from its tropical origin to temperate waterbodies has been reported (Poniedziałek et al., 2012). The cyanotoxin cylindrospermopsin (CYN), an alkaloid consisting of a hydroxymethyluracil moiety linked to a tricyclic guanidine (Ohtani et al., 1992) (Fig. 1), was first isolated from a culture of *Cylindrospermopsis raciborskii* (Ohtani et al., 1992).

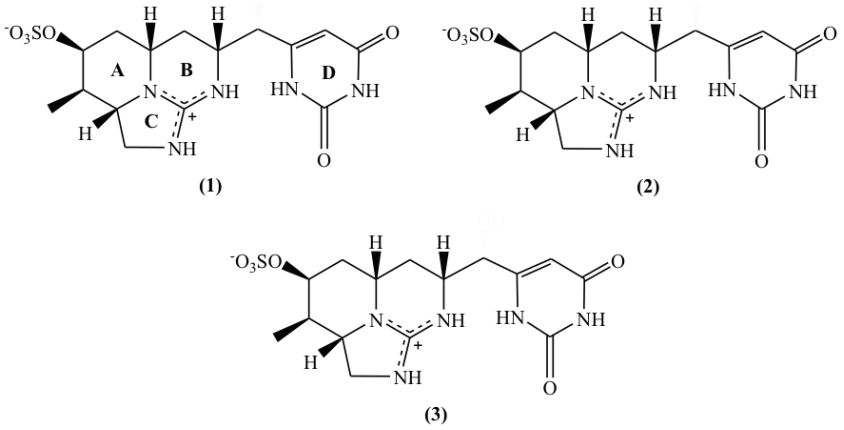

Fig. 1. The molecular structure of cylindrospermopsin **(1)** and its analogs 7-deoxy-cylindrospermopsin **(2)** and 7-epicylindrospermopsin **(3)**.

Since then, several CYN-producing cyanobacteria have been identified, including *Umezakia natans* (Terao et al., 1994), *Aphanizomenon ovalisporum* (Banker et al., 1997), *Raphidiopsis curvata* (Li et al., 2001), *Lyngbya wollei* (Seifert et al., 2007), *Anabaena bergii*, *Aphanizomenon flos-aquae* (Preussel et al., 2006), and *Anabaena lapponica* (Spoof et al., 2006). Structural variants of CYN, such as 7-epi-CYN and deoxy-CYN (Banker et al., 2000; Norris et al., 1999; Seifert et al., 2007) (Fig. 1), have also been identified. Although the molecular mechanism of CYN toxicity is not yet established, it inhibits eukaryotic protein synthesis (Froscio et al., 2008; Runnegar et al., 2002; Terao et al., 1994) and glutathione (GSH) synthesis (Runnegar et al., 1995). Furthermore, the metabolic activation of CYN by cytochrome P450 (CYP450) contributes to its toxicity (Froscio et al., 2003; Runnegar et al., 1995). Due to the high CYP450 activity in hepatocytes, the liver seems to be the main target organ of sub-chronic oral exposure (Froscio et al., 2003; Runnegar et al., 1995). Nevertheless, although to a lesser extent, toxic effects of CYN have also been reported in the kidneys, thymus, spleen, lungs, intestine and heart (Falconer et al., 1999; Froscio et al., 2003; Runnegar et al., 1995; Terao et al., 1994). Studies in mice have shown that the overall toxic effects of CYN are delayed and progressive (LD_{50} of 2100 µg/kg at 24 h and 200 µg/kg at 5–6 days, intra-peritoneal administration (Ohtani et al. 1992)) and are exerted by the following mechanisms: (1) acute toxicity produced by CYP450-generated metabolites of CYN (Froscio et al., 2001; Runnegar et al., 1995) and (2) a slower toxicity generated by the inhibition of protein synthesis, non-dependent on CYN metabolism (Froscio et al., 2001, 2003). The bioactivation of CYN by CYP450 seems to be responsible for the induction of genotoxic the effects (Falconer and Humpage, 2006)

because CYP450 inhibitors appear to prevent DNA damage in *in vitro* assays (Humpage et al., 2005; Runnegar et al., 1995). A preliminary study suggests that tumors are generated in mice by oral exposure to CYN (Falconer and Humpage, 2001); however, it remains to be elucidated whether the genotoxic effects might increase the risk of carcinogenicity *in vivo*. Despite the lack of consistent epidemiological data, consumption of contaminated water and food is the major source of chronic human exposure to CYN. The first human intoxication associated with CYN was in Palm Island (Queensland, Australia in 1979), where 149 people supplied with drinking water from a reservoir with CYN-producing *C. raciborskii* suffered a hepatoenteritis-like illness (Griffiths & Saker, 2003). Since then, public health concerns over CYN exposure have increased, and the risks associated with this toxin are still under investigation. CYN is highly water soluble due to its zwitterionic character, and contrary to the majority of cyanotoxins, as much as 90% of the total CYN is outside of the cells dissolved in the surrounding water (Chiswell et al., 1999; Rücker et al., 2007). Furthermore, CYN can persist in the water because its photodegradation is very low under natural conditions (Wörmer et al., 2010). Thus, the high levels and persistence of CYN in water can potentiate its accumulation in a wide range of aquatic organisms. Previous studies have shown that aquatic organisms, especially bivalves, can accumulate high levels of CYN without lethal effect (Gutiérrez-Praena et al., 2013; Ibelings and Chorus, 2007; Kinnear, 2010; Saker et al., 2004). Based on the potential for human health risks, a provisional tolerable daily intake (TDI) of 0.03 µg/kg of body weight has been proposed by Humpage and Falconer (2003). The human exposure assessment due to the consumption of CYN-contaminated food has been based on the direct comparison between the CYN concentration in raw edible organisms and TDI (Ibelings and Chorus, 2007). However, edible organisms are usually stored and processed before consumption, and these practices may change the concentration of CYN available in the food. Recent studies with microcystin-LR, the most studied cyanotoxin, have shown that its recovery from food matrices is changed after applying common food storage and cooking practices, as well as proteolytic digestion (Freitas et al., 2014; Guzmán-Guillén et al., 2011; Morais et al., 2008; Zhang et al., 2010). According to our knowledge, and as was recently reviewed by Gutiérrez-Praena et al. (2013) there are no studies regarding the influence of different food storage and cooking procedures on the CYN concentration in edible organisms. Furthermore, from the health risk point of view, the oral bioavailability of CYN is an important parameter to consider because it can vary from the CYN contained in food matrices. The bioavailability of a contaminant depends on its (1) bioaccessibility, (2) transport across the intestinal epithelium into the portal vein and (3) metabolism, mainly in the liver (Versantvoort et al., 2005). The

bioaccessibility corresponds to the fraction of a contaminant that is released from the food matrix by digestive juices and can be seen as an appropriate indicator of the maximal oral bioavailability. Several *in vitro* digestion models can be used to determine bioaccessibility by simulating, in a simplified manner, the digestion processes in the mouth, stomach and small intestine (Guerra et al., 2012).

CYN is highly water-soluble and stable to extreme temperatures and pH (Chiswell et al., 1999); thus, knowledge of the influence of common food storage and processing practices, as well as human digestion, is required to achieve a more accurate human health risk assessment. Therefore, the aim of this study was to assess the changes in the CYN concentration in edible mussels with storage and processing time and to assess the bioaccessibility of CYN in raw and processed (steamed) edible mussels.

2. Material and methods

2.1. Reagents and chemicals

The mammalian enzymes α-amylase (A-3176), pepsin (P7000), trypsin (T0303), chymotrypsin (C4129), pancreatin (P1750) and bile salts (B-8756) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl, 99.5-100%), sodium chloride (NaCl, ≥98%), sodium phosphate monobasic (NaH_2PO_4, 98-102%), sodium hydrogen carbonate ($NaHCO_3$, 99%), ammonium chloride (NH_4Cl, A-0171), calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$, >99%), hydrochloric acid (HCl, 37%, g/g), glucosamine hydrochloride (>99%), bovine serum albumin (BSA, 98%), magnesium chloride ($MgCl_2$, 99%), sodium sulfate (Na_2SO_4, ≥99%), potassium phosphate monobasic (KH_2PO_4, ≥99%), glucuronic acid (97.5-102.5%), glucose (99.5%), uric acid (99%) and urea were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade potassium thiocyanate (KSCN, 99%) was purchased from Merck (Germany). All solutions were prepared with ultrapure water supplied by a Millipore water purification system (0.0054 µS/cm) (MilliQ water). The acetonitrile (ACN) and water used for CYN extraction were analytical grade, and all solvents used in liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis were high-purity LC-MS grade (HiPerSolv CHROMANORM® for LC-MS, VWR). The reagents used in the Z8 medium were analytical grade. Analytical grade purity CYN was used as the reference standard (Lot# 20050531, CAS No.:

143545-90-8, National Research Council, Canada).

2.2. Biological material – cyanobacterial culture and mussels

The exposure experiment was conducted with CYN-producing cells of *Cylindrospermopsis raciborskii* (LEGE 97047). Cyanobacteria were cultured to the death phase in Z8 medium (Kotai, 1972) (6-L flask) under fluorescent light (light/dark cycle of 14/10 h) and 25 ± 1 °C. Live mussels, *Mytilus galloprovincialis* (Lamark, 1819) with a mean shell length of 6.50 ± 0.56 cm (n=90), were purchased from a Portuguese local market as for human consumption. The mussels were acclimated for 2 weeks prior to the experiment in 20-L aquaria with seawater. During this period, the mussels were fed twice a week with *Chlorella vulgaris* (10^5 cells/mL). The water was renewed weekly.

2.3. Exposure and experiment

2.3.1. Mussels intoxication

The presence of CYN and the occurrence and growth of *C. raciborskii* in marine ecosystems have not yet been documented. However, recently, Vareli et al. (2012) found microcystins (freshwater cyanotoxins) in concentrations ranging from 45 ± 2 to 141.5 ± 13.5 ng/g in specimens of *M. galloprovincialis* collected from the Mediterranean Sea, NW Greece. This raised attention for the potential negative impacts of freshwater cyanotoxins in marine aquaculture in Mediterranean estuaries. Therefore, this study attempted to simulate the contamination of estuarine or seashore ecosystems with senescent toxic cyanobacterial blooms. Mussels were intoxicated with cyanobacterial crude extract (cells and culture medium) for 4 days in static conditions (≈ 10 to 15 µg/L of CYN per day). During the exposure experiment, the physical and chemical conditions of the seawater were as follows: temperature 17.5 ± 1.3°C, salinity 32.84 ± 0.58 ‰ and pH 8.25 ± 0.23.

2.3.2. Simulation of food storage and processing practices

The common food storage and processing practices applied in this study were performed according to Freitas et al. (2014), with some modifications. Briefly, at the end of 4 days of exposure, mussels were rinsed with tap water and weighed, and sets of six animals for each treatment (two mussels per replicate, three biological replicates, *n*= 6) were submitted to common food storage (refrigeration and freezing) and processing (boiling, steaming and microwaving) procedures for different periods of time, as shown in Table 1.

Table 1. Food storage and processing practices applied in the experiment.

	Storage process			Cooking process		
Conditions	**25º C**	**4º C**	**- 20º C**	**Microwaving (550 W)**	**Boiling (100° C)**	**Steaming**
Period of time	0 h	24 h	48 h	0.5 min	5 min	10 min
	24 h	48 h	1 week	1 min	10 min	15 min
			1 month		15 min	

After exposure, one group of mussels was depurated for 8 days (the seawater was renewed every 2 days). At the end of the depuration period, mussels were analyzed raw and after steaming for 10 min. The experimental control group was not stored or processed but was immediately extracted for toxin quantification. The boiling time began after the water reached 100 °C. The ratio of water:mussels in the processing treatments was 10 mL of MilliQ water:two mussels. The remaining water in which the mussels were cooked was also analyzed, and its evaporation was not prevented throughout the experiment to simulate the usual household cooking procedures.

2.3.3. Mussels *in vitro* digestion

The *in vitro* model used to simulate the human digestion was adapted from Hur et al. (2009), Maulvault et al. (2011) and Versantvoort et al. (2005), with some modifications. Briefly, after exposure, mussels were rinsed with tap water and weighed. Uncooked and cooked mussels (steamed, 10 min as in 2.3.2) were removed from their shells and

homogenized with a blender for 1 to 2 min. Approximately 2 g (ww) of the homogenate from both treatments was transferred to a 50-mL sterile centrifuge tubes and then submitted to a chemical digestion, as shown in Fig. 2.

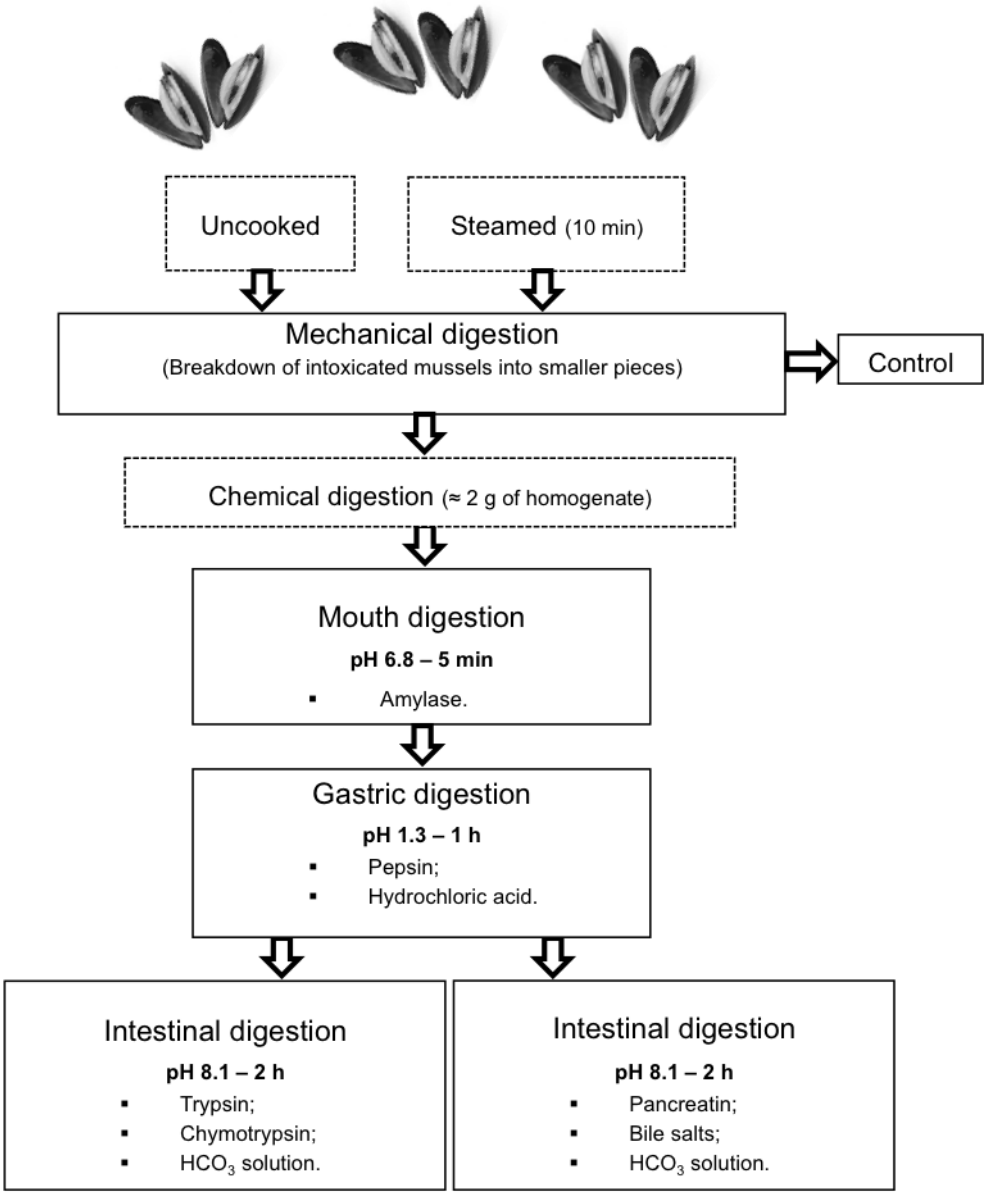

Fig. 2. Schematic representation of the *in vitro* digestion model used in the experiment.

The digestive procedure was performed in triplicate, and samples were sequentially incubated in a shaking water bath (GFL 1083, GFL, Germany) at 37 ºC for (1) 5 min with salivary juice (6 mL; 12 tubes), followed by (2) 1 h with gastric juice (12 mL; 9 tubes) and (3) 2 h with duodenal juices, which were individually tested, simulating digestion in the small intestine by the proteolytic enzymes trypsin and chymotrypsin (12

mL and 2 mL HCO_3 (1 M); 3 tubes) or by whole pancreatic juice, containing pancreatic proteolytic enzymes and pancreatic amylase and lipase (12 mL, 6 mL bile juice and 2 mL HCO_3 (1 M); 3 tubes). The composition of the digestive juices is shown in Table 2.

Table 2. Composition (constituents and concentration) of digestive juices used in the *in vitro* digestion, representing fed conditions. Adapted from Hur et al. (2009), Maulvault et al. (2011) and Versantvoort et al. (2005).

Components			**Digestive juices (g/L)**		
	Saliva	Gastric	Duodenal (pancreatin)	Duodenal (proteases)	Bile
Inorganic solution	0.9 g KCl	0.82 g KCl	0.56 g KCl	0.56 g KCl	0.38 g KCl
	0.3 g NaCl	2.75 g NaCl	7 g NaCl	7 g NaCl	5.26 g NaCl
	1.7 g $NaHCO_3$		3.39 g $NaHCO_3$	3.39 g $NaHCO_3$	5.79 g $NaHCO_3$
			0.08 g KH_2PO_4	0.08 g KH_2PO_4	
	0.89 g NaH_2PO_4	0.27 g NaH_2PO_4			
	0.2 g KSCN	0.31 g NH_4Cl	0.05 g $MgCl_2$	0.05 g $MgCl_2$	
	0.58 g Na_2SO_4				
		0.4 g $CaCl_2 \cdot 2H_2O$	0.2 g $CaCl_2 \cdot 2H_2O$	0.2 g $CaCl_2 \cdot 2H_2O$	0.22 g $CaCl_2 \cdot 2H_2O$
		6.5 mL HCl 37% g/g	180 µL HCl 37% g/g	180 µL HCl 37% g/g	150 µL HCl 37% g/g
Organic solution	0.2 g Urea	0.085 g Urea	1 g Urea	1 g Urea	0.25 g Urea
		1 g BSA	1 g BSA	1 g BSA	1.8 g BSA
		0.02 g Glucuronic acid			
		0.65 g Glucose			
		0.33 g Glucosamine hydrochloride			
Bioactive (Enzyme)	0.29 g α-Amylase	2.5 g Pepsin	9 g Pancreatin	9 g Trypsin	30 g Bile salts
	0.015 g Uric acid			9 g Chymotrypsin	
pH	6.8 ± 0.2	1.3 ± 0.02	8.1 ± 0.2	8.1 ± 0.2	8.2 ± 0.2

At the end of each digestive stage (mouth, stomach and small intestine), samples were stored at 4 °C to stop the enzymatic reactions. The soluble and non-soluble fractions from each digestive phase were obtained by centrifugation (Thermo Scientific – Legend^{TM} T/RT QUIKset^{TM}, Germany) (20 min, 4 °C, 4495 *g*). Groups of intoxicated mussels, either raw or cooked, without chemical digestion were used as experimental controls. Purified CYN in solution (100 ng/mL) was also subjected to the same enzymatic treatments.

2.4. CYN determination

2.4.1. CYN extraction and clean-up

To avoid any interference in the final concentration of the toxin through sample freezing/thawing, the CYN from all samples (except the soluble fractions from the digestion experiments, which were lyophilized) was immediately extracted after each treatment.

The mussels of section 2.3.2 and solid the fractions of section 2.3.3 were weighed, removed from their shells and homogenized with a blender for 1 to 2 min. Approximately 1 g (ww) of the homogenate was transferred to 50-mL sterile centrifuge tubes, and CYN was extracted twice in 5 mL of 90% ACN by ultrasonication (750 Watt, 20 KHz) (BioBlock Scientific, Vibracell 75041) in an ice bath for 1 min (pulse 1 second, amplitude 40) and was then centrifuged (Eppendorf, 5810R) (5 min, 4 °C, 3200 *g*). For the clean-up of the extracts, both supernatants were pooled in 0.5 g of C18 powder (Bakerbond Octadecyl 40 µm, Prep LC Packing, J.T. Backer) and homogenized for 1 min at 25 °C and 1300 rpm (thermomixer comfort Eppendorf MTP). The supernatant (9 mL) was recovered by centrifugation (1 min, 20 °C, 2000 *g*), and 900 µL was then evaporated to dryness in a SpeedVac (Eppendorf). The residue was re-suspended in 200 µL 0.1% formic acid (FA) in water (v/v). Samples were stored at -80 ºC until further LC-MS/MS analysis.

2.4.2. LC-MS/MS analysis

The quantification of CYN was conducted by the linear regression of six-point calibration curves of mussel matrix spiked (in triplicate) with a standard solution of CYN (50, 100, 200, 400, 800 and 1600 ng/g). The lowest standard of the calibration curve (50 ng/g) was the limit of quantification. The potential interference of matrix components in the LC-MS/MS chromatograms was also assessed (in three independent pools in triplicate), and the method was selective for CYN quantification (data not shown).

Samples were analyzed on an Ultimate™3000 LC system (LC Packings, Dionex) coupled to an ESI Turbo V ion source and a hybrid triple quadrupole/linear ion-trap 4000 QTrap mass spectrometer operated by Analyst 1.6.1 (AB Sciex). The chromatographic separation was performed in a 3-µm Luna NH_2 column (150 × 2.0

mm, 100 Å, Phenomenex) with a 4 × 2.0 mm NH_2 guard-column (Phenomenex). The flow rate was set to 150 µL/min, and mobile phases A and B were 0.1% FA in water and 0.1% FA in ACN, respectively. The LC program started with a linear gradient from 50% to 5% B (0 - 0.5 min), and it was maintained at 5% B for 6 minutes. After each sample analysis, a 6-minute run was performed in isocratic mode with 50% B for column equilibration. The ionization source was operated in the positive mode set to an ion spray voltage of 5500 V, 30 psi for nebulizer gas 1 (GS1), 20 psi for nebulizer gas 2 (GS2), 30 psi for the curtain gas (CUR), and the temperature was 450 °C. Cylindrospermopsin was quantified by multiple reaction monitoring (MRM) triple quadrupole scan mode at unit resolution both in Q1 and Q3, and the MRM transitions were 416.4/194.3 (used for quantification), 416.4/176.1 and 416.4/336.5 (both used for compound confirmation). The MRM parameters were 10 eV for the entrance potential (EP), 15 eV for the collision cell exit potential (CXP), and 100 V for the declustering potential (DP), and the collision gas (CAD) was set to 8 psi. The dwell time was 100 ms, and the collision energies were 50 eV for transition 416.4/176.1, 60 eV for transition 416.4/176.1 and 32 eV for transition 416.4/336.5. The peak areas were integrated using MultiQuant software (version 2.1, AB Sciex).

2.5. Statistical analysis

Statistical analysis of the data was performed by one-way ANOVA after checking the homogeneity of variance with the Levene test. When necessary, data were transformed to achieve the variance homogeneity assumption. The Tukey multiple range test was used to compare means whenever differences were detected by ANOVA at the significance level of 0.05. All analyses were performed with IBM SPSS Statistics, version 22. Four individual data groups were considered for statistical analysis: (1) CYN concentration in the control group and CYN concentration after applying the storage practices; (2) CYN concentration in the control group and CYN concentration after applying the cooking practices; (3) CYN concentration in the control group and CYN concentration in mussels depurated either raw or cooked; and (4) CYN in solution before and after *in vitro* digestion. Significant differences were also tested between different periods of time in the groups belonging to the storage and processing treatments. The results are expressed in ng/g (ww) as the mean ± standard deviation (SD) for three replicates for each treatment.

3. Results and Discussion

The estimation of human exposure to CYN by food consumption is an essential element for quantifying the risk; thus, data for the concentration of CYN in edible organisms are required. In this study, marine mussels (*M. galloprovincialis*) were able to accumulate CYN (28.1 ± 4.9 to 41.6 ± 5.7 ng/g; whole organism) when exposed to ecologically relevant concentrations (≈ 10 to 15 µg/L) for 4 days, showing neither mortality nor apparent detrimental effects on their organoleptic characteristics. As mentioned above, Vareli et al. (2012) recently found microcystins in *M. galloprovincialis* collected from the Mediterranean Sea, indicating that this edible aquatic organism could contribute to chronic human exposure to cyanotoxins. Shellfish is an important component of the human diet; thus, the study of the influence of common food storage and processing practices and the human digestion on CYN availability in these edible organisms is of major significance to provide more accurate data on human exposure to this cyanotoxin.

3.1. Effects of storage on the CYN concentration in edible mussels

The storage process is a critical step to provide safe and high-quality shellfish for human consumption. Thus, in this study, we intended to determine if different storage conditions lead to changes in the levels of CYN in edible mussels. The concentration of CYN in mussels stored unrefrigerated (25 ºC) for 24 h (43.1 ± 4.1 ng/g) and stored refrigerated (4 ºC) for 24 h (45.3 ± 0.8 ng/g) or 48 h (55.7 ± 14.6 ng/g) did not significantly differ from the control group (41.6 ± 5.7 ng/g) (Fig. 3).

The chemical stability of CYN is an important factor associated with its environmental persistence and toxicity. In aqueous media, the stability of CYN has already been demonstrated at temperatures ranging from 4 to 50 ºC for up to 4 weeks (Chiswell et al., 1999).

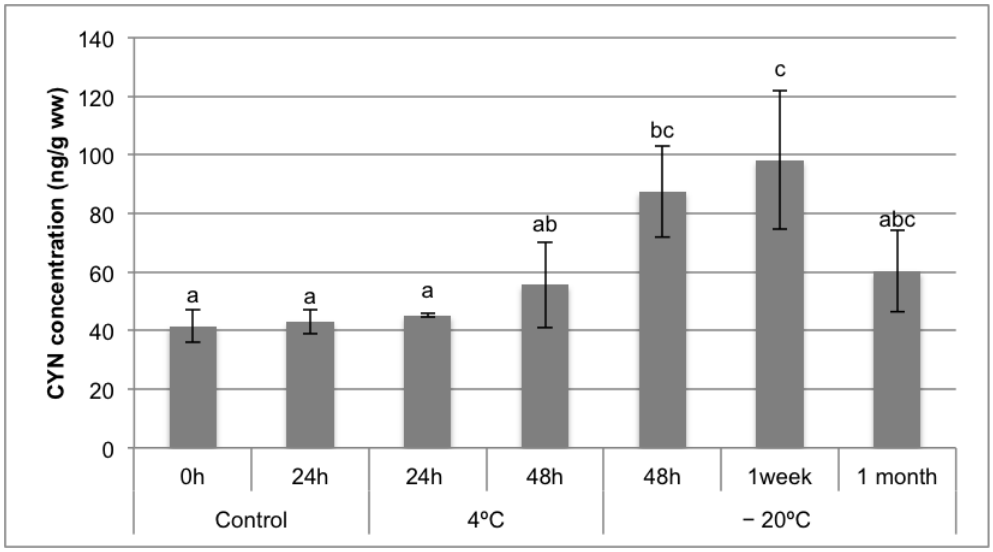

Fig. 3. The concentration of CYN (ng/g) in the mussel matrix submitted to different storage treatments at different periods of time. Values are expressed as the mean ± SD (n = 3). Different letters (a, b and c) indicate significant differences ($p < 0.05$). Columns that share the same letter are not significantly different.

In mussels stored frozen (-20 ºC), the recovery of CYN increased, especially after 48 h (87.6 ± 15.6 ng/g) and 1 week (98.2 ± 23.6 ng/g) of storage, where the concentration of CYN was significantly higher, 52.5% and 57.7%, respectively, ($p < 0.05$) than the control group (Fig. 3). Froscio et al. (2008) showed that in reticulocyte lysates [^{14}C], CYN was partially released by incubation with an excess of unlabeled CYN, suggesting that the toxin binds reversibly. Thus, our results can be associated with a more efficient extraction of CYN from mussel tissues due to the potential cell disruption and protein denaturation caused by freezing/thawing. These results may have relevance for monitoring programs of CYN in edible organisms in which the frozen storage of samples before analysis is typical. Furthermore, this increase in CYN recovery from the mussel matrix may suggest that higher levels of the toxin are available when mussels are consumed after being frozen.

3.2. Effects of cooking on the CYN concentration in edible mussels

Shellfish is generally cooked prior to consumption to enhance its microbiological safety and flavor. However, cooking treatments can also alter the availability of chemical contaminants in food (Domingo, 2011). As recently reviewed by Gutiérrez-Praena et al. (2013) and Kinnear (2010), several studies have assessed the concentration of CYN in

edible organisms; however, all of the analyses were performed in uncooked/raw matrices. In this study, we investigated the effects of boiling, steaming and microwaving for different periods of time on the CYN concentration in mussels. Overall, the application of these processing treatments, i.e., boiling (5, 10 and 15 min), steaming (10 and 15 min) and microwaving (0.5 and 1 min), did not produce significant changes in the concentration of CYN in mussels in comparison with the control group (41.6 ± 5.7 ng/g, $p < 0.05$) (Fig. 4), suggesting that cooking does not remove or produce alterations in the CYN availability in the mussel matrix (flesh).

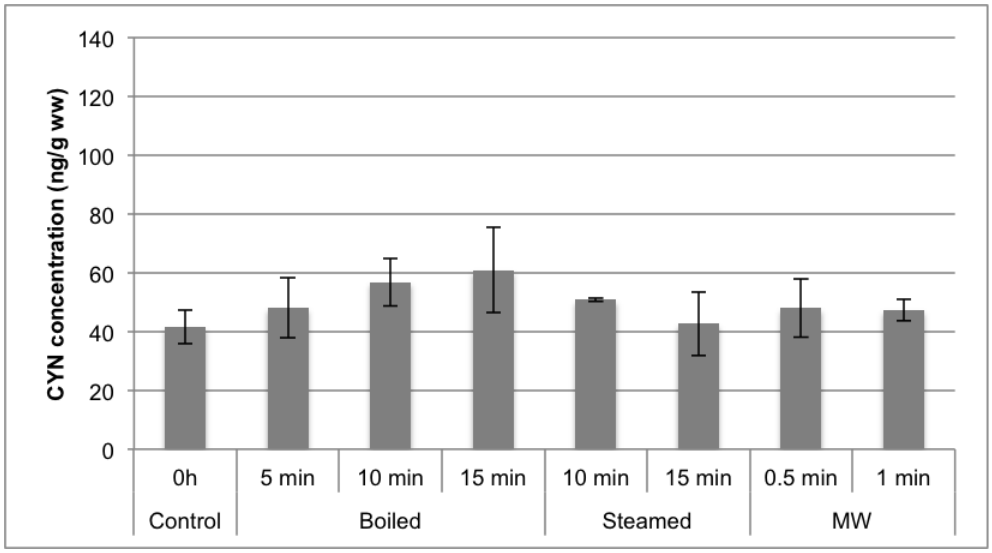

Fig. 4. The concentration of CYN (ng/g) in the mussel matrix submitted to different processing conditions at different periods of time. Values are expressed as the mean ± SD (n = 3).

However, because a substantial concentration of the toxin (also not significantly different between treatments) was found in the water in which the mussels were cooked (Fig. 5), the CYN quantified in the mussel matrix is not representative of the real amount available in these organisms.

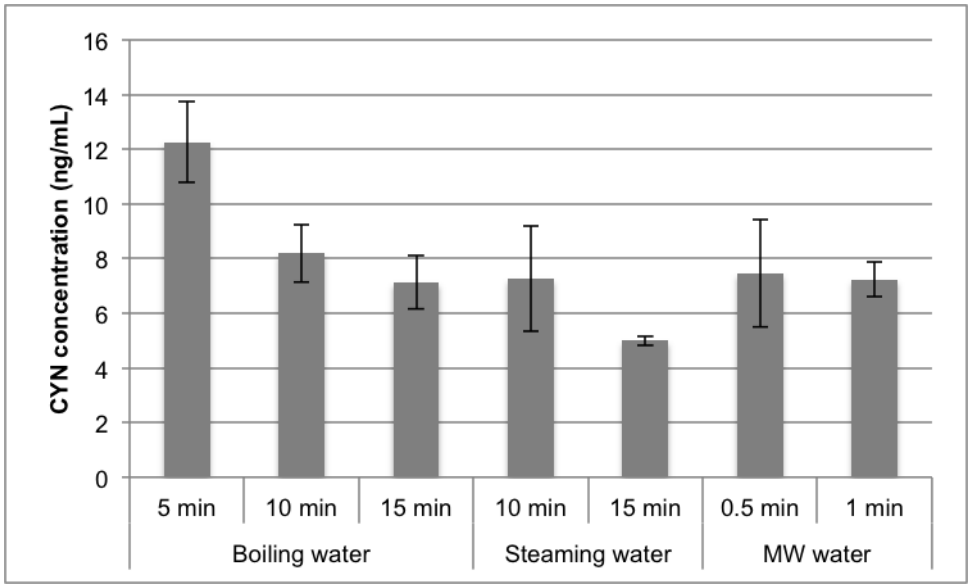

Fig. 5. The concentration of CYN (ng/mL) in the water in which the mussels were cooked. Values are expressed as the mean ± SD (n = 3).

In fact, heat processing causes a loss of water containing soluble compounds from the mussel matrix into the intervalval fluid, which leaks into the cooking water due to the shell opening shortly after the beginning (approximately 3 min) of the treatment. Therefore, it is likely that this fluid is the source of the CYN found in the cooking water. In view of this, the potential human exposure to CYN due to the consumption of contaminated mussels based on the amount of CYN in raw mussels will be under-evaluated. On the other hand, because the heat processing enhances the CYN extraction from the mussel tissues, this practice can be applied to reduce its availability in this food item by simply disposing of the cooking water. This can be relevant for other industrial processes with mussels, such as canning and brine. According to the Food and Agriculture Organization of the United Nations (FAO), canning represents approximately 40 to 50% of the mussel market. Normally, mussels are precooked in steam or boiling water to separate the edible portion from the shells, and the remaining liquor is kept for reuse in canning or brine. The latter procedure should be avoided because the sauce is usually ingested together with the mussels, which may potentially increase the human exposure to CYN. Considering the Hazard Analysis Critical Control Points (HACCP) system set by the *Codex Alimentarius Commission*, our results provide new insights to identify critical control points and to determine critical limits for the culinary preparation of mussels. The heat processing and the disposal of the cooking water are suitable procedures to at least reduce the availability of CYN in

mussels. In addition to heat processing, we hypothesized that depuration could play a role in the reduction of CYN in mussels. Thus, the effect of depuration on the CYN concentration in raw and steamed (10 min) mussels was also considered (Fig. 6).

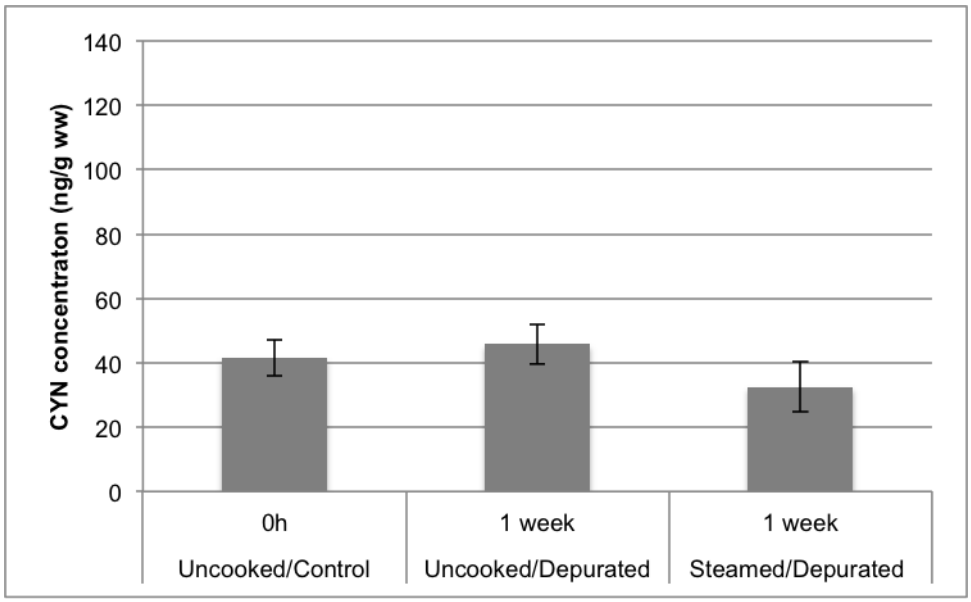

Fig. 6. The concentration of CYN (ng/g) in uncooked (control), uncooked depurated (1 week) and steamed (10 min) depurated mussels. Values are expressed as the mean ± SD (n = 3).

However, the concentration of CYN in uncooked/depurated and steamed/depurated mussels was not significantly reduced in comparison with the control group ($p < 0.05$), suggesting that this process has no effect on the CYN availability in the mussel matrix. Saker et al. (2004) studied the depuration pattern of CYN in the freshwater mussel *Anodonta cygnea* for 2 weeks and found a decrease in the CYN concentration at the beginning of the depuration period (first 4 days), followed by a rise from the 6th to the 12th day of depuration. Depuration presupposes that shellfish expel contaminants from their gills and intestinal tract by pumping clear seawater. Once CYN is mostly dissolved in the surrounding medium and not inside the cells (Chiswell et al., 1999; Rücker et al., 2007), its accumulation in the intestinal tract due to the ingestion of cyanobacterial cells is residual, which is probably why depuration was not effective in reducing CYN in mussel tissues. However, CYN was not detected in the steaming water of depurated mussels (data not shown), in contrast to the steaming water of non-depurated mussels in which the concentration of CYN was of 7.27 ± 1.94 ng/mL (Fig. 5). Although the depuration process had no effect on the CYN concentration in mussel tissues (flesh),

an amount of the toxin was removed by this procedure, most likely due to the renewal of the intervalval water of the mussels.

3.3. Bioaccessibility of CYN

The ingestion of contaminated food is an important route of chronic exposure to CYN. However, the human health risk due to the presence of CYN in food depends on its release from the food matrix by the digestive juices, i.e., its bioaccessibility. Usually, food is cooked before ingestion, which can enhance the bioaccessibility of contaminants due to the increased digestibility of the food constituents (e.g., proteins because of denaturation). In this study, the calculation of CYN bioaccessibility in raw and cooked mussels was attempted using an *in vitro* digestion model, which mimics the composition of digestive juices along the human digestive tract (mouth, stomach and small intestine) (Hur et al., 2009; Maulvault et al., 2011; Versantvoort et al., 2005).

In general, CYN was detected after all digestion steps; however, the final concentrations were below the limit of quantification (50 ng/g) in both the liquid (bioaccessible) and solid fractions (residue resulting from each digestive phase). This was probably due to the relatively low initial concentration of CYN in mussels (28.1 to 51.9 ng/g, after 4 days of exposure). Freshwater organisms can be exposed for longer periods and can accumulate much higher concentrations of CYN, as in the case of the freshwater mussel *Anodonta cygnea*, which can accumulate up to 50-100 times more CYN (2.5 µg/g) when exposed to a CYN-containing *C. raciborskii* extract for 16 days (Saker et al., 2004). Nevertheless, the peak areas of the chromatograms obtained suggest that the sequential exposure of mussels to digestive juices progressively decreases the availability of CYN to levels near zero in both fractions (Figs. 7 and 8).

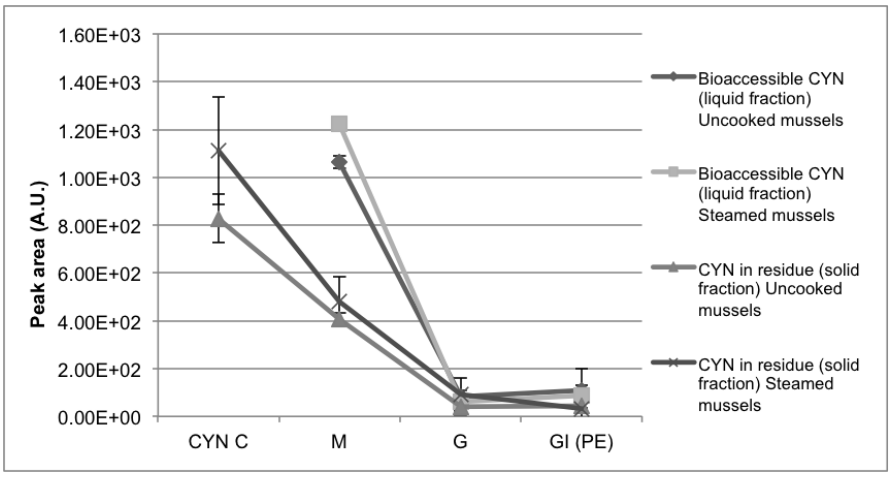

Fig. 7. Chromatographic peak area of CYN detection from uncooked and steamed mussels before (CYN C) and after digestion (for liquid and solid fractions). Letters represent each digestive step: mouth (M), gastric (G) and gastrointestinal (GI) with proteolytic enzymes (PE). Values are expressed as the mean ± SD (n = 3). A.U., Arbitrary Units.

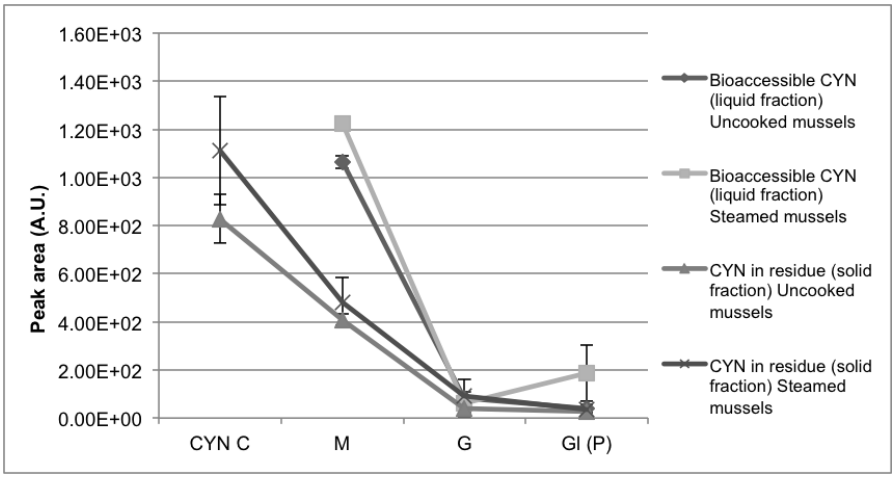

Fig. 8. Chromatographic peak area of CYN detection from uncooked and steamed mussels before (CYN C) and after digestion (for liquid and solid fractions). Letters represent each digestive step: mouth (M), gastric (G) and gastrointestinal (GI) with pancreatic juice (P). Values are expressed as the mean ± SD (n = 3). A.U., Arbitrary Units.

To confirm the decrease in the CYN content after being submitted to digestive juices and to exclude any interference of the food matrix on CYN detection in the bioaccessible fraction (toxin bound to proteins or other molecules), purified CYN in solution (100 ng/mL) was submitted to the same *in vitro* digestion model. The results show the same trend of the intoxicated mussels, where the digestive juices sequentially

reduced CYN to levels near zero (Fig. 9).

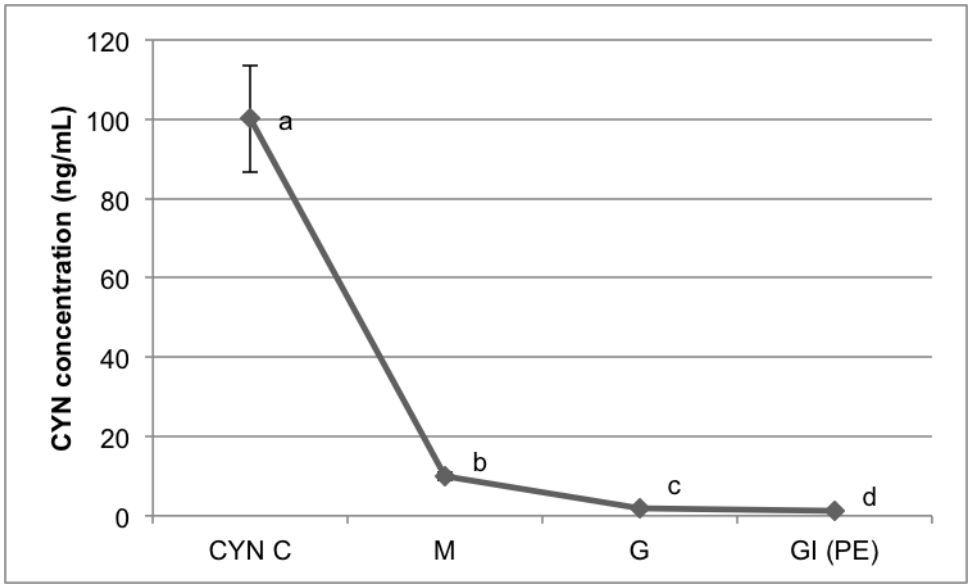

Fig. 9. The concentration of CYN (ng/mL) before (CYN C) and after digestion of the free form in solution (purified CYN). Letters represent each digestive step: mouth (M), gastric (G) and gastrointestinal (GI) with proteolytic enzymes (PE). Values are expressed as the mean ± SD (n = 3). Different letters (a, b, c and d) indicate significant differences (p<0.05). Columns that share the same letter are not significantly different.

The incubation of CYN with salivary juice promoted the most significant reduction on its concentration (90%, $p < 0.05$). Then, there was a progressive decrease in CYN availability after digestion with gastric juice (G) and intestinal proteolytic juice (PE) (98 and 99%, respectively, $p < 0.05$). This result supports the finding that CYN-producing *C. raciborskii* is at least 25 times less toxic to mice by the oral route than by the intraperitoneal route (Falconer et al., 1999). The action of the digestive juices in alkaloid-CYN degradation is unclear. Merkel et al. (2012), using a similar *in vitro* digestion model, also found degradation (to a moderate extent) of some ergot alkaloids by digestive juices which was attributed to the presence of the digestive enzymes and varying pH conditions. On the other hand, it was recently shown that some plant alkaloids can bind to bovine α-chymotrypsin (Zsila et al., 2011) and fungal α-amylase (Tintu et al., 2012). Therefore, the possibility of the decrease in the CYN concentration measured during this experiment being due to the binding of CYN to digestive enzymes and its subsequent non-detection cannot be discarded.

To our knowledge, this is the first report presenting the potential effects of human digestion on CYN contained in a food item. According to our findings, it is reasonable to assume that at the tested concentrations, CYN itself would not represent a risk to human health. The assumption that the oral bioavailability of CYN is similar to the levels quantified in raw and cooked mussels is erroneous; thus, the comparison of TDI with CYN extracted from food items is not recommended. In this way, the bioaccessibility of CYN must be integrated into the health risk assessment. In fact, as referred to above, Falconer et al. (1999), by comparing the effects of various batches of *C. raciborskii* in mice, found that oral toxicity is at least 25-fold lower than intraperitoneal toxicity. Associated with the potential effects of digestive juices, some other important factors may reduce the bioavailability of CYN, such as (1) uptake being potentially dependent on a transporter; (2) hepatic metabolism (Norris et al., 2002); (3) removal by intestinal microflora (Nybom et al., 2008); and (4) urinary and fecal excretion (Norris et al., 2001). Finally, although human digestion substantially reduces the CYN availability, the absence of health risks due to consumption of contaminated mussels should be considered cautiously because the presence of additional toxins in the *C. raciborskii* extracts has been reported (Falconer et al., 1999). Thus, the study of the effects of other toxins, as well as the enzymatic-generated metabolites of CYN, should be developed in the future.

4. Conclusion

In conclusion, this study fills a gap in the knowledge of the influence of food storage and cooking practices on the levels of CYN in edible organisms. Our results show that the recovery of CYN can be enhanced with frozen storage, and its concentration in raw mussels does not represent the total amount of toxin available. Although there were no significant differences in CYN concentration in raw, cooked and depurated mussel matrices (flesh), heat processing and depuration are suitable procedures to reduce the toxin in this edible organism through the removal/renewal of the intervalval fluid. More importantly, in the industry, these practices can be easily applied and should be considered in the implementation of HACCP systems.

This study also provides the first insights into the effects of human digestion on CYN. At the concentrations tested, the digestive juices were effective in the reduction of CYN content, whether present in solution or in the raw and steamed mussel matrices.

According to these findings, CYN itself would not represent a risk to human health, which highlights the importance in integrating the bioaccessibility of CYN as a part of the risk assessment because the amount of toxin effectively available for absorption is much lower than the amount of toxin quantified in raw and cooked mussels. Further studies should be developed to identify the contributing factors of CYN removal, particularly the role of digestive juices.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Chapter 5

General conclusions and future research

5.1. General discussion and conclusions

The occurrence of toxic cyanobacterial blooms is expected to become increasingly recurrent, which creates an important challenge for agricultural productivity, environmental management and human health.

The toxic effects of cyanotoxins on crop plants are poorly studied, especially for CYN and its mixture with MC-LR. In the present work (Chapter 3) the effects of ecologically relevant concentrations of MC-LR, CYN and their mixture were determined in an important crop plant, lettuce. The overall effects on the parameters assessed are summarized in Table 1.

Table 1. The general results obtained regarding to the effects of MC-LR, CYN and the MC-LR/CYN mixture in lettuce plants.

Concentration of exposure (µg/L)		Growth (Fw)		GST activity		GPx activity		Mineral content		Protein abundance	
		Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
MC-LR	1	↑/↑	↑/↑	↑/↑	O/O	O/O	O/↑	-	↑/O	-	-
	10	↑/O	↑/↑	↑/↑	O/↑	O/O	O/O	-	O/O	-	-
	100	↑/↑	↓/O	↑/↑	O/O	O/↓	O/O	-	↓/↓	-	-
CYN	1	O/↑	O/O	↑/↑	O/↓	O/O	O/O	-	O/↑	-	↓
	10	O/↑	O/O	↑/↑	O/↓	O/O	O/O	-	↑/↑	-	↑
	100	O/↑	O/↓	↑/↑	O/O	↓/O	↓/O	-	↑/↑	-	↑
MC-LR/CYN	1	↑/O	↑/↑	↑/↑	O/↓	O/O	O/O	-	O/O	-	↑
	10	↑/O	O/↑	↑/↑	O/↓	O/↓	O/↓	-	O/O	-	↓
	100	↑/↑	↓/↓	↑/↑	O/O	↓/↓	↓/↓	-	O/↓	-	↓

↑ Increased; ↓ Decreased; O No effects; - Not measured; 5 Days/ 10 Days of exposure.

Lettuce plants in non-early stage of development were able to cope with MC-LR, CYN and their mixture, especially at the lower concentrations and at the lowest time of exposure, ensuring the maintenance of mineral uptake/translocation and the oxidative stress under control, as indicated by the increased activity of GST in roots.

The study of lettuce proteome allowed the understanding of the metabolic pathways activated in response to cyanotoxins, providing new insights into potential protein markers of exposure (e.g., SOD and APX) and the pathways that may confer tolerance to lettuce plants (enhancement of the capacity to perform photosynthesis and to synthesize ATP). With this study we hypothesize that the potential mechanism of tolerance of lettuce plants to CYN, at 5 days of exposure, can be promoted by the activation of mineral uptake and improving photosynthesis capacity.

However, the concentration and the time of exposure are preponderant factors for the toxic effects of these cyanotoxins in lettuce plants. The exposure of lettuce plants to 100 µg/L, especially of MC-LR and the MC-LR/CYN mixture, can compromise the crop yield and productivity, as demonstrated by the decrease of the growth and the mineral content in leaves (Table 1). In the exposure to the higher concentrations of MC-LR/CYN mixture, the impairment of photosynthesis and carbon metabolism; stress/defense response, and protein synthesis and signal transduction were also observed at proteome level. Thus, the use of water for irrigation contaminated with these cyanotoxins at concentrations ≥ 100 µg/L can lead to potential economic losses in crop production.

This study led also to the discovery of some traits associated with the quality and safety of leaves of lettuce plants exposed to environmentally relevant concentrations of MC-LR, CYN and the mixture of MC-LR/CYN. Among other nutrients, lettuce provides an important source of minerals and antioxidants for human nutrition. Leaves of lettuce plants exposed to CYN had an enhanced content of minerals and probably antioxidants, and this can be of major significance facing the challenge of food security, once lettuce was able to cope with abiotic stress and also maximize its nutritional quality.

On the other hand, lettuce accumulated putative allergenic proteins and probably also cyanotoxins. Hence, the covering of various parameters allowed a broader understanding of the mechanisms of action of these cyanotoxins in crop plants, and this can be an asset to take more targeted measures in a field poorly regulated, such as the use of surface water for agricultural production. Even so, the findings of this work indicate the importance to conduct further studies to evaluate the health significance and the risks of the utilization of irrigation waters contaminated with cyanotoxins in agriculture.

Regarding to human exposure to cyanotoxins, obviously, the best measure to reduce the occurrence of cyanotoxins in edible aquatic organisms is to control the toxic cyanobacterial blooms. However, the proliferation of toxic cyanobacterial blooms has been forecasted as increasingly recurrent as a consequence of the intensification of anthropogenic pressure and climate change. Furthermore, bivalves may contain unpredictable amounts of cyanotoxins in their tissues due to bioaccumulation and long-term elimination.

This work fills also an information gap concerning the influence of food storage and cooking practices as well as human digestion on the levels of MC-LR and CYN in edible bivalves (Chapter 4). The Table 2 summarizes the results obtained.

Table 2. The general results obtained on the changes of MC-LR and CYN in bivalves after applying different practices of food storage and processing as well as the effects of digestive juices.

	Condition	Time	MC-LR	CYN
Storage	25° C	12h	○	-
		24 h	○	○
		48 h	↓	-
	4° C	24 h	○	○
		48 h	↓	○
		72 h	↓	-
	- 20 ° C	48 h	↓	↑
		1 week	↑	↑
		1 month	↑	○
Cooking	Boiling	5 min	↑	○
		10 min	-	○
		15 min	↑	○
		30 min	↑	-
	Steaming	10 min	-	○
		15 min	-	○
	Microwaving	0.5 min	↑	○
		1 min	↑	○
		3 min	○	-
		5 min	↓	-
Toxin in the cooking water			-	X
Bioaccessibility		Raw	↓	↓
		Steamed 10 min	-	↓

↑ Increased in comparison to control group; ↓ Decreased in comparison to control group;

○ There were no effects in comparison to control group; - Not measured; X The toxin was found in cooking water.

Overall the results show that the recovery of MC-LR and CYN in clams and mussels, respectively, are enhanced with frozen storage. This indicates that the toxin measured from fresh clam and mussel tissues can underestimate the real amount available.

Thus, the extraction of MC-LR and CYN can be improved with this procedure and this may have relevance for the monitoring programs and human health risk assessment.

The cooking procedures influenced the availability of MC-LR and CYN in edible bivalves. It is important to highlight that although there were no differences in the concentration of CYN in processed mussel matrices in comparison to control group, the water in which mussels were cooked contained a significant amount of CYN. Thus, it is likely that the overall increase of the MC-LR availability after processing is related to the joint determination of free MC-LR content in both matrices, i.e., boiled clams together with the water in which they were boiled.

Despite the differences in the physico-chemical properties of these two cyanotoxins, both are hydrophilic molecules, thus, it was expected that a considerable amount would be leached into the cooking water. Thus, the combination of processing methods with MeOH and ACN extraction of MC-LR and CYN, respectively, could result in a more effective and representative determination of these cyanotoxins in food products.

Under this framework, once MC-LR and CYN are recognized as relevant chemical hazards in edible aquatic organisms, the development and implementation of risk management strategies are of critical importance. Thus, the treatments applied in this study can also be considered for the definition of critical control limits, considering the HACCP approach as a promising tool for risk management. In this way, the control of the processing time and the discarding of cooking water may be suitable procedures to reduce both cyanotoxins in edible aquatic organisms.

The bioaccessibility of MC-LR and especially CYN is highly reduced due to digestion with pancreatic proteolytic enzymes and salivary juice, respectively. This strongly reduces the potential toxicological risks because the concentration determined in fresh clams and mussels might considerably overestimate the the amount of toxin available for assimilation after consumption. Further studies should be developed to identify the contributing factors of the removal of cyanotoxins from the edible tissues of these aquatic organisms, i.e., if they are related to the degradation of cyanotoxins or, for instance, to the binding of these toxins with digestive enzymes.

Because the food consumption is one of the most important routes of exposure to cyanotoxins, the incorporation of the bioaccessibility in the human exposure estimation would be of particular relevance to achieve a more realistic human health risk assessment.

In conclusion, this dissertation provides substantial contributions towards a more rational identification of the relevant hazards as well as the application of more assertive management measures in the field of the human health and environmental risks associated to toxic cyanobacterial blooms.

5.2. Future research

Several hypotheses and questions were brought to light in this thesis, requiring further supporting evidences and scientific clarification. The following matters should be addressed in future research:

1 - To analyze the concentration of MC-LR and CYN in the edible tissues of lettuce plants. This work is already in progress.

In this topic it would be also of particular interest exploring the effects of these cyanotoxins in the accumulation of other constituents that compromise food safety such as allergenic proteins.

2 - To analyze non-enzymatic substances of antioxidative system, such as carotenoids, tocopherols and phenolic compounds to better characterize the nutritional quality of lettuce plants exposed to cyanotoxins.

3 – To analyze the activity of other antioxidant enzymes, which seem to be active (by proteomic analysis) in lettuce response to cyanotoxins exposure, such as SOD, CAT, APX.

4 – To characterize and validate the function of some identified proteins, that seems to confer tolerance to lettuce plants against CYN and MC-LR/CYN.

5 – To analyze the MC-LR bioaccessibility after applying different food storage and processing methods.

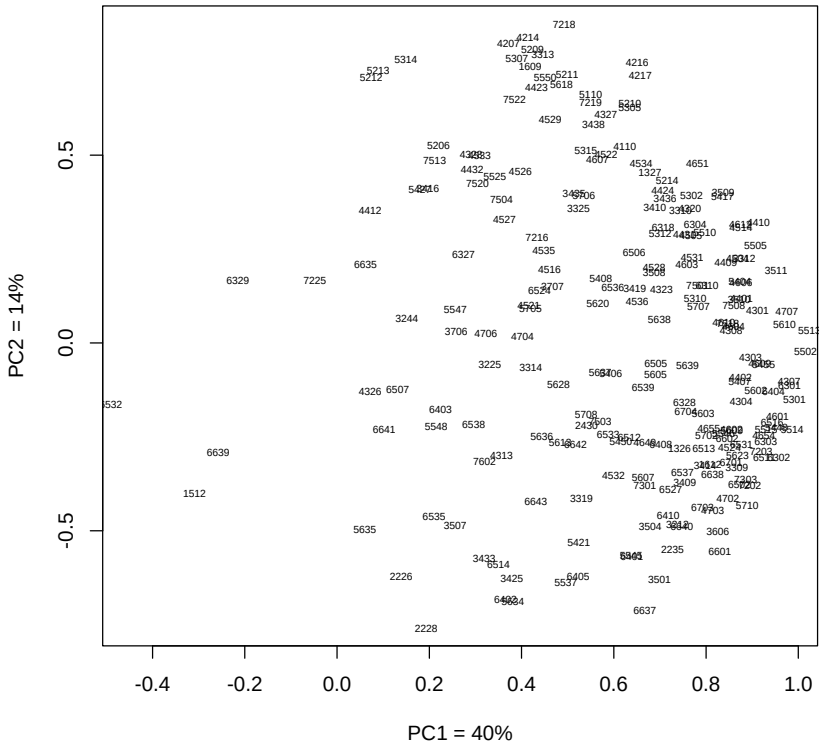
6 – To characterize the toxic potential of the products of digestion of MC-LR and CYN.

Chapter 6

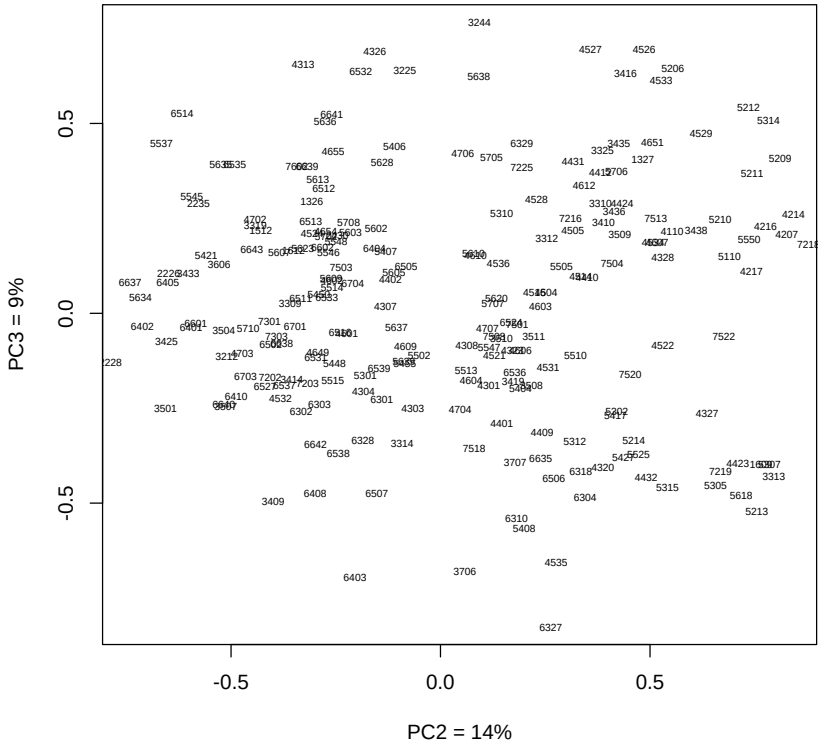
Supporting information

From the manuscript: **Marisa Freitas**, Alexandre Campos, Joana Azevedo, Aldo Barreiro, Sébastien Planchon, Jenny Renaut, Vitor Vasconcelos. Lettuce (*Lactuca sativa* L.) leaf-proteome profiles after exposure to cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture: a concentration-dependent response. Accepted for publication in the Journal Phytochemistry.

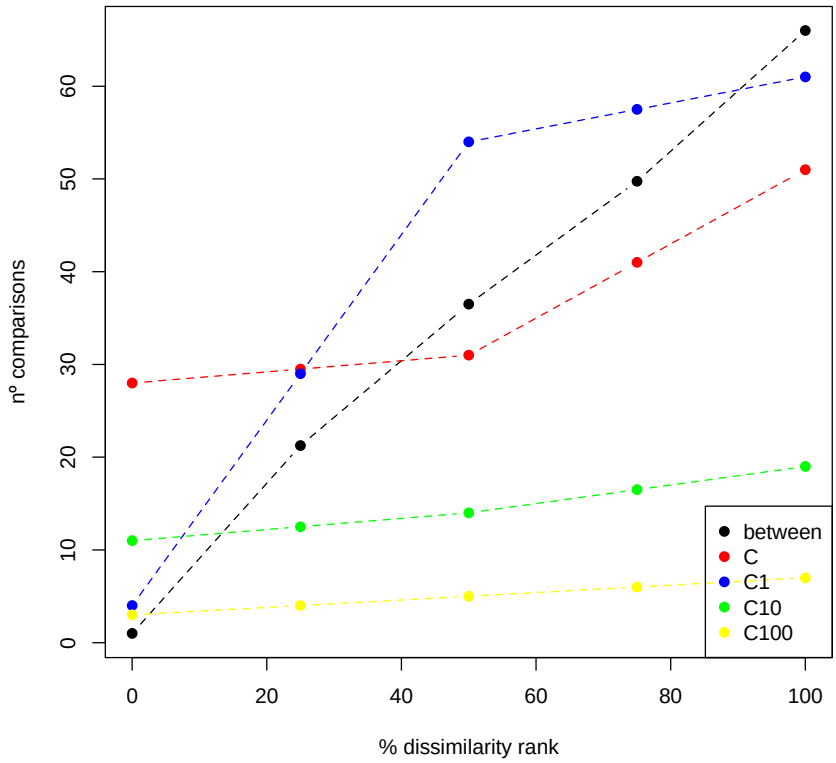
Supplementary figures associated with the CYN exposure experiment



Supplementary Figure 1: Variable representation of PCA along the first and second dimensions.

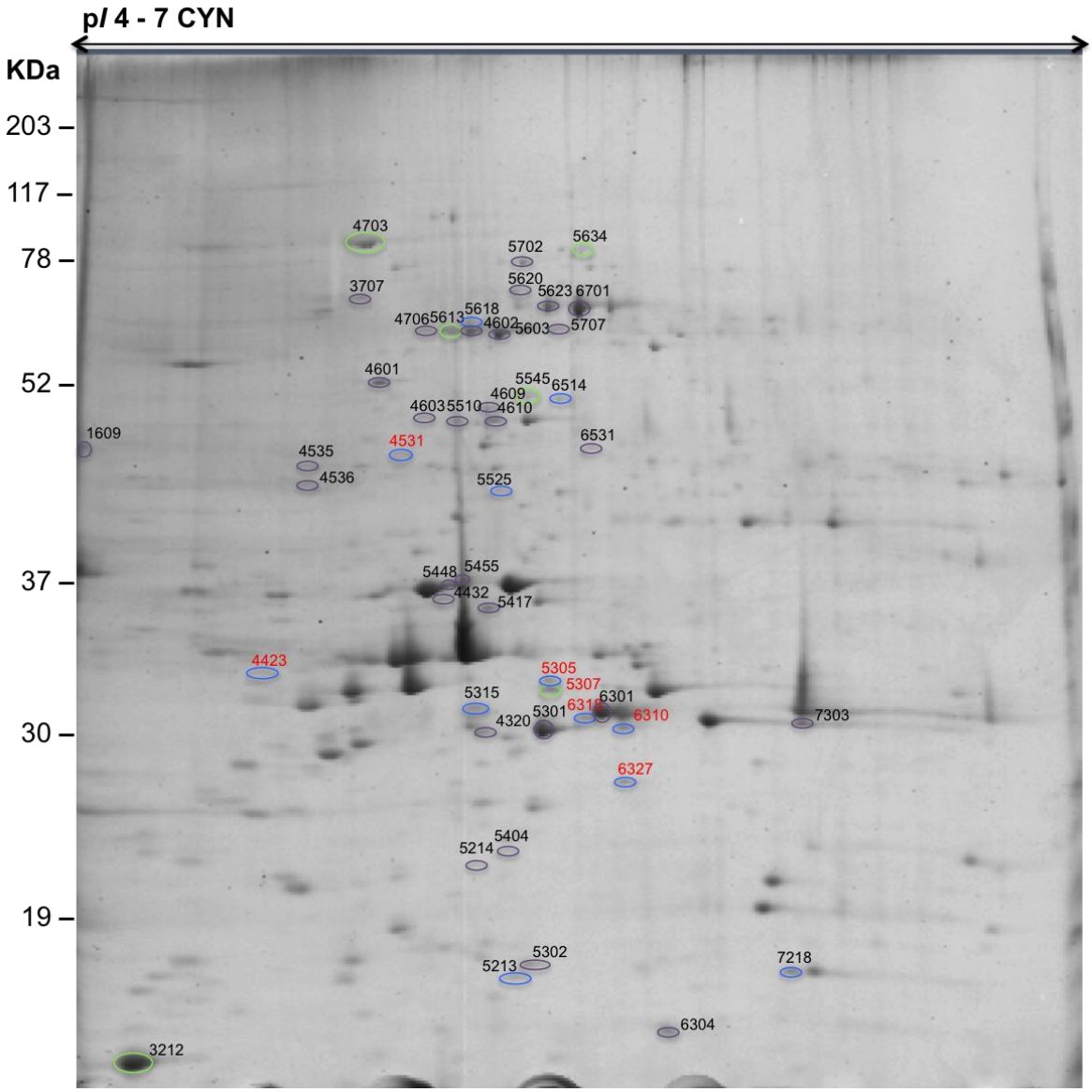


Supplementary Figure 2: Variable representation of PCA along the second and third dimensions.



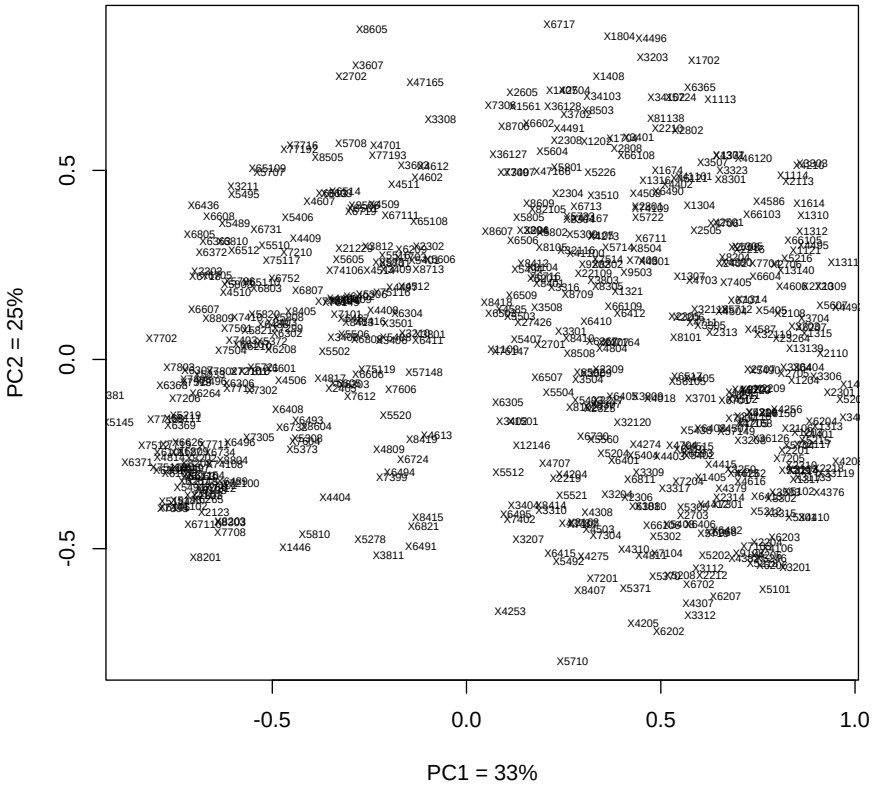
Supplementary Figure 3: Percentage of dissimilarity rank obtained with the ANOSIM test (ANOSIM statistic R = 0.3519; Significance = 0.027).

	Percentage of variance	cumulative percentage of variance	
comp 1	90.106448	39.870110	39.87011
comp 2	31.777936	14.061034	53.93114
comp 3	20.449165	9.048303	62.97945
comp 4	19.319029	8.548243	71.52769
comp 5	16.265303	7.197037	78.72473
comp 6	12.079244	5.344798	84.06952
comp 7	10.060970	4.451757	88.52128
comp 8	8.255149	3.652721	92.17400
comp 9	6.376853	2.821617	94.99562
comp 10	5.753039	2.545592	97.54121
comp 11	5.556863	2.458789	100.00000

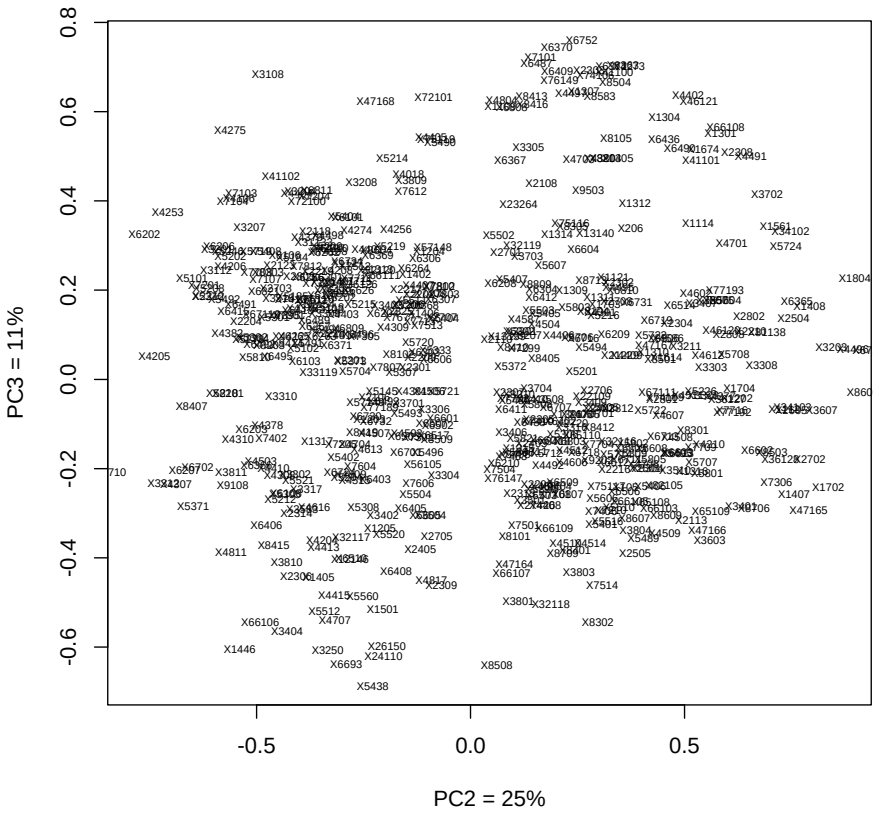


Supplementary Figure 4: 2-DE gel of lettuce leaf-protein spots that changed in abundance after CYN exposure. A protein mass of 400 µg was loaded on each Immobiline IEF gel strip (17 cm, pH 4-7) for isoelectric focusing. The SDS-PAGE was performed in 12% (w/v) polyacrylamide gels, which were stained with Colloidal Coomassie G-250. The differentially abundant proteins were identified by MALDI-TOF/TOF MS. The spots surrounded by a green color correspond to group C1, the spots surrounded by a blue color correspond to group C10, and the spots surrounded by a purple color correspond to group C100. The spot numbers labeled red indicate that the same protein exhibited changes in abundance in multiple groups.

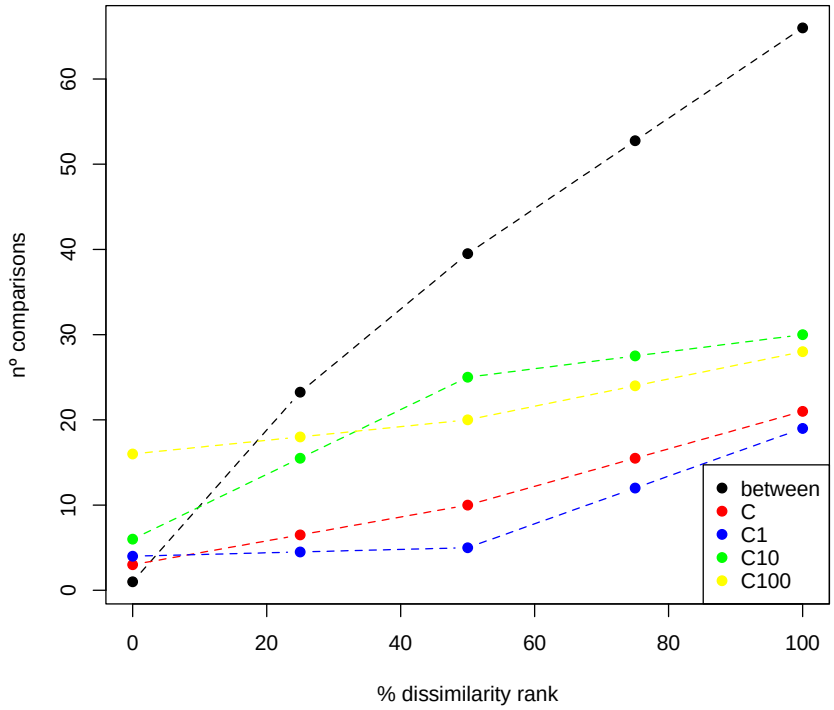
Supplementary figures associated with the MC-LR/CYN exposure experiment



Supplementary Figure 5: Variable representation of PCA along the first and second dimensions.

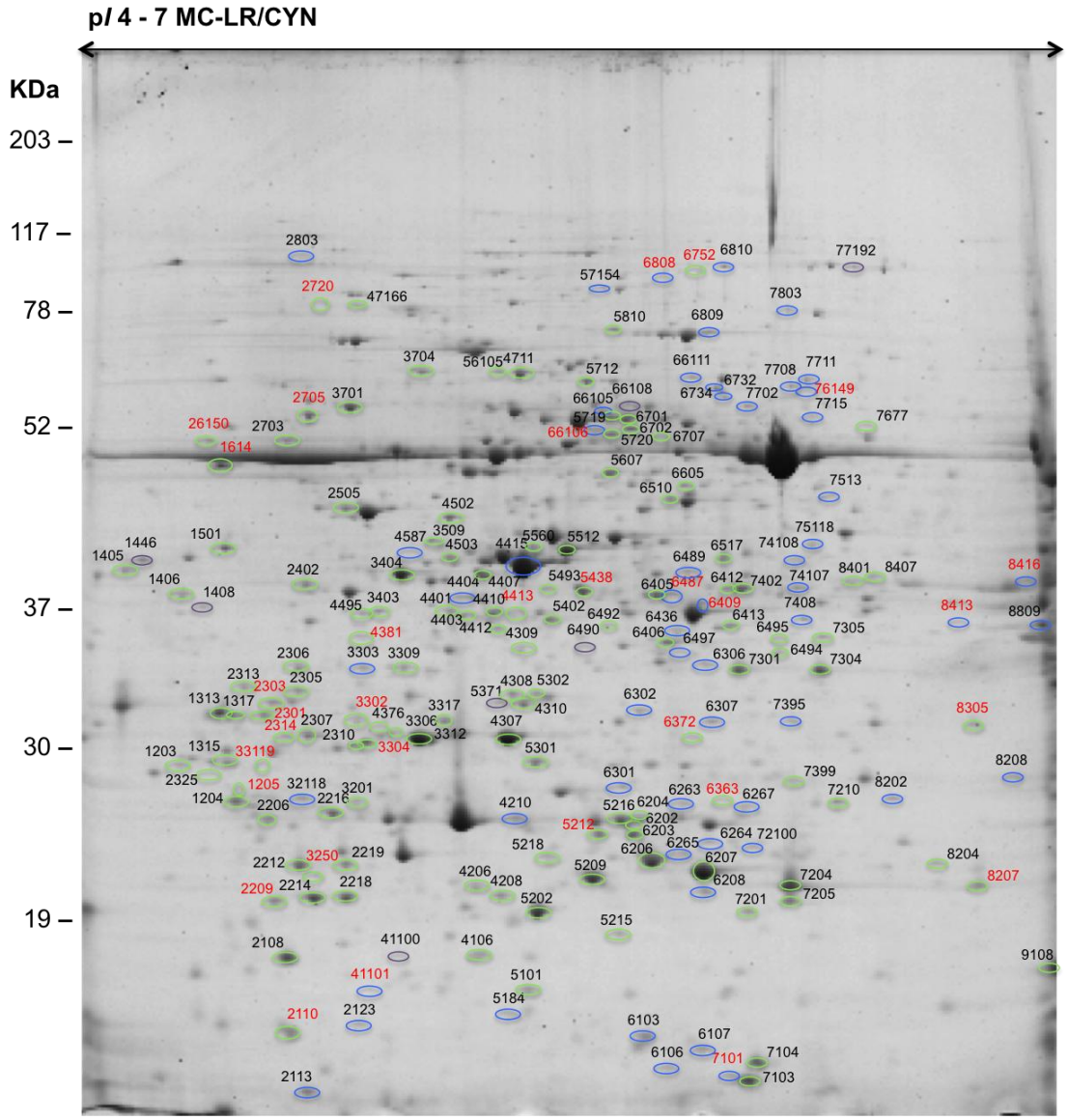


Supplementary Figure 6: Variable representation of PCA along the second and third dimensions.



Supplementary Figure 7: Percentage of dissimilarity rank obtained with the ANOSIM test (ANOSIM statistic R = 0.6636; Significance = 0.002).

	Percentage of variance	cumulative percentage of variance
comp 1	184.040890	32.516058	32.51606
comp 2	142.833375	25.235579	57.75164
comp 3	63.703200	11.254982	69.00662
comp 4	43.745250	7.728843	76.73546
comp 5	31.651982	5.592223	82.32768
comp 6	28.372424	5.012796	87.34048
comp 7	22.878164	4.042078	91.38256
comp 8	16.114324	2.847054	94.22961
comp 9	12.191268	2.153934	96.38355
comp 10	11.198727	1.978574	98.36212
comp 11	9.270396	1.637879	100.00000



Supplementary Figure 8: 2-DE gel of lettuce leaf-protein spots that changed in abundance after MC-LR/CYN exposure. A protein mass of 400 µg was loaded on each Immobiline IEF gel strip (17 cm, pH 4-7) for isoelectric focusing. The SDS-PAGE was performed in 12% (w/v) polyacrylamide gels, which were stained with Colloidal Coomassie G-250. The differentially abundant proteins were identified by MALDI-TOF/TOF MS. The spots surrounded by a green color correspond to group C1, the spots surrounded by a blue color correspond to group C10, and the spots surrounded by a purple color correspond to group C100. The spot numbers labeled in red indicate that the same protein exhibited changes in abundance in multiple groups.

Supplemental table 1: Full data regarding to the proteins identification of the CYN exposure experiment with lettuce plants.

Condition/ Function	Spot number		Proteins identified	Homologous protein	Species	Acession number	Score	Expect	No. of unique peptides	MW (Da)	Spot intensity				Fold change variation	Relative protein abundance
											Average ± SD C	Average ± SD C1	Average ± SD C10	Average ± SD C100		
Photosynthesis and carbon metabolism																
C1	3212		RecName: Full=Plastocyanin		*Lactuca sativa*	gi|130270	323	5.80E-27	3	10410	8130.3 ± 3298.6	1716.7 ± 491.8	NA	NA	4.7	**Decreased**
	5545	EST	QGF6C23.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF6C23, mRNA sequence	Chain A, Chloroplast Nadp-Dependent Malate Dehydrogenase	*Flaveria bidentis*	gi|2241358 7|gb|BQ99 4052.1|	294	7.00E-24	4	26207	90 ± 41.8	18.7 ± 11.63	NA	NA	4.8	**Decreased**
C1 and C100	5307	EST	QGF5O18.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF5O18, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 8, chloroplastic-like	*Cucumis sativus*	gi|2241349 0|gb|BQ99 3955.1|	208	2.80E-15	4	27024	NA	44.1 ± 16.4	NA	106.7 ± 65.4	Protein spot absent in C	NA
C10	5315	EST	CLRY6253.b1_J03.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY6253, mRNA sequence	Chlorophyll a/b-binding protein	*Tagetes erecta*	gi|8391546 3|gb|DW11 7543.1|	208	2.80E-15	3	31074	5.7 ± 3.2	NA	289.2 ± 145.1	NA	50.7	Increased
C10 and C100	4423		Chlorophyll a/b binding protein precursor		*Euphorbia esula*	gi|6716783	112	7.30E-06	2	28542	NA	NA	119 ± 72.1	441.4 ± 126.8	Protein spot absent in C	NA
	4531	EST	CLSS13408.b1_P16.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS13408, mRNA sequence	PREDICTED: quinone oxidoreductase-like protein At1g23740, chloroplastic-like	*Fragaria vesca subsp. Vesca*	gi|9051787 6|gb|DY979 734.1|	162	1.10E-10	2	27498	2.3 ± 1.4	NA	29 ± 15.9	49.2 ± 3.5	12.8 (C10) and 21.7 (C100)	Increased
	5305	EST	QGF5O18.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF5O18, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 8, chloroplastic-like	*Cucumis sativus*	gi|2241349 0|gb|BQ99 3955.1|	171	1.40E-11	5	27024	NA	NA	188.6 ± 111.8	224.5 ± 59.2	Protein spot absent in C	NA
	6318	EST	CLPX5655.b1_M22.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPX5655, mRNA sequence	Oxygen-evolving enhancer protein 2, chloroplastic	NA	gi|8388079 0|gb|DW08 2870.1|	260	1.80E-20	4	24725	NA	NA	210.5 ± 27.5	344.2 ± 76.7	Protein spot absent in C	NA
C100	4535		Sedoheptulose-1,7-bisphosphatase		*Cucumis sativus*	gi|2295975 43	143	5.80E-09	3	42532	15.3 ± 7.2	NA	NA	180.5 ± 79.9	11.8	Increased
	4601	EST	CLPX707.b1_F10.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPX707, mRNA sequence	Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplast precursor, putative	*Ricinus communis*	gi|8388193 1|gb|DW08 4011.1|	623	8.80E-57	7	31547	382.5 ± 83.5	NA	NA	932.8 ± 168.6	2.4	Increased

	4609	EST	CLPX707.b1_F10.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPX707, mRNA sequence	Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplast precursor, putative	*Ricinus communis*	gi|83881931|gb|DW084011.1|	444	7.00E-39	5	31547	96.2 ± 36.8	NA	NA	252.1 ± 47.8	2.6	Increased
	4610	EST	QGF12E09.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF12E09, mRNA sequence	Putative ribulose-1,5-bisphosphate carboxylase/oxygenase activase	*Olea europaea*	gi|224049981|gb|BQ987456.1|	638	2.80E-58	7	24684	151.6 ± 80.2	NA	NA	408.9 ± 57.8	2.7	Increased
	5301	EST	QGG25D19.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGG25D19, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 6A, chloroplastic-like	*Fragaria vesca subsp. vesca*	gi|224349600|gb|BU000565.1|	741	1.40E-68	7	26457	1577.2 ± 740.3	NA	NA	3095 ± 413.3	2.0	Increased
	5448	EST	CLSX2910.b1_K08.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX2910, mRNA sequence	Oxygen-evolving enhancer protein 1, chloroplastic	NA	gi|83980274|gb|DW126383.1|	662	1.10E-60	5	31297	277.5 ± 82.2	NA	NA	554.7 ± 20.4	2.0	Increased
	5455		Chloroplast light-harvesting chlorophyll a/b-binding protein		*Artemisia annua*	gi|146403796	194	4.60E-14	3	26996	941.4 ± 259.5	NA	NA	2385.7 ± 670.5	2.5	Increased
	5510		Chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase activase		*Flaveria bidentis*	gi|158726716	209	1.50E-15	4	48808	31 ± 12.9	NA	NA	153.4 ± 41	5.0	Increased
	6531	EST	CLSX4555.b1_F12.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX4555, mRNA sequence	PREDICTED: phosphoribulokinase, chloroplastic-like	*Vitis vinifera*	gi|83981925|gb|DW128034.1|	125	5.60E-07	3	34837	25.4 ± 14.3	NA	NA	58.8 ± 13.5	2.3	Increased
ATP synthesis																
C1	5613		ATP synthase CF1 beta subunit		*Lactuca sativa*	gi|81176257	747	2.30E-69	7	53590	128.9 ± 39.7	43.9 ± 26.4	NA	NA	2.9	**Decreased**
C10	5618		ATP synthase CF1 beta subunit		*Lactuca sativa*	gi|81176257	1060	1.20E-100	8	53590	NA	NA	414.5 ± 132.7	NA	Protein spot absent in C	NA
C100	4602		ATP synthase CF1 beta subunit		*Lactuca sativa*	gi|81176257	1090	1.20E-103	9	53590	496.2 ± 131.4	NA	NA	1195.8 ± 136.2	2.4	Increased
	4706		ATP synthase CF1 beta subunit		*Lactuca sativa*	gi|81176257	633	5.80E-58	7	53590	94.3 ± 39.9	NA	NA	373.6 ± 88.9	4.0	Increased
	5302		ATPase epsilon chain		*Lactuca sativa*	gi|6635347	427	2.3E-037	6	13772	61.3 ± 25.10	NA	NA	326.8 ± 4.45	5.3	Increased
	5603		ATP synthase CF1 beta subunit		*Lactuca sativa*	gi|81176257	1190	1.20E-113	9	53590	1164.7 ± 452.98	NA	NA	2981.1 ± 870.8	2.6	Increased
	5620		ATP synthase CF1 alpha subunit		*Lactuca sativa*	gi|811762404	209	1.50E-15	4	55541	NA	NA	NA	43.1 ± 7.3	Protein spot absent in C	NA
	5623		ATP synthase CF1 alpha subunit		*Lactuca sativa*	gi|811762404	935	3.70E-88	8	55541	698.6 ± 185.5	NA	NA	1507.7 ± 133.2	2.2	Increased
	5707		ATP synthase CF1 beta subunit		*Lactuca sativa*	gi|81176257	472	7.30E-42	6	53590	76.03 ± 36.98	NA	NA	272.2 ± 109	3.6	Increased
	6701		ATP synthase CF1 alpha subunit		*Citrus sinensis*	gi|114329641	108	1.80E-05	1	55509	2520.1 ± 798.97	NA	NA	5202.9 ± 1085.7	2.1	Increased
Stress response/Protein folding																

C1	4703		Heat shock protein 70		*Lactuca sativa*	gi|432140649	575	3.70E-52	7	74289	815.4 ± 150.3	323.1 ± 216.0	NA	NA	2.5	**Decreased**
	5634		PREDICTED: heat shock 70 kDa protein, mitochondrial-like		*Glycine max*	gi|356549495	112	7.30E-06	2	72555	72.2 ± 16.9	31.5 ± 7.6	NA	NA	2.3	**Decreased**
C100	3707		PREDICTED: ruBisCO large subunit-binding protein subunit alpha, chloroplastic-like		*Brachypodium distachyon*	gi|357112497	281	9.20E-23	3	61745	142.9 ± 51.3	NA	NA	585.7 ± 87.1	4.1	Increased
	5214	EST	QGJ13G20.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGJ13G20, mRNA sequence	PREDICTED: peptidyl-prolyl cis-trans isomerase FKBP16-3, chloroplastic-like	*Cucumis sativus*	gi|224448 28|gb|BU010433.1|	226	4.40E-17	5	23715	0.65 ± 0.35	NA	NA	69.5 ± 39.23	106.9	Increased
	5404	EST	CLVX8498.b1_C13.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX8498, mRNA sequence	Putative thioredoxin-dependent peroxidase	*Citrus hybrid cultivar*	gi|840191 84|gb|DW156454.1|	753	8.80E-70	7	15642	77.1 ± 42.4	NA	NA	338.4 ± 75.2	4.4	Increased
Defense response/Allergens																
C10	5525	EST	CLRY8240.b2_P19.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY8240, mRNA sequence	Eugenol synthase 1	*Petunia x hybrida*	gi|839175 45|gb|DW119625.1|	578	2.80E-52	7	32541	7.9 ± 1.41	NA	24.3 ± 4.6	NA	3.1	Increased
C100	6301	EST	CLSS12894.b1_K08.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS12894, mRNA sequence	PREDICTED: thaumatin-like protein-like	*Fragaria vesca subsp. Vesca*	gi|905173 43|gb|DY979201.1|	512	1.10E-45	5	31719	1509.9 ± 449.8	NA	NA	4074.97 ± 1082.7	2.7	Increased
Proteolysis																
C100	1609		Cysteine protease		*Lactuca sativa*	gi|239937266	95	3.70E-04	3	15878	NA	NA	NA	3564.4 ± 1925.8	Protein spot absent in C	NA
	5702		Putative zinc dependent protease		*Trifolium pratense*	gi|844683 24	628	1.80E-57	6	75406	195.7 ± 46.1	NA	NA	460.8 ± 113.7	2.4	Increased
Nucleosome assembly																
C100	6304		Histone H4		*Allium cepa*	gi|1199967	155	3.70E-10	2	6630	232.9 ± 49.7	NA	NA	722.5 ± 35.5	3.1	Increased
Transport activity																
C10 and C100	6327	EST	CLLX3611.b1_F15.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX3611, mRNA sequence	Temperature-induced lipocalin	*Populus tremula x Populus tremuloides*	gi|837906 07|gb|DW050824.1|	144	7.00E-09	4	29077	37.2 ± 7.5	NA	109.9 ± 39.4	93.97 ± 18.5	3.0 (C10) and 2.5 (C100)	Increased
Cytoskeleton formation																

C10	6514		Actin		*Stevia rebaudiana*	gi|23955912	823	5.80E-77	8	41943	134.2 ± 52.3	NA	39.1 ± 20.4	NA	3.4	**Decreased**
C100	4536		Plastid-dividing ring protein		*Solanum lycopersicum*	gi|350539589	109	1.50E-05	3	44069	107.8 ± 37.7	NA	NA	302.93 ± 69.0	2.8	Increased
	4603		Plastid-dividing ring protein		*Solanum lycopersicum*	gi|350539589	167	2.30E-11	3	44069	61.9 ± 24.7	NA	NA	313.3 ± 67.4	5.1	Increased
Unknown/Miscellaneous																
C10	5213	EST	CLLY5506.b1_D09.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY5506, mRNA sequence	PREDICTED: thylakoid lumenal 15 kDa protein 1, chloroplastic-like	*Solanum lycopersicum*	gi|83805737|gb|DW065954.1|	238	2.80E-18	5	31379	2.1 ± 0.9	NA	78.5 ± 39.3	NA	37.4	Increased
C10 and C100	6310	EST	CLRX6025.b2_A20.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX6025, mRNA sequence	PREDICTED: auxin-binding protein ABP19a-like	*Cucumis sativus*	gi|839066810|gb|DW108761.1|	204	7.00E-15	2	30319	45.1 ± 14.8	NA	274.3 ± 90.8	202.6 ± 81.4	6.1 (C10) and 4.5 (C100)	Increased
	7218	EST	CLSS2149.b1_I10.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS2149, mRNA sequence	Lettuce sativa Lactuca sativa cDNA clone CLSS2149, mRNA sequence	NA	gi|90518319|gb|DY980177.1|	592	1.10E-53	8	28094	51.97 ± 18.9	NA	206.5 ± 55.4	237.7 ± 67.9	4.0 (C10) and 4.6 (C100)	Increased
C100	4320	EST	QGA10P22.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGA10P22, mRNA sequence	Thylakoid lumen 18.3 kDa protein	*Arabidopsis lyrata subsp. lyrata*	gi|222233421|gb|BQ843556.1|	677	3.50E-62	6	27549	129.8 ± 53.3	NA	NA	439.2 ± 81.4	3.4	Increased
	4432	EST	CLLY7947.b1_E20.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY7947, mRNA sequence	PREDICTED: unknown protein DS12 from 2D-PAGE of leaf, chloroplastic-like	*Solanum lycopersicum*	gi|83807970|gb|DW068187.1|	677	3.50E-62	6	27549	16.2 ± 1.9	NA	NA	138.9 ± 24.7	8.6	Increased
	5417	EST	CLRX2340.b1_G09.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX2340, mRNA sequence	Lettuce serriola Lactuca serriola cDNA clone CLRX2340, mRNA sequence	NA	gi|83902453|gb|DW104533.1|	669	2.20E-61	6	30931	67.6 ± 36.7	NA	NA	291.5 ± 84	4.3	Increased
	7303	EST	CLRX6025.b2_A20.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX6025, mRNA sequence	PREDICTED: auxin-binding protein ABP19a-like	*Cucumis sativus*	gi|839066810|gb|DW108761.1|	510	1.80E-45	4	30319	1051.5 ± 456.2	NA	NA	4514.2 ± 639.9	4.3	Increased

NOTES

gi| Proteins identified using NCBI databasw

gi|gb| Proteins identified using EST database

NA: not applicable

Supplemental table 2: Full data regarding to the proteins identification of the MC-LR/CYN exposure experiment with lettuce plants.

Condition/ Function	Spot number		Proteins identified	Homologous protein	Species	Acession number	Score	Expect	No. of unique peptides	MW (Da)	Spot intensity				Fold change variation	Relative protein abundance
											Average ± SD C	Average ± SD C1	Average ± SD C10	Average ± SD C100		
Photosynthesis and carbon metabolism																
C1	2206	EST	CLVX6829.b1_J04.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX6829, mRNA sequence	PREDICTED: heme-binding protein 2-like	*Fragaria vesca subsp. Vesca*	gi|84017490|gb|DW154760.1|	329	2.20E-27	4	25580	170.8 ± 89.4	540.1 ± 211.7	NA	NA	3.2	Increased
	2212	EST	QGH5D12.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGH5D12, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 13, chloroplastic-like	*Cucumis sativus*	gi|224420955|gb|BU007700.1|	321	1.40E-26	3	25497	625.9 ± 194.5	1649 ± 208.8	NA	NA	2.6	Increased
	2216	EST	CLLX13763.b1_F10.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX13763, mRNA sequence	PREDICTED: probable ribose-5-phosphate isomerase-like	*Solanum lycopersicum*	gi|37868877|gb|DW047094.1|	291	1.40E-23	5	30124	366.3 ± 144.02	814.1 ± 48.4	NA	NA	2.2	Increased
	2219		Chlorophyll a/b-binding protein		*Medicago truncatula*	gi|357452427	118	1.80E-06	3	28592	254.5 ± 28.4	562.3 ± 107.2	NA	NA	2.2	Increased
	2505	EST	CLPX707.b1_F10.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPX707, mRNA sequence	Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplast precursor, putative	*Ricinus communis*	gi|83881931|gb|DW084011.1|	334	7.00E-28	6	31547	188.4 ± 77.7	554.7 ± 123.5	NA	NA	2.9	Increased
	3306	EST	CLSY1255.b1_N01.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSY1255, mRNA sequence	Oxygen-evolving enhancer protein 1, chloroplastic	NA	gi|83987671|gb|DW133780.1|	263	8.80E-21	2	31446	157.6 ± 84.9	554.03 ± 122.2	NA	NA	3.5	Increased
	3312	EST	CLSX2910.b1_K08.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX2910, mRNA sequence	Oxygen-evolving enhancer protein 1, chloroplastic	NA	gi|83980274|gb|DW126383.1|	796	4.40E-74	7	31297	4049.6 ± 1060.7	7353 ± 610.2	NA	NA	2.0	Increased
	3404	EST	CLSY9694.b1_K23.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSY9694, mRNA sequence	PREDICTED: phosphoribulokinase, chloroplastic-like	*Vitis vinifera*	gi|83995525|gb|DW141634.1|	757	3.50E-70	8	35619	1127.8 ± 486.1	2591.8 ± 689.1	NA	NA	2.3	Increased
	3704	EST	QGG12O20.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGG12O20, mRNA sequence	PREDICTED: beta-xylosidase/alpha-L-arabinofuranosidase 2-like	*Vitis vinifera*	gi|224308606|gb|BQ996470.1|	241	1.40E-18	2	24887	128.6 ± 63.98	313.5 ± 8.7	NA	NA	2.4	Increased

4208	EST	CLSX6640.b1_P03.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX6640, mRNA sequence	PREDICTED: ATP synthase subunit delta', mitochondrial-like	*Cucumis sativus*	gi|839838033|gb|DW129912.1|	214	7.00E-16	5	29178	205.97 ± 38.2	431.5 ± 36.6	NA	NA	2.1	Increased
4307	EST	CLLX6558.b1_K07.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX6558, mRNA sequence	Chloroplast PsbO4 precursor	*Nicotiana benthamiana*	gi|837937413|gb|DW053958.1|	1030	1.80E-97	6	27209	3723.8 ± 1189.6	6555.7 ± 657.5	NA	NA	2.0	Increased
4401	EST	CLSM11007.b1_M16.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM11007, mRNA sequence	PREDICTED: quinone oxidoreductase-like protein At1g23740, chloroplastic-like	*Fragaria vesca subsp. Vesca*	gi|90498807|gb|DY960665.1|	219	2.20E-16	2	30195	119.6 ± 30.5	240.5 ± 21.2	NA	NA	2.0	Increased
4403	EST	CLSS8010.b1_D12.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS8010, mRNA sequence	Fructan 1-exohydrolase IIa	*Cichorium intybus*	gi|90522869|gb|DY984727.1|	293	8.80E-24	4	32650	94.3 ± 26.1	191.8 ± 35.02	NA	NA	2.0	Increased
4407	EST	QHB20B21.yg.ab1 QH_ABCDI sunflower RHA801 Helianthus annuus cDNA clone QHB20B21, mRNA sequence	PREDICTED: photosystem II stability/assembly factor HCF136, chloroplastic-like	*Cucumis sativus*	gi|22315830|gb|BQ917049.1|	637	3.50E-58	7	25140	590.97 ± 101.8	1252.5 ± 360.2	NA	NA	2.1	Increased
4410	EST	CLSY6696.b1_P17.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSY6696, mRNA sequence	PREDICTED: quinone oxidoreductase-like protein At1g23740, chloroplastic-like	*Fragaria vesca subsp. Vesca*	gi|83992866|gb|DW138975.1|	448	2.80E-39	3	31341	146 ± 11.8	510.3 ± 34.3	NA	NA	3.5	Increased
4503		Chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase activase		*Flaveria bidentis*	gi|158726716	475	3.7E-042	7	26268	112.3 ± 18.8	489.03 ± 76.9	NA	NA	4.4	Increased
5202	EST	CLVX9487.b2_M20.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX9487, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 6A, chloroplastic-like	*Fragaria vesca subsp. Vesca*	gi|84020136|gb|DW157406.1|	604	7.00E-55	6	32745	1466.2 ± 257.4	3512.8 ± 333.1	NA	NA	2.4	Increased
5216	EST	QGI13D20.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGI13D20, mRNA sequence	PREDICTED: triosephosphate isomerase, chloroplastic-like	*Glycine max*	gi|22258490|gb|BQ871940.1|	662	1.10E-60	8	26402	436.6 ± 206.3	1019.5 ± 291.4	NA	NA	2.3	Increased
5218	EST	QGF5O18.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF5O18, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 8, chloroplastic-like	*Cucumis sativus*	gi|22413490|gb|BQ993955.1|	233	8.80E-18	7	27024	57.4 ± 24.7	294.8 ± 37.9	NA	NA	5.1	Increased
5512	EST	CLLY12657.b1_B22.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY12657, mRNA sequence	Chloroplast phosphoglycerate kinase 3	*Helianthus annuus*	gi|83798893|gb|DW060110.1|	1090	1.80E-103	8	32830	967.2 ± 541.3	2339.8 ± 512.9	NA	NA	2.4	Increased
5560	EST	QGH6C23.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGH6C23, mRNA sequence	Chloroplast phosphoglycerate kinase 3	*Helianthus annuus*	gi|224424044|gb|BU008009.1|	587	3.50E-53	8	23723	57.6 ± 31.2	440.9 ± 213.3	NA	NA	7.7	Increased

	5712	EST	CLSY5153.b1_B17.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSY5153, mRNA sequence	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	NA	gi|83991588|gb|DW137697.1|	243	8.80E-19	4	31118	125.3 ± 59.5	321.7 ± 57.5	NA	NA	2.6	Increased
	6206	EST	CLVX8096.b1_O07.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX8096, mRNA sequence	PREDICTED: chlorophyll a-b binding protein 8, chloroplastic-like	*Cucumis sativus*	gi|84018797|gb|DW156067.1|	686	4.40E-63	9	28238	1765.3 ± 434.2	4486.6 ± 372.3	NA	NA	2.5	Increased
	6207	EST	CLPX5655.b1_M22.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPX5655, mRNA sequence	Oxygen-evolving enhancer protein 2, chloroplastic	NA	gi|83880790|gb|DW082870.1|	777	3.50E-72	7	24725	3298.4 ± 1085.4	8651.5 ± 1053.2	NA	NA	2.6	Increased
	6510	EST	QGB14A10.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB14A10, mRNA sequence	PREDICTED: dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 2, mitochondrial-like	*Cucumis sativus*	gi|22236425|gb|BQ850956.1|	112	1.10E-05	3	24729	98.7 ± 56.1	458.4 ± 168.7	NA	NA	4.7	Increased
	6517		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit		*Cullen australasicum*	gi|212901232	506	2.90E-45	6	47514	242.7 ± 4.9	563.2 ± 167.2	NA	NA	2.3	Increased
	6707		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)		*Potentilla erecta*	gi|340511870	405	3.70E-35	6	50644	96.7 ± 9	348.97 ± 87.7	NA	NA	3.6	Increased
	7301	EST	CLRY4414.b1_L23.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY4414, mRNA sequence	PREDICTED: ferredoxin--NADP reductase, leaf-type isozyme, chloroplastic-like	*Solanum lycopersicum*	gi|83914232|gb|DW116312.1|	526	4.40E-47	8	34178	381.8 ± 28.3	1297 ± 206.7	NA	NA	3.4	Increased
	7304	EST	CLSS2231.b1_N06.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS2231, mRNA sequence	Ferredoxin-NADP reductase	*Medicago truncatula*	gi|90518401|gb|DY980259.1|	556	4.4E-050	7	3238 7	584.6 ± 274.99	1937 ± 520.9	NA	NA	3.3	Increased
	7399	EST	CLPZ4124.b1_G24.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPZ4124, mRNA sequence	Gamma carbonic anhydrase-like 2	*Arabidopsis thaliana*	gi|83899361|gb|DW101441.1|	320	1.80E-26	5	27735	63.1 ± 28.4	135.7 ± 9.8	NA	NA	2.2	Increased
	9108	EST	CLLZ3823.b2_N20.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLZ3823, mRNA sequence	PSI reaction center subunit II	*Citrus sinensis*	gi|83812168|gb|DW072385.1|	687	3.50E-63	6	25274	1135.3 ± 509.8	3650.03 ± 297.9	NA	NA	3.2	Increased
C1 and C10	3304	EST	CLRX2804.b1_H05.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX2804, mRNA sequence	PREDICTED: soluble inorganic pyrophosphatase 1, chloroplastic-like	*Solanum lycopersicum*	gi|83902911|gb|DW104991.1|	125	5.60E-07	2	34588	212.3 ± 71.6	706.9 ± 79.6	511.5 ± 138.2	NA	3.3 (C1) and 2.4 (C10)	Increased
	4413	EST	CLSM14093.b1_I20.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM14093, mRNA	PREDICTED: transaldolase-like	*Vitis vinifera*	gi|90502001|gb|DY963859.1|	315	5.60E-26	4	32274	59.7 ± 3.8	151.5 ± 35.4	116.8 ± 31.3	NA	2.5 (C1) and 2.0 (C10)	Increased

			sequence												
C1 and C100	26150		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)		*Hypochaeris radicata*	gi|340511438	277	2.30E-22	4	50720	NA	318.8 ± 127.5	NA	104.3 ± 13.1	Protein spot absent in C	NA
	3250	EST	CLSS6504.b1_P18.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS6504, mRNA sequence	Thylakoid lumenal 21.5 kDa protein, chloroplast precursor, putative	*Ricinus communis*	gi|90522050|gb|DY983908.1|	571	1.40E-51	7	33524	NA	109.2 ± 30.4	NA	68.8 ± 36.2	Protein spot absent in C	NA
C10	2123	EST	QGE11K18.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGE11K18, mRNA sequence	PREDICTED: ATP synthase delta chain, chloroplastic-like	*Solanum lycopersicum*	gi|22398180|gb|BQ980657.1|	733	8.80E-68	7	23331	70.6 ± 35.3	NA	NA	NA	Protein spot absent in C10	NA
	2803		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)		*Aquilegia vulgaris*	gi|340510868	84	4.60E-03	1	50983	37.3 ± 18.97	NA	NA	NA	Protein spot absent in C10	NA
	4210		Chloroplast light-harvesting chlorophyll a/b-binding protein		*Artemisia annua*	gi|146403796	314	4.6E-026	6	26996	367.7 ± 118.1	NA	1243.2 ± 129.94	NA	3.4	Increased
	4415	EST	QGF12E09.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF12E09, mRNA sequence	Putative ribulose-1,5-bisphosphate carboxylase/oxygenase activase	*Olea europaea*	gi|224049861|gb|BQ987456.1|	961	1.40E-90	8	24684	1549.8 ± 152.4	NA	3150.03 ± 841.8	NA	2.0	Increased
	6208	EST	CLLZ5472.b1_O23.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLZ5472, mRNA sequence	PREDICTED: thylakoid lumenal 19 kDa protein, chloroplastic-like	*Fragaria vesca subsp. vesca*	gi|838135577|gb|DW073774.1|	632	1.10E-57	7	30058	103.1 ± 19.3	NA	20.2 ± 10.5	NA	5.1	**Decreased**
	6436	EST	CLLY4312.b1_O21.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY4312, mRNA sequence	PREDICTED: pyruvate dehydrogenase E1 component subunit beta-like	*Fragaria vesca subsp. vesca*	gi|838044486|gb|DW064665.1|	582	1.1E-052	9	30299	NA	NA	30.2 ± 7.7	NA	Protein spot absent in C	NA
	66111	EST	QGC7N23.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGC7N23, mRNA sequence	putative cytosolic NADP-malic enzyme	*Flaveria pringlei*	gi|222518665|gb|BQ866400.1|	406	4.40E-35	7	28303	52.5 ± 23.8	NA	NA	NA	Protein spot absent in C10	NA
	6809	EST	QGB23P16.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB23P16, mRNA sequence	Transketolase, putative	*Ricinus communis*	gi|222401644|gb|BQ854699.1|	329	2.20E-27	3	25634	69.5 ± 19.1	NA	NA	NA	Protein spot absent in C10	NA
	7395	EST	CLRX606.b1_K08.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX606, mRNA sequence	Oxygen-evolving enhancer protein 1, chloroplastic	NA	gi|839067248|gb|DW108804.1|	253	8.80E-20	3	30973	60.8 ± 30.7	NA	NA	NA	Protein spot absent in C10	NA
	7702	EST	QGI12N22.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGI12N22, mRNA sequence	Alpha isopropylmalate synthase	*Ipomoea purpurea*	gi|222583536|gb|BQ871806.1|	174	7.00E-12	2	24375	52.5 ± 9.4	NA	5 ± 2.5	NA	10.5	**Decreased**
	7708		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)		*Hypochaeris radicata*	gi|340511438	385	3.70E-33	6	50720	52 ± 20.7	NA	NA	NA	Protein spot absent in C10	NA

	7711		Succinate dehydrogenase		*Litchi chinensis*	gi|326467055	325	3.70E-27	4	69574	66.5 ± 19.3	NA	NA	NA	Protein spot absent in C10	NA
	7715	EST	CLSM11592.b1_O17.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM11592, mRNA sequence	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta	NA	gi|90499383|gb|DY961241.1|	114	7.00E-06	2	32973	70.9 ± 10.05	NA	NA	NA	Protein spot absent in C10	NA
C10 and C100	6409	EST	CLSM12308.b1_G05.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM12308, mRNA sequence	Isocitrate dehydrogenase (NAD+)	*Nicotiana tabacum*	gi|90500129|gb|DY961987.1|	639	2.20E-58	6	32255	102.2 ± 37.6	NA	NA	NA	Protein spot absent in C10 and C100	NA
	8413	EST	CLVX7336.b1_O09.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX7336, mRNA sequence	Malate dehydrogenase	*Glycine max*	gi|84018031|gb|DW155301.1|	303	8.80E-25	5	29634	52.7 ± 24.94	NA	NA	NA	Protein spot absent in C10 and C100	NA
	8416	EST	QGF20O02.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF20O02, mRNA sequence	Glyceraldehyde-3-phosphate dehydrogenase	*Helianthus annuus*	gi|22410236|gb|BQ990701.1|	513	8.80E-46	5	22206	124.4 ± 53.4	NA	NA	NA	Protein spot absent in C10 and C100	NA
ATP synthesis																
C1	4106		ATP synthase D chain, mitochondrial, putative		*Ricinus communis*	gi|255577651	309	1.50E-25	5	19728	316.6 ± 71.4	618.8 ± 108.6	NA	NA	2.0	Increased
	5719		ATP synthase CF1 alpha subunit		*Lactuca sativa*	gi|81176244	920	1.2E-086	9	55541	247.3 ± 39.8	674.5 ± 257.4	NA	NA	2.7	Increased
	7402	EST	CLSM20020.b1_G13.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM20020, mRNA sequence	PREDICTED: ATP synthase gamma chain, chloroplastic-like isoform 1	*Glycine max*	gi|90507111|gb|DY968969.1|	727	3.50E-67	8	34799	216.2 ± 79.8	797 ± 305.2	NA	NA	3.7	Increased
	7677		ATP synthase CF1 alpha subunit		*Lilium superbum*	gi|338825720	134	4.60E-08	2	55342	NA	78.5 ± 25.98	NA	NA	Protein spot absent in C	NA
C10	6489	EST	CLSM9831.b1_M10.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM9831, mRNA sequence	PREDICTED: V-type proton ATPase subunit C-like	*Solanum lycopersicum*	gi|90514256|gb|DY976114.1|	239	2.20E-18	3	33428	32.2 ±8.7	NA	NA	NA	Protein spot absent in C10	NA
	66105		ATP synthase CF1 alpha subunit		*Lactuca sativa*	gi|81176244	147	2.30E-09	3	55541	79.3 ± 11.7	NA	NA	NA	Protein spot absent in C10	NA
C10 and C100	6487	EST	CLSM20020.b1_G13.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM20020, mRNA sequence	PREDICTED: ATP synthase gamma chain, chloroplastic-like isoform 1	*Glycine max*	gi|90507111|gb|DY968969.1|	102	NA	2	34799	55.9 ± 6.8	NA	NA	NA	Protein spot absent in C10 and C100	NA
Stress response/Protein folding																

C1	1406	EST	QGD5L16.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGD5L16, mRNA sequence	PREDICTED: protein grpE-like	*Vitis vinifera*	gi|2225504 7|gb|BQ86 9290.1|	341	1.40E-28	4	2408 7	176.2 ± 69.8	369.97 ± 36.1	NA	NA	2.1	Increased
	2214	EST	CLPX555.b1_F19.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPX555, mRNA sequence	PREDICTED: 2-Cys peroxiredoxin BAS1, chloroplastic-like	*Cicer arietinu m*	gi|8388067 8|gb|DW08 2758.1|	636	4.40E-58	9	29061	800 ± 217.3	2179.5 ± 120.6	NA	NA	2.7	Increased
	2218	EST	QGE11L19.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGE11L19, mRNA sequence	Peroxiredoxin 2	*Tamarix hispida*	gi|2239820 2|gb|BQ98 0679.1|	828	2.80E-77	7	19803	568.3 ± 52.4	1457.1 ± 83.97	NA	NA	2.6	Increased
	2402	EST	QGB6M09.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB6M09, mRNA sequence	PREDICTED: peptidyl-prolyl cis-trans isomerase CYP38, chloroplastic-like	*Solanum lycopersi cum*	gi|2224262 5|gb|BQ85 7160.1|	360	1.80E-30	5	27902	163.3 ± 90.8	383 ± 8.6	NA	NA	2.3	Increased
	3201	EST	QGJ5C14.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGJ5C14, mRNA sequence	14-3-3-like protein GF14 lambda	*Arabidop sis thaliana*	gi|2244802 0|gb|BU013 625.1|	498	2.80E-44	4	21628	116.03 ± 27.95	305.8 ± 66.95	NA	NA	2.6	Increased
	3701		Putative rubisco subunit binding-protein alpha subunit precursor (60 kDa chaperonin alpha subunit)		*Oryza sativa* Japonica Group	gi|3119391 9	197	4.50E-13	2	61477	587.6 ± 170.15	1130.1 ± 183.5	NA	NA	1.9	Increased
	4310	EST	CLVX12121.b1_A08.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX12121, mRNA sequence	PREDICTED: 30S ribosomal protein 1, chloroplastic-like	*Vitis vinifera*	gi|8400976 5|gb|DW14 7035.1|	679	2.20E-62	7	34266	94.3 ± 9.7	454.3 ± 110.7	NA	NA	4.8	Increased
	5101	EST	CLVX8498.b1_C13.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX8498, mRNA sequence	Putative thioredoxin-dependent peroxidase	*Citrus hybrid cultivar*	gi|8401918 4|gb|DW15 6454.1|	805	5.60E-75	6	15642	194.3 ± 48.3	458.9 ± 52.1	NA	NA	2.4	Increased
	6202	EST	CLLY12292.b1_G01.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY12292, mRNA sequence	Ferritin	*Conyza canaden sis*	gi|8379950 1|gb|DW05 9718.1|	370	1.80E-31	4	35167	237.4 ± 122.96	551.3 ± 141.4	NA	NA	2.3	Increased
	6203	EST	CLRY6208.b1_O15.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY6208, mRNA sequence	PREDICTED: 20 kDa chaperonin, chloroplastic	*Vitis vinifera*	gi|8391541 4|gb|DW11 7494.1|	1020	1.80E-96	8	30195	270 ± 127.9	931 ± 178.4	NA	NA	3.4	Increased
	6204	EST	CLLX6922.b1_C03.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX6922, mRNA sequence	Ferritin	*Conyza canaden sis*	gi|8379412 9|gb|DW05 4346.1|	346	4.40E-29	3	31830	255.77 ± 90.91	665.4 ± 38.5	NA	NA	2.6	Increased
	6412	EST	CLSS5984.b1_O08.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS5984, mRNA sequence	Oxidoreductase, putative	*Ricinus communi s*	gi|9052149 6|gb|DY983 354.1|	707	3.50E-65	10	29359	95.2 ± 16.69	272.1 ± 82.2	NA	NA	2.9	Increased

	6605	EST	CLVY2521.b1_A08.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY2521, mRNA sequence	PREDICTED: protein disulfide isomerase-like 2-3-like	*Fragaria vesca subsp. Vesca*	gi|8402672 8|gb|DW16 3998.1|	221	1.40E-16	4	31144	25.9 ± 5.9	92.4 ± 37.7	NA	NA	3.6	Increased
	7103	EST	CLSS12787.b1_F05.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS12787, mRNA sequence	Superoxide dismutase [Cu-Zn], chloroplastic	NA	gi|9051722 9|gb|DY979 087.1|	469	2.20E-41	3	31353	972.7 ± 103.1	2495.8 ± 66.24	NA	NA	2.6	Increased
	7104	EST	CLSL2553.b1_A16.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSL2553, mRNA sequence	Cu/Zn superoxide dismutase	*Mikania micrantha*	gi|9049814 8|gb|DY960 006.1|	298	2.80E-24	6	23614	783.2 ± 387.2	1547.2 ± 50.4	NA	NA	2.0	Increased
C1 and C10	1614	EST	QGF6G12.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF6G12, mRNA sequence	RecName: Full=Calreticulin; Flags: Precursor	NA	gi|2241367 0|gb|BQ99 4135.1|	212	1.10E-15	4	27889	673.4 ± 251.5	1552.2 ± 65.7	1732.7 ± 281.3	NA	2.3 (C1) and 2.6 (C10)	Increased
	2705	EST	CLSM12761.b1_A23.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM12761, mRNA sequence	Protein disulfide isomerase	*Ipomoea batatas*	gi|9050061 3|gb|DY962 471.1|	577	3.5E-052	4	33987	146.7 ± 59.5	488.8 ± 83.3	430.5 ± 134.3	NA	3.3 (C1) and 2.9 (C10)	Increased
	2720		HSP90		*Pelargonium peltatum*	gi|4043330 12	64	0.44	2	80529	NA	51.5 ± 13.94	49.1 ± 17.9	NA	Protein spot absent in C	NA
	4381	EST	CLSS2836.b1_H13.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS2836, mRNA sequence	Late embryogenesis abundant protein Lea14-A, putative	*Ricinus communis*	gi|9051899 1|gb|DY980 849.1|	165	5.60E-11	5	34653	14.1 ± 1.7	NA	NA	NA	Protein spot absent in C1 and C10	NA
	5438	EST	CLRY3470.b1_K03.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY3470, mRNA sequence	PREDICTED: probable aldo-keto reductase 2-like	*Fragaria vesca subsp. Vesca*	gi|8391325 8|gb|DW11 5338.1|	280	1.80E-22	2	31192	NA	310.8 ± 134.3	103.7 ± 49.3	NA	Protein spot absent in C	NA
C1, C10 and C100	6752		PREDICTED: chaperone protein ClpB3, chloroplastic-like		*Glycine max*	gi|3565510 74	188	1.80E-13	5	11031 9	24.6 ± 3.1	8.4 ± 3.2	NA	NA	2.9 (C1); Protein spot absent in C10 and C100	**Decreased**
C10	2113	EST	CLLX709.b1_J10.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX709, mRNA sequence	PREDICTED: peroxiredoxin-2E, chloroplastic	*Vitis vinifera*	gi|8379430 7|gb|DW05 4524.1|	131	1.40E-07	3	32594	299.1 ± 121.6	NA	1418.9 ± 341.7	NA	4.7	Increased
	57154		PREDICTED: chaperone protein ClpC, chloroplastic-like		*Glycine max*	gi|3565164 95	347	2.30E-29	7	10260 5	16.5 ± 6.5	NA	NA	NA	Protein spot absent in C10	NA
	6263		Glutaredoxin S16		*Populus trichocarpa*	gi|2241391 36	137	2.30E-08	2	23823	28.8 ± 10.8	NA	NA	NA	Protein spot absent in C10	NA
	6302	EST	QGB5N21.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB5N21, mRNA sequence	Thylakoid-bound ascorbate peroxidase	*Tamarix hispida*	gi|2224229 0|gb|BQ85 6825.1|	207	3.50E-15	3	23088	80.9 ± 21.6	NA	27.5 ± 6.4	NA	2.9	**Decreased**

	6306	EST	CLVY3468.b1_G03.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY3468, mRNA sequence	PREDICTED: thioredoxin reductase 2-like	*Vitis vinifera*	gi|84027650|gb|DW164920.1|	388	2.80E-33	5	28534	69.7 ± 21.1	NA	11.8 ± 4.4	NA	5.9	**Decreased**
C10 and C100	6808		PREDICTED: chaperone protein ClpB3, chloroplastic-like		*Glycine max*	gi|356551074	242	7.30E-19	7	110319	9.3 ± 3.9	NA	NA	NA	Protein spot absent in C10 and C100	NA
C100	41100	EST	QGJ5C18.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGJ5C18, mRNA sequence	PREDICTED: PITH domain-containing protein At3g04780	*Vitis vinifera*	gi|224480234|gb|BU013628.1|	486	4.40E-43	6	27305	48.8 ± 21.1	NA	NA	NA	Protein spot absent in C100	NA
Defense response/Allergen																
C1	4309	EST	CLRY8240.b2_P19.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY8240, mRNA sequence	Eugenol synthase 1	*Petunia x hybrida*	gi|83917545|gb|DW119625.1|	625	5.60E-57	7	32541	71.8 ± 9.1	167.8 ± 13.2	NA	NA	2.3	Increased
	5209	EST	CLSS12894.b1_K08.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS12894, mRNA sequence	PREDICTED: thaumatin-like protein-like	*Fragaria vesca subsp. Vesca*	gi|90517343|gb|DY979201.1|	630	1.80E-57	6	31719	2100.5 ± 506.8	4521.9 ± 38.5	NA	NA	2.2	Increased
	5301	EST	QGE15G19.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGE15G19, mRNA sequence	Harpin binding protein 1	*Solanum lycopersicum*	gi|223995778|gb|BQ982053.1|	237	3.50E-18	3	17337	242.5 ± 48.93	727.2 ± 55.3	NA	NA	3.0	Increased
C10	7408	EST	CLSZ2508.b1_G04.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSZ2508, mRNA sequence	Quinone oxidoreductase	*Arabidopsis thaliana*	gi|83997140|gb|DW143249.1|	286	4.40E-23	4	32689	66.9 ± 27.7	NA	11.6 ± 2.03	NA	5.8	**Decreased**
Proteolysis																
C1	1405	EST	CLSM17819.b1_F15.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM17819, mRNA sequence	CND41, chloroplast nucleoid DNA binding protein	*Nicotiana tabacum*	gi|90504806|gb|DY966664.1|	345	5.60E-29	5	31369	73.1 ± 20.3	613.1 ± 180.3	NA	NA	8.4	Increased
	4711		Putative zinc dependent protease		*Trifolium pratense*	gi|84468324	416	1.4E-059	6	75406	231.1 ± 78.1	720.1 ± 173.3	NA	NA	3.1	Increased
	5302	EST	CLSX9289.b2_A19.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX9289, mRNA sequence	PREDICTED: proteasome subunit alpha type-1-B	*Vitis vinifera*	gi|83986515|gb|DW132624.1|	502	1.10E-44	8	29870	81.5 ± 34.7	241.8 ± 7.3	NA	NA	3.0	Increased
	5402	EST	QGF12O11.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF12O11, mRNA sequence	PREDICTED: aspartic proteinase nepenthesin-2	*Vitis vinifera*	gi|224072177|gb|BQ987683.1|	350	1.80E-29	5	26408	152.6 ± 57.1	504.8 ± 49.5	NA	NA	3.3	Increased

	5607	EST	CLSS8038.b1_L18.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS8038, mRNA sequence	PREDICTED: mitochondrial-processing peptidase subunit alpha	*Vitis vinifera*	gi|90522897|gb|DY984755.1|	604	7.00E-55	6	29302	355.4 ± 128.6	618.1 ± 94.3	NA	NA	1.7	Increased
	56105		Cell division protein ftsH, putative		*Ricinus communis*	gi|255558698	301	9.20E-25	4	75504	32.97 ± 13.8	100.6 ± 23.4	NA	NA	3.1	Increased
	7210	EST	QGB21F17.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB21F17, mRNA sequence	PREDICTED: proteasome subunit alpha type-3-like	*Solanum lycopersicum*	gi|222392099|gb|BQ853744.1|	273	8.80E-22	2	21613	93.6 ± 35.1	189 ± 38.9	NA	NA	2.0	Increased
	8407	EST	CLVY11124.b1_H22.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY11124, mRNA sequence	PREDICTED: probable 26S proteasome non-ATPase regulatory subunit 7	*Vitis vinifera*	gi|84021736|gb|DW159006.1|	584	7.00E-53	7	35681	66.8 ± 57.4	268.7 ± 111.96	NA	NA	4.0	Increased
C10	8202	EST	CLLX11780.b1_G18.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX11780, mRNA sequence	PREDICTED: proteasome subunit alpha type-6 isoform 1	*Vitis vinifera*	gi|83784749|gb|DW044966.1|	796	4.40E-74	10	28002	54 ± 20.3	NA	NA	NA	Protein spot absent in C10	NA
Glutathione metabolism															
C1, C10 and C100	6372	EST	CLPY6462.b1_L08.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPY6462, mRNA sequence	S-formylglutathione hydrolase	*Gossypium hirsutum*	gi|83893284|gb|DW095364.1|	410	1.80E-35	5	34458	22.3 ± 2.3	NA	NA	NA	Protein spot absent in C1, C10 and C100	NA
C10	6307	EST	CLSL2511.b1_M04.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSL2511, mRNA sequence	PREDICTED: S-formylglutathione hydrolase isoform 1	*Vitis vinifera*	gi|90498118|gb|DY959976.1|	380	1.80E-32	5	34557	64.4 ± 25.0	NA	NA	NA	Protein spot absent in C10	NA
C100	5371	EST	CLRY8401.b2_B14.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY8401, mRNA sequence	PREDICTED: protein IN2-1 homolog B-like	*Vitis vinifera*	gi|83917705|gb|DW119785.1|	294	7.00E-24	6	33528	14.1 ± 1.3	NA	NA	46.6 ± 2.9	3.3	Increased
Protein synthesis and signal transduction (Transcription, RNA processing and translocation, and translation)															
C1	1203	EST	CLVZ4611.b1_E01.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVZ4611, mRNA sequence	PREDICTED: nascent polypeptide-associated complex subunit alpha-like	*Vitis vinifera*	gi|84038261|gb|DW173580.1|	403	8.80E-35	6	27008	59.85 ± 9.5	405.3 ± 166.2	NA	NA	6.8	Increased
	1204	EST	QGF21O10.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF21O10, mRNA sequence	Ribonucleoprotein, chloroplast, putative	*Ricinus communis*	gi|224106116|gb|BQ991081.1|	677	3.50E-62	7	27801	453.2 ± 214.8	886.2 ± 117.2	NA	NA	2.0	Increased
	1313	EST	QGF25M12.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGF25M12, mRNA	PREDICTED: 31 kDa ribonucleoprotein, chloroplastic-	*Vitis vinifera*	gi|224120281|gb|BQ992493.1|	429	2.20E-37	8	28277	756.97 ± 230.4	1953.4 ± 247.5	NA	NA	2.6	Increased

		sequence	like												
1315	EST	C06B09 Lactuca sativa PI251246 leaf reverse SSH library Lactuca sativa cDNA similar to chloroplast RNA binding protein, mRNA sequence	PREDICTED: 31 kDa ribonucleoprotein, chloroplastic-like	*Vitis vinifera*	gi|317383543|gb|HS586847.1|	497	3.50E-44	7	34900	235.3 ± 121.5	637.6 ± 44.3	NA	NA	2.7	Increased
1317	EST	CLSZ3508.b1_G13.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSZ3508, mRNA sequence	PREDICTED: nascent polypeptide-associated complex subunit alpha-like protein 2-like	*Vitis vinifera*	gi|399812 6|gb|DW14 4235.1|	654	7.00E-60	7	19076	212.5 ± 51.3	771.3 ± 316.6	NA	NA	3.6	Increased
2305	EST	CLSZ1479.b1_N10.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSZ1479, mRNA sequence	Elongation factor 1-beta, putative	*Ricinus communis*	gi|399614 6|gb|DW14 2255.1|	446	4.40E-39	6	32012	135.8 ± 44.5	344.6 ± 94.7	NA	NA	2.5	Increased
2306		Proliferating cell nuclear antigen		*Nicotiana benthamiana*	gi|1094642 7	246	2.90E-19	5	29509	84.1 ± 20.3	280.1 ± 77.1	NA	NA	3.3	Increased
2307	EST	CLLY9318.b1_L01.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY9318, mRNA sequence	PREDICTED: 14-3-3-like protein D-like	*Vitis vinifera*	gi|380928 8|gb|DW06 9505.1|	568	2.80E-51	7	30462	202.4 ± 80.2	486.3 ± 145.1	NA	NA	2.4	Increased
2313	EST	CLVY12319.b1_M07.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY12319, mRNA sequence	PREDICTED: eukaryotic translation initiation factor 3 subunit F-like	*Solanum lycopersicum*	gi|840229 6|gb|DW16 0236.1|	645	5.60E-59	8	34924	101.3 ± 50.5	300.7 ± 55.3	NA	NA	3.0	Increased
3509	EST	CLSM19483.b1_E24.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM19483, mRNA sequence	PREDICTED: 30S ribosomal protein S1, chloroplastic	*Vitis vinifera*	gi|905065 5|gb|DY968 413.1|	169	2.20E-11	3	31145	80.6 ± 37.6	210.9 ± 26.6	NA	NA	2.6	Increased
4412	EST	CLRY8789.b1_J14.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY8789, mRNA sequence	PREDICTED: transcription factor Pur-alpha 1-like	*Fragaria vesca subsp. Vesca*	gi|839180 8|gb|DW12 0168.1|	635	5.60E-58	9	32651	70.7 ± 23.1	184 ± 27.3	NA	NA	2.6	Increased
4502	EST	CLSX4289.b1_A17.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX4289, mRNA sequence	PREDICTED: 30S ribosomal protein S1, chloroplastic-like	*Solanum lycopersicum*	gi|839816 3|gb|DW12 7748.1|	651	1.40E-59	7	26268	164.03 ± 70.3	555.4 ± 70.98	NA	NA	3.4	Increased
5810		PREDICTED: eukaryotic translation initiation factor 3 subunit D-like		*Cucumis sativus*	gi|224079205	209	1.50E-15	2	63145	36.4 ± 12.8	131.6 ± 31.91	NA	NA	3.6	Increased
6406	EST	CLVY10437.b1_I17.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY10437, mRNA sequence	PREDICTED: 60S acidic ribosomal protein P0	*Vitis vinifera*	gi|840210 5|gb|DW15 8325.1|	747	3.50E-69	9	36869	156.97 ± 44.3	723.9 ± 56.2	NA	NA	4.6	Increased
6492	EST	CLSY5357.b1_J20.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone	U2 small nuclear ribonucleoprotein A, putative	*Ricinus communis*	gi|399180 8|gb|DW13 7917.1|	360	1.80E-30	6	33901	19 ± 7.98	58.1 ± 20.3	NA	NA	3.1	Increased

			CLSY5357, mRNA sequence												
	6495	EST	CLLY4453.b1_I10.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY4453, mRNA sequence	Zinc finger protein, putative	*Ricinus communis*	gi|83804602|gb|DW064819.1|	225	5.60E-17	3	29073	29.8 ± 10.3	185.9 ± 52.8	NA	NA	6.2	Increased
	8204	EST	CLSZ675.b1_F02.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSZ675, mRNA sequence	Minor allergen Alt a, putative	*Ricinus communis*	gi|83998732|gb|DW144841.1|	572	1.1E-051	6	28441	163.2 ± 30.7	358.7 ± 20.43	NA	NA	2.2	Increased
C1 and C10	2110	EST	QGB18O24.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB18O24, mRNA sequence	50S ribosomal protein L12, chloroplastic	NA	gi|222382332|gb|BQ852767.1|	327	3.5E-027	4	17289	468.97 ± 90.3	1610.1 ± 164.5	1381.4 ± 246.4	NA	3.4 (C1) and 2.9 (C10)	Increased
	3302	EST	QGC12N17.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGC12N17, mRNA sequence	PREDICTED: eukaryotic translation initiation factor 3 subunit J-like	*Solanum lycopersicum*	gi|222448499|gb|BQ859384.1|	203	8.80E-15	4	24723	58.9 ± 9.8	215.6 ± 35.7	144.1 ± 48.2	NA	3.7 (C1) and 2.4 (C10)	Increased
	33119	EST	CLVX13217.b1_B17.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX13217, mRNA sequence	PREDICTED: 14-3-3 protein 1-like	*Vitis vinifera*	gi|840109|gb|DW148188.1|	351	1.40E-29	5	31315	30.1 ± 13.8	74.9 ± 13.1	122.3 ± 54.0	NA	2.5 (C1) and 4.1 (C10)	Increased
	8207	EST	QGJ15D06.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGJ15D06, mRNA sequence	PREDICTED: auxin-binding protein ABP20-like	*Cucumis sativus*	gi|224048037|gb|BQ987278.1|	475	5.60E-42	4	23032	543.7 ± 66.3	2041.4 ± 234.2	1395.4 ± 194.7	NA	3.8 (C1) and 2.6 (C10)	Increased
C1 and C100	2314	EST	QGJ14P03.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGJ14P03, mRNA sequence	PREDICTED: 31 kDa ribonucleoprotein, chloroplastic-like	*Fragaria vesca subsp. vesca*	gi|224453647|gb|BU010969.1|	216	4.40E-16	5	18596	50.2 ± 15.6	189.9 ± 72.2	NA	127.4 ± 30.3	3.8 (C1) and 2.5 (C100)	Increased
	5212	EST	CLLX400.b1_O03.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX400, mRNA sequence	Ribonucleoprotein, chloroplast, putative	*Ricinus communis*	gi|837909977|gb|DW051214.1|	549	2.20E-49	5	34181	128.4 ± 42.3	739.8 ± 186.1	NA	348.8 ± 90.7	5.8 (C1) and 2.7 (C100)	Increased
C1, C10 and C100	2303	EST	CLVY12319.b1_M07.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY12319, mRNA sequence	PREDICTED: eukaryotic translation initiation factor 3 subunit F-like	*Solanum lycopersicum*	gi|840229|gb|DW160236.1|	545	5.6E-049	7	34924	77.8 ± 27.8	NA	NA	NA	Protein spot absent in C1, C10 and C100	NA
C10	32118	EST	CLSY3618.b1_D17.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSY3618, mRNA sequence	Ribonucleoprotein, chloroplast, putative	*Ricinus communis*	gi|839899|gb|DW136061.1|	324	7.00E-27	6	24824	101.3 ± 26.7	NA	495.1 ± 107.9	NA	4.9	Increased
	3303	EST	CLSX9852.b1_G16.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX9852, mRNA sequence	14-3-3 protein, putative	*Ricinus communis*	gi|839870|gb|DW133168.1|	226	4.4E-017	3	25891	133.1 ± 58.8	NA	278.2 ± 35.1	NA	2.1	Increased
	4404	EST	CLRY3990.b2_L13.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA	PREDICTED: 40S ribosomal protein SA-like	*Solanum lycopersicum*	gi|839137|gb|DW115860.1|	245	5.60E-19	3	28095	105.5 ± 33.4	NA	27 ± 9.97	NA	3.9	**Decreased**

			clone CLRY3990, mRNA sequence													
	4587		30S ribosomal protein S1, chloroplastic		NA	gi|133872	133	5.80E-08	4	45044	46.5 ± 23.6	NA	155.5 ± 58.2	NA	3.3	Increased
	5184	EST	CLPY2072.b1_P13.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPY2072, mRNA sequence	Regulator of ribonuclease activity A, putative	*Ricinus communis*	gi|8388728|gb|DW090808.1|	270	1.80E-21	5	30579	72.73 ± 36.92	NA	NA	NA	Protein spot absent in C10	NA
	6103		RecName: Full=Eukaryotic translation initiation factor 5A; Short=eIF-5A		NA	gi|20138786	295	3.70E-24	4	17483	78.3 ± 32.93	NA	NA	NA	Protein spot absent in C10	NA
	6106	EST	CLVX7115.b1_E04.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX7115, mRNA sequence	PREDICTED: 40S ribosomal protein S12-like	*Solanum lycopersicum*	gi|84017797|gb|DW155067.1|	135	5.60E-08	3	20018	69 ± 27.6	NA	NA	NA	Protein spot absent in C10	NA
	6264	EST	CLRY2358.b1_K13.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY2358, mRNA sequence	PREDICTED: abscisic acid receptor PYR1-like	*Vitis vinifera*	gi|83912166|gb|DW114246.1|	238	2.80E-18	6	26470	42.7 ± 7.07	NA	NA	NA	Protein spot absent in C10	NA
	6265	EST	CLSS13118.b1_K15.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS13118, mRNA sequence	PREDICTED: eukaryotic translation initiation factor 3 subunit K-like	*Fragaria vesca subsp. Vesca*	gi|90517578|gb|DY979436.1|	787	3.50E-73	9	29066	28.2 ± 5.0	NA	NA	NA	Protein spot absent in C10	NA
	8809	EST	QGG8O06.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGG8O06, mRNA sequence	Poly(A)-binding protein	*Medicago truncatula*	gi|22440105|gb|BU005710.1|	267	3.50E-21	5	27410	30.3 ± 12.74	NA	NA	NA	Protein spot absent in C10	NA
C100	77192	EST	CLSX11037.b1_I24.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX11037, mRNA sequence	Elongation factor 2	*Aegilops tauschii*	gi|83978469|gb|DW124578.1|	147	3.50E-09	3	33612	56.5 ± 6.6	NA	NA	32.85 ± 7.4	2	**Decreased**
Transport activity																
C1	4376	EST	QGE12M22.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGE12M22, mRNA sequence	PREDICTED: chloroplast processing peptidase-like	*Solanum lycopersicum*	gi|223986|gb|BQ981083.1|	559	2.20E-50	8	25375	44 ± 4.07	146.6 ± 22.6	NA	NA	3.3	Increased
	5215	EST	CLLX3611.b1_F15.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX3611, mRNA sequence	Temperature-induced lipocalin	*Populus tremula x Populus tremuloides*	gi|83790607|gb|DW050824.1|	153	8.80E-10	5	29077	58 ± 10.6	117.9 ± 19.15	NA	NA	2.0	Increased
	6702	EST	QGG12J01.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGG12J01, mRNA sequence	PREDICTED: protein TIC 62, chloroplastic-like	*Cucumis sativus*	gi|22430737|gb|BQ996341.1|	338	2.80E-28	3	24768	44.97 ± 26.93	363.4 ± 42.62	NA	NA	8.1	Increased

	7201	EST	CLSS5192.b1_O02.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS5192, mRNA sequence	Apolipoprotein d, putative	*Ricinus communis*	gi|90521021|gb|DY982879.1|	20311	3.50E-54	8	20311	87.3 ± 31.2	216.9 ± 22.25	NA	NA	2.5	Increased
C10	8208		RecName: Full=GTP-binding nuclear protein Ran1A		NA	gi|1710007	489	1.50E-43	7	24114	118.5 ± 56.8	NA	NA	NA	Protein spot absent in C10	NA
C10 and C100	66106	EST	CLLY11583.b1_M15.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY11583, mRNA sequence	PREDICTED: protein TIC 62, chloroplastic-like	*Cucumis sativus*	gi|837987378|gb|DW058955.1|	182	1.10E-12	3	32021	13.7 ± 1.4	NA	46.7 ± 4.1	62.5 ± 20.9	3.4 (C10) increased and 4.6 (C100) decreased	**Decreased**
Cytoskeleton formation																
C1	3403		Plastid-dividing ring protein		*Solanum tuberosum*	gi|47156057	409	1.50E-35	4	44089	103.9 ± 23.36	245.4 ± 16.29	NA	NA	2.4	Increased
C1 and C10	2301	EST	CLVY7509.b1_I06.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY7509, mRNA sequence	Fibrillin	*Coffea canephora*	gi|84033889|gb|DW169208.1|	267	3.5E-021	4	31814	146.6 ± 73.7	385.6 ± 84.3	415.6 ± 137.7	NA	2.6 (C1) and 2.8 (C10)	Increased
C10	6107	EST	QGE6O14.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGE6O14, mRNA sequence	Actin depolymerizing factor 2	*Gossypium hirsutum*	gi|22402995|gb|BQ985470.1|	744	7.00E-69	8	20038	96.2 ± 10.0	NA	NA	NA	Protein spot absent in C10	NA
Cell wall biogenesis/degradation																
C1	8401	EST	CLPY6271.b1_N07.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPY6271, mRNA sequence	PREDICTED: UDP-arabinopyranose mutase 1	*Vitis vinifera*	gi|83893082|gb|DW095162.1|	181	1.40E-12	4	32689	62.6 ± 16.6	235.4 ± 103.9	NA	NA	3.8	Increased
C1 and C100	8305	EST	CLSS6180.b1_G09.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS6180, mRNA sequence	Xyloglucan endotransglucosylase/hydrolase	*Tagetes patula*	gi|90521702|gb|DY983560.1|	557	3.50E-50	8	36297	295.7 ± 66.5	924.2 ± 266.6	NA	107.4 ± 53.60	3.1 (C1)increased and 2.8 (C100) decreased	**Decreased**
Amino acid metabolism																
C1	6701	EST	CLVY7678.b1_L24.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVY7678, mRNA sequence	ketol-acid reductoisomerase	*Elaeis guineensis*	gi|84034071|gb|DW169390.1|	674	7.00E-62	6	31229	189.3 ± 78.10	635.4 ± 105.4	NA	NA	3.4	Increased
C10	6497	EST	QGB27B24.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB27B24, mRNA sequence	PREDICTED: N-carbamoylputrescine amidase-like	*Fragaria vesca subsp. Vesca*	gi|222410605|gb|BQ855595.1|	212	1.10E-15	4	26768	39.9 ± 5.2	NA	NA	NA	Protein spot absent in C10	NA
	6732		Acetohydroxyacid synthase 1		*Helianthus annuus*	gi|469488552	348	1.80E-29	4	71154	29.3 ± 4.5	NA	9.5 ± 2.7	NA	3.1	**Decreased**

	7513	EST	CLRY2510.b1_K04.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY2510, mRNA sequence	PREDICTED: diaminopimelate decarboxylase 2, chloroplastic isoform 1	*Vitis vinifera*	gi|83912316|gb|DW114396.1|	368	2.8E-031	6	31648	50.4 ± 24.3	NA	NA	NA	Protein spot absent in C10	NA
	7803		Vitamin-b12 independent methionine synthase, 5-methyltetrahydropteroyltriglutamate-homocysteine		*Populus trichocarpa*	gi|224104961	210	1.20E-15	4	85033	51.5 ± 19.5	NA	NA	NA	Protein spot absent in C10	NA
C100	6490	EST	CLSS4535.b2_N06.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS4535, mRNA sequence	Thiosulfate sulfertansferase, putative	*Ricinus communis*	gi|90520693|gb|DY982551.1|	301	1.40E-24	5	26299	55 ± 21.94	NA	NA	NA	Protein spot absent in C100	NA
Pigment metabolism																
C1	6405	EST	CLRY6323.b1_F21.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY6323, mRNA sequence	PREDICTED: coproporphyrinogen-III oxidase, chloroplastic-like	*Vitis vinifera*	gi|83915540|gb|DW117620.1|	436	4.40E-38	9	33519	145.9 ± 49.8	452.7 ± 119.43	NA	NA	3.1	Increased
C10	74107	EST	CLRX3506.b1_C13.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX3506, mRNA sequence	Uroporphyrinogen decarboxylase, chloroplastic	NA	gi|83903592|gb|DW105672.1|	232	1.10E-17	5	28920	103.1 ± 35.33	NA	NA	NA	Protein spot absent in C10	NA
C100	66108	EST	QGB6J15.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB6J15, mRNA sequence	Polyphenol oxidase precursor (chloroplast)	*Taraxacum officinale*	gi|222425622|gb|BQ857097.1|	147	3.50E-09	2	26774	83.2 ± 19.54	NA	NA	NA	Protein spot absent in C100	NA
Lipid metabolism																
C1	4495	EST	CLSS2981.b1_J02.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS2981, mRNA sequence	PREDICTED: GDSL esterase/lipase At5g45670	*Vitis vinifera*	gi|90519132|gb|DY980990.1|	634	7.00E-58	7	33181	47.6 ± 16.28	104.7 ± 8.9	NA	NA	2.2	Increased
	6413	EST	CLLY11826.b1_D06.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY11826, mRNA sequence	Enoyl-ACP reductase 1	*Helianthus annuus*	gi|83799000|gb|DW059217.1|	527	3.50E-47	6	33037	106.5 ± 10.9	339.4 ± 38.9	NA	NA	3.2	Increased
	6494	EST	CLSM18468.b1_G09.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM18468, mRNA sequence	PREDICTED: GDSL esterase/lipase LTL1-like	*Glycine max*	gi|90505494|gb|DY967352.1|	267	3.50E-21	4	34501	46.3 ± 21.6	141.1 ± 27.5	NA	NA	3.0	Increased
	7305	EST	CLSY7797.b1_J05.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSY7797, mRNA sequence	PREDICTED: bifunctional epoxide hydrolase 2-like	*Solanum lycopersicum*	gi|83994039|gb|DW140148.1|	522	1.10E-46	8	32235	76.8 ± 22.2	127.7 ± 9.1	NA	NA	1.7	Increased
C10	6734	EST	QGC4h07.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGC4h07, mRNA sequence	PREDICTED: 2-hydroxyacyl-CoA lyase-like	*Glycine max*	gi|22250919|gb|BQ865454.1|	335	5.60E-28	4	23074	29.3 ± 5.8	NA	NA	NA	Protein spot absent in C10	NA

	75118	EST	CLSM7866.b1_D23.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM7866, mRNA sequence	Acetyl-CoA C-acetyltransferase protein	*Camellia oleifera*	gi|90512340|gb|DY974198.1|	356	4.40E-30	4	28386	95.9 ± 28.6	NA	NA	NA	Protein spot absent in C10	NA
Ascorbic acid biosynthesis																
C1, C10 and C100	6363	EST	CLPY2939.b1_E16.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPY2939, mRNA sequence	Phosphomanno mutase	*Glycine max*	gi|83889580|gb|DW091660.1|	138	2.80E-08	2	33644	24.3 ± 7.01	NA	NA	NA	Protein spot absent in C1, C10 and C100	NA
C10	6267	EST	CLPY3087.b1_M03.ab1 CLP(XYZ) lettuce perennis Lactuca perennis cDNA clone CLPY3087, mRNA sequence	Phosphomanno mutase	*Glycine max*	gi|83889719|gb|DW091799.1|	453	8.80E-40	5	33650	32.8 ± 2.5	NA	NA	NA	Protein spot absent in C10	NA
Vitamin B1 biosynthesis																
C1	3309	EST	CLLX1473.b1_B10.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX1473, mRNA sequence	PREDICTED: thiamine thiazole synthase, chloroplastic-like	*Fragaria vesca subsp. Vesca*	gi|83787896|gb|DW048113.1|	194	7.00E-14	2	29123	158.5 ± 5.5	327.8 ± 89.95	NA	NA	2.1	Increased
	4308	EST	CLLX1473.b1_B10.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX1473, mRNA sequence	PREDICTED: thiamine thiazole synthase, chloroplastic-like	*Fragaria vesca subsp. Vesca*	gi|83787896|gb|DW048113.1|	297	3.50E-24	4	29123	118.8 ± 37.8	417 ± 154.7	NA	NA	3.5	Increased
Inositol biosynthesis																
C1	5720		L-myo-inositol-1-phosphate synthase		*Actinidia deliciosa*	gi|409194966	528	1.80E-47	5	56733	156.5 ± 103.94	538.03 ± 101.08	NA	NA	3.4	Increased
Unknown/Miscellaneous																
C1	1501	EST	QGB19G06.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB19G06, mRNA sequence	TGB12K interacting protein 2	*Nicotiana tabacum*	gi|22238401|gb|BQ852936.1|	238	2.8E-018	4	21973	99.7 ± 39.92	378.8 ± 128.28	NA	NA	3.8	Increased
	2108	EST	CLRY589.b1_I04.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY589, mRNA sequence	Drought-induced protein SDi-6 - common sunflower (fragment)	NA	gi|83915068|gb|DW117148.1|	397	3.50E-34	4	16020	1273.6 ± 452.4	2375.9 ± 62.7	NA	NA	2.0	Increased
	2310	EST	CLLY12528.b1_O12.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY12528, mRNA sequence	PREDICTED: probable plastid-lipid-associated protein 13, chloroplastic-like	*Glycine max*	gi|83799755|gb|DW059972.1|	427	3.50E-37	4	32179	172.4 ± 32.43	345.8 ± 81.15	NA	NA	2.0	Increased
	2325	EST	QGB15I23.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGB15I23, mRNA sequence	Alpha chain of nascent polypeptide associated complex	*Nicotiana benthamiana*	gi|22236981|gb|BQ851516.1|	466	4.40E-41	5	24262	NA	243.5 ± 53.43	NA	NA	Protein spot absent in C	NA

	2703	EST	CLLY8910.b1_K19.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY8910, mRNA sequence	Lettuce saligna Lactuca saligna cDNA clone CLLY8910, mRNA sequence	NA	gi|83808894|gb|DW069111.1|	193	8.80E-14	3	31562	155.1 ± 60.99	535.03 ± 116.1	NA	NA	3.5	Increased
	3317	EST	CLLY3938.b1_D01.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY3938, mRNA sequence	PREDICTED: protein CbbY-like	*Fragaria vesca subsp. Vesca*	gi|83804044|gb|DW064261.1|	492	1.10E-43	5	30930	90.43 ± 10.1	340.7 ± 97.5	NA	NA	3.8	Increased
	4206	EST	QGA10P22.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGA10P22, mRNA sequence	PREDICTED: UPF0603 protein At1g54780, chloroplastic	*Vitis vinifera*	gi|222233421|gb|BQ843556.1|	1090	1.80E-103	8	27549	194.93 ± 61.2	451.5 ± 78.56	NA	NA	2.3	Increased
	47166	EST	CLRX4336.b1_P03.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRX4336, mRNA sequence	Lettuce serriola Lactuca serriola cDNA clone CLRX4336, mRNA sequence	NA	gi|83904547|gb|DW106627.1|	112	1.10E-05	2	28027	47.5 ± 15.6	95 ± 13.98	NA	NA	2.0	Increased
	5493	EST	QGG12H07.yg.ab1 QG_EFGHJ lettuce serriola Lactuca serriola cDNA clone QGG12H07, mRNA sequence	Lettuce serriola Lactuca serriola cDNA clone QGG12H07, mRNA sequence	NA	gi|22430696|gb|BQ996300.1|	155	5.60E-10	2	23846	40.43 ± 12.72	108.6 ± 33.6	NA	NA	2.7	Increased
	7204	EST	CLLY3304.b1_O10.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY3304, mRNA sequence	PREDICTED: auxin-binding protein ABP19a-like	*Cucumis sativus*	gi|83803421|gb|DW063638.1|	560	1.80E-50	4	25783	3082.4 ± 1133.5	7286.43 ± 343.12	NA	NA	2.4	Increased
	7205	EST	CLVX5952.b1_P23.ab1 CLV(XYZ) lettuce virosa Lactuca virosa cDNA clone CLVX5952, mRNA sequence	PREDICTED: auxin-binding protein ABP19a-like	*Cucumis sativus*	gi|84016559|gb|DW153829.1|	420	1.80E-36	4	28613	240.93 ± 26.8	1231.8 ± 443.6	NA	NA	5.1	Increased
C1 and C10	1205	EST	CLLX562.b1_D21.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX562, mRNA sequence	Lettuce saligna Lactuca saligna cDNA clone CLLX562, mRNA sequence	NA	gi|83792742|gb|DW052959.1|	230	1.80E-17	3	30363	75.9 ± 39.2	316.3 ± 78.34	389.6 ± 125.04	NA	4.2 (C1) and 5.1 (C10)	Increased
	2209	EST	CLSS4038.b1_K02.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSS4038, mRNA sequence	Lettuce sativa Lactuca sativa cDNA clone CLSS4038, mRNA sequence	NA	gi|90520166|gb|DY982024.1|	421	1.40E-36	6	29179	276.6 ± 39.7	654.8 ± 60.78	498.17 ± 93.0	NA	2.4 (C1) and 2.0 (C10)	Increased
C10	6301		PREDICTED: stem-specific protein TSJT1		*Vitis vinifera*	gi|225432548	215	3.70E-16	3	25409	75.17 ± 21.17	NA	NA	NA	Protein spot absent in C10	NA
	6810	EST	CLLX9942.b1_L14.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLX9942, mRNA sequence	PREDICTED: aconitate hydratase 2, mitochondrial	*Vitis vinifera*	gi|83797130|gb|DW057347.1|	293	8.80E-24	4	28360	63.5 ± 16.99	NA	15.05 ± 5.39	NA	4.2	**Decreased**
	72100	EST	CLRY2138.b1_C08.ab1 CLR(XYZ) lettuce serriola Lactuca serriola cDNA clone CLRY2138, mRNA sequence	Lettuce serriola Lactuca serriola cDNA clone CLRY2138, mRNA sequence	NA	gi|83911952|gb|DW114032.1|	83	0.0088	4	33177	59.4 ± 19.20	NA	NA	NA	Protein spot absent in C10	NA

	74108	EST	CLLY3754.b1_D04.ab1 CLL(XYZ) lettuce saligna Lactuca saligna cDNA clone CLLY3754, mRNA sequence	PREDICTED: uncharacterized oxidoreductase At4g09670-like	*Vitis vinifera*	gi|83803854|gb|DW064071.1|	181	1.40E-12	4	30511	50.8 ± 23.64	NA	NA	NA	Protein spot absent in C10	NA
C10 and C100	41101	EST	QGA2d11.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGA2d11, mRNA sequence	Lettuce salinas Lactuca sativa cDNA clone QGA2d11, mRNA sequence	NA	gi|222317055|gb|BQ847436.1|	649	2.20E-59	8	25396	44.1 ± 13.17	NA	NA	NA	Protein spot absent in C10 and C100	NA
	7101		PREDICTED: 40S ribosomal protein S12-like isoform 2		*Vitis vinifera*	gi|225465502	179	1.50E-12	2	15359	98.83 ± 1.89	NA	NA	NA	Protein spot absent in C10 and C100	NA
	76149	EST	CLSM8393.b1_B12.ab1 CLS(LMS) lettuce sativa Lactuca sativa cDNA clone CLSM8393, mRNA sequence	PREDICTED: U-box domain-containing protein 72	*Vitis vinifera*	gi|90512855|gb|DY974713.1|	160	1.80E-10	5	33215	33.37 ± 10.98	NA	NA	NA	Protein spot absent in C10 and C100	NA
C100	1408	EST	QGC21I20.yg.ab1 QG_ABCDI lettuce salinas Lactuca sativa cDNA clone QGC21I20, mRNA sequence	Plastid transcriptionally active 6	*Arabidopsis thaliana*	gi|22248068|gb|BQ862603.1|	421	1.40E-36	5	23478	105.8 ± 4.02	NA	NA	61.83 ± 8.28	2.0	**Decreased**
	1446	EST	CLSX9917.b1_J08.ab1 CLS(XYZ) lettuce sativa Lactuca sativa cDNA clone CLSX9917, mRNA sequence	PREDICTED: probable plastid-lipid-associated protein 3, chloroplastic-like	*Vitis vinifera*	gi|83987123|gb|DW133232.1|	138	2.80E-08	4	31742	NA	NA	NA	202.97 ± 44.35	Protein spot absent in C	NA

NOTES

gi| Proteins identified using NCBI databasw

gi|gb| Proteins identified using EST database

NA: not applicable

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