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ECOTOXICOLOGICAL CHARACTERIZATION OF CYANOBACTERIA AND INVASIVE ALGAE FROM THE GENUS *CAULERPA* USING THE CONCEPT OF EFFECTS-DIRECTED ANALYSES (EDA)

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Ekotoksikološka karakterizacija cijanobakterija i invazivnih algi roda *Caulerpa* primjenom koncepta učincima usmjerenih analiza

Petra Marić

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Kratki sažetak doktorske disertacije:
Koncept učincima usmjerenih analiza predstavlja suvremeni multidisciplinarni dijagnostički alat znanosti o okolišu koji omogućava identifikaciju, karakterizaciju i prioritizaciju toksikanata prisutnih u složenim okolišnim uzorcima. U ovom istraživanju koristili smo ovaj koncept za provedbu detaljne ekotoksikološke karakterizacije dvaju tipova bioloških uzoraka, invazivne tropske alge roda *Caulerpa* i više cijanobakterijskih sojeva. U oba tipa uzoraka otkriveni su biološki relevantni mehanizmi toksičnosti sekundarnih metabolita na fazu 0. i I. staničnog detoksikacijskog mehanizma.

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Ecotoxicological characterization of cyanobacteria and invasive algae from the genus *Caulerpa* using the concept of effects-directed analyses (EDA)

Petra Marić

Thesis performed at: Ruđer Bošković Institute, Zagreb, Croatia
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Short abstract:
The concept of effects-directed analyses (EDA) is a modern multidisciplinary diagnostic tool in the environmental science that enables the identification, characterization and prioritization of toxicants present in complex environmental samples. In this study we used the modified EDA concept for detailed ecotoxicological characterization of two types of biological samples, invasive tropical algae from the genus *Caulerpa* and cyanobacterial strains. Relevant mechanisms of toxicity of secondary metabolites present in biological samples were discovered to phase 0 and I of the cellular detoxification pathway.

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Petra Marić

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1. INTRODUCTION

1.1. EFFECTS-DIRECTED ANALYSES

1.1.1. Background

Ecotoxic effects are constantly present in the environment, either naturally or more often caused by the human impact. These toxic effects are typically caused by highly diverse mixtures of known and unknown chemicals that differ in their toxic potency and mechanism of action (Brack et al., 2013). Furthermore, these complex environmental mixtures contain compounds whose concentrations usually range over several orders of magnitude and that can produce various congeners and isomers during spontaneous chemical reactions in the environment. In addition, these compounds can interact with each other and modulate the toxic potential, sometimes becoming even more toxic (Brack, 2011). This has become a great issue in modern human society especially since the beginning of the industrial and technological development from the mid-20th century that is marked by large-scale production and multitude use of a vast number of chemicals. As a consequence, many new environmental pollutants are constantly being introduced into the environment. Together with toxins of natural origin they significantly affect surface and ground waters, sediment, soil and biota to which humans and wildlife are continuously exposed. Most of these pollutants are unknown and non-regulated contaminants. Therefore, in order to preserve and protect environmental and human health, characterization and identification of toxins that cause various adverse effects has become a constant struggle for ecotoxicology and environmental chemistry.

Due to multilevel complexity of environmental mixtures it is especially hard to predict biological effects of the related chemical classes (Altenburger et al., 2000; Silva et al., 2002). It was clear early on that biological and chemical aspects of these issues should be addressed equally. However, for most of potentially toxic compounds and their degradation products no analytical methods or standards were available as they were not previously analytically identified. But most importantly, there was little or no knowledge about their effects even if these contaminants have been identified. Therefore, in the past two decades the focus of environmental science has been to develop bioanalytical concepts that could tackle this analytical challenge and enable filtering and identification of the chemicals responsible for the observed effect.

To date, two fundamentally distinct but prominent concepts have been used for environmental diagnostics of complex environmental matrices (municipal and industrial

effluents, ground, surface and interstitial waters, sediment and biota). They are well known as "Effects-directed Analyses" (EDA) and "Toxicity Identification Evaluation" (TIE) (Samoiloff et al., 1983; Brack, 2003; Brack et al., 2007). It all started in the late 1970s when first EDA study was conducted and developed in Europe in order to identify mutagenic compounds in cigarette smoke, diesel exhaust particles, drinking water and consumer products (Schuetzle and Lewtas, 1986). As a response to European approach, the US Environmental Protection Agency (US EPA) introduced in the late 1980s the so called TIE protocol to regulate effluent waters in the United States (Mount and Anderson-Carnahan, 1988; Mount and Anderson-Carnahan, 1989; Mount, 1989). Occasionally, European environmental researchers and managers used TIE in the late 1990s (Hollert et al., 2002a). Both EDA and TIE aim to identify chemical causes of measurable effects by a step-by-step toxicity assessment of environmental samples in order to establish cause-effect relationships.

1.1.2. EDA and TIE

EDA and TIE have been developed based on (1) understanding that environmental matrices mostly consist of a vast and versatile number of chemicals; and (2) that only a tiny fraction of them can be analyzed by target analysis. For a reliable hazard and risk assessment both of these concepts combine biological and analytical analysis. But they also act on two different strategic approaches. Firstly, TIE is based on the removal of toxicity from the tested matrix and the determination of the cause of toxicity in the removed fraction (Samoiloff et al., 1983). EDA procedure filters the chemicals by determination of the toxic response of each fraction that is already an extract of the original sample (Brack, 2003). Further, EDA and TIE detect different types of compounds and use different types of tests to observe the biological effect. EDA generates sophisticated fractions which are subjected to chemical analysis in order to detect organic compounds mainly in solid samples (soil, sediment) with high degree of specificity rather than bioavailability where confirmation step is not strictly defined (ter Laak et al., 2007). By applying both *in vivo* and *in vitro* assays the toxicity and toxic mode of action of detected target and non-target compounds can be provided by EDA studies. On the other hand, TIE study was originally developed on water samples where during fractionation and chemical analyses bioavailability is maintained and considered critical for accurate identification of a causative compound as well as further toxicity confirmation step. Later on, TIE studies were also applied for sediment bound contaminants (Traunspurger et al., 1997;

Duft et al., 2007; Stesevic et al., 2007). TIE is less scientific approach then EDA as it is guided by standardized USA EPA protocol with focus on inorganic and organic toxicants and uses only *in vivo* tests to monitor biological effects (Mount and Anderson-Carnahan, 1988; Mount, 1989). Therefore, more detailed insights into relevant cellular mechanisms of toxicity are not included in TIE studies. Furthermore, EDA includes extraction procedures of the original sample that enables separation of chemical compounds while TIE excludes chemical compounds using retrospective step by step approach (Brack, 2011). Although EDA proposes certain advantages over TIE by providing specific biological toxicity and analytical pathways, higher ecological relevance of TIE studies lays in preservation of a test sample by avoiding extraction procedures and direct contact with toxic mixture by using whole organism biological testing. In conclusion, these fundamentally different approaches have unique aspects that are advantageous or deficient to each other and therefore are complementary.

Nevertheless, both of these concepts include characterization, identification and confirmation phases in their studies. TIE phase I includes the removal of the compound until the toxicity disappears. Suspected compounds are chemically identified in fractions that are lacking toxic response during the phase II and finally by using the same bioassays toxicity is confirmed in the phase III (Ankley et al., 1991a, 1991b). EDA phase I measures the bioassay response of the entire extract or fraction, then the activity assessment and identifications of the extracts or fractions with toxic response are made in the phase II and finally original extract compound is tested for the confirmation step (Fig. 1.1.) (Barceló and Petrovic, 2007).

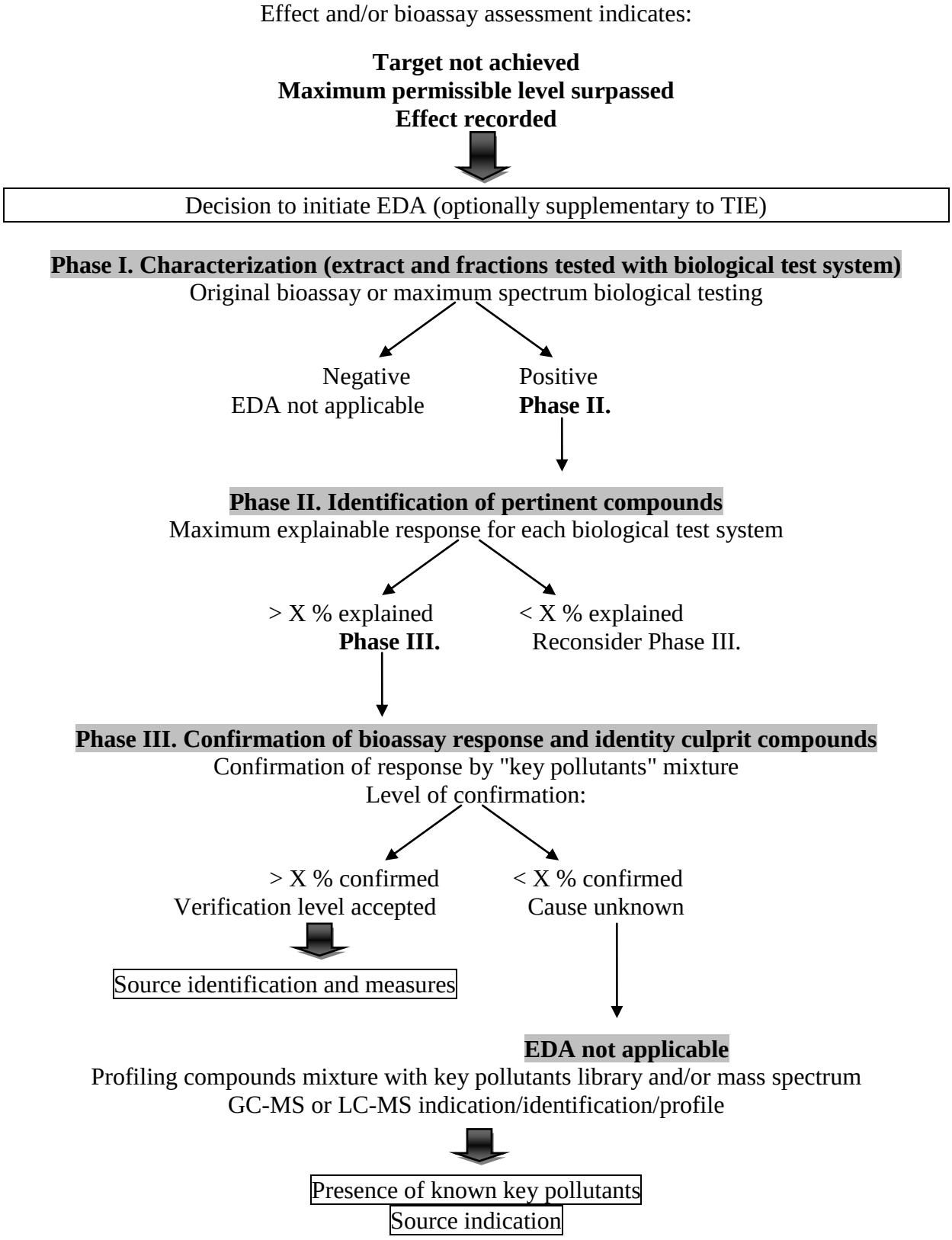


Figure 1.1. Schematic presentation of the EDA phases: I – characterization, II – identification, and III – confirmation (adapted from Barceló and Petrovic, 2007).

1.1.3. Principle of EDA

EDA, sometimes also called effect-based or -oriented analysis, integrates stepwise fractionation procedures together with the biotesting and chemical analysis in order to reduce sample complexity, detect the toxic effects and identify chemicals of concern (Fig. 1.2.) (Brack, 2003; Brack et al., 2003; Brack et al., 2005). The first step of EDA toxicant identification is the extraction that separates the toxicants from the chemical mixture. This initial step is already selective as it is focused on extraction of the organic compounds excluding the heavy metals and inorganic anions from the further process. Organic compounds are lipophilic substances to which many of priority compounds belong and they had been typical target groups in EDA studies, while polar and ionic chemicals have been studied by EDA later on (Castillo and Barceló, 2001). Further on, these extracts are usually tested with appropriate bioassay in order to screen the toxicity of the tested sample. The type of bioassay that is used for the first screening is determined according to the biological effect of interest. Selected bioassays are preferably sensitive, representative, specific, cost effective, high-throughput test such as *in vitro* assays that utilize bacteria or specific cell lines (Barceló and Petrovic, 2007). Complex environmental extracts that show toxic response in this first screening are subjected to the first step fractionation for separation of different groups of compounds or individual toxins that are present in the mixture. The aim is to narrow down the range of compounds responsible for the observed effect by grouping them according to their physico-chemical properties such as polarity, hydrophobicity, planarity, molecular size and the presence of functional groups (Brack, 2003; Brack et al., 2003). The nature of the sample (water, sediment, biota) is highly related to the fractionation technique that is applied. This separation step provides initial information about the basic compound characteristics that are useful during later phases of identification and confirmation.

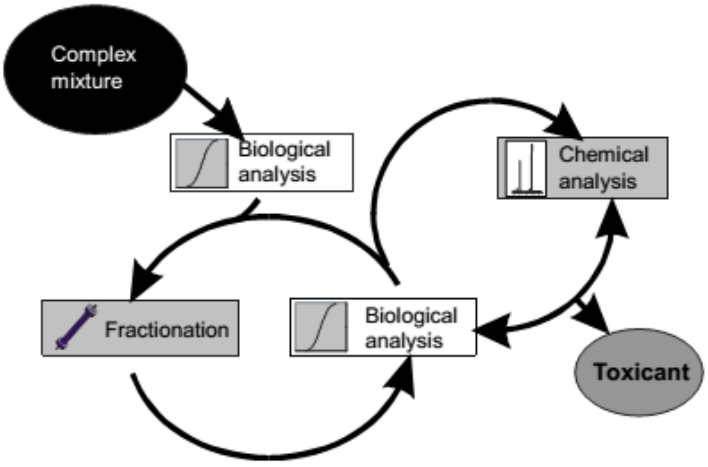


Figure 1.2. Schematic display of the Effects-directed Analyses (from Brack, 2003).

After the initial separation step the fractions are tested with the same bioassay(s) as used before for selection of active fractions that will be further fractionated by chemical-analytical techniques such as high-performance liquid chromatography (HPLC) (Holmbom et al., 1984; Brack and Schirmer, 2003). Most often these two fractionation steps are used in EDA protocols that enable elimination of nontoxic components and provide guidance to the remaining toxicants. Furthermore, elimination of non-toxic compounds minimizes the appearance of synergistic, antagonistic or additive toxicity that often occur in such complex mixtures (Warne and Hawker, 1995; Altenburger et al., 2003). Again, these sub-fractions are being tested with the same bioassays and the few individual compounds or group(s) of compounds that are left are subjected to chemical identification and quantification by instrumental analytical techniques such as gas or liquid chromatography–mass spectrometry (GC-MS/LC-MS) (Lukasewycz and Durhan, 1992; Castillo and Barceló, 1999, 2001; Dobiáš et al., 1999). Conformation of key pollutants is then achieved by testing uncontaminated standards at concentrations measured in the fractions. In the end, what remains in the specific fraction is a small number of toxicants that are responsible for the observed effects in the bioassays.

Therefore, the overall EDA process includes the following key elements: biotests that enable detection of the toxic effect; chromatographic fractionation that reduces the complexity of the environmental mixture and separates candidate toxins (i.e. chemicals of potential relevance for the observed effect); and multi-target and non-target chemical analysis to identify the candidate toxins. Primary uses of EDA studies are the assessment and prioritization of complex contaminants and the clarification of exposure to toxic stressors in a given ecosystem. The described combination of specific biomarkers, fractionation methods, chemical analysis and *in situ* investigations represent a most suitable tool available in environmental science today for ecotoxicological characterization and risk assessment related to complex environmental matrices.

1.1.4. Previous and current EDA studies

Since 1980s numerous EDA studies have been conducted on different environmental matrices for evaluation of various biological effects. Mutagenicity was one of the first toxicological endpoints evaluated by EDA where Rosenkratz et al. (1980) identified nitropyrenes as mutagenic impurities in xerographic toners. This is regarded as one of the

earliest and most successful EDA studies that demonstrated EDA as a powerful new approach. Through the following years mutagenicity as well as genotoxicity were among the most studied biological effects by EDA that enabled identification of new non-regulated major mutagens such as polycyclic aromatic hydrocarbons (PAHs), but also more polar components such as polycyclic quinones and nitroquinones, as well as nitro-PAHs in estuary and coastal European river sediments (Fernández et al., 1992; Thomas et al., 2002a; Brack et al., 2005). Many other toxicological endpoints were also successfully studied by EDA. From the beginning of the 1990s, EDA studies on the aryl hydrocarbon receptor (AhR)-mediated effects identified dioxin-like compounds (Safe, 1990; Van den Berg et al., 1998). Extensive EDA studies on AhR-mediated effects were made on European sediments from river basins and costal areas that identified polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyles (PCBs), dinaphthofurans and naphthalenyl - benzothiophene as key pollutants (Brack et al., 2002; Brack and Schirmer, 2003; Vondrácek et al., 2004). Estrogenic effects of contaminated European sediments revealed nonylphenol and tributyltin as major toxic compounds (Brack et al., 1999; Schulte-Oehlmann et al., 2001; Céspedes et al., 2005; Hollert et al., 2005). Estrogenic potency of water and biota samples were also investigated by EDA where substances like 17β-estradiol, ethinylestradiol and alkylphenolic compounds accounted for the majority of the estrogenic potential (Desbrow et al, 1998; Routledge et al., 1998; Khim et al., 1999; Snyder et al., 2001; Thomas et al., 2001; Sheahan et al., 2002; Brack and Schirmer, 2003; Houtman et al, 2004). EDA studies on anti-androgenic and androgenic effects confirmed the presence of various hormones in European estuaries (Thomas et al., 2002b; Thomas et al., 2009; Weiss et al., 2009). Table 1.1. shows all contaminants identified so far in European rivers by EDA studies.

Through the years, the use of EDA studies significantly increased where number of publications rose by 7 times compared to the 1990s (Hecker and Hollert, 2009). Significant improvements in EDA approach have been made during the last 10 years (Brack, 2011). Fractionation methods for more polar compounds have been included into EDA studies to overcome the bioavailability issue in these studies, and new toxicological endpoints were introduced to broaden the scope of the monitored biological effects (Hecker et al., 2007; Kosmehl et al., 2007; Meinert et al., 2007; Schwab and Brack, 2007; Lübcke-von Varel et al., 2008; Scholz et al., 2008; Bandow et al., 2009). The focus has been also aimed at development of *de novo* identification strategies using high-resolution mass-spectrometry (Weiss et al., 2011).

Table 1.1. Major contaminants identified by EDA studies in European river basins (adapted from Brack et al., 2007).

Compound	Priority substance (by EU-WFD)	Confirmation (cause of the measured effect)	Site/Basin
Mutagenicity/Genotoxicity			
benzo[*a*]pyrene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, benzo[ghi]perylene	Yes	Yes	Many sites worldwide
perylene, benz[*a*]fluoranthene	No	Yes	Neckar basin (Germany)
11H-indeno[2,1,7-*cde*]pyrene, methyl benz[*e*]anthracenes, perylenes	No	No	Neckar basin (Germany)
polar polycyclic compounds: benz[*a*]anthracen - quinone, pyrenequinone, nitropyrenequinone, nitroanthraquinone, nitrobenzanthracenedione, 6-nitrochrysene, nitrobenzo[*a*]-pyrenes, nitro - indeno[1,2,3-*cd*]pyrene	No	No	Mediterranean Sea, coastal zone at Barcelona (Spain)
Ah-receptor-mediated effects			
PCDD/Fs, PCBs	No	Yes	Western Scheldt (The Netherlands), Spittelwasser (Elbe basin, Germany)
benzo[*a*]pyrene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene	Yes	Yes	Morava river (Danube basin, Czech Republic)
dinaphthofurans, 2-(2-naphthalenyl) - benzothiophene, 9-methylbenz[*a*] –anthracene, 1-methylchrysene	No	Yes	Spittelwasser (Elbe basin, Germany)
Estrogenicity			
nonylphenol	Yes	Yes	Llobregat (Spain), river Neckar
benzophenone, phthalates, dehydroabietic acid sitosterol, 3-(4-methylbenzylidine)camphor, 6-acetyl-1,1,2,4,4,7-hexamethyltetralin	No	No	Rivers Neckar, Rhine (Germany), Thames (United Kingdom)
tributyltin	Yes	Yes	Elbe (Germany)
17β-estradiol, estrone, estriol	No	Yes	United Kingdom estuaries, different rivers in the Netherlands, Swiss wastewater treatment plant effluents
Androgenicity			
dehydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone, epi-androsterone	No	Yes	United Kingdom estuaries
Green algae			
N-phenyl-2-naphthylamine, prometryn	No	Yes	Spittelwasser (Elbe basin, Germany)
priority PAHs, tributyltin	Yes	Yes	Spittelwasser (Elbe basin, Germany)
Invertebrates			
methyl parathion	No	Yes	Spittelwasser (Elbe basin, Germany)
pentachlorophenol, atrazine	Yes	No	United Kingdom estuaries
tri-, tetra-chlorphenol, 4-chloro-3,5-dimethyl - phenol, nonylphenol, 4-chloro-3,5-xylenol, dieldrin, carbophenothion methylsulfoxide	No	No	United Kingdom estuaries

For assessing the mixture toxicity EDA studies also started to focus on the development of methodologies for substances with unknown mode of action and heterogeneity of concentration-response curves (Brack et al., 2008). Furthermore, EDA was utilized in environmental monitoring programs for the environmental risk assessment (ERA) purposes (Biselli et al., 2005; Brack et al., 2005; Kammann et al., 2005a; Wölz et al., 2008; Phillips et al., 2009; von der Ohe et al., 2009; Weiss et al., 2009).

In the last 5 years there is still much focus on the estrogenic toxic effects and the identification of environmental ligands of steroid estrogen receptors (ER) in sediment samples (Grund et al., 2011; Schmitt et al., 2012; Wang et al., 2012; Creusot et al., 2013, 2014). Recent EDA studies also continue to identify androgenic, mutagenic as well as dioxin-like compounds as indicated in recent articles on newly identified alkylated chrysenes and picenes (Meyer et al., 2014). But the main focus for recent EDA studies are *in vivo* studies and development of new research models. Although *in vitro* assays provide the useful information about the toxicity mechanism of the toxic compound they do not reflect the complexity of an organism due to limitations of pharmacokinetics, tissue distribution and biotransformation that may occur *in vivo*. Limited metabolic activity of the cell cultures further disables the detection of potentially active substances that need bioactivation (Brack, 2011). So far, most EDA studies were primarily *in vitro* oriented but recently novel methodologies have been developed for the detection and evaluation of toxic compound by use of new *in vivo* models such as zebrafish embryo (*Danio rerio*) and mudsnail (*Potamopyrgus antipodarum*) (Schmitt et al., 2011; Lagler et al., 2011; Fetter et al., 2014). So far, these models were used for assessing estrogenic activity of compounds present in soil and sediment samples (Lager et al., 2011). Previous EDA *in vivo* studies used mostly fish species such as sanddab (*Citharichthys stigmaeus*), California halibut (*Paralichthys californicus*), flounder (*Platichthys flesus*) and trout (*Oncorhynchus mykiss*) for assesing estrogenic and dioxin-like toxins in sediment (Munkittrick et al., 1995; Gunther et al., 1998; Schlenk et al., 2005; Viganò et al., 2011). Clam *Scrobicularia plana* was also used for scanning estrogenic effects (Langston et al., 2007).

1.1.5. EDA on biota samples

EDA studies have been traditionally used and successful in identifying active biological contaminants mostly in abiotic environmental samples such as soil, sediment and

water (Thomas et al., 2002b; Grote et al., 2005; Urbatzka et al., 2007). Most of EDA studies have been applied to sediment and soil samples, as EDA approach was unlike TIE initially oriented on solid material (Brack et al., 1999, 2002; Hilscherova et al., 2001; Brack and Schirmer, 2003). Biotic samples have also been used for EDA identification and evaluation of toxicants but much rarely due to difficulties with proper sampling and preparation techniques prior to bio-chemical analysis which is much more challenging than those of the abiotic samples. Although sample treatment of the biota is more complicated it provides valuable information of the bioavailability, bioaccumulation and possible metabolization of the toxic compounds that is not present in abiotic samples or components. Furthermore, analytically identified target compound cannot always provide the complete explanation for the measured effects that are present in complex biological samples. Therefore, EDA testing of these two fundamentally different environmental samples provides insights into different aspects of compound toxicity.

Biota samples such as tissue, whole body homogenate, blood and urine have only recently gained more attention in EDA studies with novel extraction and clean-up methodologies available (Simon et al., 2010). Thyroid-hormone-like and anti-androgenic activities have been studied in muscle tissue of three different fish extracts: eel (*Anguilla anguilla*), flathead mullet (*Mugil cephalus*), pangasius (*Pangasius hypophthalmus*) (Simon et al., 2010). Several appropriate bioassays have been identified for EDA studies on body fluids and tissues of exposed organisms by using biomarkers such as vitelogenin, ER and androgen receptors (AR), steroid binding protein and ER-chemically activated luciferase gene expression assay (ER-CALUX) for the assessment of the total estrogenic activity (Hewitt et al., 2003; Houtman et al., 2004).

1.1.6. Advantages and limitations of EDA

Many groups of toxicants have never been considered of any importance until the development of EDA. Chemicals such as diazinon and certain surfactant compounds from municipal effluent waters were not thought to be of any concern prior to their discovery achieved by EDA studies (Brack, 2011). TIE and especially EDA are considered to be the most advanced methodologies for addressing complex environmental toxicity issues that provide valuable information about the identity of the toxic compound, mechanisms of toxicity and their effects, and finally synergistic or antagonistic interactions among toxic

compounds present in the chemical mixtures (Hilscherova et al., 2000; Körner et al., 2000). These two effects-related approaches are the only currently available diagnostic tools that successfully respond to complex environmental mixtures. Although EDA requires the use of various bio-chemical methodologies and set-ups which are often time and cost intensive and not easily standardized, EDA provides the broadest spectrum of possible resolutions and insights into environmentally relevant toxicity. Nevertheless, new improvements of this approach are clearly required. That in turn motivated recently organized EDA-EMERGE consortium, a Marie Curie ITN project initiative funded by the European Commission within the 7th framework programme (http://www.ufz.de/eda-emerge/) in developing innovative mode-of-action biognostic tools and introducing transgenic organisms and 'omics' techniques into EDA studies. Furthermore, nowadays EDA is directed towards the development of powerful and new bio-analytical detection tools for improvement of structure elucidation technologies, enhancement of EDA throughput and reduction of sample complexity (Brack et al., 2013).

Bioassays are of essential importance in EDA because they enable the detection of toxic effects in samples of concern and guide further chemical fractionation and analysis steps of separated bioactive fractions. Bioassays also play a significant role in the final phase of EDA where conformation step for pure substance(s) identified as potential bioactive compound(s) is required (Brack, 2003; Weller, 2012; Burgess et al., 2013). Therefore, their selection and application is crucial for identifying the right causative compound or a group of compounds. Furthermore, high sensitivity and specificity of bioassays is required for the successful EDA, because low volume of sample needed for a particular bioassay is often a critical prerequisite due to often limited sample amount and large number of testing fractions (Burgess et al., 2013). While *in vivo* bioassays give insights into the whole organism toxic effect with multiple complex metabolic pathways present, *in vitro* studies provide specific receptor-mediated mechanisms of toxic action. Various *in vitro* bioassays and cell lines have been used in EDA studies (Table 1.2.). Rat hepatoma cells and rainbow trout liver cells (RLT-W1) have been widely used to assess the exposure to dioxin-like chemicals that bind to the AhR in EROD (7-etoxyresorufin *O*-deethylase) and H4IIE-Luc (rat hepatoma cell line) bioassays (Engwall et al., 1999; Hollert et al., 2002b; Brack et al., 2005, 2008; Kammann et al. 2005b). Comet test, micronucleus formation and mutations were most often applied as bioassays for determination of genotoxicity and mutagenicity (Ames et al., 1973; Mortelmans and Zeiger, 2000; Da Rocha et al., 2009). Ames test was also frequently used for assessing

mutagenic compounds such as PAHs in environmental samples that uses the special strain of mutated *Salmonella typhimurium*. For estimation of acute toxicity inhibition of bioluminescent bacteria *Vibrio fischeri* (now *Aliivibrio fischeri*) were also used mainly for water samples (Parvez et al., 2006). CALUX bioassay and yeast estrogen screen (YES) system were used for examination of estrogenic compounds (Murk et al., 1996; Coldman et al., 1997; Takamura-Enya et al., 2003; Houtman et al., 2004; Thomas et al., 2004; Rastall et al., 2006; Vigano et al., 2008). Besides bacteria, yeast and animal cells, human breast cancer cell lines (MCF-7, T-47D, MDA) have also been used in EDA studies (Brack et al., 2011).

Table 1.2. *In vitro* cell lines and bioassays applied in EDA studies (adapted from Brack et al., 2011).

Effect type	Cell line	Source	Toxicity endpoints
AhR receptor ligands	H4IIE-Luc	Rat hepatoma cell line	Ah receptor-mediated luciferase reporter gene assay
	RTL-W1	Rainbow trout liver cell line	7-ethoxyresorufin Odeethylase (EROD) activity (CYP1A1)
Genotoxic chemicals	DNA-repair deficient DT40	Chicken DT40 B- lymphocyte line	Screening and characterizing the genotoxicity
	Ames test	Salmonella TA98 strain	Prokaryote cell assay to assess the potential of chemical compounds to cause point mutations
		Salmonella TA100 strain	Prokaryote cell assay to assess the potential of chemical compounds to cause frame shift mutations
Endocrine disrupting chemicals	H295R	Human adrenal cancer cells	Endocrine disrupting activities: modulation of steroidogenesis
	MVLN assay	Transformed MCF-7 human breast cancer cell line	Estrogen receptor-mediated luciferase reporter gene assay
	T47DKBluc	Transformed T-47D human breast cancer cell line	Estrogen receptor-mediated luciferase reporter gene assay
	MDA-KB	Transformed MDA human breast cancer cell line	Androgen receptor mediated luciferase reporter gene assay
	YES YAS	Transformed yeast cell Transformed yeast cell	Estrogen Lac-Z reporter gene assay Androgen Lac-Z reporter gene assay

Although the use of various bioassays enabled monitoring of different toxicological effects they are still regarded as limiting factor in EDA studies. In majority of studies EDA follows only one or two biological effects for identification and evaluation of toxic compounds. This could lead to restricted detection of susceptible compounds and the highly realistic risk that certain toxicants remain undiscovered. Therefore, there is a need to extend

the range of testing during the EDA by applying multiple bioassays to monitor diverse toxicological effects. Combination of new advanced *in vivo* models such as zebrafish embryos or transgenic organisms and multiple *in vitro* bioassay approach could further improve EDA.

1.1.7. Modifications of the EDA protocol proposed in this study

Based on the EDA shortages described above, in this study we demonstrated the use of a series of six different and biologically relevant *in vitro* bioassays for identification and evaluation of complex environmental toxicants by assessing their cytotoxicity and interaction with basic cellular detoxification mechanisms. Accordingly, the name "effect-directed analysis" changes to "effects-directed analyses". As in the standard EDA, the first step requires clean-up and extraction of the original sample prior to testing. This preliminary extraction includes the use of adequate organic solvent that is compatible with the cell systems used for testing. Initial screening of the test samples is done by applying acute and chronic toxicity bioassays, as well as specific bioassays for all four phases of the cellular detoxification mechanism. This phase I of the EDA modified protocol aims to characterize the sample(s) of concern. Sample(s) with positive response to the observed effect(s) is(are) further processed to the first fractionation step that includes the use of liquid chromatography on silica gel column and three different organic solvents, n-hexane (HEX), dichloromethane (DCM) and methanol (MeOH) for obtaining fraction A, B and C. These three fractions are again tested with the same bioassays that demonstrated positive response from the previous phase. Each of the fractions that exert positive response to the observed effect are selected for the second fractionation step that uses high-performance liquid phase chromatography to obtain 40 new sub-fractions. This fractionation step also uses specialized silica gel columns and solvents as stationary and mobile phases. Again, the series of bioassays that showed positive response from the previous phase is used to test these sub-fractions for the toxic effect. Fraction(s) of concern is(are) further subjected to gas or liquid chromatography-mass spectrometry most frequently applied for toxicant identification. The two step fractionations and chemical analysis make the phase II of the modified EDA protocol that aims to identify the fractions with toxic response. The compound that is detected is further tested for the observed effect(s) from previous bioassay(s). This represents the final phase that aims to confirm the effects of the detected compound. The entire EDA modified workflow is presented on Figure 1.3.

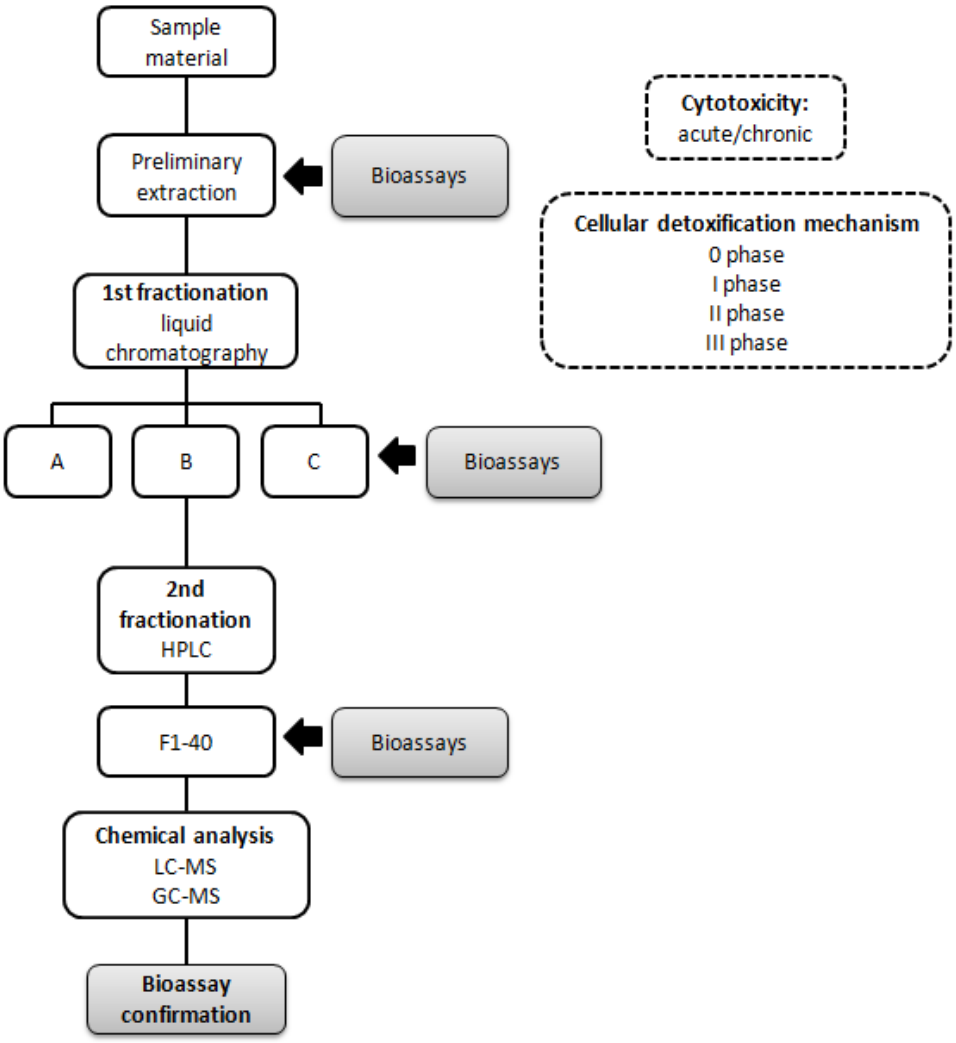


Figure 1.3. Schematic display of the modified EDA protocol.

1.2. BIOLOGICAL MATERIAL

1.2.1. Invasive algae from the genus *Caulerpa*

Genus *Caulerpa* J.V. Lamouroux belongs to the *Caulerpaceae* family and *Bryopsidophyceae* order of the marine green algae division (*Chlorophyta*). This order is characterized by the presence of chlorophyll a, b and carotinoides, with starch as a food reserve stored in unpigmented plastids called amyloplasts (Raven et al., 1992). *Caulerpa* species are also characterized by specific active photosynthetic pigments, siphonaxanthin and siphonein (Silva, 2002). Xylan is the major skeletal constituent and within the chloroplasts there are specific organelles called pyrenoids with light refracting properties (Debelius and

Baensch, 1997). Within the *Caulerpa* genus there are approximately 97 species (Guiry and Guiry, 2015). During the last 25 years the genus *Caulerpa* has gained substantial research attention due to its invasive behavior when introduced to foreign habitats. Two tropical *Caulerpa* species, *Caulerpa taxifolia* (M. Vahl) C. Agardh and *Caulerpa racemosa* (Forsskål) J. Agardh, have been distinguished as one of the most invasive algae organisms present in the environment. *C. racemosa* is also known as "sea grapes" while *C. taxifolia* was publicized as the "killer algae" (Meinesz, 1999). In the genus *Caulerpa* there are several different complexes including *C. racemosa* complex that comprises of several distinct varieties. In the Mediterranean Sea there are three existing intraspecific taxa of *C. racemosa* including: a taxon corresponding to *C. racemosa* var. *turbinata* (J. Agardh) Eubank and var. *uvifera* (C. Agardh) J. Agardh; *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f. *requienii* (Montagne) Weber-van Bosse; and *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (hereafter *C. racemosa*) (Verlaque et al., 2000; Verlaque et al., 2003).

1.2.1.1. Main features of *C. racemosa* and *C. taxifolia*

Genus *Caulerpa* has specific anatomical, morphological, ecological and physiological characteristics that strongly distinct it from the other seaweeds but most importantly enables invasive properties (Silva, 2002). Anatomical structure of *Caulerpa* species features multinucleated cells called coenocytes that are not separated with intracellular membranes from the continuous cytoplasm but have branching ingrowths of the wall called trabeculae. One of the major characteristics of *Caulerpales* is that they are one of the largest known single celled organisms (Meinesz, 1999). Although their vegetative body is not separated by cell walls, their morphology is quite complicated because they generate roots and leaves like-organs that are in fact pseudo-organs. Morphological characteristics of *Caulerpa* genus include uniaxial siphonous thallus that is divided into a horizontal crawling axis called stolon from which rhizoids grow downwards and fronds (pinnae) come out upwards. Rhizoids are thin and short, root like extensions that enable fixation to the substrate and absorb water, food and nutrients. Fronds are vertical shoots that differ in their morphological appearance within species being either nude, leaf-like or with grape- or feather-like branchlets. *C. racemosa* possesses spherical, club-shaped or mushroom- to disc-shaped branchlets while *C. taxifolia* has feather-like fronds that are flattened laterally (Fig. 1.4.). These organisms exhibit

polymorphism as the size of fronds and stolons differ between these two species and can vary according to the depth, season and region which sometimes can make them difficult to identify (Meinesz, 1995). Usually the stolons are 1 to 1.5 m in length. Primary fronds of *C. racemosa* grow up to 11 cm (or exceptionally 19 cm) while *C. taxifolia* fronds are 2-15 cm (Meinesz, 1995). Branchlets or pinnules are usually 1 cm long, radially or distichously arranged and they are oppositely attached to the midribs (rachis, main axis of the frond), and for *C. taxifolia* they are slightly curved upwards on the basal and terminal side (Verlaque et al., 2003). In shallow waters these morphological structures are usually smaller or even absent while in deeper waters they are much longer. Significant changes in size, length and growth rate are present between the native and the invasive forms of these two *Caulerpa* species (Boudouresque et al., 1995). While tropical *C. taxifolia* has narrow and smaller fronds, stolons and pinnules, the invasive form exhibits larger and broader morphological structures (Meinesz et al., 1995; Benzie et al., 2000). The primary fronds of invasive Mediterranean *C. taxifolia* vary from 5 cm to 80 cm in height and their stolons can even grow up to 2.8 m in length (Meinesz and Hesse, 1991; Meinesz et al., 1995). However, the sizes and growth rate of invasive forms of *Caulerpa* species can also vary due to season, region, substrate, temperature and other ecological factors (Debelius and Baensch, 1997).

In most cases *Caulerpa* species reproduce asexually but they can also reproduce sexually (Debelius and Baensch, 1997). *C. racemosa* and *C. taxifolia* differ in their reproductive mechanisms; *C. racemosa* often creates sexual zygotes while *C. taxifolia* uses almost exclusively vegetative reproduction (Meinesz and Hesse, 1991). In general, the knowledge of their clonal and/or sexual reproductive biology is still not entirely understood. Their most efficient dispersal mechanism includes the aspects of vegetative reproduction that occurs in three forms: growth pattern, fragmentation and formation of propagules. Fragmentation occurs randomly in any part of the alga by currents, grazing or human activities (vessels, nets, aquacultural products) and formed fragments can survive for days drifting in the waters before attaching to a suitable substrate (Ceccherelli and Piazzi, 2001). For *C. racemosa* this mechanism is mostly observed during the summer season (Ceccherelli et al., 2000; Ceccherelli and Piazzi, 2001). Propagules are globular, cylindrical or club-shaped branchlets (ramuli) that could be even few millimeters in size and when detached they grow into a new plant after only 5 days (Ceccherelli and Piazzi, 2001). Sexual reproduction occurs when diploid macroscopic alga stage produces haploid gametes by the process of holocarpy, meaning that the whole protoplast gives rise to produce biflagellate, motile anisogametes that

are liberated simultaneously thereby causing the death of the alga individual. Prior to the gamete release a small papillae is formed and the cytoplasm transforms into brown-green network-like structure. Male and female gametes are released approximately 14 min before sunrise forming a green cloud (Panayotidis and Žuljević, 2001). Both *C. racemosa* and *C. taxifolia* are monoecious producing both male and female gametes on the same gametophyte (Goldstein and Morall, 1970; Panayotidis and Žuljević, 2001). A protonema is developed from the zygote and new diploid thallus begins to grow (Raven et al., 1992; Silva, 2002). The study on invasive Mediterranean strain of *C. taxifolia* using nuclear and cytoplasmic sequences reveled that this species spreads mainly clonally and that sexual reproduction is a rare and an unpredictable event (Meusnier et al., 2002). It is possible that certain ecological factors, such as higher water temperature (25°C) could induce sexual reproduction in invasive *C. taxifolia* (Žuljević and Antolić, 2000). Only male gametes have been found for *C. taxifolia* (Žuljević and Antolić, 2000). On the contrary, *C. racemosa* uses sexual reproduction and generates both male and female gametes whose genome can even be subjective to sexual recombination that results in creation of hybrid strains among its varieties (Panayotidis and Žuljević, 2001; Durand et al., 2002). Consequently, this contributes to existence of few *C. racemosa* complexes unlike *C. taxifolia* genome that varies relatively little due to its asexual and clonal propagation (Meinesz et al., 1993a). Furthermore, this successful sexual production of *C. racemosa* is probably the cause of its massive spread all over the Mediterranean unlike *C. taxifolia* whose spread is discontinuous in certain parts of the Mediterranean Sea (Meinesz et al., 2001; Panayotidis and Žuljević, 2001; Piazzi et al., 2005).

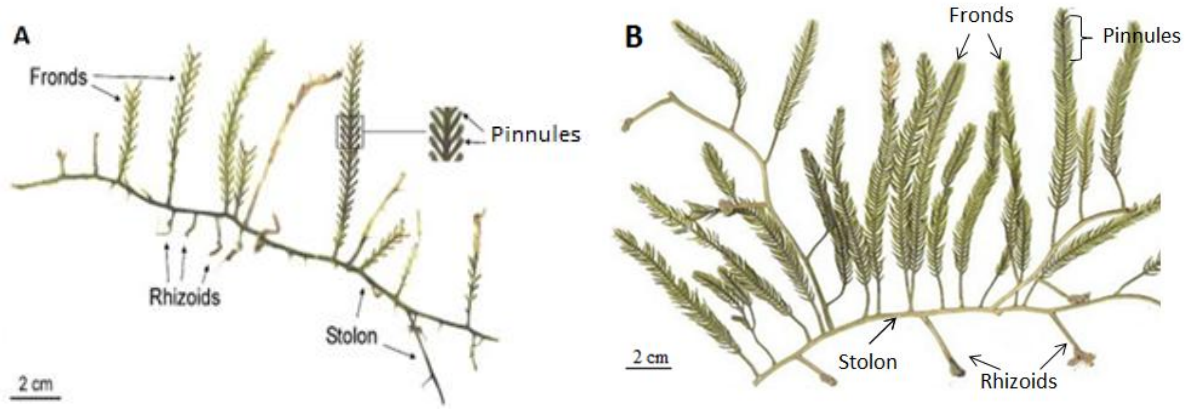


Figure 1.4. Thallus of *C. racemosa* (**A**) (adapted from Klein and Verlaque, 2008) and *C. taxifolia* (**B**) (adapted from Creese et al., 2004).

Native *C. racemosa* is present in south-western coast of Australia from shallow intertidal areas to 6 m deep reef flats and pools (Womersley, 1984; Carruthers et al., 1993) with temperatures ranging from 14°C to 16°C during winter and 22.5°C during summer season (Verlaque et al., 2003). Tropical *C. racemosa* grows under certain environmental conditions of salinity (30–40‰) and higher light intensity (20–60 $\mu Em^{-2}s^{-1}$) (Carruthers et al., 1993). Invasive strain of this species grows on pebbles, rocks, sand, mud, detritic structures from 0 to 70 m of depth in the Mediterranean Sea surviving from lower 8°C to higher 28°C temperature (Žuljević, 2005). Deeper habitats of invasive *C. racemosa* are tolerable to low light condition due to the ability of alga to photoacclimate (Raniello et al., 2004; Raniello et al., 2006). Furthermore, *C. racemosa* meadows are found in sheltered areas or exposed shores, as well as polluted and pristine areas (Ballesteros et al., 1999; Žuljević et al., 2004; Ruitton et al., 2005; Mifsud and Lanfranco, 2007). *C. taxifolia* is native in subtidal waters in subtropical and tropical areas including the Caribbean, Indonesia, Southeast Asia, Australia and Hawaii (Phillips and Price, 2002). Both *Caulerpa* species do not exert their invasive behavior in their native habitats due to presence of other competing species and herbivores (Carpenter, 1986; Hay, 1997).

Seasonal variation in size, growth rate, and biomass production is present for both *Caulerpa* species, as mentioned earlier. These changes are closely related to certain environmental factors, such as temperature and light. From March the intensity of growth starts to increase up to summer and autumn period which are characterized by the highest production of algal biomass and stolon length (Klein and Verlaque, 2008). In winter time growth rate decreases rapidly as the seasonal temperature drop occurs (Klein and Verlaque, 2008). *C. racemosa* probably survives the winter temperatures by generating zygotes, fragments and propagules (Piazzi et al., 2001; Verlaque et al., 2003; Verlaque et al., 2004).

1.2.1.2. Toxic secondary metabolites

One of the greatest advantages of *Caulerpa* species is that they produce various secondary metabolites most of which are toxic or repulsive compounds that largely attribute to their invasive behavior. It is believed that these secondary metabolites serve as a chemical defense mechanism of genus *Caulerpa* against herbivores and for interspecific competition (Klein and Verlaque, 2008). Their chemical structures consist mainly of bisindole alkaloids, sesquiterpenoids and diterpenoids, with aldehyde and/or enol acetate functional groups to

which high biological activity is attributed (Guerrerio et al., 1992; Guerrerio et al., 1993; Smyrniotopoulos et al., 2003) (Fig. 1.5.). Various bioactivities have been recorded for these compounds such as antimicrobial, insecticidal, antifouling, ichthyotoxic, feeding deterrent, anti-inflammatory, cytotoxic, and growth regulatory properties (Paul and Fenical, 1982; Paul et al., 1987; Raub et al., 1987; Fischel et al., 1995; Smyrniotopoulos et al., 2003; De Souza et al., 2009; Alarif et al., 2010; Nagaraj and Osborne, 2014). Major secondary metabolite of *Caulerpa* species is a unique acetylenic sesquiterpen caulerpenyne (CYN) which was first isolated from Mediterranean *C. prolifera* (Amico et al., 1978). CYN is found to be abundant both in *C. taxifolia* and *C. racemosa* but higher levels of CYN are contained especially in *C. taxifolia* (McConnell et al., 1982; Boudouresque et al., 1996; Jung et al., 2002). The amount of CYN that *C. taxifolia* invasive strain produces in the Mediterranean is significantly higher than in its original Australian habitat (Guerriero et al., 1992; Amade et al., 1995). Genus *Caulerpa* can generate other terpenes that mostly develop during the CYN transformation processes such as oxytoxin-1 and -2 that are highly instable and reactive but probably even more toxic than CYN (Guerriero et al., 1992; Jung et al., 2002). By hydrolysis, degradation, deacetylation or oxidation processes other CYN derived products have been recorded in *C. taxifolia* such as taxifolial A-D, taxifolione, 10,11-epoxycaulerpenyne and caulerpenynol (Guerriero et al., 1992; Lemée et al., 1993; Guerriero and D'Ambrosio, 1999) (Fig. 1.5.). These minor metabolites are significantly generated during the algal wound healing process by activation of esterases that transform CYN to 1,4-dialdehyde (oxytoxin-2) that together with protein cross-linking enable wound-plug formation (Adolph et al., 2005; Pohnert, 2005). When released into the sea, CYN and its metabolites are harmful to marine organisms and even when degraded, toxic by-products of CYN and its metabolites can form. Furthermore, it was demonstrated that CYN is sensitive to light and reacts with amines (Schauder and Krief, 1982; Guerreiro et al., 1995). Antiproliferative, antiviral, antimicrobial and apoptotic effects of CYN have been demonstrated on different cell lines (Fischel et al., 1995; Nicoletti et al, 1999; Barbier et al., 2001). Furthermore, it was shown that CYN induces stress to *Cymodocea nodosa* by causing alternations in seagrass photosynthesis, and blocks the cleavage of sea urchin eggs by inhibition of microtubule polymerization (Paul and Fenical, 1986; Pesando et al., 1996; Barbier et al., 2001; Raniello et al., 2007). By inhibiting the Na^+/K^+-ATPase CYN exhibits the neurological activity as well, but CYN can also inhibit the mitogen-activated protein kinase (MAPK) pathway and mitosis block (Mozzachiodi et al., 1997; Brunelli et al., 1998). Most of CYN is being produced in fronds then in stolons of *Caulerpa* with seasonal variability present (Dumay et al., 2002). CYN is biosynthesized by the methyl-erythritol-4-

phosphate (MEP) pathway which occurs in the chloroplasts where CO_2 is fixed by photosynthesis (Pohnert and Jung, 2003). Certain tropical herbivorous reef fish and sacoglossan opisthobranch molluscs feed on *Caulerpa* species in its natural habitat such as rabbitfish (*Siganidae*), surgeonfish (*Acanthuridae*) and species of *Elysia*, *Oxynoe*, *Volvatella*, and *Lobiger* (Jensen, 1983; Paul and Hay, 1986; Paul et al., 1990). Furthermore, it was shown that these species accumulate CYN and by hydrolytic enzymes can metabolize CYN to highly toxic oxytoxins 1 and 2 that are excreted with mucous thereby functioning as a chemical defense (Cutignano et al., 2004; Marin and Ros, 2004).

Other important metabolites of *Caulerpa* are caulerpin (CLP), caulersin and caulerpicin that were first isolated from three different *Caulerpa* species including *C. racemosa* (Fig. 1.5.) (Aguilar-Santos and Doty, 1968, 1971; Doty and Aguilar-Santos, 1966; Aguilar-Santos, 1970; Maiti et al., 1978; Mahendran et al., 1979, Nielsen et al., 1982). CLP is an orange-red chlorophyte pigment that possesses a bisindole structure which is present in *C. taxifolia* but is more abundant in *C. racemosa*. CLP analogues have also been discovered such as 10-keto-3,7,11-trimethyldodecanoic acid, as well as caulerpinic acid that is an alkaline hydrolysis product of CLP (Fig. 1.5.) (Anjaneyulu et al., 1991; Alarif et al., 2010). It was shown that CLP can: (i) regulate plant growth and act like an indole auxin (Raub et al., 1987); (ii) inhibit human protein tyrosine phosphatase 1B (Mao et al., 2006); (iii) restore the growth of a yeast strain that overexpresses the human indoleamine 2,3-dioxygenase gene (Vottero et al., 2006); (iv) act as an anticancer agent that disrupts mitochondrial ROS-regulated hypoxia-inducible factor-1 (HIF-1) activation and HIF-1 downstream target gene expression by inhibiting the transport or delivery of electrons to mitochondrial complex III (Liu et al., 2009). Furthermore, CLP can stimulate plant root growth and more interestingly, exhibit prominent anti-inflammatory and antinociceptive activities that are thought to be caused by the indol functional group that possesses antioxidative activity (De Souza et al., 2009). In general, CLP is regarded as a low toxicity compound (Vidal et al., 1984).

Discovery of new biologically active substances in *Caulerpa* species is still very much in focus. Recent studies revealed the identity of four new bisindole alkaloids and two new prenylated para-xylenes in *C. racemosa* from South China Sea, caulerchlorin, racemosins A-C, caulerprenylols A and B, and a new polyacetylenic acid (8E,12Z,15Z)-10-hydroxy-8,12,15-octadecatrien-4,6-diynoic acid) (Fig. 1.5.) (Liu et al., 2012, 2013a, 2013b; Yang et al., 2014). Antifungal activity was observed for prenylated para-xylenes and caulerchlorin

while neuroprotective and inhibitory activity was observed for racemosins A and C (Liu et al., 2012, 2013a, 2013b).

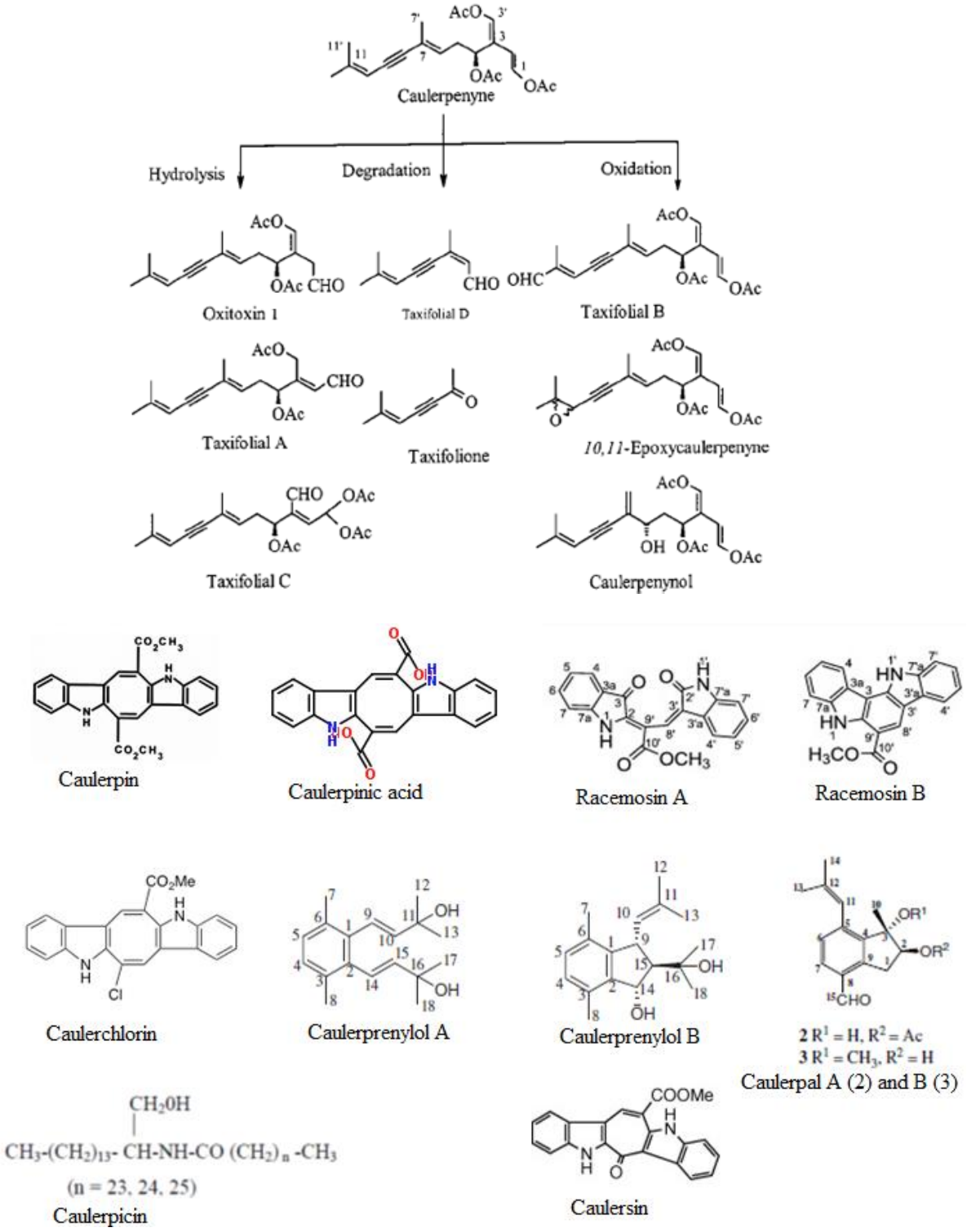


Figure 1.5. Chemical structures of identified secondary metabolites from algae *Caulerpa* (adapted from Doty and Aguilar-Santos, 1966; Guerriero and D'Ambrosio, 1999; Mao et al., 2006; Miki et al., 2006; Liu et al., 2012, 2013a, 2013b; http://biophysics.sbg.ac.at/ct/scans/fig4f.jpg; http://www.chemspider.com/Chemical-Structure.10474432.html).

The most recent study on *C. racemosa* active metabolites isolated three new diterpenoids (racemobutenolids A, B, 40,50-dehydrodiodictyonema A), one new α-tocopheroid (α-tocoxylenoxy), and one new 28-oxostigmastane steroid ((23E)-3b-hydroxy-stigmasta-5,23-dien-28-one) (Yang et al., 2015). The structure of two aromatic valerenane-type sesquiterpenes from *C. taxifolia* have been elucidated, caulerpal A and B (Fig. 1.5.) (Mao et al., 2006).

1.2.1.3. Spread and impact

C. racemosa first appeared in 1926 when it was observed in Sousse Harbor in Tunisia by Hamel. Its features were not considered invasive until its appearance in Libya 1990 (Nizamuddin, 1991). The findings of *C. racemosa* sources of introduction and propagation in the Mediterranean Sea are not as straightforward as *C. taxifolia* due to existence of several different strains of *C. racemosa* that might also be distinctive species (Benzie et al., 1997; Famà et al., 2002). At first *C. racemosa* was regarded as a Lessepsian migrant that was introduced to the Mediterranean from the Red Sea (Alongi et al., 1993; Giaccone and Di Martino, 1995; Piazzi et al., 2001). However, morphological, bibliographical and molecular studies later on revealed that *C. racemosa* is a hybrid species originating from south-western Australia that was introduced into the Mediterranean Sea in the early 1900s (Famà et al., 2000; Verlaque et al., 2000; Verlaque et al., 2003). That new taxon was classified as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (Verlaque et al., 2003) and it started to spread even faster than *C. taxifolia* due to mass production of gametes spreading widely across the Mediteranean Sea (Ceccherelli et al., 2001; Piazzi et al., 2001). Additional genetic studies confirmed that species from Croatia, Cyprus, France, Greece, Italy, Turkey and the Canary Islands match the new established *C. racemosa* taxon (Famà et al., 2000; Verlaque et al., 2000; Verlaque et al., 2003; Nuber et al., 2007). However, the way that this alga was introduced into Mediterranean still remains unclear, but so far ballast waters, ship hull fouling and aquaria are considered to be possible carries (Frisch Zaleski and Murray, 2006; Stam et al., 2006; Walters et al., 2006). Furthermore, the total surface area affected by *C. racemosa* in the Mediterranean Sea is still not completely known (Klein and Verlaque, 2008). From its native region in Australia between Perth and Hopetoun, *C. racemosa* was recorded to spread in 2001 to other Australian coastline in Adelaide (Womersley, 2003; Collings et al., 2004). *C. racemosa* was also detected in the Atlantic where it was found in the Canary Islands since late 1990s (Verlaque et al., 2004).

C. taxifolia appearance outside its native habitat was first recorded in 1984 by Meinesz in the coastal waters of the northern part of the Mediterranean Sea, near the Oceanographic Museum in Monaco (Cote d'Azur) (Meinesz and Hesse, 1991). It was used as a decorative plant in tropical seawater public aquariums first in Germany and then in France and Monaco as its invasive properties were still unknown. Apparently, *C. taxifolia* escaped or was accidentally released from an aquarium into the Mediterranean. As soon as *C. taxifolia* was introduced into the Mediterranean waters the aggressive and invasive behavior of the alga became apparent with more rapid and extensive growth than in the original tropical area. During the next 16 years the alga engulfed around 131 km^2 of benthos spreading across the 191 km of coastline of France, Italy, Spanish Balearic Islands, Croatia, Egypt and Tunisia (Meinesz et al., 2001). Furthermore, in 2000 it was recorded off the Pacific coasts of California (USA) and in 2002 in New South Wales (Australia). In some areas it covers 100% of the surface between 5 m and 25m (Meinesz et al., 1993).

The secondary spreading of both *Caulerpa* species is caused by shipping activities through ballast waters, anchors, fishing nets or other fishing equipment (Verlaque et al., 2003). Once it reaches the new microhabitat, alga forms dense clumps of rhizomes and stolons conquering the new habitats sometimes even within six months of entry. The impact of *C. taxifolia* and *C. racemosa* spreading caused loss of indigenous seagrass beds and consequent reduction of the infralittoral communities and general negative effects on the coastal ecosystem (Boudouresque et al., 1992, 1995; Verlaque and Fritayre, 1994; Thomsen et al., 2009). Uniform and dense meadows of *C. racemosa* and *C. taxifolia* that formed influenced the feeding habits of demersal species, alternated the predator-prey interactions and led to a decline of the fish populations, overall resulting in homogenized microhabitats (Schröder et al., 1998; Longepierre et al., 2005; Wallentinus and Nyberg, 2007; Vázquez-Luis et al., 2010). They replaced the dominant seagrass species such as *Cymodocea nodosa* and *Posidonia oceanica* and consequently reduced the number of gastropod and crustacean species that use them as a source of food and refuge (Ruitton et al., 2005).

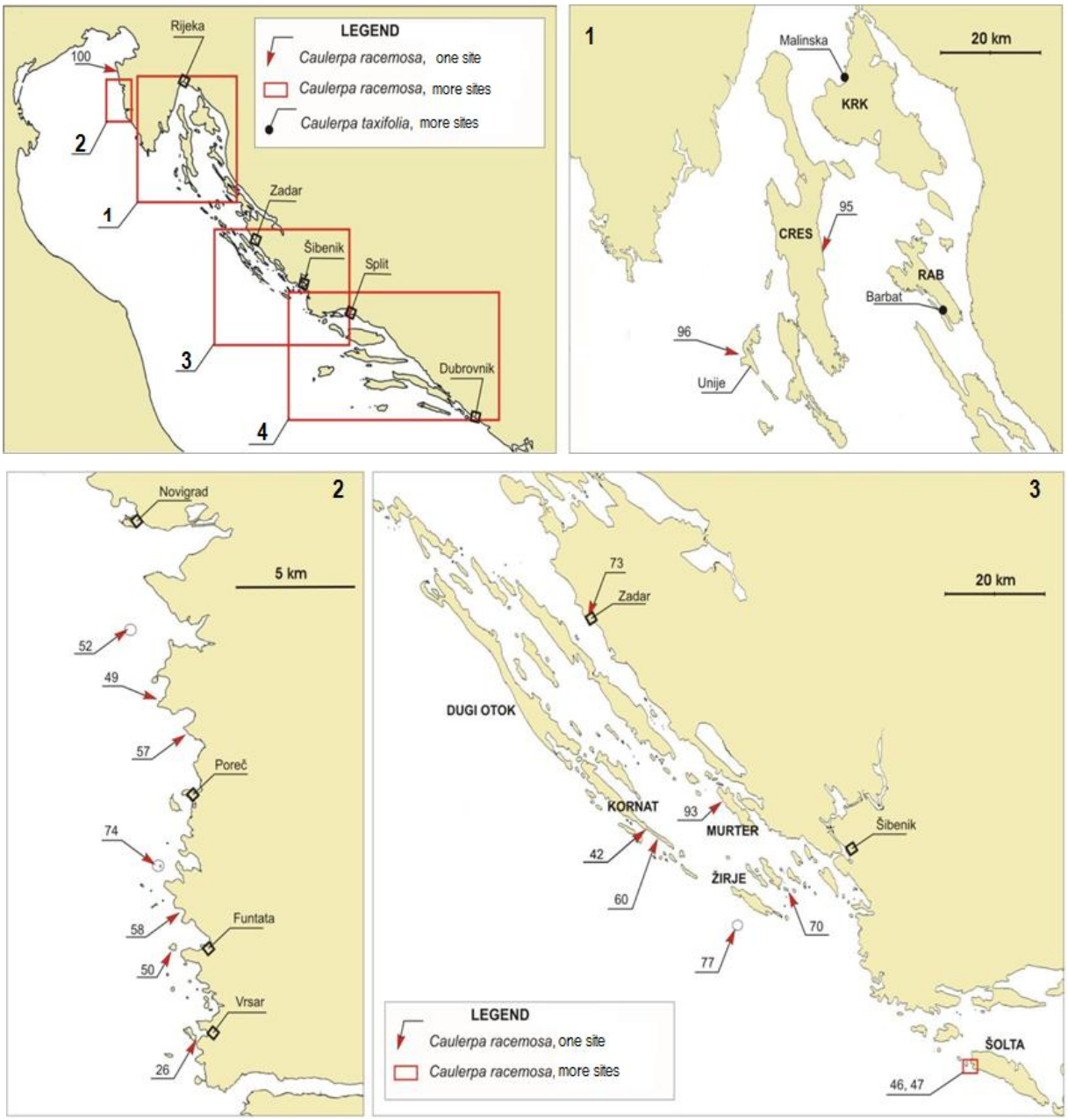
1.2.1.4. *C. racemosa* and *C. taxifolia* in the Adriatic Sea

In Croatia both *Caulerpa* species have settled causing significant damage to benthic flora and fauna in the Adriatic Sea. *C. racemosa* has been first observed in Croatia in autumn of 2000 on Pakleni Islands, just off the southwest coast of the Hvar Island (Žuljević et al.,

2003). By the end of 2005 it spread throughout the Adriatic coastline with 43 locations most of which inhabited south Adriatic waters from the island of Vis to Cavtat (Žuljević et al., 2003; Žuljević et al., 2010). Only one location of *C. racemosa* was found in north Istrian peninsula on the coast of Vrsar village. The efforts to remove *C. racemosa* were much more challenging than *C. taxifolia* due to its efficient production of gametes that uncontrollably spread by currents and smaller fragments that are often hard to spot. Furthermore, this alga is of a great danger for Croatian National and Nature Parks most of which are islands with unique ecosystems. Unsuccessful removal attempts have been made in 2006 that unfortunately contributed to *C. racemosa* spread, where by 2007 it was observed in 13 more locations. Removal procedures were started again at Mljet National Park. By 2010 *C. racemosa* was distributed in 100 different locations including the Kvarner area in the north Adriatic Sea where this alga was never before found (Žuljević et al., 2010). From the 2010 the official monitoring of *C. racemosa* in the Adriatic Sea by Croatian Environmental Agency was terminated but findings from local people indicated that the alga continued to spread with more than 100 localities that was estimated by the end of 2012 (Žuljević et al., 2012). The total distribution of *C. racemosa* in Adriatic Sea from 2010 is shown on the Figure 1.6.

In late 1994 *C. taxifolia* was first recorded along the Croatian coastline more than 965 km away from its original site in Monaco at a site close to a mooring dock in Stari Grad on the island of Hvar (Žuljević et al., 2010). Only a year later it was observed at another site in a port at Malinska in the island of Krk (Zavodnik, 1995; Špan et al., 1998; Zavodnik et al., 1998a, 1998b, 2001; Žuljević et al.; 1998). Although the control measures in the Stari Grad Bay have been applied to stop the spread, *C. taxifolia* quickly expanded throughout the whole Hvar region and in 1996 reached its third location in the Adriatic Sea, the Barbat Channel near the island of Dolin (Žuljević and Antolić, 2001). In September that same year the alga was manually removed, however it appeared there again in June 2001. Nevertheless, due to removal efforts and low winter temperatures the alga retreated from the Barbat Channel in 2002 and since then it was never recorded there again. The polluted areas by beds of *C. taxifolia* were 40 ha for Hvar Island and 1.3 ha for Krk Island by the end of the 2000 (Meinesz et al., 2001). By the end of the 2005 fronds and stolons of *C. taxifolia* were reduced to 1 m^2 in the Malinska site while only two localities were left within the Stari Grad Bay. In 2007 Stari Grad Bay was the only site of *C. taxifolia* in the Adriatic Sea with three locations within, the original site, Veli Zeleminec and Sv. Ante Bay with algal coverage area of 75 ha, 250 m^2 and 5000m^2, respectively (Žuljević et al., 2007). In all these localities the alga was

found to be the most abundant and dominant at depth range from 5 m to 12 m on silted and sandy bottoms together with *Cymodocea nodosa* seagrass (Žuljević et al., 2007). During 2009 it was located only on the main founding site and one allocated site within the Stari Grad Bay (Žuljević et al., 2007). As it was the case for *C. racemosa*, the official monitoring of *C. taxifolia* in the Adriatic Sea by Croatian Environmental Agency was terminated from 2010 and all further data was obtained exclusively by reports from the local people (Žuljević et al., 2007). *C. taxifolia* remained in the Stari Grad Bay original site until today. In 2012 the alga was not found in shallow waters but only at depths from 8 m to 15 m (Žuljević et al., 2012). The total distribution of *C. taxifolia* in the Adriatic Sea from 2010 is shown on the Figure 1.6.



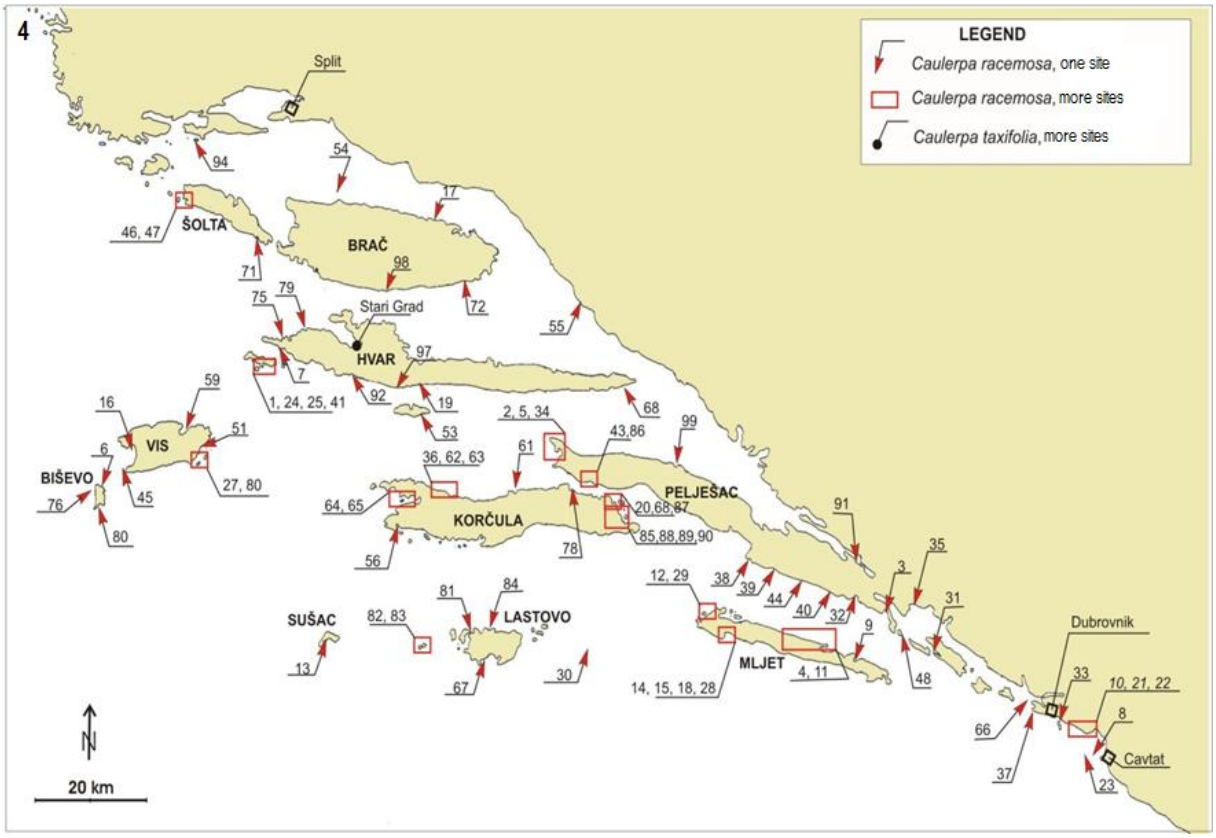


Figure 1.6. Distribution and spread of *C. racemosa* and *C. taxifolia* in the Adriatic Sea in 2010 (from Žuljević et al., 2010).

1.2.2. Cyanobacterial strains

1.2.2.1. General

Cyanobacteria are one of the most diverse groups of gram-negative photosynthetic prokaryotic microorganisms with specific metabolic, biochemical, physiological and ecological features that enabled their existence on Earth for over 3.5 billions of years (Codd, 1995) (Fig. 1.7.). They are considered to be the critical components of the Earth's biosphere as they were major creators of the oxygen atmosphere in the Archaean and Proterozoic Eras and are evidenced in formation of stromatolites fossil record (Wilmotte, 1994; Falconer, 2005). For a long time they were classified as blue-green algae because they largely resembled to eukaryotic green algae. These ancient organisms are also primary colonizers and cosmopolites occupying diverse types of habitats, from polar to tropical regions, in both terrestrial and aquatic biotopes (Whitton and Potts, 2000). However, they are most commonly present in alkaline limnic and marine ecosystem where they are a part of the phytoplankton seasonal cycle (Luuc et al., 1999). Cyanobacteria possess multiple adaptation mechanisms

that enabled them broad geographical distribution and long evolution, such as atmospheric nitrogen fixation through specialized structures called heterocystes, formation of resistant cells that work as spores (acinets) and ability to regulate buoyancy (Kaebernick and Neilan, 2006). They are also tolerable to high oscillations of various ecological factors such as high ultraviolet (UV) exposure, high salinity and acidity, high metal concentration, low oxygen levels and wide range of temperatures (Kaebernick and Neilan, 2006). Furthermore, they possess a special environmental adaptation for surviving in low CO_2 environment, the CO_2 concentrating mechanism. Cyanobacteria are regarded as oxygenic phototrophs that contain diverse light harvesting pigments such as chlorophyll a, and two other accessory pigments, phycoerythrin and phycocyanin, that give cyanobacteria a blue-green appearance after which they were named. By the theory of endosymbiosis, cyanobacteria were responsible for development of chloroplasts in plant and algal cells and they were probably the first organisms that used photosystems I and II. Their morphological appearance differs as they can be unicellular, colonial or filamentous and use fission to reproduce asexually. Except the heterocystes and acinetes their vegetative cells are undifferentiated from each other. They can be symbionts living with plants and fungi, in the benthos or in water column.

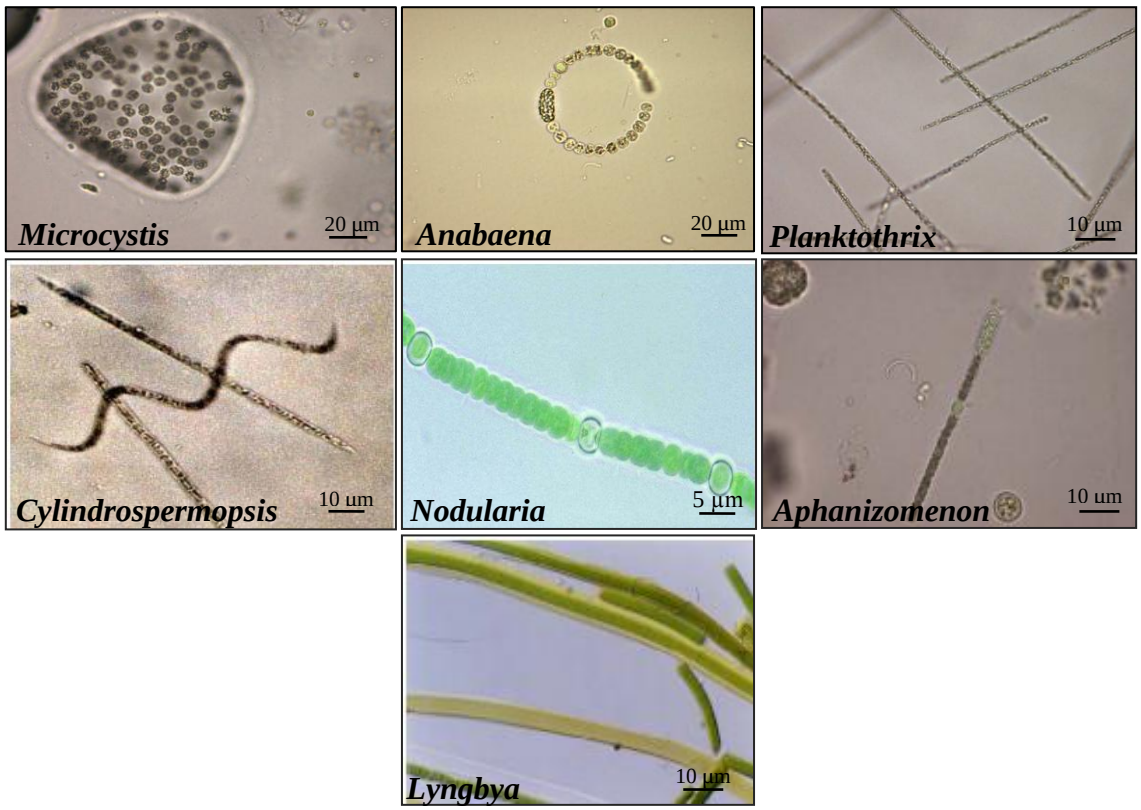


Figure 1.7. Morphology of various cyanobacterial strains (adapted from Simeunović, 2005, 2010; http://microbes.arc.nasa.gov/images/content/gallery/lightms/publication/lyngbya.jpg; https://microbewiki.kenyon.edu/index.php/File:Nodularia2.png).

In recent times cyanobacterial strains are attracting strong attention in basic, as well as applied research. They are considered to be potential new sources of renewable energy as they are able to produce photobiological H_2 (Sakurai et al., 2015). The nitrogenase-based photobiological H_2 biofuel production is solar-driven and carbon-neutral that can be used to generate electricity at high efficiencies and minimal pollution (Sakurai et al., 2015).

1.2.2.2. Cyanotoxins

The most advantageous feature of cyanobacteria is that they are able to produce toxic secondary metabolites called cyanotoxins that enable them to modify their habitats making them more suitable under environmental stress conditions (Ehrenreich et al., 2005). Cyanobacteria generate cyanotoxins within their cells that can be released into the environment either during the cell life or after cell lysis (Lam et al., 1995; Chorus, 2001; Haider et al., 2003; De la Cruz et al., 2011). Cyanotoxins are divided on the basis of their target organ of toxicity to hepatotoxins (microcystins, nodularin, cylindrospermopsin), neurotoxins (anatoxin-a, anatoxin-a(S), homoanatoxin-a, saxitoxins, neosaxitoxins), dermatotoxins and irritants (lyngbyatoxins, aplysiatoxin) (Carmichael, 1992; Chorus and Bartram, 1999; Codd et al., 1999). Furthermore, they can be divided according to their chemical structure to cyclic peptides (microcystins, nodularin), alkaloids (anatoxin-a, anatoxin-a(S), saxitoxin, cylindrospermopsin, aplysiatoxin and lyngbiatoxin-a) and lipopolysaccharides (Fig 1.8.) (Carmichael, 1992; Chorus and Bartram, 1999; Codd et al., 1999). Most commonly produced cyanotoxins are hepatoxins and neurotoxins (Chorus and Bartram, 1999). One cyanobacterial genera usually produces several types of cyanotoxins.

Microcystins are the most studied cyanotoxins present in cyanobacterial genera *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Anabaenopsis*, *Nostoc* and *Hapalosiphon* (Chorus and Bartram, 1999) (Table 1.9.). The chemical structure of microcystins is characterized by the presence of two specific amino acids, N-methyldehydroalanine (Mdha) and hydrophobic β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) that are responsible for their toxic properties (Botes et al., 1985; Rao et al., 2002). Furthermore, microcystins are comprised of more than 90 structural variants called congeners that differ in amino acid composition on the second and fourth peptide (Pearson et al., 2010). Microcystin-LR is the most toxic congener and the most studied (De la Cruz et al., 2011). These cyclic heptapeptides are non-ribosomally synthetized and act as

inhibitors of serine/threonine protein phosphatases 1 and 2a (PP1/2a) in hepatocytes causing oxidative stress, DNA damage and tumor proliferation (Eriksson et al., 1990; Matsushima et al., 1990; Yoshizawa et al., 1990; Toivola et al., 1994). They are extremely stabile structures in the environment resistant to hydrolysis, oxidation or heat (De la Cruz et al., 2011). Nodularin is a monocyclic pentapeptid that has Adda moiety in its structure together with two additional amino acids, D-erythro-β-methylaspartic acid (D-Masp) and N-methyldehydrobutyrine (Mdhb) (Rinehart et al., 1994) (Table 1.9.). Only *Nodularia spumigena* has been shown to produce this toxin and so far with four identified nodularins (Botes et al., 1982a, 1982b; Meriluoto et al., 1989; Namikoshi et al., 1992; Merel et al., 2010). It also inhibits protein serine-threonine phosphatases that lead to excessive phosphorylation and carcinogenicity (Bouaïcha and Maatouk, 2004). Similar as microcystins, they are very stable and are degraded only under intense UV irradiation, ozone and chlorine presence (Nicholson et al., 1994; Tsuji et al., 1995, 1997; Hoeger et al., 2002; Acero et al., 2005; Merel et al., 2009; Brooke et al., 2006). Cylindrospermopsin is found in several cyanobacterial genera mainly found in (sub)-tropical areas including *Cylindrospermopsis raciborskii* (Ohtani and Moore, 1992), *Umezakia natans* (Harada et al., 1994), *Anabaena bergii*, *Aphanizomenon ovalisporum, A. flos-aquae* and *Raphidiopsis curvata* (Falconer, 2005) (Table 1.3.). Unlike other hepatotoxins, cylindrospermopsin is concentrated several meters below the water surface and it is an alkaloid that contains a tricyclic guanidine moiety combined with hydroxymethyl uracil (Carmichael and Gorham, 1978). This toxin is highly soluble in water and it induces liver toxicity by suppressing glutathione and protein synthesis (Terao et al., 1994; Runnegar et al., 1995).

Anatoxin-a is the first described cyanotoxin that is found in many cyanobacterial genera such as *Anabaena, Aphanizomenon, Cylidrospermopsis, Oscillatoria (Planktothrix), Microcystis, Raphidiopsis, Nostoc, Phormidium* and *Arthrospira* (Carmichael et al., 1975; Park et al., 1993; Sivonen, 1996; Westrick et al., 2010) (Table 1.3.). Anatoxin-a is an alkaloid, bicyclic secondary amine 2-acetyl-9-azabycyclo[4,2,1]non-2-ene that mimics acetylcholine and binds to the nicotinic-acetylcholine receptors with higher affinity than acetylcholine (Devlin et al., 1977). Once bound to the nicotinic receptors it inhibits the transmission at the neuromuscular junctions resulting in overstimulation of the muscle as it is not degradable by any enzyme present in eukaryotes. It is unstable in the environment and transforms to the non-toxic products dihydroanatoxin-a and epoxyanatoxin-a (Smith and Lewis, 1987). *Anabaena lammermannii, Anabaena flos-aquae* can also produce another

variant of anatoxin-a which causes hypersalivation in vertebrates (Matsunaga et al., 1989). Consequently, this neurotoxin was named anatoxin-a(S) that is a phosphate ester of a cyclic N-hydroxyguanine which is an anticholinesterase and more potent than anatoxin-a (Table 1.3.).

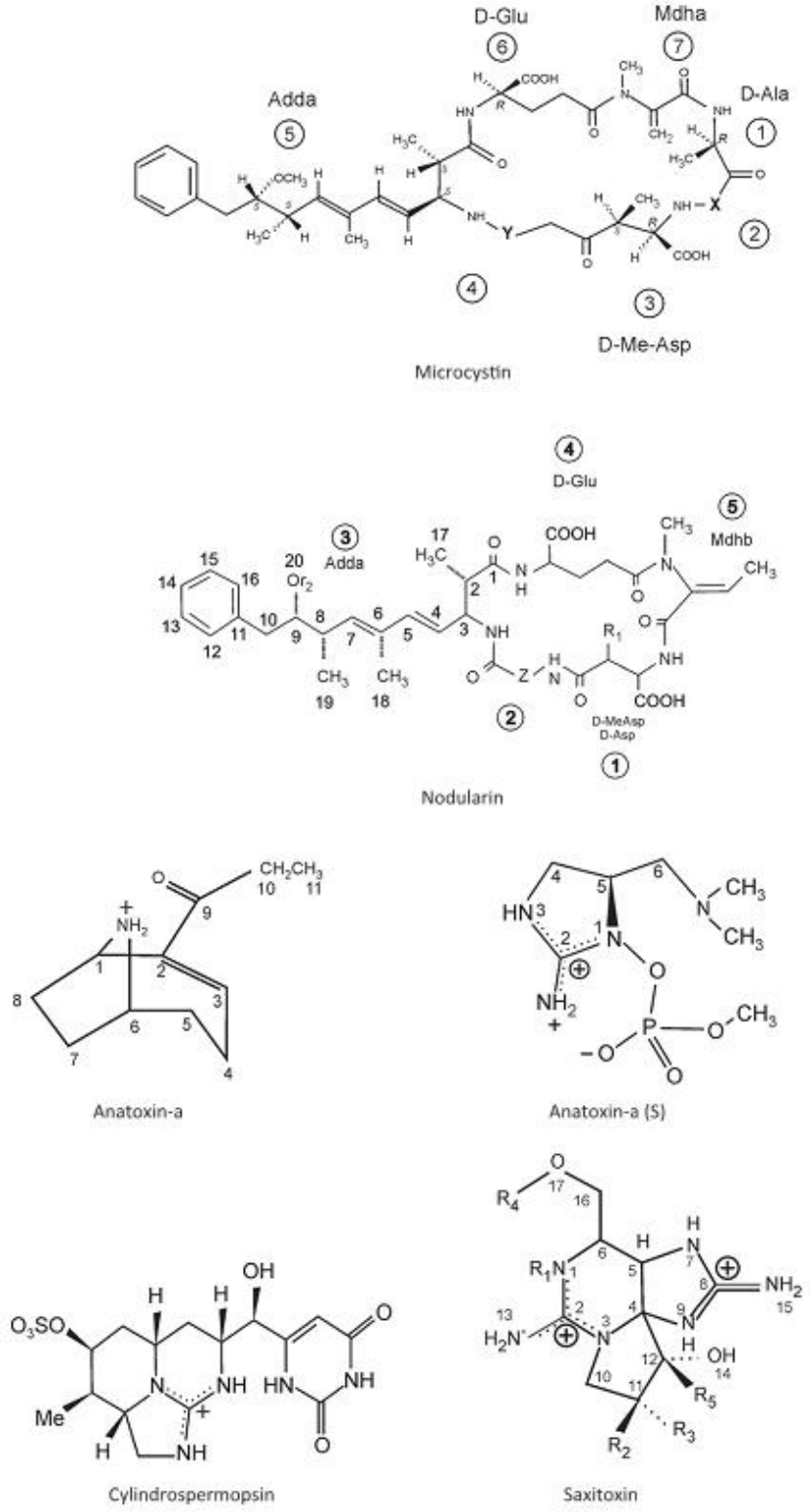


Figure 1.8. Chemical structures of the most common cyanotoxins (from Pantelić et al., 2013.).

Anabaena circinalis, *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii* and *Lyngbya wollei* are main producers of saxitoxins and neo-saxitoxins, a carbamate alkaloids that exists as non-sulfated (saxitoxins), singly sulphated (gonyautoxins) or doubly sulphated (C-toxins) (Table 1.3.). They cause a phenomenon called paralytic shellfish poisoning (PSP) and act as inhibitors of the nerve conduction by blockage of neuronal sodium channels (Sivonen and Jones, 1999; Pomati et al., 2000). Certain dinophlagellate species are also producers of these toxins that are most famous for toxic 'red tide' events (Carmichael, 1994).

1.2.2.3. Harmful algal blooms

Cyanobacterial strains have the ability to bloom and become dominant over other phytoplanktonic groups, especially in water ecosystems. Due to variations of certain environmental factors these blooms can occur naturally but most commonly are developed in eutrophic waters additionally polluted by anthropogenic activities. Increased proliferation of cyanobacterial strains have been accounted as harmful algal blooms (HABs) or cyanobacterial blooms (Carmichael, 2001, 2008; Hudnell et al., 2008; Paerl, 2008). Although these HABs have been first recorded 130 years ago, their incidence in both fresh and costal environments became more frequent and severe in modern human society (Francis, 1878; Chorus and Bartram, 1999; Carmichael, 2001, 2008; Heisler et al., 2008; Hudnell, 2008; Hoagland et al., 2002; Paerl, 2008; Paerl and Huisman, 2008; Paul, 2008). Concentrations of cyanobacteria above 10000 cells/mL signify the phenomenon of HABs while concentrations above 1000 cells/mL are considered hazardous and potentially harmful to animal and human health that require further observations (WHO, 1999; Graham et al., 2009). HABs are characterized by the appearance of the blue green layer on the water surfaces caused by the phycobilins and chlorophyll a, and production of volatile compounds, geosmin (GSM) and 2-methylisoborneol (MIB) (Falconer et al., 1999; Fleming et al., 2002). But more importantly, higher amounts of various cyanotoxins are released during HABs into the waters with severe impacts on humans and animals (Codd et al., 2005).

Table 1.3. Main groups of cyanotoxins with their acute toxicities, structures and known producers (adapted from Blàha et al., 2009).

Toxins (LD50 - acute toxicity)	**Structure**	**Activity**	**Toxigenic genera**
Hepatotoxins			
Microcystins (25 to ~ 1000)	Cyclic heptapeptides	Hepatotoxic, protein phosphatase inhibition, membrane integrity and conductance disruption, tumor promoters	*Microcystis, Anabaena, Nostoc, Planktothrix, Anabaenopsis Hapalosiphon*
Nodularins (30 to 50)	Cyclic pentapeptides	Hepatotoxic, protein phosphatase inhibition, membrane integrity and conductance disruption, tumor promoters, carcinogenic	*Nodularia*
Cylindrospermopsins (200 to 2100)	Guanidine alkaloids	Necrotic injury to liver (also to kidneys, spleen, lungs, intestine), protein synthesis inhibitor, genotoxic	*Cylindrospermopsis, Aphanizomenon, Anabaena, Raphidiopsis, Umezakia*
Neurotoxins			
Anatoxin-a (250)	Tropane-related alkaloids	Postsynaptic, depolarising neuromuscular blockers	*Aphanizomenon, Anabaena, Raphidiopsis, Oscillatoria, Planktothrix, Cylindrospermopsis*
Anatoxin-a(S) (40)	Guanidine methyl phosphate ester	Acetylcholinesterase inhibitor	*Anabaena*
Saxitoxins (10 to 30)	Carbamate alkaloids	Sodium channel blockers	*Aphanizomenon, Anabaena, Planktothrix, Lyngbya, Cylindrospermopsis*
Dermatotoxins (irritants) and cytotoxins			
Lyngbyatoxin-a	Alkaloid	Inflammatory agent, protein kinase C activator	*Lyngbya, Schizotrix, Oscillatoria*
Aplysiatoxin	Alkaloids	Inflammatory agent, protein kinase C activators	*Lyngbya, Schizotrix, Oscillatoria*
Endotoxins (irritants)			
Lipopolysaccharides	Lipopoly-saccharides	Inflammatory agents, gastrointestinal irritants	All cyanobacteria?

HABs are regarded as complex environmental events that are caused by multiple factors occurring simultaneously in marine, estuarine and fresh ecosystems (Heisler et al., 2008). These favoring factors for bloom formation include low turbulence and high temperature, light intensity, pH value and nutrient input, especially through increased aquaculture and industrial production, detergent and sewage wastes, and intensive farming where substantial amounts of phosphorus and nitrogen are released into the waters (Paperzak, 2003; de Figueiredo et al., 2004; Heisler et al., 2008; Pearl and Huisman, 2008; Paul, 2008).

As a consequence of cyanobacterial extensive biomass growth, hypoxic conditions arise that are lethal to invertebrate and fish species, the ambient light levels reduce affecting submerged aquatic vegetation, the composition of phytoplanctonic species decreases, and cyanotoxins contamination increases. Humans are exposed to hazardous cyanotoxins primarily by drinking polluted water or by consuming toxic sea or freshwater food (Chorus and Bartram, 1999). Recreational activity in such waters is also considered as potential route of exposure of humans to cyanotoxins through inhalation and dermal contact (Granéli and Turner, 2006). The most notorious case of cyanotoxin poisoning in humans is recorded in Brasil where 76 patients in hemodialysis clinic died after being intoxicated by microcystins polluted water during renal dialysis treatment in 1996, the phenomenon now called "Caruaru Syndrom" (Carmichael et al., 2001).

It has been estimated that 50 to 75% of these cyanobacterial blooms are toxic and that more than 50 different cyanobacterial strains produce cyanotoxins (Chorus, 2001; Rapala and Lahti, 2002; Bláhlová et al., 2007, 2008). Eutrophication is increasing worldwide with more than 40% of eutrophic lakes and reservoirs that are susceptible to cyanobacterial mass development (Bartram et al., 1999). The most common cyanobacteria occurring blooms in European freshwaters include genus *Microcystis, Anabaena, Aphanizomenon, Nodularia, Oscillatoria* (*Planktothrix)* and *Nostoc* (Sivonen and Jones, 1998). Toxic cyanobacterial HABs have been present in the region of Vojvodina in Serbia where studies indicated constant increase of blooming sites since 1980s (Svirčev et al., 2007; Simeunović et al., 2010). Around 70% of all reservoirs, water flows and lakes, mostly in agricultural areas of the Vojvodina, are now polluted by cyanobacterial blooms and one third of them are drinking water reservoirs (Svirčev et al., 2007). Dominant genus in Vojvodina cyanobacterial blooms include *Microcystis, Aphanizomenon, Anabaena* and *Oscillatoria* (*Planktothrix*) which are all producers of cyanotoxins where microcystin was found to be the most dominant one (Simeunović et al., 2010). A significant increase of primary liver cancer incidence has been reported in the areas of Vojvodina with polluted drinking water supplies and has been linked to increased concentrations of microcystins present in the waters (Svirčev et al., 2009, 2013).

1.2.2.4. Novi Sad Cyanobacterial Culture Collection

A long history of research on the biodiversity, occurrence and toxicity of cyanobacteria in Serbian rivers, lakes, ponds and reservoirs is recorded. Drinking reservoirs

have been studied the most, as well as effects of cyanobacteria on water quality and human health. The long lasting research resulted in the establishment of the Serbian Cyano Database (SCD). Numerous cyanobacterial strains have been isolated from aquatic and terrestrial environments and are a part of a unique and diverse collection of cyanobacterial cultures at the Department of Biology and Ecology, University of Novi Sad (UNS) in Serbia. The Novi Sad Cyanobacterial Culture Collection (NSCCC) has been founded 35 years ago and it is regarded as the most extensive and valuable collection of cyanobacterial strains from the Eastern Europe. The major portion of the NSCCC constitutes the terrestrial cyanobacterial strains that have been isolated from Serbia, as well as from other countries. Production of various types of secondary metabolites is present in these strains including proteins, lipids, pigments, polysaccharides, antimicrobial compounds, antioxidative substances, anticancer substances and cyanotoxins. The collection includes over 300 strains that belong to the genus *Microcystis, Nostoc, Anabaena, Rivularia, Tolypothrix, Calothrix, Scytonaema, Oscillatoria, Phormidium, Aphanizomenon and Spirulina* (Simeunović, 2005). Certain numbers of strains in this collection are also extremophiles.

1.3. CELLULAR DETOXIFICATION MECHANISM

Cells possess basic detoxification mechanisms that are essentially a build-in defense system against potentially harmful effects of various endo- and xenobiotics. Major barriers for uptake, distribution, metabolism and elimination of endo- and xenobiotics are biological membranes (Simkiss, 1995). However, once absorbed into the cell and/or organism, endogenous or potentially toxic xenobiotics undergo a detoxification pathway in the cells which comprises of several phases including: 1) phase 0 that is responsible for uptake of compounds into the cells via membrane uptake transporters (Organic Anion Transporting Polypeptides (OATPs), Organic Cation Transporters (OCTs), and Organic Anion Transporters (OATs)); 2) phase I that includes biotransformation of entered compounds by addition of different functional groups (-OH, -COOH, -NO_2, etc.) via cytochrome P450 superfamily of monooxygenases (CYPs), NADPH-dependent cytochrome P450 reductase (NADPH-CPR), monoamine oxidases (MAOs), alcohol- and aldehyde dehydrogenases (ADHs, ALDHs), and flavin-containing monooxygenases (FMOs); 3) phase II that includes conjugation of transformed compounds with more polar groups via glutathione S-transferases (GSTs), uridine 5'-diphospho-glucuronosyltransferase (UDP-GT), NAD(P)H-menadione

oxidoreductase (NMO), epoxide hydrolases (EPHs), N-acetyltransferases (NETs), and sulfotransferase (SULT) superfamilies enzymes in order to facilitate their excretion from the cell; 4) phase III that is responsible for the elimination of the metabolized compounds via membrane efflux transporters (ATP-binding Cassette superfamily (ABC), Multidrug and Toxic compound Extrusion family (MATE)) (Fig. 1.9.). For the intake of chemically diverse compounds into the cells, the uptake transporters have to be polyspecific, thereby enabling the upcoming phase of biotransformation by phase I and II enzymes that alter the chemistry of non-polar lipophilic chemical to polar–water soluble metabolites and finally lead to the detoxification and elimination of the parent compound before it exerts toxic effects (Van Der Oost et al., 2003). The most studied phases of cellular detoxification pathway are the biotransformation phases I and II. However, in the past decades much attention has been directed to studying uptake and efflux membrane transporters as they are equally important in the detoxification process (Szakács et al., 2008).

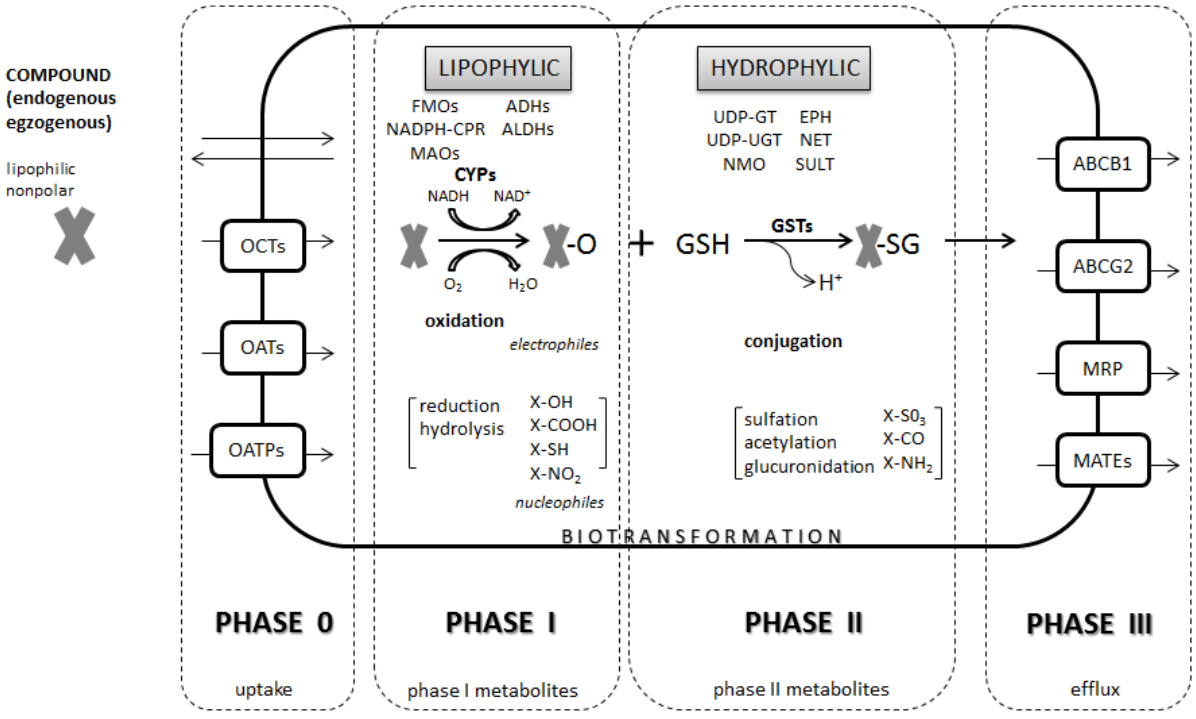


Figure 1.9. Schematic presentation of the cellular detoxification mechanism.

1.3.1. Current knowledge on *Caulerpa* and cyanobacterial interaction with the cellular detoxification mechanism

The toxic effects of two major secondary metabolites from genus *Caulerpa*, CYN and CLP have been studied on cellular detoxification pathways, especially their interaction with the biotransformation phases. The influence of CYN metabolite on the activity of phase II biotransformation enzymes demonstrated that it induces activation of GSTs in a gastropod *Bittium reticulatum* that consumed *C. taxifolia* and in the scorpio fish *Scorpaena porcus* that consumed prey that grazed on *C. taxifolia* (Uchimura et al., 1999a; Sureda et al., 2009). The scorpio fish exposed to *C. taxifolia* demonstrated modulation of CYP enzymes indicating that CYN could act as a phase I inducer (Uchimura et al., 1999a). Later study by Uchimura et al. (1999b) showed that CYP activity is in fact inhibited by CYN. The induction of GST activity, as well as antioxidative enzymes (glutathione peroxidase (GP) and glutathione reductase (GR)) was also recorded in teleost species *Coris julis* indicating that phase II enzymes are involved in metabolization of CYN toxic metabolite (Sureda et al., 2006). Recently, study on the effects of the *C. racemosa* on the sea urchin *Paracentrodus lividus* demonstrated higher concentration of glutathione (GSH) and increased GST activity (Tejada et al., 2013). Although more research has been done on the toxicity mechanisms of CYN, the effects of CLP form *C. racemosa* on cellular detoxification pathway have been investigated as well. A recent study on the toxicity mechanisms of *C. racemosa* from the Mediterranean showed the involvement of CYP family in the metabolism of CLP that induced the activity of EROD enzyme in white seabream *Diplodus sargus* (Feline et al., 2012). Except the activation of the phase I, CLP induced the activity of GST enzymes in white seabream (Feline et al., 2012). CLP exhibited inhibitory effect towards ABC efflux transmembrane transporter, P-glycoprotein (P-gp) form the phase III of the cellular detoxification metabolism in sponges and mussels (Schröder et al., 1998). Nevertheless, the knowledge of the influence of secondary metabolites from *Caulerpa* on the uptake and efflux membrane transporters of detoxification metabolism is still scarcely investigated. CYN and CLP mechanisms of toxicity are presented in Table 1.4.

Relevant mechanisms of cyanobacterial toxicity on cellular detoxification pathways have been studied most commonly for microcystin since it is the most dominant cyanotoxin in HABs. The studies on microcystins transport into the cell have been conducted identifying several OATP transporters in liver responsible for the uptake of microcystins. The first indicated uptake transporter for microcystin was Oatp1d1 in the liver of little skate *Leucoraja*

erinacea (Meier-Abt et al., 2007). Further studies on *Xenopus laevis* oocytes with high expression of rat and human transporters indicated the higher activity of rat Oatp1b2 and human OATP1B1, OATP1B3 and OATP1A2 transporter exposed to microcystin (Fischer et al., 2005). The uptake of two microcystin congeners (microcystin-LR, microcystin-RR) on Caco-2 cell line revealed the increased activity of two uptake transporters, OATP3A1 and OATP4A1 (Zeller et al., 2011). Furthermore, the interaction of microcystins with phase III transporters has been conducted that showed the significant increase in the expression levels of P-gp in *Jenynsia mutidentata* and *Dreissena polymorpha* (Contardo-Jara et al., 2008; Amé et al., 2009). Recently, zebrafish Abcb4 has been indicated as potential efflux transporter for microcystin-LR (Lu et al., 2015). Biotransformation enzymes are also included in the metabolization of microcystins where more studies have been made on interaction of this cyanotoxin with GST enzymes. Studies on Japanease quail *Coturnix japonica* exhibited increased activity of EROD enzyme in the heart and brain of the bird indicating the influence of microcystins to phase I of the detoxification pathway (Skocovska et al., 2007; Paskova et al., 2008). The activity of various cytosolic and microsomal GSTs has been increased after exposure to microcystins in different species such as plants, invertebrates, birds, fish and fish eggs (Pflugmacher et al., 1998; Takenaka, 2001). The interaction of cellular detoxification pathways with other cyanotoxins has been studied as well. Cylindrospermopsin is considered to passively diffuse to the cell due to its low molecular weight and the activation of CYP enzymes is considered to be included in the cylindrospermopsin metabolization (Humpage et al., 2005). Presence of anatoxin-a, anatoxin-a(S) and saxitoxins have also been linked to increased activities of GST and/or CYP enzymes (Osswald et al., 2013). Mechanisms of detoxification of cyanotoxins are presented in Table 1.4.

Table 1.4. Interaction of toxic secondary metabolites from *Caulerpa* algae and cyanobacteria with the cellular detoxification mechanisms.

Toxic secondary metabolite	Detoxification phase
Caulerpenyne	cytochrome P-450, GST
Caulerpin	cytochrome P-450, GST, P-glycoprotein
Microcystin	Oatp1d1, Oatp1b2, OATP1B1, OATP1B3, OATP1A2, OATP3A1, OATP4A1, cytochrome P-450, GST, P-glycoprotein, Abcb4
Cylindrospermopsin	cytochrome P-450
Nodularin	GST
Analoxin-a	cytochrome P-450, GST
Anatoxin-a(S)	cytochrome P-450, GST
Saxitoxin	GST

1.4. RESEARCH AIMS AND HYPOTHESES

According to the overview presented in previous chapters, the EDA approach represents a powerful multidisciplinary research tool developed in the field of environmental science that combines the use of advanced chemical and biological methods in order to prioritize toxicants present in complex environmental samples. Yet, it is clear that traditional EDA studies based on the use of 1-2 biological assays cannot offer a comprehensive, detailed and non-target characterization of (eco)toxicological properties of complex samples, or identification of potential toxic substances with various mechanisms of toxic action. Furthermore, although there are no theoretical obstacles preventing the use of EDA studies in characterizing complex biological samples, it has not been done yet.

Therefore, in this study we developed a modified EDA protocol and demonstrated the application of a series of *in vitro* bioassays targeting different mechanisms of toxicity on a detailed ecotoxicological characterization of complex biological samples. A modified protocol applied in our EDA study was based on the use of six *in vitro* and/or small-scale bioassays, addressing basic acute and toxic properties of tested biological samples, and all four phases of cellular detoxification. Two different but environmentally highly relevant types of complex biological samples were addressed: (1) various types of cyanobacterial

(prokaryotic) strains, and (2) two invasive green algae (eukaryotic) species from genus *Caulerpa*.

More specifically, the main aim of this research was to:

a) Perform a detailed ecotoxicological characterization of complex biological samples by developing and applying a modified EDA protocol;

b) Identify dominant mechanisms of toxicity by applying the EDA study that is based on a series of *in vitro* and small-scale bioassays;

c) Enable preliminary identification of biologically active compounds responsible for the observed effect by applying non-selective and non-target preparation of the samples.

Ultimately, based on the declared aims we believe this study will:

a) Improve the EDA concept as a reliable multidisciplinary diagnostic tool in the environmental science;

b) Offer a modified EDA protocol that can be reliably used for characterization of environmentally relevant complex biological samples and potential identification of new biologically active substances.

2. MATERIALS AND METHODS

2.1. SAMPLING AND EXTRACTION OF BIOLOGICAL MATERIAL

2.1.1. Invasive algae from the genus *Caulerpa*

2.1.1.1. Source of *C. racemosa* and *C. taxifolia*

Sampling of the two green algae was conducted on two different localities along the Adriatic Sea coast at depths ranging from 5-10 meters. *C. racemosa* was collected in winter season, in December 2012. Location site was the seabed near small fishing town called Vrsar on the west side of the Istrian peninsula in Croatia. It is the most northern habitat of this algal species in the Adriatic Sea and in the whole world (Figs. 2.1. and 2.3.). *C. taxifolia* was collected in summer season, in September 2013. Location site was a large deep bay on the northern part of the Hvar island called the Stari Grad Bay where this alga was first recorded in the Adriatic Sea in Croatia (Figs. 2.2. and 2.3.). Smaller fronds and stolons of *C. racemosa* were taken together with the benthic sediment while larger fronds and stolons of *C. taxifolia* were washed and cleaned from sediment impurities prior to freezing. After the collection, algal samples were stored at -20°C until further extractions procedures.

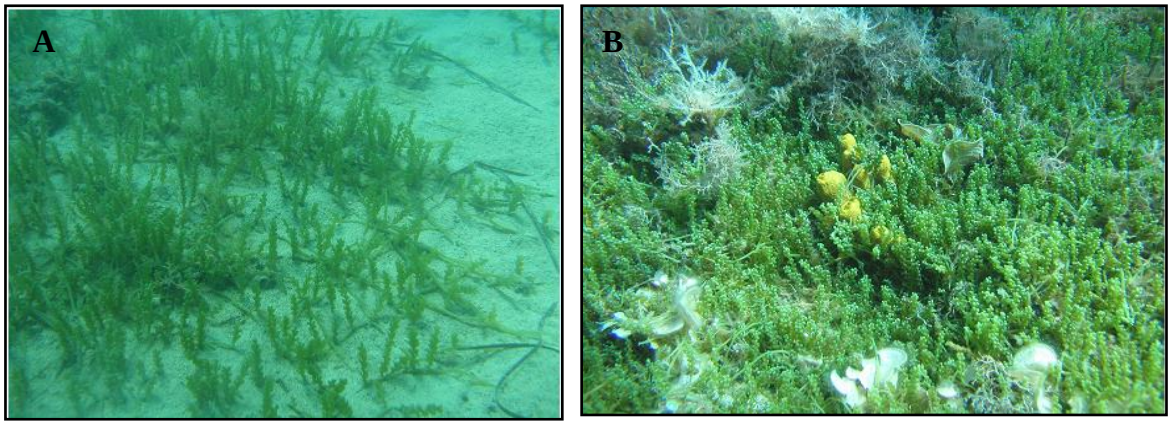


Figure 2.1. *Caulerpa racemosa* on a sandy (**A**) and rocky (**B**) bottom in Vrsar (from Project Adriatic, 2005).

Figure 2.2. *Caulerpa taxifolia* in the Stari Grad Bay (from Project Adriatic, 2005).

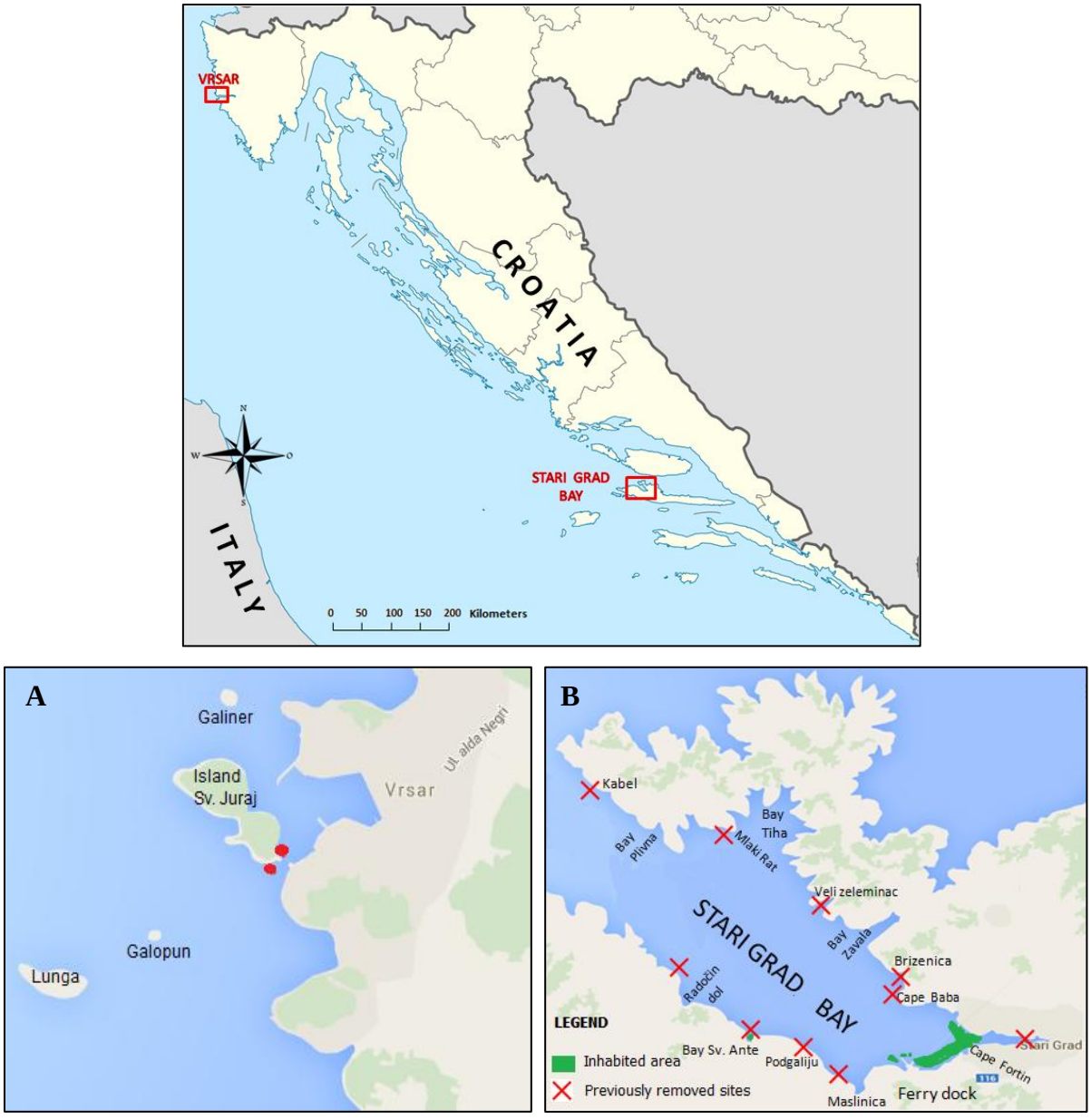


Figure 2.3. Sampling location sites for *C. racemosa* and *C. taxifolia* and their distribution (marked red and green) across the Vrsar seabed (**A**) and Stari Grad Bay (**B**) during 2009 (adapted from Žuljević et al., 2009).

2.1.1.2. Preliminary extraction

Algal material was thawed and dried. *C. racemosa* was additionally washed to remove the sediment and other remains prior to weighing. Ten g of algal fronds were weighed and separated into two 50 mL Falcon tubes (5 g in each tube) where 30 mL of solvent mixture DCM : PrOH (2-propanol) (1:1) was added. Samples were homogenized for 1 min using the Polytron homogenizer at speed of 0.5. Samples were manually mixed for 1 min, put on ice and then vortexed for 5 min on maximum speed. Afterwards samples were centrifuged (Hettich Zentrifugen, Germany) for 5 min at 2500 x *g* and supernatant was decanted into glass or plastic tubes, centrifuged again for 5 min at 2500 x *g*. Water/lipophilic layer was removed by pipetting. The resulting extracts were evaporated under nitrogen stream using a TurboVap system (Caliper Life Sciences, Hopkinton, MS, USA) at 45°C temperature. Dry residues obtained from the extracts were weighed and finally amounted to 70 mg for *C. racemosa* and 100 mg for *C. taxifolia*, respectively. Dry residue was dissolved in dimethyl sulfoxide (DMSO) and transferred to 4 mL screw-cap glass vials for further biological testing. Final concentration was 8.5 mg/mL DMSO for *C. racemosa* extract and 30 mg/mL for *C. taxifolia* extract, respectively.

2.1.2. Cyanobacterial strains

2.1.2.1. Source of cyanobacterial material

Different strains of cyanobacteria were acquired from NSCCC that belongs to the UNS, Department of Biology and Ecology in Serbia. Cyanobacterial strains selected for detailed ecotoxicological extraction were aquatic and terrestrial strains that belong to the following genuses: *Anabaena, Nostoc, Oscillatoria* and *Phormidium* (Fig. 2.4.). Two *Anabaena* strains (Č2 and Č5), two *Nostoc* strains (Z1 and S8), one *Oscillatoria* strain (K3) and one *Phormidium* strain (Z2) were selected for further ecotoxicological characterization (Simeunović, 2005, 2010). *Anabaena* Č2/Č5 and *Nostoc* S8 strains are terrestrial while *Nostoc* Z1, *Oscillatoria* K3 and *Phormidium* Z2 are aquatic strains. Designations used represent UNS codes labeled by the location site from which strains were isolated. Cyanobacterial lyophilized material was delivered from UNS, with the amount of material expressed in mg (Table 2.1.).

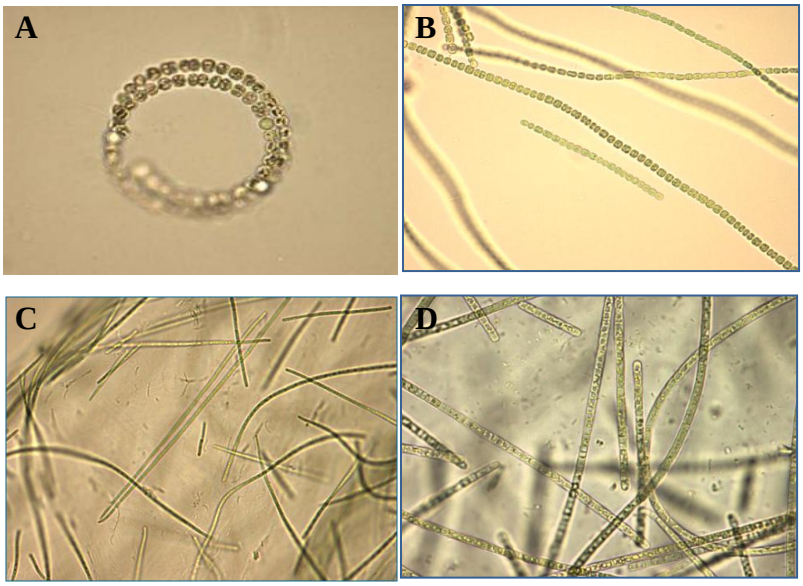


Figure 2.4. Cyanobacterial strains: *Anabaena* (**A**), *Nostoc* Z1 (**B**), *Phormidium* Z2 (**C**) and *Oscillatoria* K3 (**D**) (from Simeunović, 2005, 2010).

Table 2.1. Basic ID data for cyanobacterial strains obtained from UNS.

	Cyanobacterial strain	**Amount (mg)**	**Cultivation (month, year)**
TERRESTRIAL	*Anabena* Č2	30	January, 2013 March, 2014
	Anabena Č5	30	January, 2013 March, 2014
	Nostoc S8	33	August, 2013 March, 2014
AQUATIC	*Nostoc* Z1	14.1	March, 2014 September, 2014
	Phormidium Z2	27.3	March, 2014 September, 2014
	Oscillatoria K3	29.1	September, 2014

The selected strains belong to the dominant species in the Vojvodina region (Republic of Serbia) blooms whose locations have increased by 18% from 2003 to 2007 (Svirčev et al., 2007). Some of those are microcystin producing strains that is the most common cyanotoxin in Vojvodina region, and whose increase in concentration has been recorded especially in Vojvodina waters from 2005 to 2007 (Simeunović et al., 2010). Also, they belong to potentially toxic cyanobacterial strains that make 75% of all strains in Vojvodina blooms. Initial ecotoxicological studies showed that *Anabaena* and *Nostoc* terrestrial strains have antibacterial and antifungal biological activities (Drobac-Čik et al., 2007). Epidemiological studies showed that high primary liver cancer incidence is related to the sites in Serbia with

occurrence of cyanobacterial blooms in water supply reservoirs (Svirčev at al., 2009, 2013).

2.1.2.2. Preliminary extraction

Lyophilized cyanobacterial strains were dissolved in 1 mL of solvent mixture DCM : MeOH (1:1) and sonicated (MSE sonicator, UK). Sonication was carried out in two cycles, one with the low amplitude (4 microns) and one with the high amplitude (6 microns) during 10 sec interval. Samples were vortexed twice for 3 min at maximum speed and put back on ice. Extracts were centrifuged (Hettich Zentrifugen, Germany) at 10 000 x *g* for 5 min at room temperature. Supernatant was carefully transferred into 2 mL screw-cap glass vials. Extracts were evaporated under nitrogen stream using a TurboVap system (Caliper Life Sciences, Hopkinton, MS, USA) on 37°C temperature until dry matter was left. Cyanobacterial extracts were dissolved in DMSO for further biological testing. Final concentrations are shown in Table 2.2.

Table 2.2. Final concentrations of cyanobacterial strains expressed obtained upon preliminary extraction.

Cyanobacterial strain	Concentration (mg/mL DMSO)
Anabena Č2	150
Anabena Č5	150
Nostoc S8	165
Nostoc Z1	70.5
Phormidium Z2	136.5
Oscillatoria K3	145.5

2.2. BIOLOGICAL *IN VITRO* ASSAYS

2.2.1. Cytotoxicity assays

2.2.1.1. AlgaeTox test

The chronic toxicity of biological samples was evaluated using ISO validated freshwater algal growth inhibition test with the unicellular green alga *Scenedesmus subspicatus* Chodat. (alternative name: *Desmodesmus subspicatus*), 89.81 SAG (Sammlung

von Algenkulturen/Culture Collection of Algae) (ISO/FDIS 8692, 2004). The green alga culture was obtained from Dr. Knut-Erik Tollefsen from Norwegian Institute for Water Research. Cyanobacteria and *Caulerpa* samples were tested for their potential to inhibit the growth rate of *Scenedesmus subspicatus*.

2.2.1.1.1. Chemicals

- Ammonium chloride;
- Magnesium chloride hexahydrate**;**
- Calcium chloride dihydrate;
- Magnesium sulphate heptahydrate;
- Monopotassium phosphate;
- Iron(III) chloride hexahydrate;
- Ethylenediaminetetraacetic acid disodium salt dihydrate;
- Boric acid;
- Sodium hydroxide;
- Manganese(II) chloride tetrahydrate;
- Zinc chloride;
- Cobalt(II) chloride hexahydrate;
- Copper(II) chloride dihydrate;
- Sodium molybdate dihydrate;
- Sodium bicarbonate;
- Potassium dichromate.

Chemicals listed above are purchased from Sigma-Aldrich (St. Louis, MO, SAD) and Kemika (Zagreb, Croatia).

2.2.1.1.2. Materials and instruments

- sterile 96-well microplates, transparent flat bottom - STARLAB GmbH, Hamburg, Germany;
- sterile hood with laminar flow - Heraeus Instruments GmbH, Hanau, Germany;
- microplate reader - Infinite M200, Tecan, Salzburg, Austria;
- single and multi-channel micropipettes - Eppendorf, Hamburg, Germany;
- sterile plastic tips, glass pipettes and bottles, Erlenmeyer flasks;
- membrane filters (pore diameter 0.22 μm);
- hemocytometer;
- dry sterilizer and autoclave;

- water bath (37°C);
- analytical balance - BP-61, Sartorius, Goettingen, Germany;
- plastic weighing plates or dishes;
- pH meter - PB-11, Sartorius, Goettingen, Germany;
- magnetic stirrer;
- inverted microscope - Wilovert Standard HF40, Helmut Hund GmbH, Wetzlar, Germany.

2.2.1.1.3. Preparation of growth media and cultivation of alga

Growth media was prepared by mixing nutrient stocks solutions according to Table 2.3 and sterilized by membrane filtration (pore diameter 0.22 μm). Ten mL of stock solution 1, 1 mL of stock solution 2, 1 mL of stock solution 3 and 1 mL of stock solution 4 was added to water, and pH was set to 8.1 ± 0.2. Phytoplanktonic alga *Scenedesmus subspicatus* (Fig. 2.5.) was incubated at 24 ± 2°C under continuous white light with an intensity of 60-120 μmol $m^{-2}s^{-1}$.

Table 2.3. Concentration of nutrients in the test solution (from ISO/FDIS 8692, 2004).

Stock solutions	Nutrient	Concentration in stock solution	Final concentration in test solution
Stock solution 1 macronutrients	NH_4Cl	1.5 g/L	15 mg/L
	$MgCl_2 \cdot 6H_2O$	1.2 g/L	12 mg/L
	$CaCl_2 \cdot 2H_2O$	1.8 g/L	18 mg/L
	$MgSO_4 \cdot 7H_2O$	1.5 g/L	15 mg/L
	KH_2PO_4	0.16 g/L	1.6 mg/L
Stock solution 2 Fe-EDTA	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 μg/L
	$Na_2EDTA \cdot 2H_2O$	100 mg/L	100 μg/L
Stock solution 3 trace elements	H_3BO_3	185 mg/L	185 μg/L
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 μg/L
	$ZnCl_2$	3 mg/L	3 μg/L
	$CoCl_2 \cdot 6H_2O$	1.5 mg/L	1.5 μg/L
	$CuCl_2 \cdot 2H_2O$	0.01 mg/L	0.01 μg/L
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 μg/L
Stock solution 4 $NaHCO_3$	$NaHCO_3$	50 g/L	50 mg/L

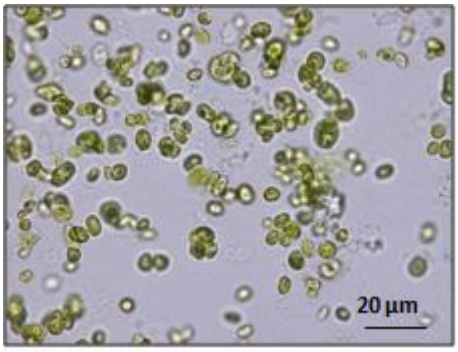


Figure 2.5. Freshwater unicellular green alga *Scenedesmus subspicatus* in a culture (adapted from: http://enfo.agt.bme.hu/drupal/node/12576).

2.2.1.1.4. AlgaeTox biotest – experimental procedure

In order to maintain the stock culture, 2 mL of the previous starter culture was aseptically transferred to 250 mL Erlenmeyer flask containing 50 mL of growth medium, and shaken manually 3 times/day for the next 3-5 days. In that period the alga culture reaches exponential growth phase at which point the alga was used for preparation of algal inoculum and toxicity testing. Alga was harvested from the liquid stock culture and 100 µL of 2 x 10^4 cells/well was inoculated in 96-well microplates. Serial dilutions of test samples were prepared in algal growth medium and 100 µL/well of each dilution was added in duplicate making the total reaction volume of 200 µL. Deionized water was used as a negative control and potassium dichromate ($K_2Cr_2O_7$) was used as a positive control. Cell density of algal inoculum was determined at zero time point by measuring fluorescence of chlorophyll a pigment ($\lambda_{ex}/\lambda_{em}$=440/680 nm) by a microplate reader. Test algal samples were incubated in previously described conditions for 96 ± 2 h after which the cell density was measured again. Inhibition was measured as decrease in growth rate of the alga exposed to biological extracts relative to control cultures grown under identical conditions.

2.2.1.1.5. Data analysis

Results were expressed as concentration-dependent percentages of algal growth inhibition, calculated from duplicates with mean ± SD (standard deviation) values. Mean and SD was first calculated from the obtained fluorescent values of duplicates. Then, average specific growth rate (μ) was calculated from the obtained cell density (N) mean values at a certain time point (t), using the following equation:

$$\mu = \frac{\ln N_{96} - \ln N_0}{t_{96} - t_0}$$

where N_{96} and N_0 are the cell densities obtained after 96 h (t_{96}) and 0 h (t_0).

Based on calculated μ of test samples and control, percent of inhibition was further calculated according to the following equation:

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} * 100$$

where $I_{\mu i}$ is the percentage of inhibition for test concentration i, μ_i is the mean growth rate for test concentration i and μ_c is the mean growth rate for control.

Serial dilutions were log transformed prior to statistical analysis. Non-linear regression method was used for obtaining sigmoidal-dose response curves and for calculations of LC50

values (lethal concentration that causes 50% of the maximal lethal effect). Calculations were performed in Microsoft Office Excel 2007 and statistical analyses were done in GraphPad Prism 5 Software for Windows.

2.2.1.2. MTT test

The acute toxicity was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay for determining cell viability. This well-established method was done according to Mosmann procedure (1983). Potential acute toxicity of cyanobacteria and *Caulerpa* samples was assessed by measuring the inhibition of PLHC-1/wt cell viability.

2.2.1.2.1. Chemicals

- Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 HAM (DMEM F-12) (L-glutamine, 15 mM *Hepes*, without sodium bicarbonate, powder), Sigma-Aldrich, St. Louis, MO, USA;
- Fetal bovine serum (FBS) (Invitrogen, CA, USA);
- Trypsin-EDTA solution, Sigma-Aldrich, St. Louis, MO, USA;
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma-Aldrich, St. Louis, MO, USA;
- Cyclosporin A (CYC), Sigma-Aldrich, St. Louis, MO, USA;
- Dulbecco's Phosphate Buffer Saline (PBS), Gibco Invitrogen, Life technologies, CA, USA;
- Sodium bicarbonate, Kemika, Zagreb, Croatia;
- 2-propanol, Kemika, Zagreb, Croatia.

2.2.1.2.2. Materials and instruments

- sterile tissue culture 96-well microplates, transparent flat bottom - STARLAB GmbH, Hamburg, Germany;
- sterile hood with laminar flow - Heraeus Instruments GmbH, Hanau, Germany;
- 28°C incubator;
- microplate reader - Infinite M200, Tecan, Salzburg, Austria;
- plate thermo-shaker – PST-60HL-4, Biosan, Riga, Latvia;
- single and multi-channel micropipettes - Eppendorf, Hamburg, Germany;

- sterile cell culture flasks 75 cm^2, TPP Techno Plastic Products AG, Switzerland;
- sterile plastic tips, glass pipettes, 50 mL Falcon tubes, 1.5 mL tubes;
- membrane filters (pore diameter 0.2 μm);
- hemocytometer;
- dry sterilizer and autoclave;
- water bath (37°C);
- analytical balance - BP-61, Sartorius, Goettingen, Germany;
- plastic weighing plates or dishes;
- pH meter - PB-11, Sartorius, Goettingen, Germany;
- magnetic stirrer;
- inverted microscope - Wilovert Standard HF40, Helmut Hund GmbH, Wetzlar, Germany.

2.2.1.2.3. PLHC-1/wt cell culture and maintenance

Fish hepatoma cell line derived from topminnow (*Poeciliopsis lucida*) was used for *in vitro* acute toxicity assessment (Fig. 2.6.). Cells were obtained from the American Type Culture Collection (ATCC® CRL-2406™; LGC Promochem, Teddington, UK). The PLHC-1/wt (wild type) cells were grown in a cell incubator at 28°C in DMEM F-12 (pH 7.4) medium containing L-glutamine, 15 mM *Hepes* and 5% FBS. Cells were passaged every 3-4 days with trypsin/EDTA. Number of passages varied between 15-30.

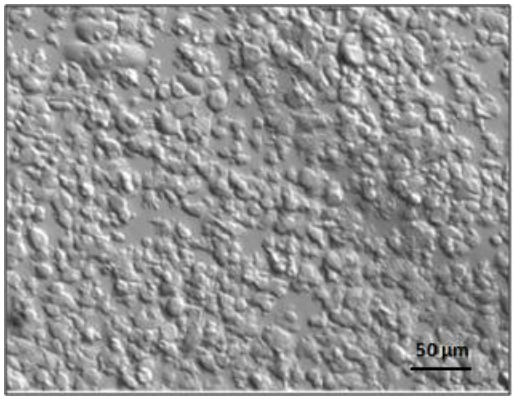


Figure 2.6. Fish hepatoma PLHC-1/wt cell line (adapted from Zaja et al., 2008).

2.2.1.2.4. MTT biotest – experimental procedure

Cells were seeded in 96-well plates with 25 x 10^4 cells/mL cultivation density and 200 μL cells/well seeding volume. Following the 24 h incubation, the cells were exposed to serial dilutions of the samples that were made in DMEM F-12 medium without FBS. Hundred μL of medium was extracted and replaced with 100 μL of sample dilutions in duplicates. DMEM F-

12 was used as a negative control and CYC was used as a positive control. PLHC-1/wt cells were exposed 72 h to sample concentrations after which the medium was removed from the wells and cells were washed with 100 μL of PBS. Five mg/mL stock solution of MTT in PBS was made, then diluted 10 times in DMEM F-12 medium and 100 μL was added to the wells. Incubation period was 3 h, at which point MTT was removed from the wells and 100 μL of PrOH was added to the cells to dissolve formed formazan salts. For the next 15 min plates were shaken at 350 rpm and then absorbance was measured using microplate reader at 570 nm with reference filter set to 750 nm.

2.2.1.2.5. Data analysis

Calculated difference between the measurement filter and the reference filter values were done in Microsoft Office Excel 2007. Serial dilutions were log transformed and the obtained values were normalized to percentages prior to statistical analysis. Sigmoidal dose-response curves were obtained using non-linear regression model in GraphPad Prism 5 software for Windows.

Results were expressed as percentages of viability from obtained duplicates with mean ± SD values. Classical four parameters sigmoidal dose-response curves were used to obtain the response (y) where b is the minimum (bottom) of response, a represents the maximum (top) response, hillslope (h) is slope of the curve, logLC50 is halfway response from bottom to top and x is the logarithm of inhibitor concentration. The equation is listed below.

$$y = b + (a - b)/(1 + 10^{((LogLC50 - x)*h)})$$

2.2.2. Interaction with basic phases of the cellular detoxification mechanism

2.2.2.1. Phase 0 – the uptake assay

For testing transport activity of the uptake transport proteins, human embryonic kidney HEK239 cell line overexpressing two different types of transporters was used. The cells were obtained from Dr. Silva Katušić Hećimović from the Ruđer Bošković Institute (ATCC® CRL-1573™). Cloned sequences of organic anion transporter polypeptide DrOatp1d1 (accession number: NP_001082802) and organic cation transporter DrOct1 (accession number: NP_998315.1) from zebrafish liver (*Danio rerio*) were used for monitoring the change in

transport activity while exposed to cyanobacteria and *Caulerpa* samples. *DrOatp1d1* and *DrOct1* genes sub-cloned into eukaryotic vector pcDNA3.1(+)/His were used for high expression in human cell line HEK293 and functional assay. Cells were seeded to 48-well plates with density between 1.9 – 2.0 x 10^5 cells/mL in a final volume of 250 µL/well. Forty-eight hours after cell seeding, transporter gene sequences cloned into pcDNA3.1(+)/His plasmid were transiently transfected to HEK293 cells using polyethyleneimine (PEI) reagent in a 1:1 ratio (Tom et al., 2008). After rapid votrexting (3 x 3 sec) and incubation (15 min) at room temperature, 25 µL/well of PEI and plasmid solution was added to the cells containing 225 µL/well of DMEM (without FBS). Incubation with transporter gene sequences lasted 4 h after which the medium was replaced with 250 µL/well of DMEM with 10% FBS. Transfection efficiency was typically in the range of 60-70%, as was evaluated using the LacZ staining protocol (Sambrook and Russell, 2001) 24 h after transfection of pcDNA3.1/His/LacZ plasmid to HEK293 cells. Potential mechanism of toxicity of cyanobacteria and *Caulerpa* extracts was determined by measuring inhibition of DrOatp1d1 and DrOct1 transport activity of proteins involved in the phase 0 of the cellular detoxification mechanism.

2.2.2.1.1. Chemicals

- Dulbecco's Modified Eagle's Medium (DMEM) (Powder, High Glucose, Pyruvate) Gibco Invitrogen, Life technologies, CA, USA;
- FBS, Invitrogen, CA, USA;
- Trypsin-EDTA solution, Sigma-Aldrich, St. Louis, MO, USA;
- PBS, Gibco Invitrogen, Life technologies, CA, USA;
- Lucifer Yellow (LY), Sigma-Aldrich, Taufkirchen, Germany;
- 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+), Sigma-Aldrich, St. Louis, MO, USA;
- Sodium chloride;
- Sodium bicarbonate;
- Potassium chloride;
- Calcium chloride;
- Magnesium chloride;
- *D*-(+)-*Glucose;*
- *Hepes,* Sigma-Aldrich, St. Louis, MO, USA;
- Sodium dodecyl sulfate 0.1% (SDS), Roth, Karlsruhe, Germany.

All used salts were purchased from Kemika, Zagreb, Croatia.

2.2.2.1.2. Materials and instruments

- sterile tissue culture 48-well microplates, transparent flat bottom – STARLAB GmbH, Hamburg, Germany;
- non-sterile 96-well plates, flat bottom - Greiner Bio-One GmbH, Frickenhausen, Germany;
- non-sterile 96-well microplates, black flat bottom - Sigma-Aldrich, Taufkirchen,Germany;
- sterile cell culture flasks 25 cm^2, TPP Techno Plastic Products AG, Switzerland;
- single and multi-channel micropipettes - Eppendorf, Hamburg, Germany;
- sterile plastic tips, glass pipettes, 50 mL Falcon tubes, 1.5 mL tubes;
- sterile hood with laminar flow - Heraeus Instruments GmbH, Hanau, Germany;
- humidified CO_2 incubator;
- vacuum pump;
- hemocytometer;
- dry sterilizer and autoclave;
- membrane filters (pore diameter 0.2 μm)
- water bath (37°C);
- analytical balance - BP-61, Sartorius, Goettingen, Germany;
- plastic weighing plates or dishes;
- pH meter - PB-11, Sartorius, Goettingen, Germany;
- magnetic stirrer;
- inverted microscope - Wilovert Standard HF40, Helmut Hund GmbH, Wetzlar, Germany;
- microplate reader - Infinite M200, Tecan, Salzburg, Austria.

2.2.2.1.3. Preparation of incubation media and maintenance of HEK293 cell line

Incubation medium was prepared according to Table 2.4 and sterilized on a 0.22 μm membrane filter whose pH was previously set to 7.4. HEK293 cells (Fig. 2.7.) were maintained in DMEM growth medium (pH 7.4) with high glucose and 10% FBS at 37°C and 5% CO_2 humidity. Cells were passaged every 3 days with trypsin/EDTA. Number of passages varied between 15 and 30.

Table 2.4. Composition of the incubation medium (pH 7.4).

Compounds	Concentration (mM)
NaCl	145
KCl	3
$CaCl_2$	1
$MgCl_2$	0.5
D-glucose	5
HEPES	5

Figure 2.7. Human embryonic kidney HEK293 cell line (adapted from: http://www.sonidel.com/sonidel/electroporation/nepa21-electroporator/cell-lines-293t-human-embryonic-kidney-cells/).

2.2.2.1.4. Uptake assay – experimental procedure

Following 24 h transfection, HEK293 cells overexpressing DrOatp1d1 and DrOct1 were exposed to 200 µL/well of the incubation medium for 15 min at 37°C. After preincubation, 100 µL/well of medium was removed and replaced with 50 µL/well of sample serial dilution in duplicate. Then, 50 µL/well of the model substrate, diluted in incubation medium, was added to the wells. Final concentration of DrOatp1d1 specific substrate LY was set to 10 µM and of DrOct1 specific substrate ASP+ to 2 µM, respectively. Later on, final concentration of ASP+ was increased to 15 µM. Incubation time for LY and ASP+ was 30 and 10 min, respectively, that was later on shortened to 5 min for each substrate at 37°C. After incubation the cells were washed two times with ice-cold incubation medium (250 µL/well) and incubated with 250 µL/well of 0.1% SDS for 30 min at 37°C for cell lysis. Fluorescence of transport specific substrates was measured in 96-well black microplates using a microplate reader at the specific wavelengths: LY 425/540 nm, ASP+ 485/590 nm. Eukaryotic vector pcDNA3.1(+)/His without cloned genes (mock cells) was also transfected into the HEK293 cells in order to determine the transporter-specific uptake. DrOatp1d1 and DrOct1 transfected cells exposed only to incubation medium were used as a negative control.

2.2.2.1.5. Data analysis

Results were expressed as inhibition percentages of LY/ASP+ uptake from obtained duplicates with mean ± SD values. Concentration-dependent LY and ASP+ uptake was calculated by using the equation listed below, where I_i is the percentage of inhibition for test concentration i, F_i is the mean fluorescent value for test concentration i, F_m is the mean fluorescent value for mock and F_c is the mean fluorescent value for control.

$$I_i = \frac{F_i - F_m}{F_c - F_m} * 100$$

Results were measured and calculated in Microsoft Office Excel 2007 and statistical analysis was made in GraphPad Prism 5 for Windows. Serial dilutions were log transformed and results were analyzed by non-linear regression method used for obtaining dose - response curves, with 95% confidence intervals (CI). Results were expressed as IC50 values that designate the concentrations that cause 50% of maximal observed inhibition. IC50 values were calculated from sigmoidal curves using the equation described in chapter 2.2.1.2.5.

2.2.2.2. Phase I - EROD biotest

This bioassay was conducted using the PLHC-1/wt cell line that is previously described in chapter 2.2.1.2.3. The assay was performed according to the protocol initially described by Hahn et al. (1996). Potential mechanism of toxicity of cyanobacteria and *Caulerpa* extracts was determined by measuring induction of CYP1A1 monooxygenase activity indicative for the phase I of the cellular detoxification mechanism.

2.2.2.2.1. Chemicals

- DMEM F-12 (L-glutamine, 15 mM *Hepes*), Sigma-Aldrich, St. Louis, MO, USA;
- FBS, Invitrogen, CA, USA;
- Trypsin-EDTA solution, Sigma-Aldrich, St. Louis, MO, USA;
- PBS, Gibco Invitrogen, Life technologies, CA, USA;
- 7-etoxiresorufine (7-ER), Sigma-Aldrich, St. Louis, MO, USA;
- Fluorescamine, Sigma-Aldrich, St. Louis, MO, USA;
- Bovine Serum Albumin (BSA) protein standard, Carl Roth GmbH + Co. KG, Karlsruhe, Germany;
- 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), St. Louis, MO, USA;

- Sodium bicarbonate, Kemika, Zagreb, Croatia;
- Disodium hydrogen phosphate, Kemika, Zagreb, Croatia;
- Sodium dihydrogen phosphate, Kemika, Zagreb, Croatia.

2.2.2.2.2. Materials and instruments

- sterile tissue culture 96-well microplates, transparent flat bottom - STARLAB GmbH, Hamburg, Germany;
- sterile cell culture flasks 75 cm^2, TPP Techno Plastic Products AG, Switzerland;
- sterile hood with laminar flow - Heraeus Instruments GmbH, Hanau, Germany;
- 28°C incubator;
- microplate reader - Infinite M200, Tecan, Salzburg, Austria;
- single and multi-channel micropipettes - Eppendorf, Hamburg, Germany;
- sterile plastic tips, glass pipettes, 50 mL Falcon tubes, 1.5 mL tubes;
- membrane filters (pore diameter 0.22 μm);
- dry sterilizer and autoclave;
- water bath (37 °C);
- analytical balance - BP-61, Sartorius, Goettingen, Germany;
- plastic weighing plates or dishes;
- pH meter - PB-11, Sartorius, Goettingen, Germany;
- magnetic stirrer;
- hemocytometer;
- inverted microscope - Wilovert Standard HF40, Helmut Hund GmbH, Wetzlar, Germany.

2.2.2.2.3. EROD biotest – experimental procedure

PLHC-1/wt cells were seeded into 96-well plates 24 h before performing the experiment. Seeding volume was 200 μL/well and cultivation density was 50 x 10^4 cells/mL. The next day, cells were exposed to serial dilutions of samples that were prepared in DMEM F-12 growth medium (pH 7.4) without FBS. Hundred μL/well of sample concentration was placed in plate in duplicates from which 100 μL/well of DMEM F-12 was previously removed. Exposure of cells to potential CYP1A1 inducers lasted for 24 h at 28°C after which EROD measurement proceeded. TCDD was used as a positive control and DMEM F-12 medium was used as a negative control.

Phosphate buffer (Na_2HPO_4, 50 mM) was prepared in distilled water and pH was set to 8 with NaH_2PO_4. The medium was removed from the plate and the cells were washed once

with 100 µL of PBS. Six mM stock of 7-ER was diluted 3000 times in phosphate buffer to gain the final concentration of 2 µM. The reaction was initiated by adding 100 µL/well of the substrate, after which the plate was immediately put into the microplate reader. Conversion of 7-ER to resorufin was monitored fluorometrically at specific wavelengths (535/590 nm) for 10 kinetic cycles with 1 min time interval. After 10 min of kinetic measurement plates were kept for further protein measurement and frozen at -20°C.

Using the reactive compound fluorescamine, protein content was measured for the normalization of the EROD activity (Udenfriend et al, 1972). The next day plates were taken from -20°C and left to thaw. Prior to protein quantification, 10 mg/mL of BSA solution was made and serial dilutions were prepared in phosphate buffer (50 mM, pH 8) to obtain the protein standard curve. Fluorescamine stock was prepared in acetone (1.5 mg/mL) and further diluted 10 times in acetonitrile (ACN) to gain the final concentration of 0.15 mg/mL. Hundred µL/well of fluorescamine was added to the plate and incubated for 3-5 min. Plates were shaken for 5 sec and fluorescence intensity was measured at specific wavelengths (390/465 nm).

2.2.2.2.4. Data analysis

"Least square" method (linest function) in Microsoft Office Excel 2007 was used to produce the best fit of the linearized equation $y = mx + b$ from the obtained fluorescence values at given time points. Data was further expressed per min, and then adapted to resorufin calibration curve data and protein quantification data. The activity of the CYP1A1 enzyme was finally expressed in pmol resorufin/min/mg protein with mean ± SD from duplicates. Results were expressed as EC50 values that designate the concentrations that cause 50% of maximal observed effect. Also, for obtaining CYP1A1 induction potential, EROD values of biological samples were expressed as EC5 and EC10 TCDD equivalents.

Protein content was determined by measuring fluorescence of fluorescamine product. Given data were adapted to previously obtained protein standard BSA calibration curve, using $x = (y-b)/a$ equation.

Serial dilutions were log transformed and data was further statistically analyzed using non-linear regression method for obtaining sigmoidal dose-response curves with 95% CIs as described in chapter 2.2.1.2.5. Statistical analysis was done in GraphPad Prism 5 for Windows.

2.2.2.3. Phase II - CDNB biotest

CDNB assay was used to monitor the change in the activity of 6 cytosolic GSTs (m3, m2, p1, p2, a3 and Rho) purified from zebrafish liver in the presence of cyanobacteria and *Caulerpa* samples. Method was done according to Habig et al. (1974) with certain modifications. Potential mechanism of toxicity of cyanobacteria and *Caulerpa* extracts was determined by measuring induction or inhibition of phase II biotransformation enzyme activity.

2.2.2.3.1. Chemicals

- Glutathione, Merck KGaA, Darmstadt, Germany;
- Phosphate buffer;
- 1-chloro-2,4- dinitrobenzene (CDNB), Sigma-Aldrich, St. Louis, MO, USA;
- Absolute ethanol, Kemika, Zagreb, Croatia.

2.2.2.3.2. Materials and instruments

- non-sterile 96-well microplates, black flat bottom - Sigma-Aldrich, Taufkirchen,Germany;
- single and multi-channel micropipettes - Eppendorf, Hamburg, Germany;
- plastic tips, 1.5 mL tubes, plastic weighing plates;
- analytical balance - BP-61, Sartorius, Goettingen, Germany;
- pH meter - PB-11, Sartorius, Goettingen, Germany;
- magnetic stirrer;
- microplate reader - Infinite M200, Tecan, Salzburg, Austria.

2.2.2.3.3. CDNB biotest – experimental procedure

GSH and CDNB solutions were made in phosphate buffer (pH 6.5) and absolute ethanol, respectively. Reaction volume was 250 µL/well and it was prepared in 96-well plates with the following components whose final concentrations were: 0.1 M phosphate buffer (pH 6.4), 1 mM GSH (50 µL/well), 1 mM CDNB (10 µL/well), 2 µg/mL GST enzyme, and sample (1:100 dilution). The reaction was initiated by adding CDNB model substrate after which the plate was rapidly put into the microplate reader. Control activity of each GST was also measured without sample present. Reaction without GST and samples was blank control. Absorbance of resulting CDNB conjugate was measured during 40 kinetic cycles with 15 s time interval. Overall, the measurement lasted for 10 min.

2.2.2.3.4. Data analysis

Results were expressed as absorbance values with mean ± SD values from duplicates. The activity of each enzyme was subtracted from the control reactions to obtain the change in the activity that was presented using a liner slope. All data calculations and measurements were analyzed in Microsoft Office Excel 2007.

2.2.2.4. Phase III - MXR biotest

Transport activity of the P-gp efflux transporter was determined using calcein acetoxymethyl ester (Ca-AM) method on mouse embryonic fibroblast NIH 3T3-R/dox cell line (Kurelec et al., 2000). Potential mechanism of toxicity of cyanobacteria and *Caulerpa* extracts was determined by measuring inhibition of P-gp transport activity as indication of the phase III of cellular detoxification mechanism.

2.2.2.4.1. Chemicals

- DMEM (Powder, High Glucose, Pyruvate) Gibco Invitrogen, Life technologies, CA, USA;
- FBS, Invitrogen, CA, USA;
- Trypsin-EDTA solution, Sigma-Aldrich, St. Louis, MO, USA;
- PBS, Gibco Invitrogen, Life technologies, CA, USA;
- Doxorubicin hydrochloride, Sigma-Aldrich, St. Louis, MO, USA;
- Sodium bicarbonate, Kemika, Zagreb, Croatia;
- Verapamil (VER), Sigma-Aldrich, St. Louis, MO, USA;
- CYC, Sigma-Aldrich, St. Louis, MO, USA;
- Valspodar (PSC 833), Novartis Pharma AG, Basel, Switzerland;
- MK571, Cayman Chemicals Co., Michigan, OR, USA;
- Calcein acetoxymethyl ester, Molecular Probes (Eugene, OR, USA).

2.2.2.4.2. Materials and instruments

- sterile tissue culture 96-well microplates, transparent flat bottom - STARLAB GmbH, Hamburg, Germany;
- non-sterile 96-well microplates, black flat bottom - Sigma-Aldrich, Taufkirchen,Germany;
- sterile cell culture flasks 75 cm^2, TPP Techno Plastic Products AG, Switzerland;
- sterile hood with laminar air flow - Heraeus Instruments GmbH, Hanau, Germany;

- humidified CO_2 incubator;
- microplate reader - Infinite M200, Tecan, Salzburg, Austria;
- single and multi-channel micropipettes - Eppendorf, Hamburg, Germany;
- sterile plastic tips, glass pipettes, 50 mL Falcon tubes, 1.5 mL tubes;
- dry sterilizer and autoclave;
- water bath (37 °C);
- analytical balance - BP-61, Sartorius, Goettingen, Germany;
- plastic weighing plates or dishes;
- pH meter - PB-11, Sartorius, Goettingen, Germany;
- magnetic stirrer;
- membrane filters (pore diameter 0.22μm);
- hemocytometer;
- inverted microscope - Wilovert Standard HF40, Helmut Hund GmbH, Wetzlar, Germany.

2.2.2.4.3. NIH 3T3-R/dox cell line

Mouse embryonic fibroblast cell line NIH 3T3 is permanently transfected with human MDR1 gene that codes for P-gp. NIH 3T3-R/dox cells are a sub-clone of NIH 3T3/MDR1 cells that have been cultured in the presence of a potent chemotherapeutic doxorubicin for increase of their multidrug resistance (Fig. 2.8.). NIH 3T3/MDR1 cells (ATCC® CRL-1658™) were kindly acquired from Dr. Balasz Sarkadi (National Institute for Hematology, Blood Transfusion and Immunology, Budapest, Hungary) with approval from Dr. Michael M. Gottesman (National Institute for Health, Bethesda, MA, USA). Cells were maintained in DMEM growth medium (pH 7.4) with 10% FBS in CO_2 incubator at 37°C. Cells were passaged every 3-4 days with trypsin/EDTA. Number of passages varied between 15 and 30.

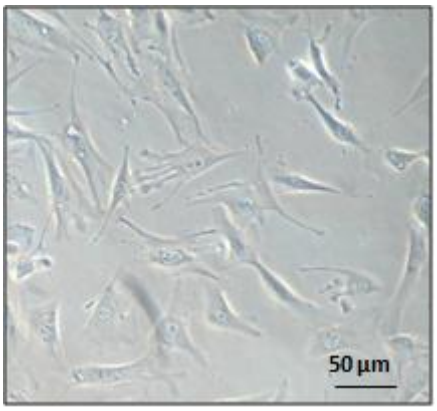


Figure 2.8. Mouse fibroblast NIH 3T3/MDR1 cell line (adapted from: https://www.nacalaiusa.com/product.php?id=72).

Before performing the MXR bioassay, cells were treated with chemotherapeutic doxorubicin (1 μM) and tested for multidrug resistance. Non-transfected cells that arise in the culture after freezing were eliminated with doxorubicin, leaving only cells with high content of human P-gp to grow in the culture. For resistance evaluation the cells were tested with competitive P-gp model inhibitors VER, CYC, MK571 and PSC833. Fold change of 5-10 times in accumulation of the model substrate Ca-AM was considered significant.

2.2.2.4.4. MXR biotest – experimental procedure

NIH 3T3-R/dox cells were seeded in 96-well plates with seeding volume of 200 μL and cultivation density of 30 x 10^4 cells/mL. Following 24 h after seeding, serial dilutions of biological samples were made in DMEM medium (pH 7.4) in non-sterile 96-well plates. The medium above the cells was removed and replaced with 100 μL/well of DMEM medium without FBS. Then 50 μL/well of serial dilutions was added and incubated for 5 min, after which 50 μL/well of Ca-AM was added to initiate the reaction. Upon 45 min incubation the medium was extracted and cells were washed with 100 μL/well of ice-cold PBS. Another 100 μL/well of ice-cold PBS was added to the cells and the fluorescence of calcein product was measured using microplate reader at 485/530 nm wavelengths. Hundred μL/well of 0.5% Triton was added to lyse the cells 1 min after adding the detergent, and also 10 min after. Positive control was model competitive inhibitor for P-gp, VER. Negative control was reaction volume without the samples.

2.2.2.4.5. Data analysis

Results were expressed as fluorescence units with mean ± SD values calculated from duplicates. Concentration-dependent P-gp inhibition was expressed as fold change compared to P-gp activity of non-treated cells (negative control). All data calculations were analyzed in Microsoft Office Excel 2007.

2.3. CHEMICAL ANALYTICAL METHODOLOGIES

2.3.1. ABC fractionation

Liquid (adsorption) chromatography (LC) with 15% silica gel was used for the separation of chemical compounds from cyanobacteria and *Caulerpa* extracts that showed potent biological responses in the preliminary testing phase. Silica gel particles, size 0.063-

0.200 mm, were used as stationary phase while three different solvents (HEX, DCM, and MeOH) were used as mobile phases for column chromatography. First, column was washed with A (HEX), B (DCM) and C (MeOH) solvents in a reverse order. Prior to filling, column was dried using nitrogen stream flow, then washed and filled with HEX. Silica gel was deactivated with mQH_2O (milli-Q H_2O) and 5 mL of 15% silica gel was carefully applied onto the chromatography column filled with HEX avoiding the formation of air bubbles. Silica gel and solvents were purchased from Kemika, Zagreb, Croatia.

Dry residues of the cyanobacterial and *Caulerpa* samples were obtained by the preliminary extraction protocols as previously described in chapters 2.1.1.2. and 2.1.2.2., respectively (Table 2.5.). Extracts were dissolved in 700 μL of HEX and transferred to the 15% silica gel column. This procedure was repeated three times after which the column was washed with 25-30 mL of HEX to obtain the A fraction. The same procedure was applied with DCM and MeOH solvents for obtaining B and C fraction, respectively. All three fractions were collected in 30 mL glass tubes that were previously weighed and labeled.

Table 2.5. Dry matter obtained after preliminary extraction for *C. racemosa*/C. *taxifolia* lyophilized cyanobacterial material and used for further ABC extraction.

Sample	mass (mg)
C. racemosa	100
C. taxifolia	190
Anabaena Č2	19
Anabaena Č5	32.7
Nostoc S8	35.3
Nostoc Z1	36.7
Phormidium Z2	46.5
Oscillatoria K3	30.2

Use of different solvents enabled the separation of compounds according to their polarity thus providing A (non-polar), B (medium polar) and C (polar) fraction. These fractions were evaporated under nitrogen stream flow using a TurboVap system (Caliper Life Sciences, Hopkinton, MS, USA) at the following temperatures: 40°C for DCM, and 50-60°C for HEX and MeOH. Obtained dry residues for A, B, C fractions of cyanobacterial and *Caulerpa* samples are shown in Table 2.6. Dry residues were dissolved in DMSO for performing further biological testing (Table 2.6.). For obtaining TCDD Eq, *Nostoc* Z1 A

fraction and *Phormidium* Z2 B fraction were adapted to EC20 TCDD while *Phormidium* Z2 C fraction was adapted to EC5 TCDD value. All other fractions of cyanobacterial strains were adapted to EC10 TCDD values.

Table 2.6. Dry residue and final concentrations expressed in mg/mL in DMSO.

Sample	A		B		C	
	mg	**mg/mL**	**mg**	**mg/mL**	**mg**	**mg/mL**
C. racemosa	1	10	7	10	27	20
C. taxifolia	12	10	12	20	68	20
Anabaena Č2	1.73	10	1.48	10	2.9	10
Anabaena Č5	1.15	10	1.59	10	3.84	10
Nostoc S8	0.66	10	1.33	10	3.25	10
Nostoc Z1	0.9	10	1.3	10	12.3	20
Phormidium Z2	1.9	10	3	10	6	10
Oscillatoria K3	1.8	10	2.4	10	5.2	10

2.3.2. High-performance liquid chromatography (HPLC)

C. taxifolia and *C. racemosa* B and C fractions were further separated into 40 sub-fractions by preparative high-performance liquid chromatography (HPLC), using a Varian ProStar instrument with a Model 410 autosampler, Model 330 photodiode array detector and Model 704 fraction collector. Octadecyl silica (C18) column (250 x 10 mm) was used for detailed separation of B and C fractions. Dry residues of fractions were obtained by previously described preliminary extraction protocols, weighing 100 mg for *C. racemosa* and 190 mg for *C. taxifolia*. Residues were dissolved in 1.3 mL of ACN : H_2O (1:1) mixture, vortexed and centrifuged (Hettich Centrifugen, Germany) for 5 min at 1000 x *g*. One mL of fraction extract was applied to the HPLC column. In case of *C. taxifolia* B fraction, only 1 mL of supernatant was successfully removed due to rising of non-dissolved pellet. Therefore, additional 300 µL of ACN/H_2O mixture had to be added before applying the sample to the HPLC column.

The mobile phase A was used as a mixture of H_2O : ACN (9:1), while pure ACN was used as the mobile phase B. The composition of H_2O and ACN varied during the elution flow by alternation of A and B phases. Gradient elution mode started at low elution strength with 100% A and 0% B from 0-10 min, then followed the linear increase of B by 10% and lowering A to 90% from 10-15 min. High elution strength at 100% B and 0% A continued

from 15-30 min and ended again with 100% A and 0% B from 30-40. Gradient flow was 5 mL/min with 40 min flow time. Twelve mL glass tubes were used for collection of the fractions that were previously weighed for measuring dry matter residues.

Resulting fractions were evaporated under nitrogen stream flow using a TurboVap system (Caliper Life Sciences, Hopkinton, MS, USA) at the following temperatures: 40°C for ACN and 60°C for water. Sub-fractions were weighed again using analytical balance and dissolved in DMSO to final concentration of 0.5 mg/100 µL as shown in Table 2.7. To dissolve the whole dry matter obtained in sub-fractions B24, C27, C28 from *C. racemosa* and C29 from *C. taxifolia*, additional volume of DMSO was added. Final concentrations were: 0.83 mg/100 µL, 0.14 mg/100 µL and 0.25 mg/100 µL for *C. racemosa* B24, C27 and C28, respectively, and 0.25 mg/100 µL for *C. taxifolia* C29 (Table 2.7.). Some fractions were additionally sonicated (MSE sonicator, UK) due to poor elution (3 x 10 sec, amplitude low/5). All fractions were centrifuged (Hettich Zentrifugen, Germany) at 1000 x g for 1-2 min to spin down. Before measurement of the samples, a blank (H_2O/ACN) sample was run through the HPLC system.

2.3.3. Liquid chromatography-mass spectrometry (LC-MS)

ABC fractions and selected HPLC sub-fractions of *C. racemosa* and *C. taxifolia* were further analyzed by highly sensitive analytical liquid chromatography coupled to high-resolution mass spectrometry (LC-MS). ABC fractions were made by procedure previously described in chapter 2.3.1. Around 50-60% of the initial dry residue obtained after preliminary extraction was applied on the 15% silica gel column, 30 and 100 mg for *C. racemosa* and *C. taxifolia*, respectively. The residue was dissolved in 2 mL of HEX and transferred to the column. After obtaining 25-30 mL extracts in ABC solvents, the fractions were evaporated by N_2 stream until the remaining volume of around 5 mL. For the LC-MS analyses 1:10 dilution of each fraction was evaporated to obtain the dry residue and dissolved in 1 mL of MeOH. HPLC sub-fractions dissolved in DMSO were used for the LC-MS analyses with the amount of sub-fractions between 3-10 mg/mL in MeOH, as presented in Table 2.8.

Waters Acquity ultra-performance liquid chromatography (UPLC, Waters Corp., Milford, MA, USA) system coupled to hybrid quadrupole-time-of-flight/mass spectrometry (QTOFMS Premier, Waters Corp., Milford, MA, USA) equipped with an electrospray

ionisation source was used to perform the separation and detection of present constituents (Terzic and Ahel, 2011). A 50 mm x 2.1 mm column filled with 1.7 μm BEH C_{18} stationary phase was used for chromatographic separation (Waters Corp., Milford, MA, USA). The analysis was conducted in the positive ionization mode (PI) and the eluents used were 0.1% (v/v) formic acid in water (A) and ACN (B). For the eluation, binary solvent gradients were used where gradient flow was 0.4 mL/min. The eluation started at 5% B for 1 min after which the percentage of B increased linearly in several steps up to 95% B in 19 min and was kept at those conditions for another 2 min. The duration of the run-time together with the column conditioning was 23 min. QTOFMS Premier Instrument with orthogonal Z-spray-electrospray interface was operated in a V mode with TOFMS data being collected between m/z 100-1000, applying collision energy of 5 eV. Extended dynamic range (DRE) was used for spectra recording and the data were collected in the centroid mode with a scan time of 0.2 s. The conditions used for mass spectrometry on a QTOFMS Premier instrument were: the desolvation gas flow was set to 650 L/h at a temperature of 280°C, the cone gas flow was adjusted to 24 L/h, and the source temperature to 120°C. The capillary voltage in the PI was 3500 V while the cone voltage was set to 30 V. An independent reference spray via the lock spray interference was used for conducting all acquisitions thereby ensuring maximum accuracy of the system. Leucine enkephalin was applied as a lock mass in PI (m/z 556.2771). Elemental composition of the precursor and fragment ions were calculated using the MassLynx software incorporated in the instrument.

The chromatograms, recorded in the total ion current (TIC) mode, were systematically examined by manually generating mass spectra of each individual peak using background-subtraction option. The generated TOF mass spectra were studied in detail in order to detect key ions suitable for the structural elucidation of the detected compounds. In addition, when a tentative identification, based on TOF mass spectra, indicated the presence of a given constituent, extracted ion chromatograms (XICs) were applied to facilitate their visualization and subsequent confirmation.

2.3.3. Analytical standard of caulerpenyne

Purified CYN standard (10 mg) was acquired from Dr. Philipe Amade (Equipe Molécules Bioactives, University of Nice-Sophia Antipolis, France). Stock solutions were prepared in MeOH for analytical LC-MS analysis (1 mg/mL) and bioassays (10 mg/mL).

Aliquotes were made and stored at -80°C. For LC-MS analyses CYN standard was diluted 1:100 in MeOH, resulting in 10 ng/μL CYN solution. Furthermore, 1% of DMSO was added to 1 mL of MeOH. The CYN standard was tested by uptake bioassay on DrOatp1d1 and DrOct1 transporter with a series of 12 concentrations starting at maximum concentration of 267 μM (or 1:100 dilution) up to minimal concentration of 0.0267 μM (or 1:1000000 dilution).

Table 2.7. Final volumes (μL DMSO) of B and C HPLC sub-fractions for *C. racemosa* and *C. taxifolia* (final concentration 0.5 mg/100 μL). Final concentrations of sub-fractions B24, C27, C28 from *C. racemosa* and C29 from *C. taxifolia* were: 0.83 mg/100 μL, 0.14 mg/100 μL, 0.25 mg/100 μL and 0.25 mg/100 μL.

	C. taxifolia		***C. racemosa***	
Fraction	**B**	**C**	**B**	**C**
1	32	20	540	20
2	50	140	20	304
3	104	2060	202	1278
4	46	80	500	42
5	20	120	308	140
6	102	120	330	20
7	64	60	100	70
8	70	100	1140	144
9	98	60	532	70
10	54	80	642	144
11	20	20	20	20
12	64	40	20	68
13	40	20	2098	20
14	20	20	330	20
15	132	80	20	192
16	40	180	20	194
17	20	160	20	46
18	114	480	76	24
19	20	20	26	58
20	20	200	22	68
21	20	240	36	20
22	42	220	20	20
23	20	120	20	44
24	20	20	120	236
25	42	260	58	20
26	20	220	20	114
27	56	140	36	70
28	20	580	26	40
29	20	40	56	86
30	60	80	70	56
31	20	300	22	46
32	32	60	20	56
33	20	40	0	76
34	20	160	20	20
35	20	20	20	102
36	20	20	20	20
37	20	60	20	62
38	72	280	70	20
39	20	20	24	20
40	20	80	70	20

Table 2.8. LC-MS analysed B and C sub-fractions of *C. racemosa* and *C. taxifolia*.

C. taxifolia			
HPLC sub-fraction	**Final volume (µL DMSO)**	**Tested by bioassays**	**For LC-MS (% in MeOH)**
B22	42	+	10
B23	20	-	10
B24	20	-	10
B25	42	+	10
B26	20	-	10
B27	56	+	10
B28	20	-	10
B29	20	-	10
B30	60	+	10
C16	180	+	10
C17	160	+	10
C18	480	+	3
C19	20	-	10
C20	200	+	5
C21	240	+	5
C22	220	+	5
C23	120	+	10
C24	20	-	10
C25	260	+	5
C26	220	+	5
C27	140	+	10
C28	580	+	3

C. racemosa			
HPLC sub-fraction	**Final volume (µL DMSO)**	**Tested by bioassays**	**For LC-MS (% in MeOH)**
B24	120	+	10
B25	58	+	10
B26	20	-	10
B27	36	+	10
B28	26	-	10
B29	56	+	10
B30	70	+	10
C19	58	+	10
C20	68	+	10
C21	20	-	10
C22	20	-	10
C23	44	+	10
C24	236	+	5
C25	20	-	10
C26	114	+	10
C27	70	+	10
C28	40	+	10
C29	86	+	10
C30	56	+	10

A schematic display of EDA workflow with extraction procedures used for *Caulerpa* species and cyanobacterial strains are presented on Figs. 2.9. and 2.10., respectively.

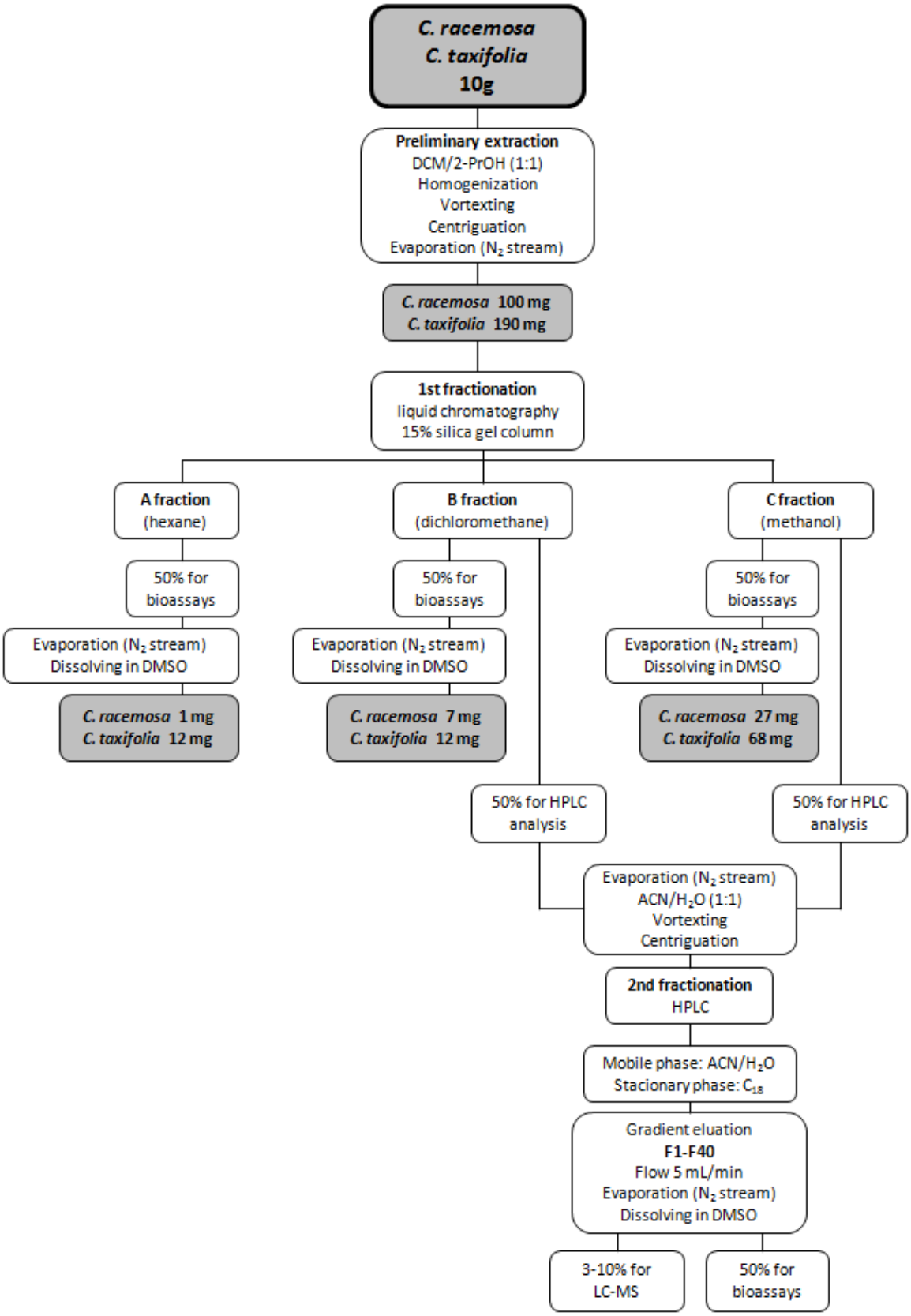


Figure 2.9. Extraction procedure for *Caulerpa* species used in our modified EDA study.

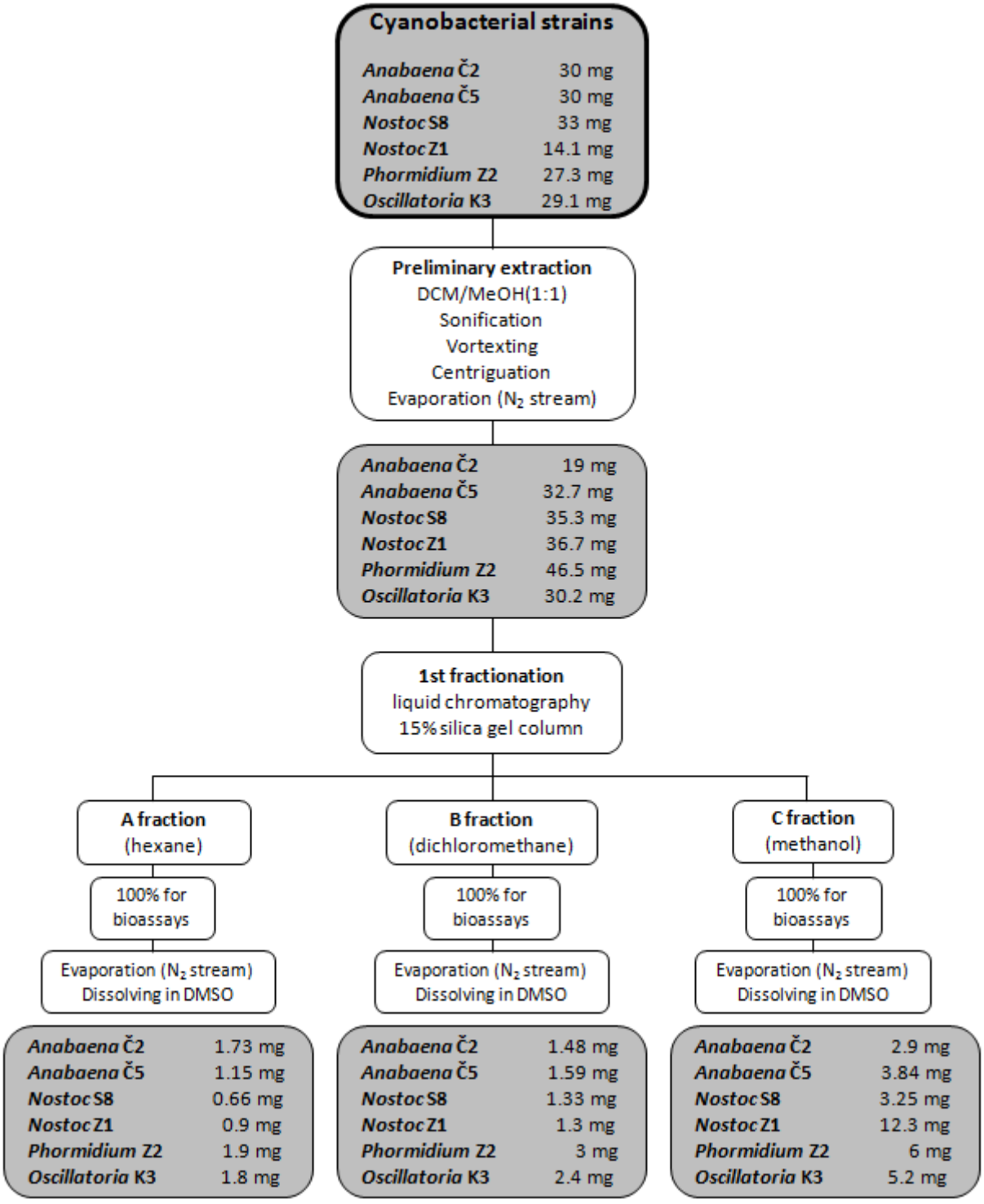


Figure 2.10. Extraction procedure for cyanobacterial strains used in our modified EDA study.

3. RESULTS

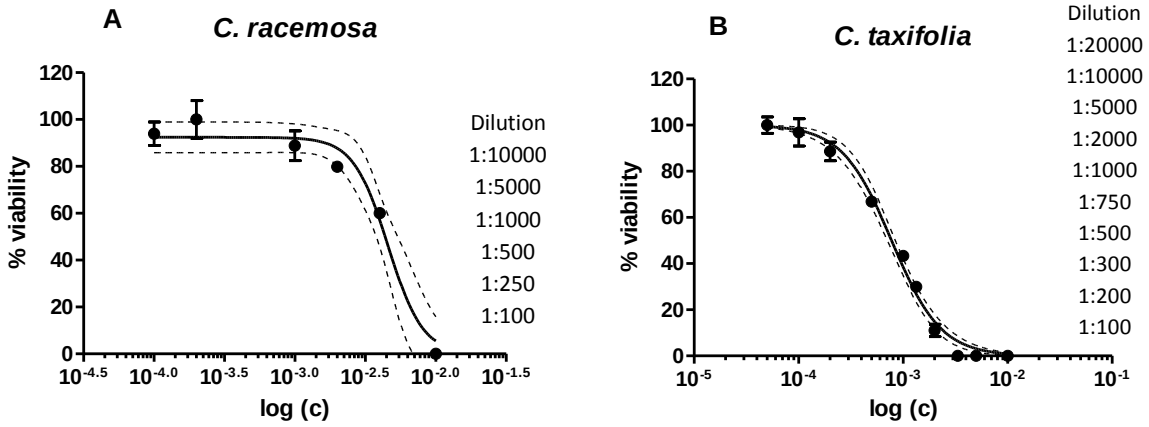
3.1. TOXICOLOGICAL CHARACTERIZATION OF PRELIMINARY EXTRACTS

Resulting concentration of *C. racemosa* and *C. taxifolia* preliminary extracts were 8.5 and 30 mg/mL, respectively, while concentrations of cyanobacterial preliminary extracts are listed in Table 2.3. Maximal amount of DMSO that was used as solvent for all tested extracts never exceeded 1% (or dilution 1:100) in *in vitro* assays.

3.1.1. Cytotoxic potential of *Caulerpa* and cyanobacterial extracts

3.1.1.1. Chronic toxicity potential

After 92 h of exposure, growth of *Scenedesmus subspicatus* alga was inhibited by *Caulerpa* and cyanobacterial preliminary extracts. Six concentrations of *C. racemosa* and 10 concentrations of *C. taxifolia* were tested, with dilutions that ranged from 1:100 to 1:10000 and 1:100 to 20000, respectively. In the case of *C. taxifolia* a reliable dose-response curve was obtained with LC50 value of 23 μg/mL (dilution 1:1290) while *C. racemosa* extract showed weaker inhibition with 39 μg/mL LC50 value (dilution 1:218) (Fig. 3.3.). Top 3 minimum dilutions of *C. taxifolia* extract resulted in maximal, 100% inhibition of the algal growth while only the highest concentration of *C. racemosa* extract showed maximum inhibition rate (Fig. 3.1.).



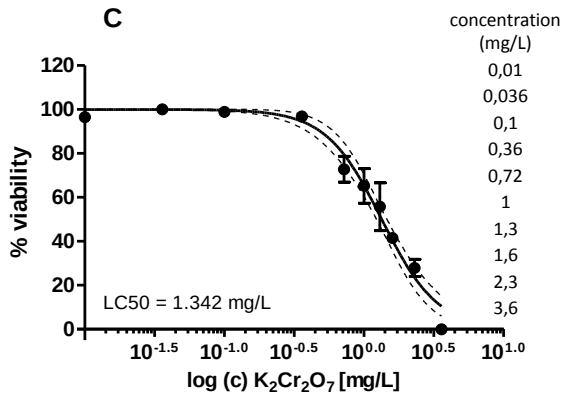
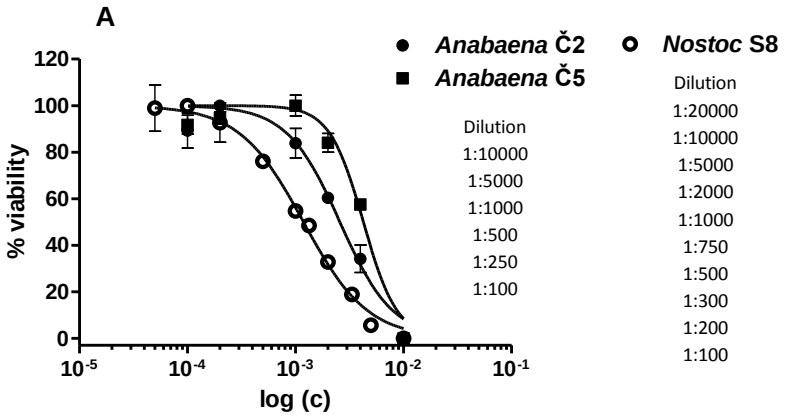


Figure 3.1. Inhibition of *Scenedesmus subspicatus* growth by either *C. racemosa* (**A**) or *C. taxifolia* (**B**) preliminary extracts, or positive control ($K_2Cr_2O_7$) (**C**). Data are expressed as percentages of viability with mean ± SD and 95% CI calculated from duplicates. Dilution ratios are shown on the right side of each graph.

Between 6 and 10 concentrations of cyanobacterial strain extracts were tested with dilutions that ranged from 1:100 to 1:20000. Both terrestrial and aquatic strains inhibited the growth of freshwater alga but *Nostoc* strains showed the most potent toxic potential (Fig. 3.2.). The lowest LC50 value, i.e. the highest toxicity, was obtained for *Nostoc* Z1 (163 µg/mL) while *Oscillatoria* K3 had the highest LC50 value (738 µg/mL) (Fig. 3.2.). The highest concentrations of all of the strain extracts demonstrated the maximal growth inhibition.



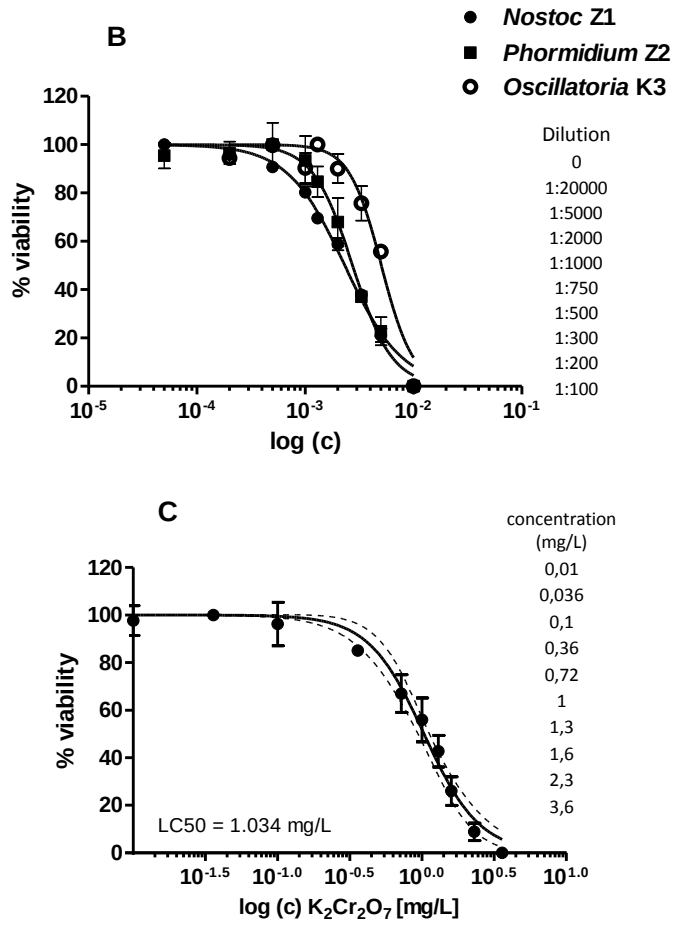


Figure 3.2. Inhibition of algal growth by cyanobacterial preliminary extracts. Terrestrial strains (*Anabaena* Č2 and Č5, *Nostoc* S8) are shown on the upper panel (**A**), aquatic strains (*Nostoc* Z1, *Phormidium* Z2, *Oscillatoria* K3) on the middle panel (**B**) and positive control ($K_2Cr_2O_7$) on the graph **C**. Data are expressed as percentages of viability with mean ± SD calculated from duplicates. Dilution ratios are shown on the right side of each graph.

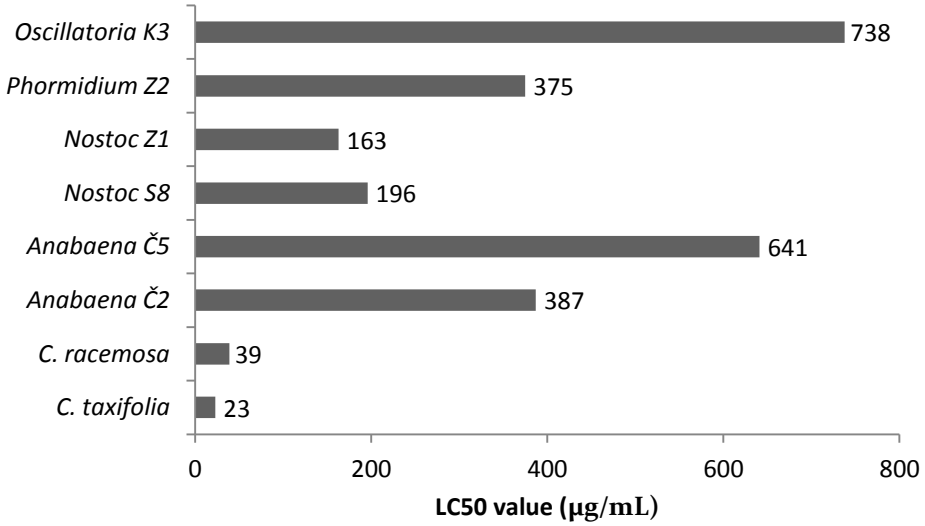


Figure 3.3. Summary presentation of the chronic inhibitory potential of *Caulerpa* and cyano - bacterial preliminary extracts, respectively, expressed as LC50 values (µg/mL).

3.1.1.2. Acute toxicity potential

C. taxifolia extract had an effect on survival of PLHC-1/wt cells showing significant reduction of mitochondrial dehydrogenase activity and production of formazan in the highest tested concentration of the extract (Fig. 3.4.). On the contrary, *C. racemosa* did not show any toxicity to PLHC-1/wt cell line (Fig. 3.4.). Ten concentrations ranging from 1:100 to 1:100000 were tested for *Caulerpa* toxicity. Obtained LC50 value for *C. taxifolia* was 47 µg/mL (or dilution 1:637) and the highest two dilutions caused 90% mortality. No decrease of PLHC-1/wt cells survival was observed also for all cyanobacterial strains (Fig. 3.5.). *Anabaena* Č2 and Č5 showed 70% viability in top 4 concentrations. From 6 to 10 concentrations were tested for all cyanobacterial extracts, in the dilution range from 1:100 to 1:100000.

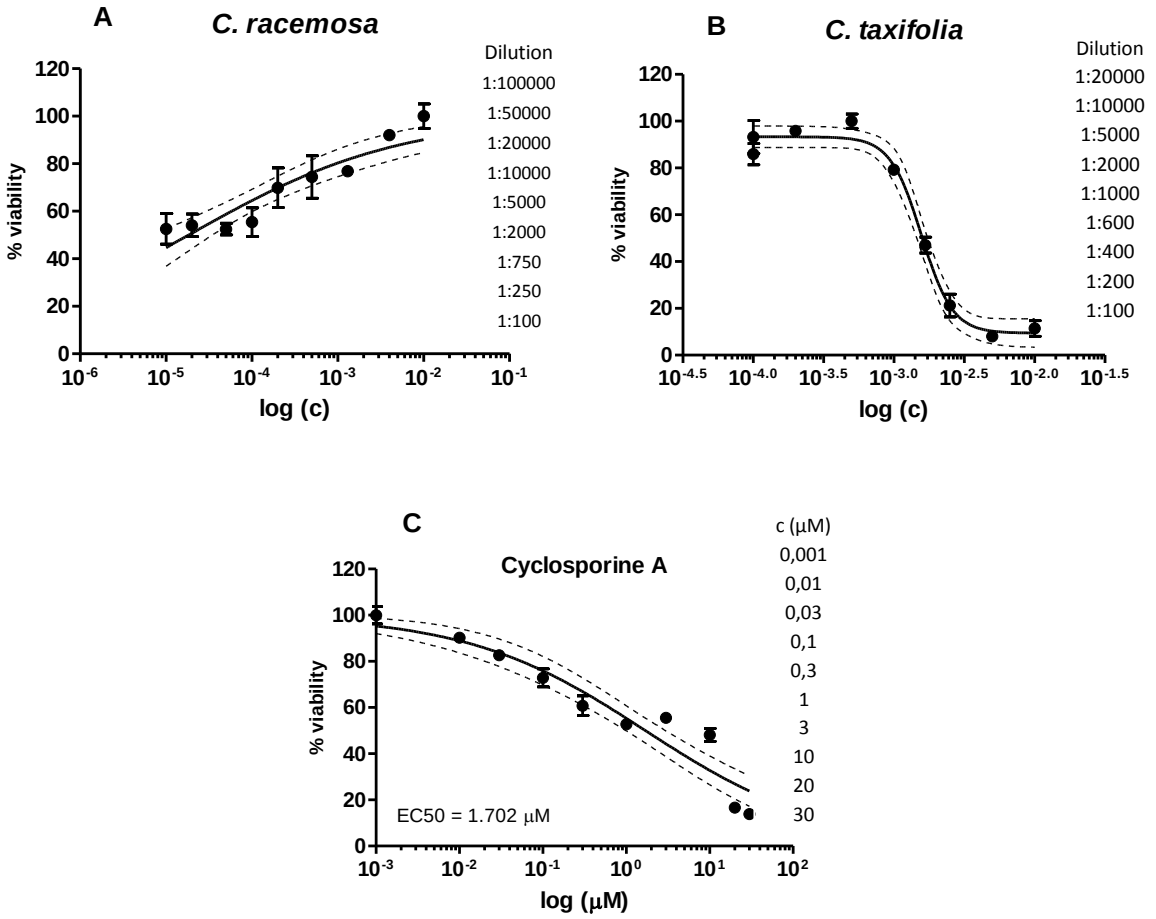


Figure 3.4. Survival of PLHC-1/wt cells by *C. racemosa* (**A**) and *C. taxifolia* (**B**) preliminary extracts. Positive control (CYC) is shown on the graph **C**. Data are expressed as percentages of viability with mean ± SD and 95% CI calculated from duplicates. Dilution ratios are shown on the right side of each panel.

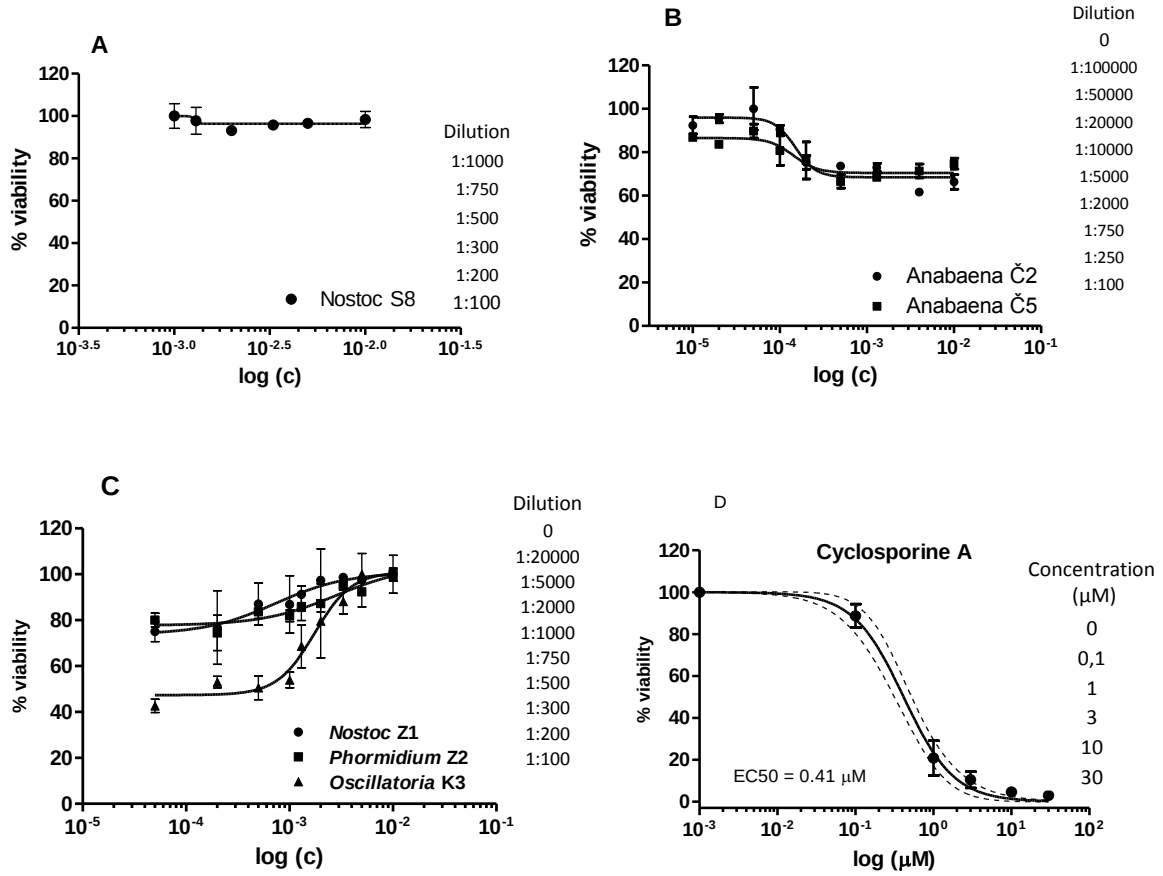


Figure 3.5. Survival of PLHC-1/wt cells after exposure to cyanobacterial preliminary extracts. Terrestrial strains (*Anabaena* Č2 and Č5, *Nostoc* S8) are shown on the graph **A** and **B**, aquatic strains (*Nostoc* Z1, *Phormidium* Z2, *Oscillatoria* K3) on the graph **C** and positive control ($K_2Cr_2O_7$) on the graph **D**. Data are expressed as percentages of viability with mean ± SD calculated from duplicates. Dilution ratios are shown on the right side of each panel.

3.1.2. Effect of *Caulerpa* and cyanobacteria on cellular detoxification phases

3.1.2.1. Inhibition of DrOatp1d1 and DrOct1 uptake transporters

The uptake activity of ecotoxicologically relevant organic anion and cation transporters, present in zebrafish, respectively, has been inhibited by *C. racemosa* and *C. taxifolia* extracts. Clear dose-response curves of DrOatp1d1 activity were obtained for both *Caulerpa* species showing a decrease in uptake of model substrates to only 6% of the uptake determined in non-treated cells at their highest concentrations (Fig. 3.6.). Nevertheless, there were clear differences in inhibition potency – *C. taxifolia* dilution range that showed response to DrOatp1d1 activity was between 1:1000 and 1:20000, while response of *C. racemosa* extracts was in a lower range from 1:100 to 1:1000 dilutions. Again, DrOct1 activity was significantly inhibited by *C. taxifolia* extracts showing a 100% uptake inhibition at its highest

concentration, while *C. racemosa* extract had weaker maximal inhibition (70%) of DrOct1 transporter (Fig. 3.6.). Nevertheless, stronger inhibition of DrOatp1d1 compared to DrOct1 transporter was obtained for both *Caulerpa* species. From 6 to 7 concentrations were used for testing uptake activity by *Caulerpa* extracts.

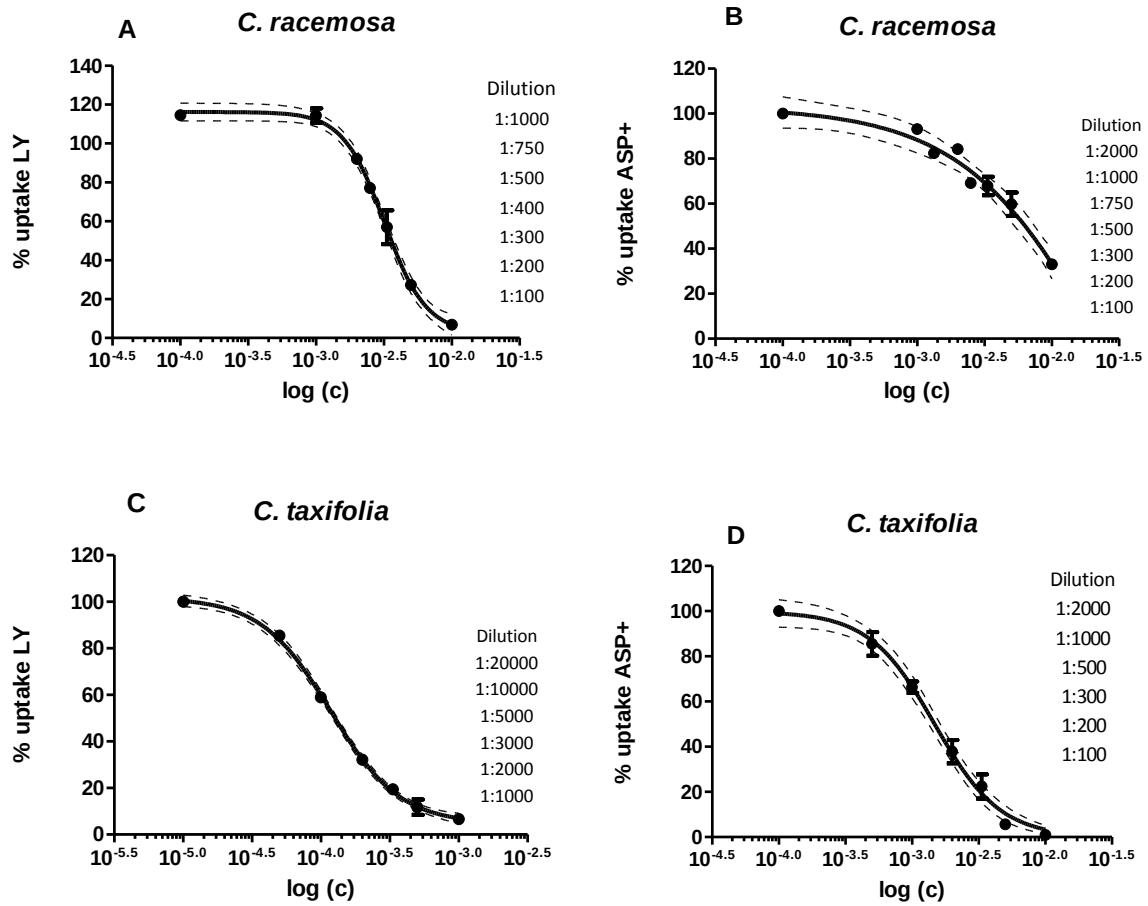


Figure 3.6. Concentration-dependent inhibition of DrOatp1d1 (**A**, **C**) and DrOct1 (**B**, **D**) transport activities by *C. racemosa* (**A**, **B**) and *C. taxifolia* (**C**, **D**) preliminary extracts. Results are shown as percentages of LY and ASP+ uptake in HEK293 cells. Mean ± SD values with 95% CI are shown as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

The uptake inhibition dose-response curves were obtained for terrestrial and aquatic cyanobacterial strains as well. *Oscillatoria* K3 extract had the strongest inhibition of DrOatp1d1 activity with only 3% of uptake determined at maximal sample concentration (Fig. 3.7.). Other strains had similar, 70-80% maximal reduction of DrOatp1d1 uptake activity (Fig. 3.7.). Five to seven concentrations of cyanobacterial extracts that ranged from 1:100 to 1:50000 were used for testing the effect on uptake activity.

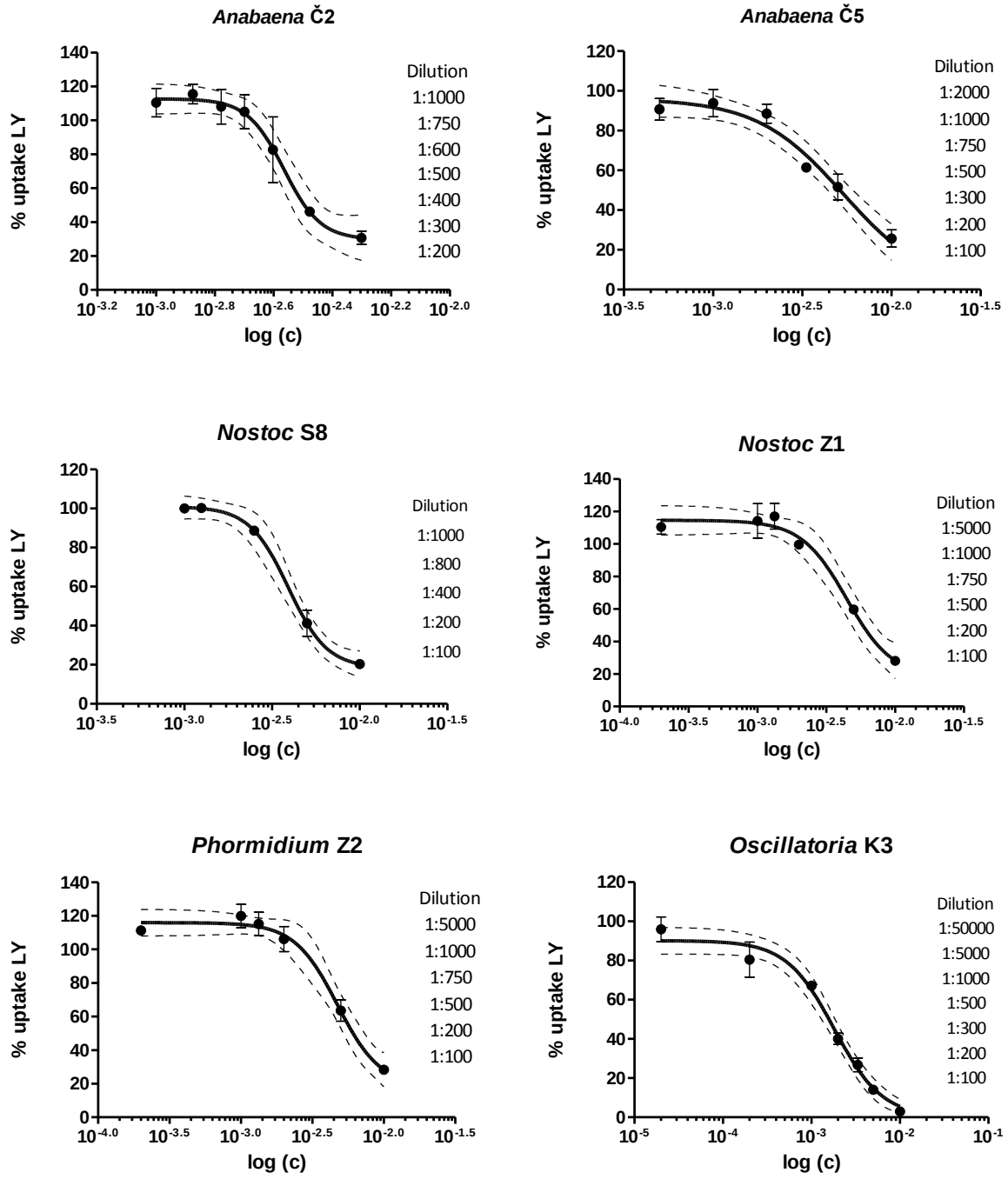


Figure 3.7. Concentration-dependent inhibition of DrOatp1d1 uptake by cyanobacterial preliminary extracts. Results are shown as percentages of LY substrate uptake in HEK293 cells. Mean ± SD values with 95% CI are shown as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

As in the case of *Caulerpa* species, cyanobacterial samples caused weaker response (inhibition) on DrOct1 then DrOatp1d1. Some strains showed no decrease in DrOct1 activity, such as *Anabaena* Č2, while the highest decline of uptake was recorded for *Nostoc* S8 with 70% inhibition at its maximum concentration (Fig. 3.8.). Most of cyanobacterial samples

reduced DrOct1 uptake activity to 40-50% of the uptake determined in non-treated cells (Fig. 3.8.). From 6 to 7 concentrations of cyanobacterial samples were used for uptake inhibition testing with dilution range from 1:100 up to 1:100000.

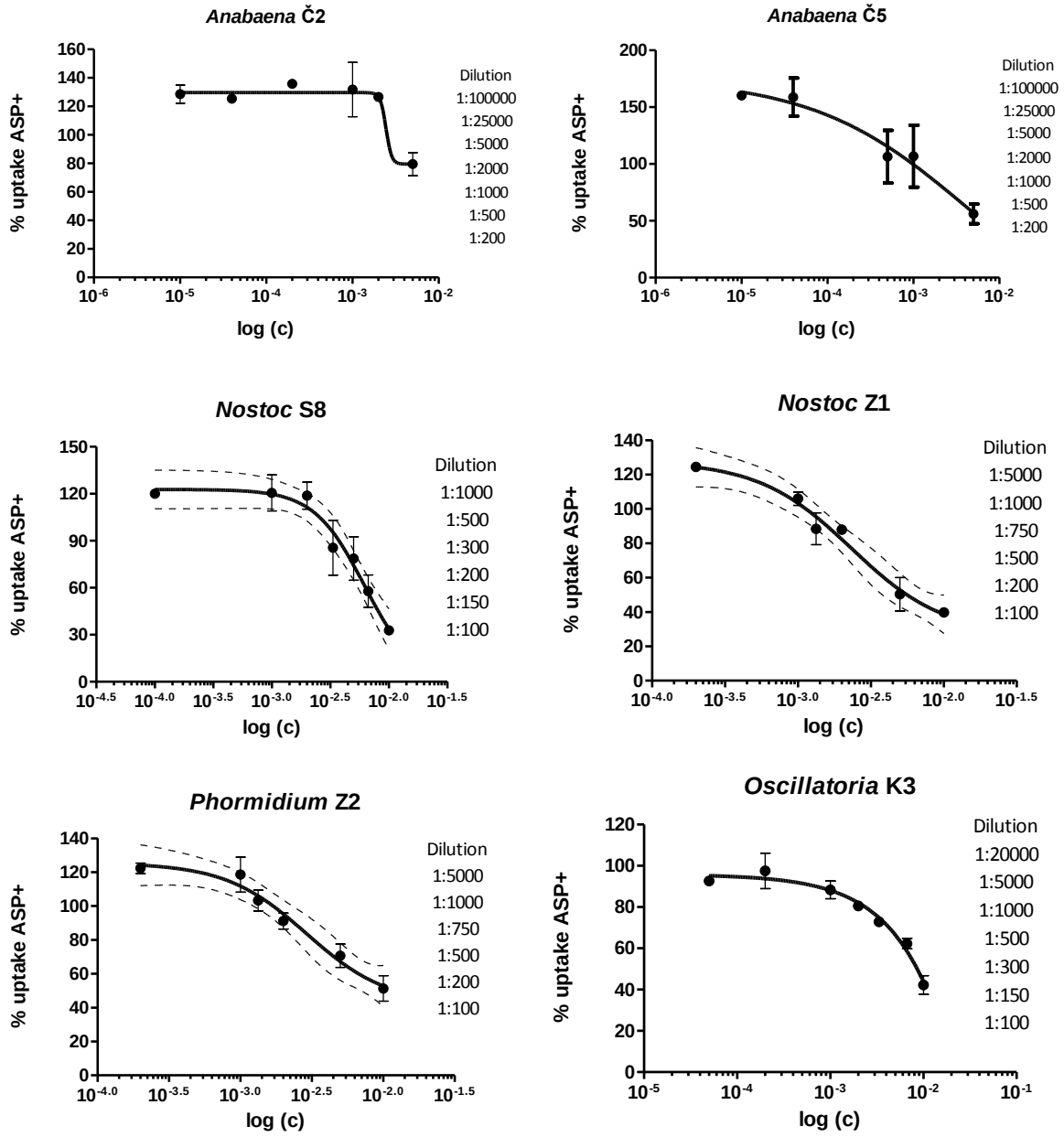


Figure 3.8. Concentration-dependent inhibition of DrOct1 uptake by cyanobacterial preliminary extracts. Results are shown as percentages of ASP+ substrate uptake in HEK293 cells. Mean ± SD values with 95% CI are shown as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

3.1.2.1.1. IC50 values

From the obtained results and calculated IC50 values we can observe that *Caulerpa* species have the strongest inhibitory effect on uptake transporters among all tested samples. The most potent inhibitory potential was determined for *C. taxifolia* extract where only 3.5 μg/mL and 44 μg/mL caused 50% of DrOatp1d1 and DrOct1 inhibition, respectively (Fig. 3.9.). *C. racemosa* extract was the second in potency of the inhibition, with 25.15 μg/mL (DrOatp1d1) and 265 μg/mL (DrOct1) IC50 values, respectively (Fig. 3.9.). *Oscillatoria* K3 had the lowest and *Anabaena* Č5 the highest IC50 value for DrOatp1d1 inhibition among all cyanobacterial strains (Fig. 3.9.). Except for *Nostoc* S8, IC50 values for DrOct1 inhibition of other cyanobacterial strains could not be calculated due to weak inhibition effects. Inhibition of DrOatp1d1 transport activity compared to DrOct1 is more pronounced among all tested samples.

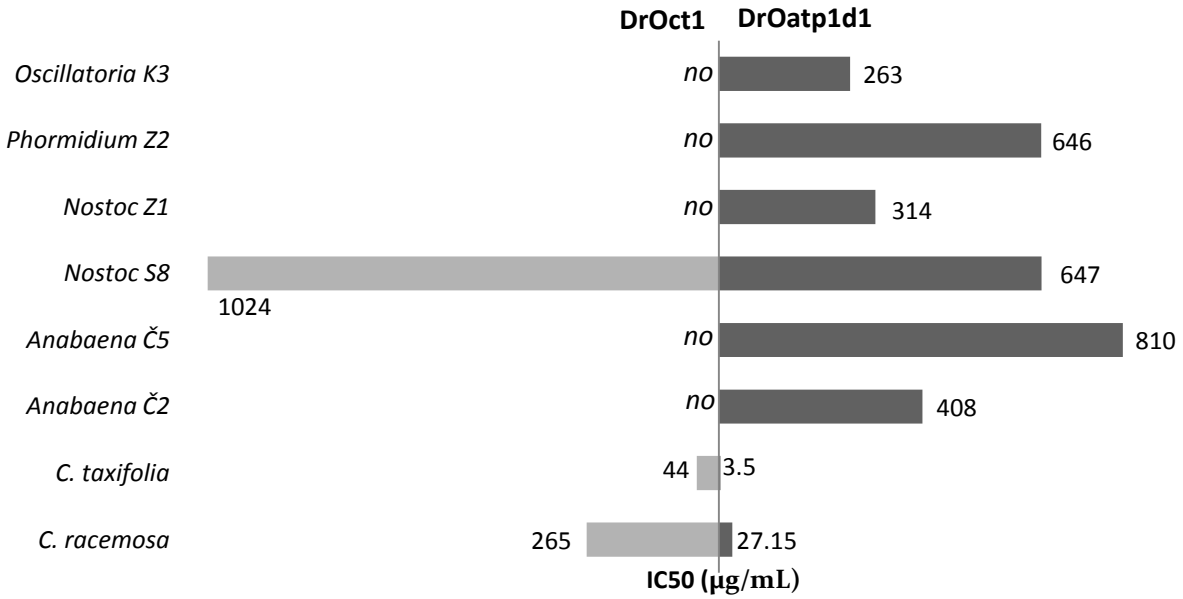


Figure 3.9. Inhibitory potential of *Caulerpa* and cyanobacterial preliminary extracts. Results are shown as IC50 values expressed in μg/mL for DrOatp1d1 transporter (right) and DrOct1 (left) transporter. *no* - not obtained.

3.1.2.2. Induction of CYP1A1 enzyme activity

Caulerpa extracts activated Ah receptor and induced transcription of CYP1A1 enzyme in PLHC-1/wt cells. A range of 6 to 8 concentrations was evaluated for EROD induction with dilution range from 1:100 to 1:40000. The fluorescence of CYP1A1 product resorufin was increasing during 10 min kinetic reaction and dose-response curves were obtained, especially

in *C. taxifolia* exposed cells. Determined CYP1A1 activity at the highest sample concentrations was 1.6 pmol/min/mg and 2.4 pmol/min/mg for *C. racemosa* and *C. taxifolia*, respectively (Fig. 3.10.). *C. taxifolia* induction was in much higher dilution range, from 1:3000 to 1:40000 compared to *C. racemosa* sample whose response was indicated from 1:100 to 1:10000 dilution (Fig. 3.10.).

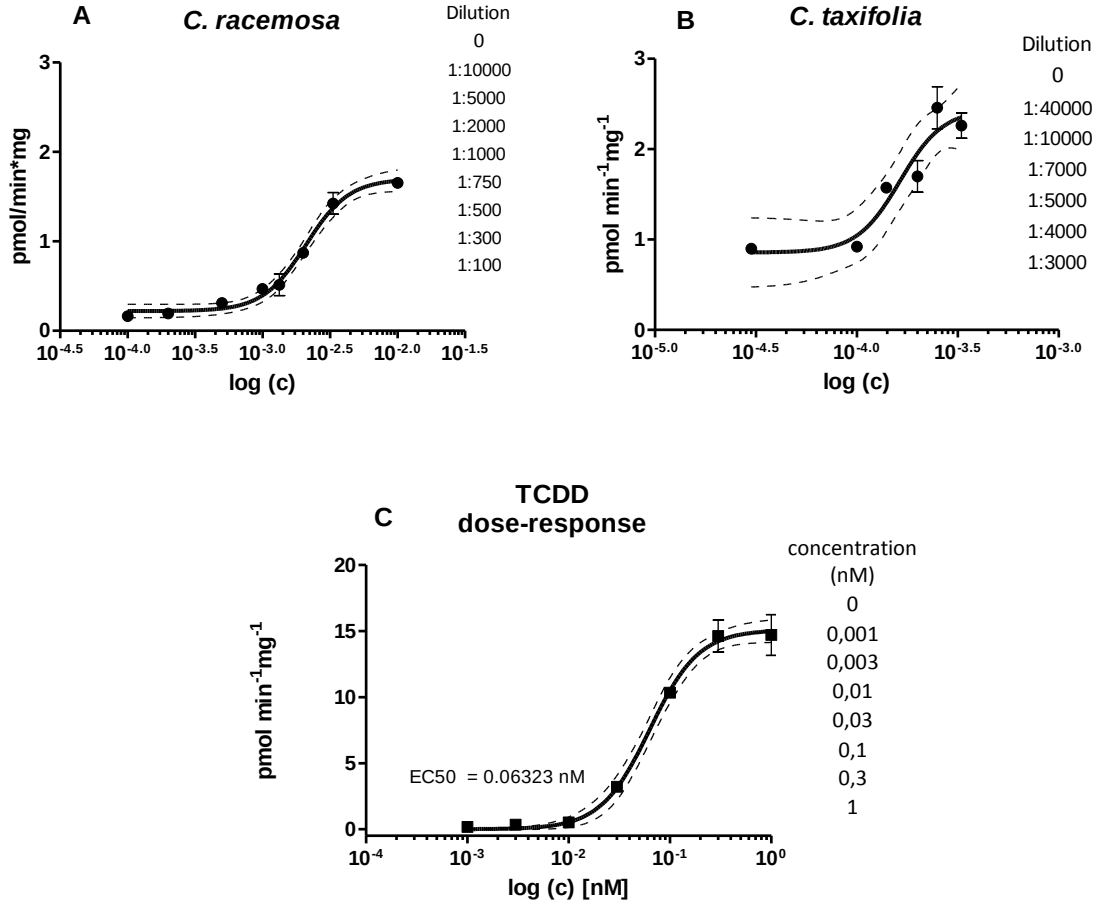
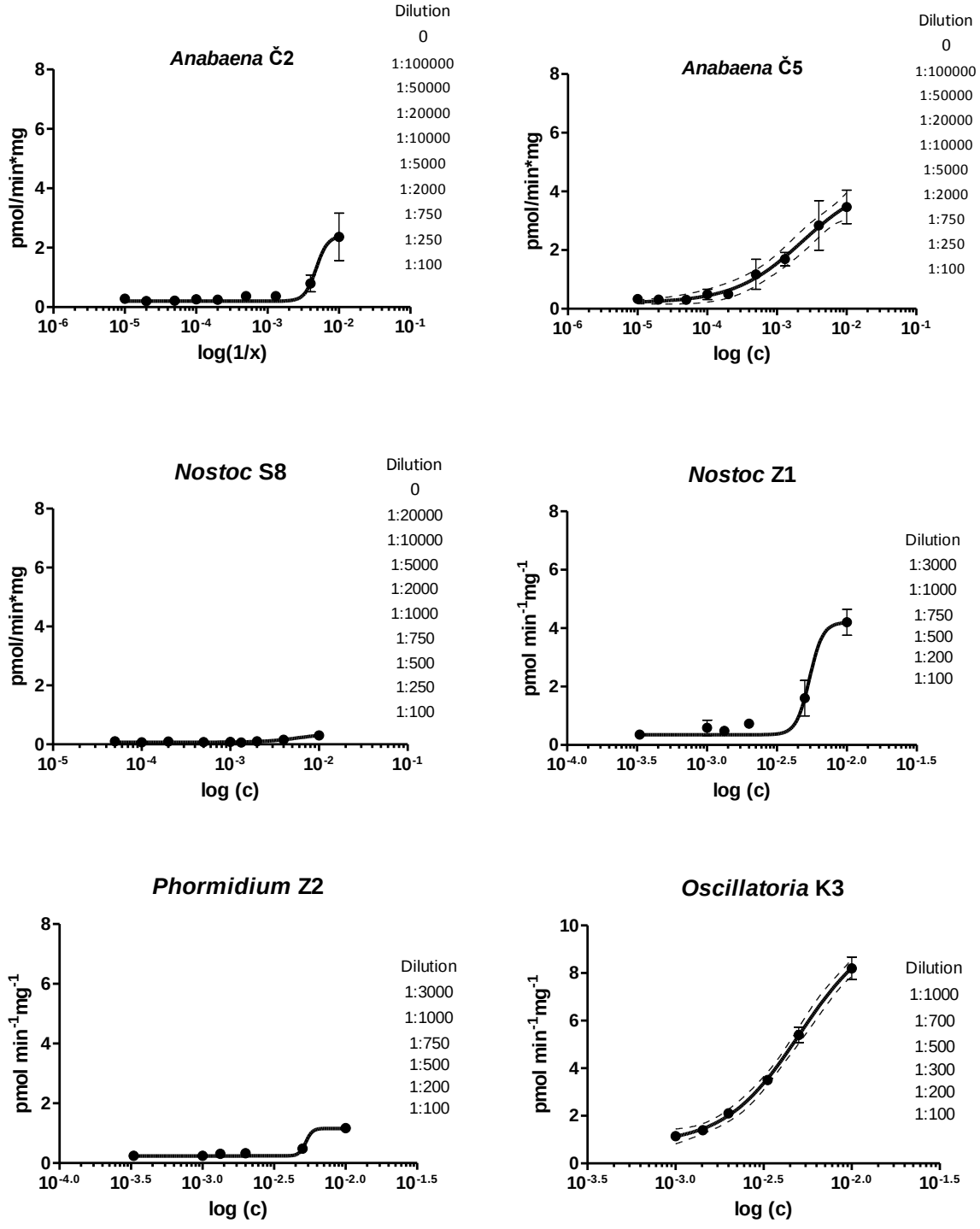


Figure 3.10. Concentration-dependent induction of CYP1A1 enzyme activity in PLHC-1/wt cells exposed to *C. racemosa* (**A**) and *C. taxifolia* (**B**) preliminary extracts. Positive control (TCDD) dose-response is shown on the graph **C**. Results are expressed as pmol/min/mg with mean ± SD values and 95% CI calculated from duplicates. Dilution ratios are shown on the right side of each graph.

Most cyanobacterial strains significantly induced CYP1A1 enzyme activity in PLHC-1/wt cells, and most of them resulted in stronger induction than *Caulerpa* species. A range of 6 to 9 concentrations was used for EROD measurement, with dilution range from 1:100 up to 1:100000. The highest dose-response value was obtained for aquatic *Oscillatoria* K3 strain whose CYP1A1 activity at the 1:100 dilution was 8.2 pmol/min/mg (Fig. 3.11.). *Anabaena* Č5 also indicated high CYP1A1 activity with 3.5 pmol/min/mg induction value within a broader

dilution range from 1:100 to 1:5000. *Nostoc* S8 showed no induction at all, while *Phormidium* Z2 and *Anabaena* Č2 induction potency at the top sample concentration was 1.1 pmol/min/mg and 2.3 pmol/min/mg, respectively. CYP1A1 activity was induced only at the two maximal concentrations of the *Nostoc* Z1 extract, resulting in 4.2 pmol/min/mg (Fig. 3.11.).



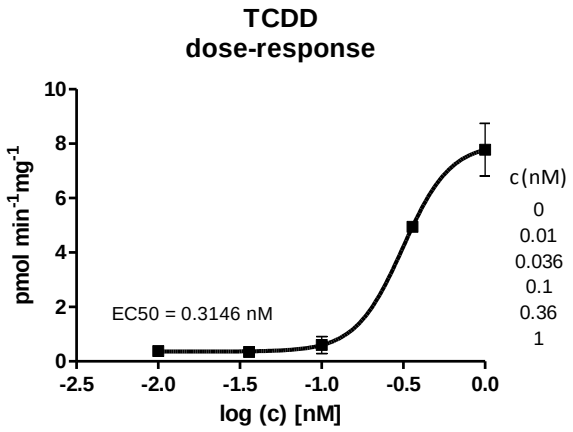


Figure 3.11. Concentration-dependent induction of CYP1A1 enzyme activity in PLHC-1/wt cells exposed to cyanobacterial preliminary extracts. Positive control (TCDD) dose-response is shown at the bottom graph. Results are expressed as pmol/min/mg with mean ± SD values calculated from duplicates. Dilution ratios are shown on the right side of each panel.

3.1.2.2.1. Comparison of CYP1A1 induction potencies by TCDD equivalents

From the four-parameter logistic equation of TCDD sigmoidal dose-response curve, EC5 and EC10 values were calculated and expressed as EROD activity in pmol/min/mg protein. Four-parameter values of *Caulerpa* and cyanobacterial species were then adapted to EC5 and EC10 values of TCDD positive control to obtain the concentration of samples that is equivalent to the corresponding TCDD EROD activity. Finally, TCDD Eq of tested samples were expressed in pg/mL of TCDD. The highest TCDD Eq were obtained for *Caulerpa* species, especially for *C. taxifolia* whose induction potential responded to 900 pg TCDD eq/mg (Fig. 3.12.). *Oscillatoria* K3 had the highest TCDD equivalent value among cyanobacterial extracts with 225 pg TCDD eq/mg (Fig. 3.12.). Other cyanobacterial samples had TCDD Eq values below 150, while *Anabaena* Č2 induced EROD activity was equivalent to 2.6 pg TCDD eq/mg (Fig. 3.12.).

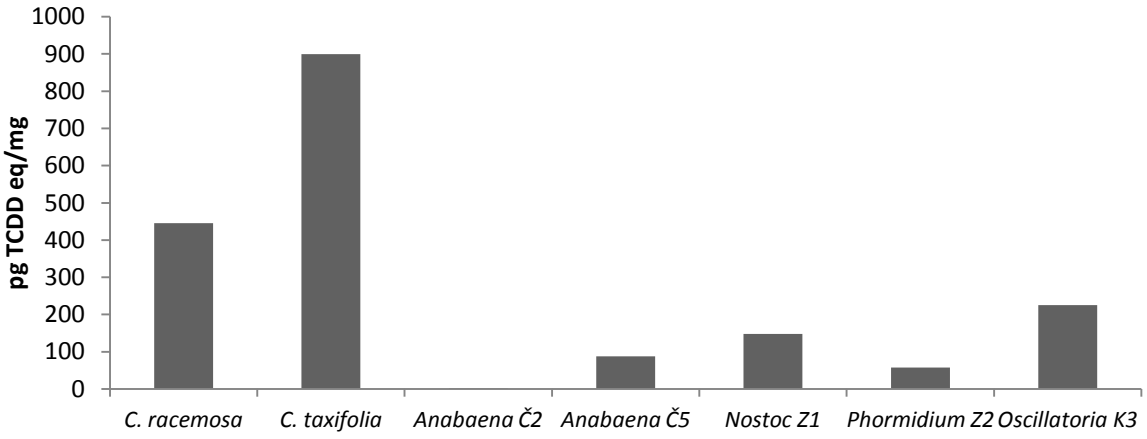


Figure 3.12. Calculated TCDD Eq of *Caulerpa* and cyanobacterial samples as expressed in pg TCDD eq/mg.

3.1.2.2.2. Comparison of CYP1A1 induction potencies by EC50 values

Although the obtained enzyme activities, expressed as pmol/min/mg values, were higher for certain cyanobacterial strains, the obtained EC50 values for *Caulerpa* species were lower than those of cyanobacterial extracts. Dilution range of the observed response was higher for *Caulerpa* extracts so the CYP1A1 induction potential was the most potent for *C. taxifolia*, followed by *C. racemosa* extracts, with 4.9 μg/mL and 17.78 μg/mL EC50 values, respectively (Fig. 3.13.). Although *Oscillatoria* K3 maximum concentrations induced CYP1A1 activity above 8 pmol/min/mg, the calculated EC50 value was actually the highest, indicating the lowest induction potency out of all samples. Cyanobacteria *Anabaena* Č5 showed the most potent CYP1A1 induction potential out of all strains (Fig. 3.13.).

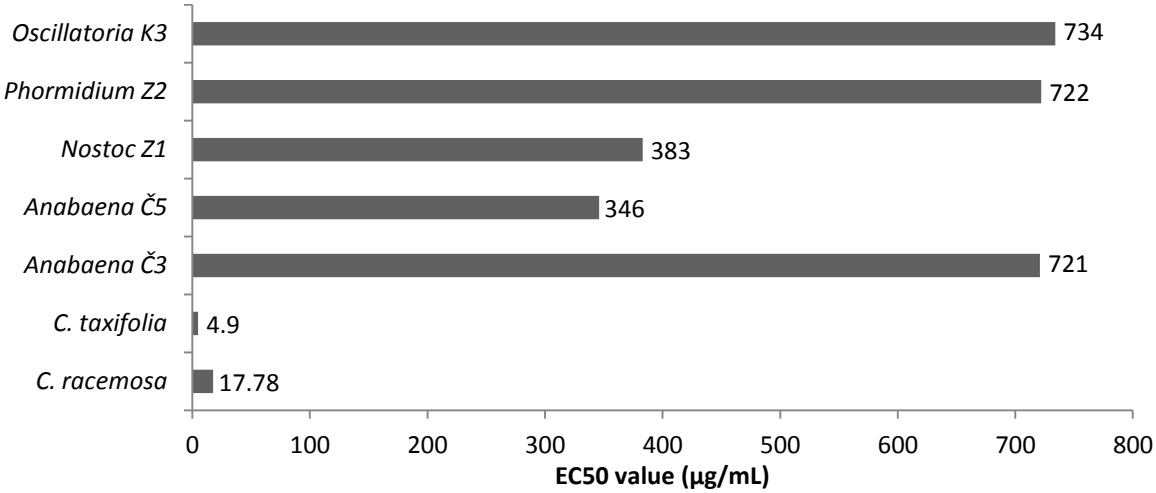


Figure 3.13. CYP1A1 induction potential of *Caulerpa* and cyanobacterial preliminary extracts expressed as EC50 value in μg/mL.

3.1.2.3. Cytosolic GST activity changes

Caulerpa and cyanobacterial extracts had no influence to the conjugation rate of 6 cytosolic GST enzymes when CDNB was used as a model substrate. Final concentration of samples in the reaction mixture was 1:100 and 1:1000 dilution for *C. racemosa* and *C. taxifolia* extract, respectively. A 20-30% decrease of Gsta3 activity was determined during the first 3-4 min of the kinetic measurement in the presence of *C. racemosa* extract after which reaction maintained on a constant rate. Figure 3.14. shows no significant induction or inhibition of cytosolic enzyme activity for both *Caulerpa* species.

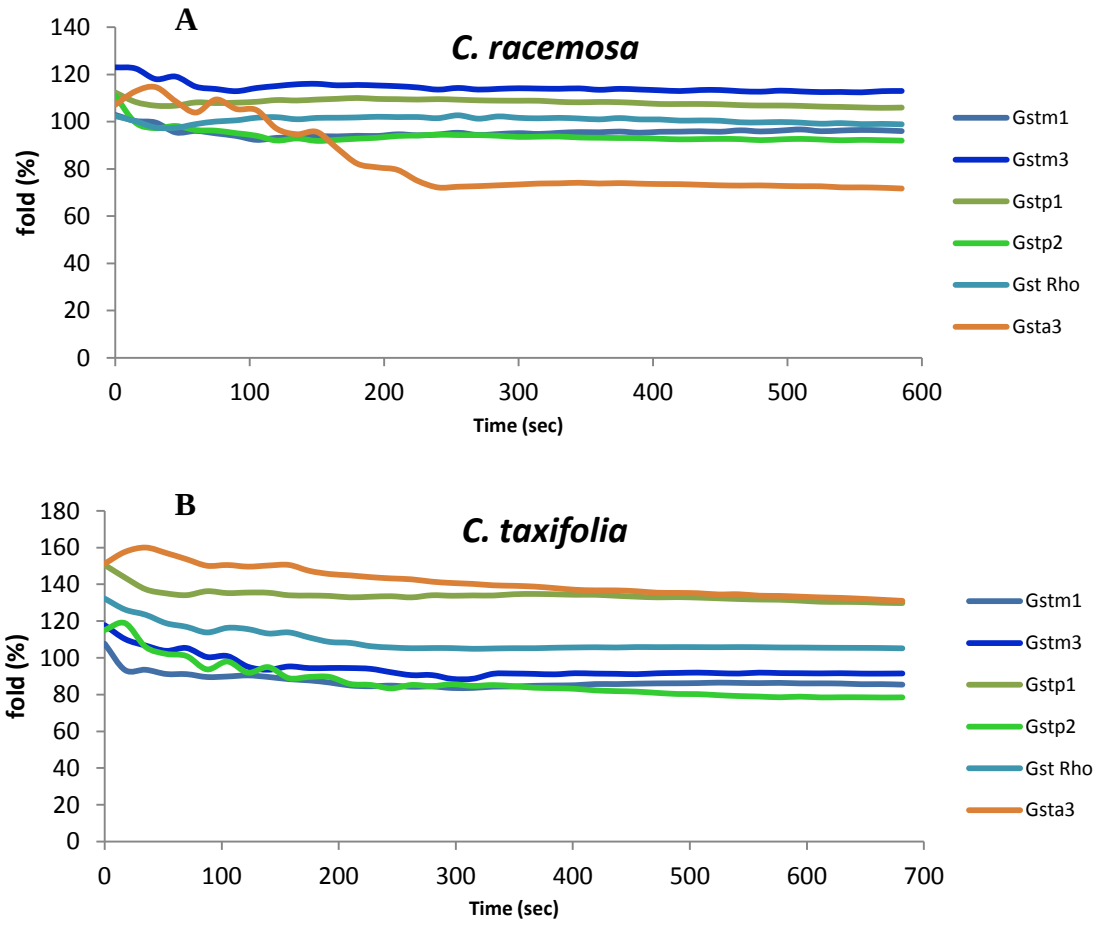
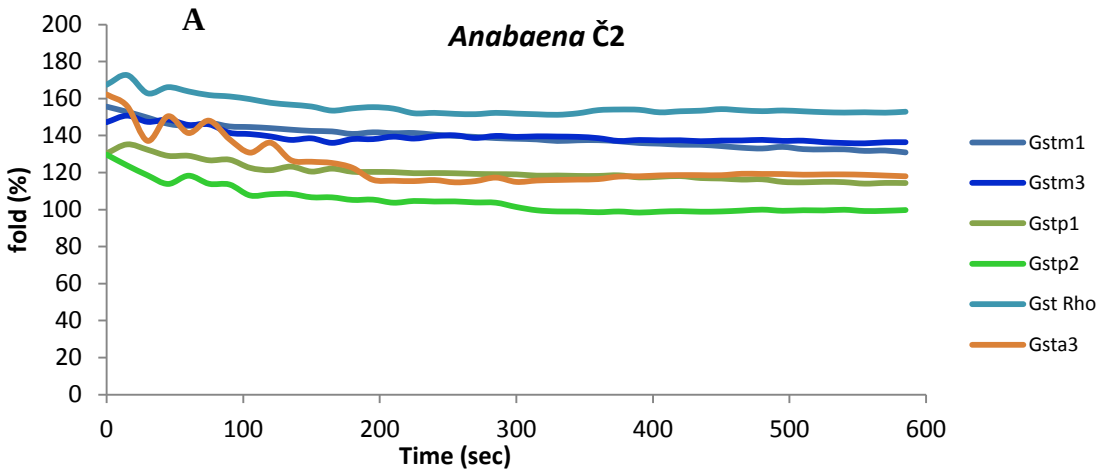
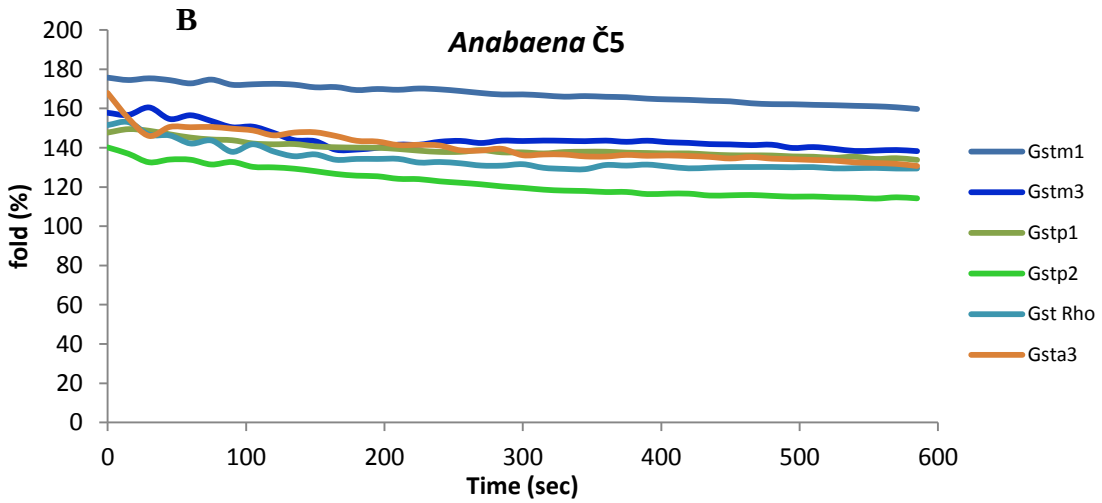
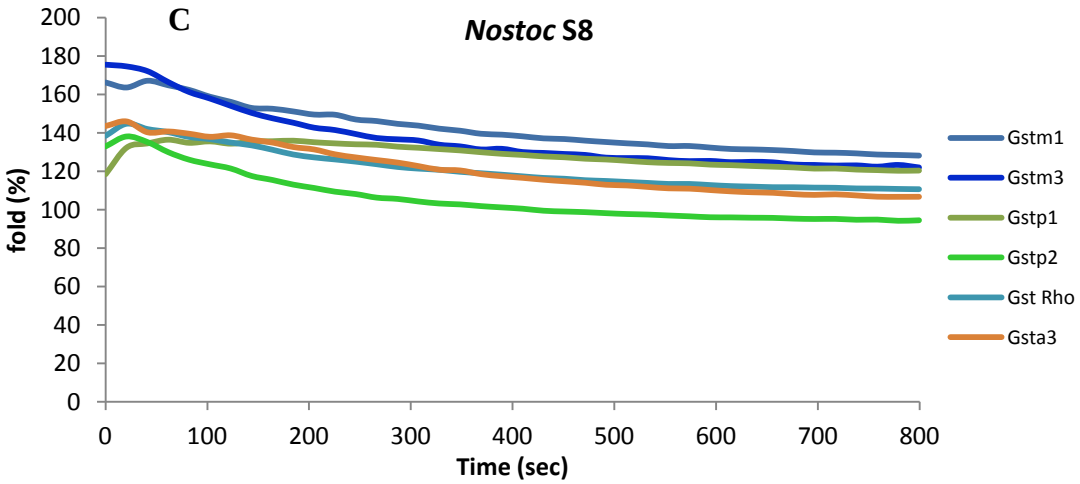


Figure 3.14. Enzyme kinetics of 6 cytosolic GSTs (m1, m3, p1, p2, Rho and a3) in conjugation reaction with CDNB model substrate as determined in the presence of *C. racemosa* (**A**) and *C. taxifolia* (**B**) preliminary extract. Results are expressed as fold (%) compared to control.

Likewise, no change in enzyme activity of 6 cytosolic GSTs at maximal sample concentration (1:100 dilution or 1%) was observed for cyanobacterial extracts (Fig. 3.15.).


A
Anabaena Č2
fold (%)
Time (sec)
Gstm1
Gstm3
Gstp1
Gstp2
Gst Rho
Gsta3


B
Anabaena Č5
fold (%)
Time (sec)
Gstm1
Gstm3
Gstp1
Gstp2
Gst Rho
Gsta3


C
Nostoc S8
fold (%)
Time (sec)
Gstm1
Gstm3
Gstp1
Gstp2
Gst Rho
Gsta3

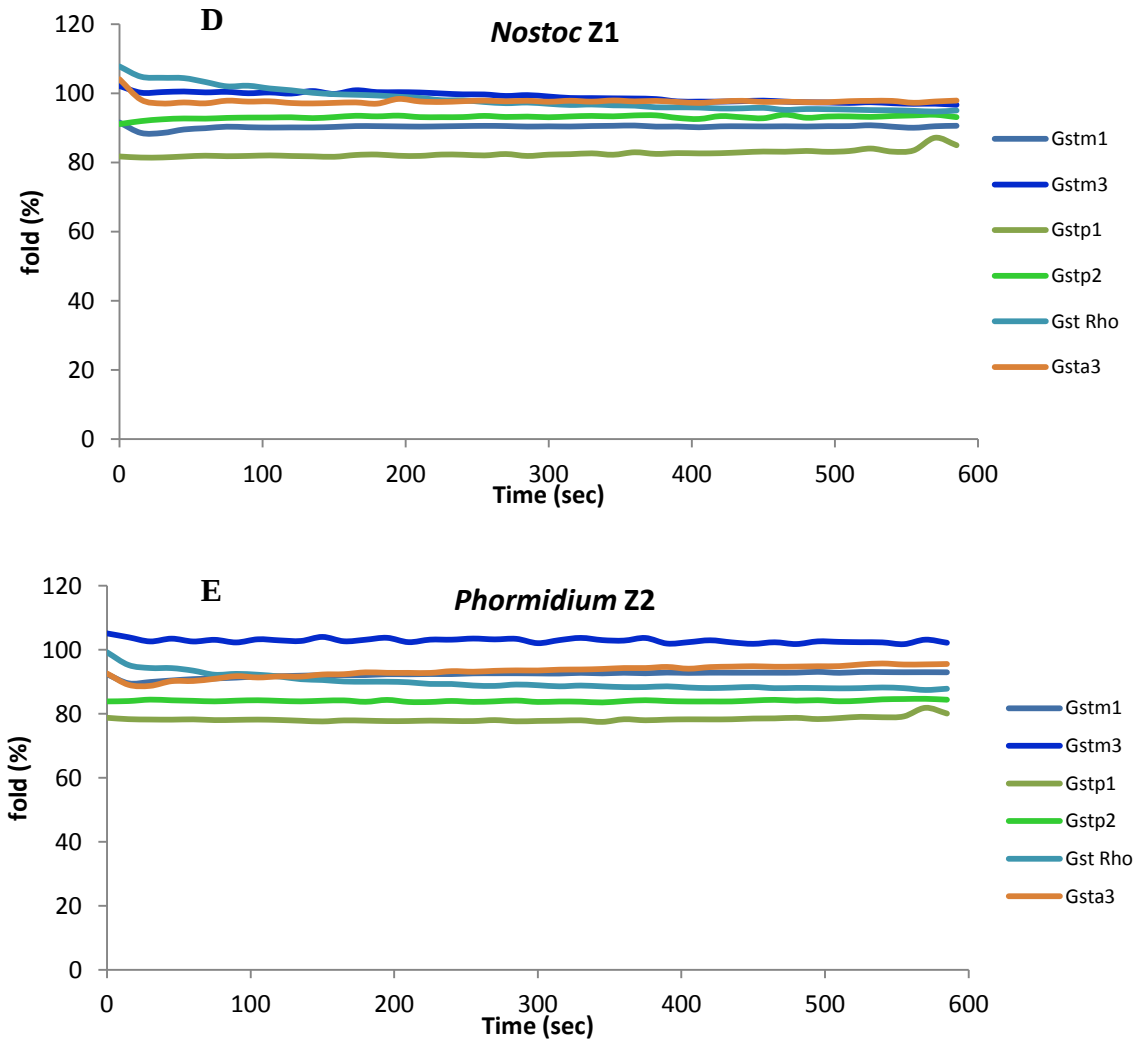


Figure 3.15. Enzyme kinetics of 6 cytosolic GSTs (m1, m3, p1, p2, Rho and a3) in conjugation reaction with CDNB model substrate as determined in the presence of cyanobacterial preliminary extracts of *Anabaena* Č2 (**A**), *Anabaena* Č5 (**B**), *Nostoc* S8 (**C**), *Nostoc* Z1 (**D**), and *Phormidium* Z2 (**E**). Results are expressed as fold (%) compared to control.

3.1.2.4. Inhibition of P-gp efflux transporter

No significant accumulation of fluorescent calcein was recorded in NIH3T3-R/dox cells after exposure to *Caulerpa* and cyanobacterial extracts. A range of 6 to 7 concentrations of extracts were applied to the cells, within 1:100 to 1:20000 dilution range. On the Fig. 3.16. fold-changes in accumulation of model substrate are expressed for minimum dilutions (i.e. the highest sample concentrations) only: 1:250 (*Anabaena* Č2 and Č5, *C. racemosa*) and 1:200 (*Nostoc* S8 and Z1, *Phormidium* Z2, *Oscillatoria* K3, *C. taxifolia*). As can be seen, at the highest concentrations the fold-changes are around or below 1 for most of the samples except *C. taxifolia* that showed 2.21-fold value. Therefore, apart from the *C. taxifolia* the obtained

data indicate no significant inhibition of P-gp efflux transporter in all tested concentrations and samples.

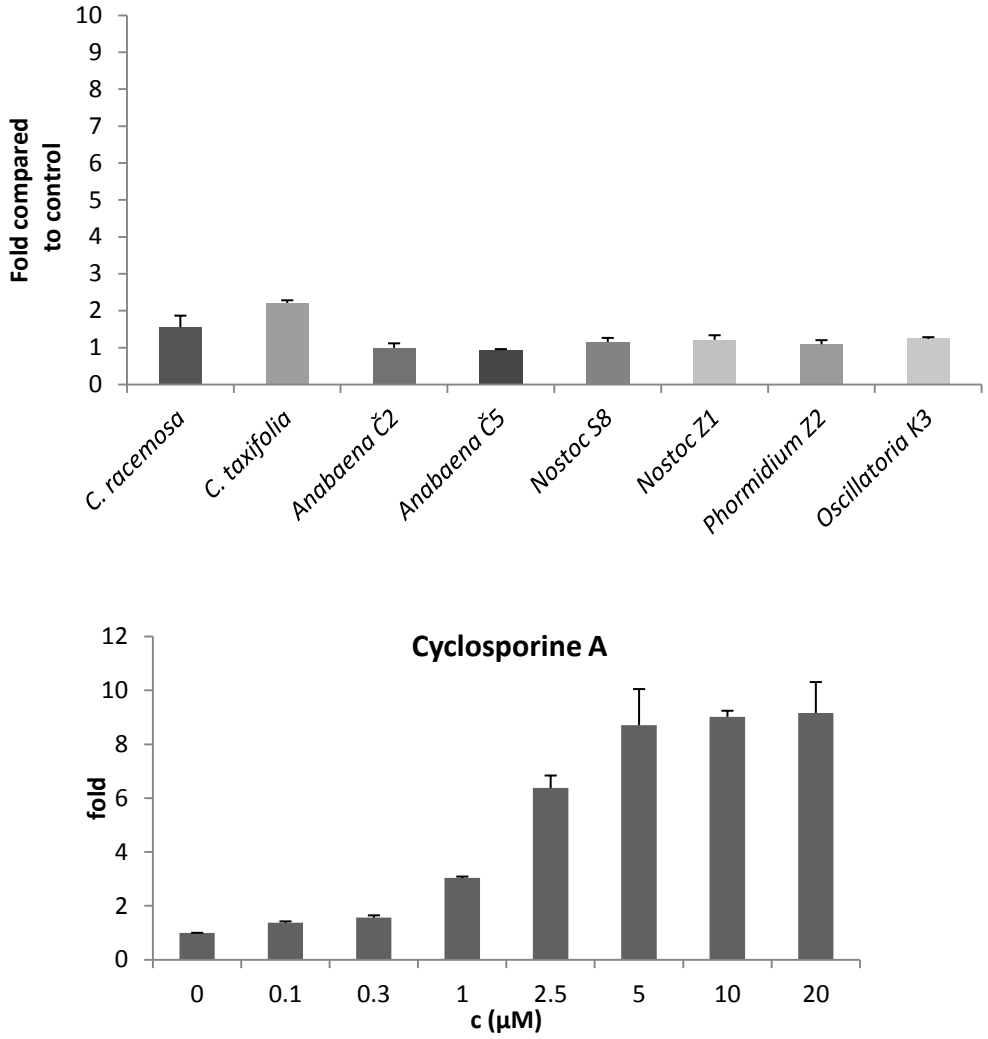


Figure 3.16. Inhibition of P-gp efflux transporter in NIH3T3-R/dox cells after exposure to *Caulerpa* and cyanobacterial extracts. Results are expressed as fold-changes in accumulation of model substrate Ca-AM obtained by comparing fluorescence of treated versus non-treated cells. Data are expressed as mean ± SD values calculated from duplicates. The effect of CYC (0.1-20 μM) used as the positive control is shown below.

3.2. TOXICOLOGICAL CHARACTERIZATION OF ABC FRACTIONS

Concentrations of *C. taxifolia*, *C. racemosa* and cyanobacterial strains in corresponding A, B, and C fractions are listed in Table 2.6. Maximal concentration of DMSO in extracts/fractions used for *in vitro* assays never exceeded 1%.

3.2.1. DrOatp1d1 and DrOct1 inhibitory potential

3.2.1.1. *C. racemosa* and *C. taxifolia* fractions

A range of 6 concentrations was used for testing DrOatp1d1 and DrOct1 inhibition. All of the three fractions of *C. taxifolia*, and the C fraction of *C. racemosa*, respectively, showed significant inhibition of DrOatp1d1 uptake activity (Fig. 3.17.).

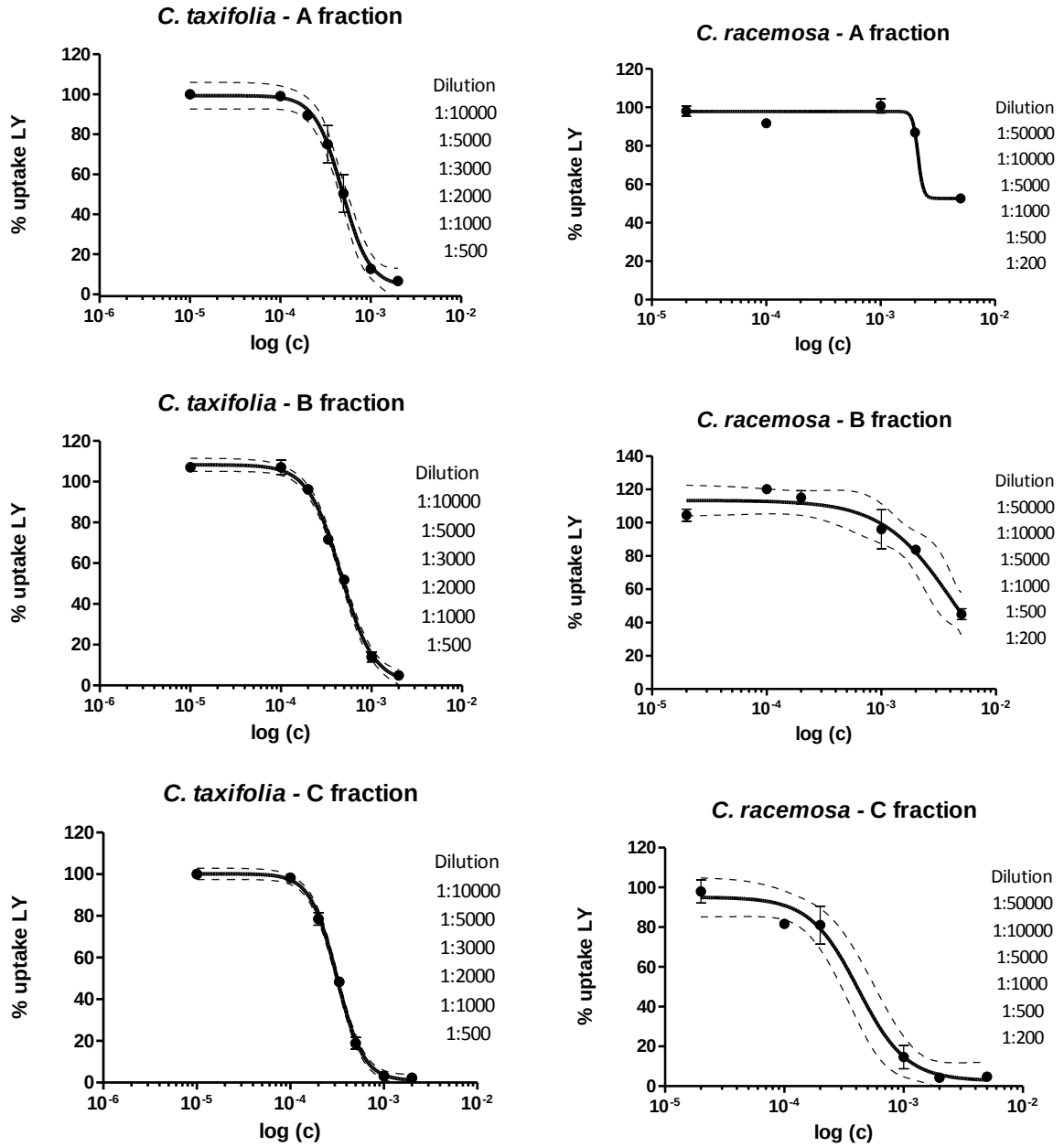


Figure 3.17. Inhibition of DrOatp1d1 transporter by *C. racemosa* (right) and of *C. taxifolia* (left) ABC fractions. Results are shown as percentages of LY substrate uptake in HEK293 cells. Mean ± SD values with 95% CI are shown, as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

ABC fractions obtained from *C. taxifolia* reduced the anion transporter activity to below 10% uptake, as was determined at the highest fraction concentrations. The lowest uptake value was recorded in *C. taxifolia* C fraction with only 2% uptake. The transporter inhibition response was observed between 1:500 and 1:10000 dilution range. A fraction of *C. racemosa* had no inhibitory effect on DrOatp1d1 while B fraction retained 45% of transporter activity. Only 4% uptake was observed in HEK293 cells exposed to *C. racemosa* C fraction within dilution range from 1:200 up to 1:50000.

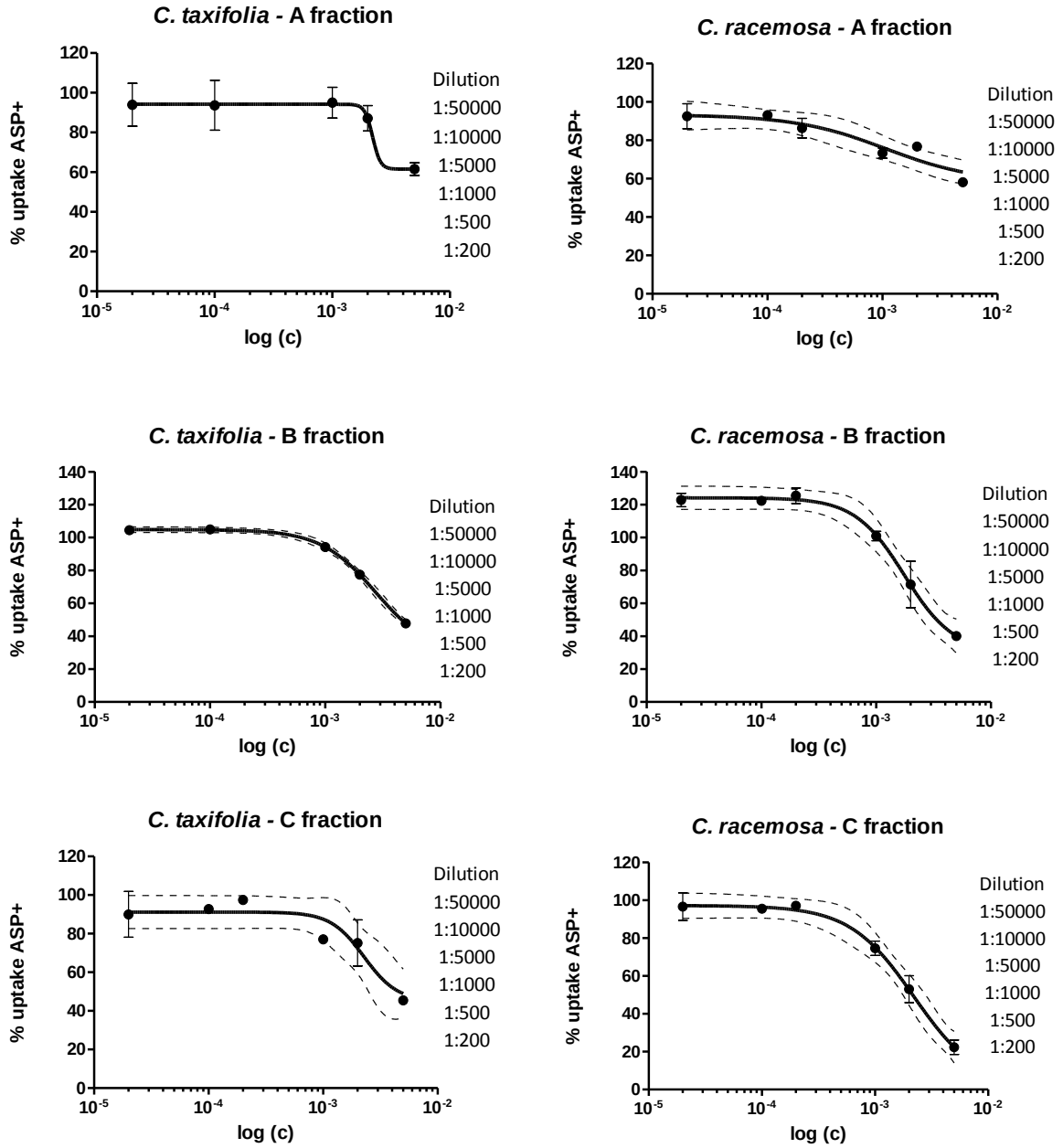


Figure 3.18. Inhibition of DrOct1 transporter by *C. racemosa* (right) and *C. taxifolia* (left) ABC fractions. Results are shown as percentages of ASP+ substrate uptake in HEK293 cells. Mean ± SD values with 95% CI are shown, as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

Inhibition of DrOct1 uptake activity was much weaker than for DrOatp1d1 for both *Caulerpa* species. Fraction A of both *Caulerpa* species did not show effect on DrOct1 transporter, while B and C fractions reduced uptake activity to around 50% at maximal concentrations (Fig. 3.18.). The strongest inhibition was observed for *C. racemosa* C fraction whose DrOct1 uptake capacity dropped to 20% (Fig. 3.18.). The observed response was within 1:200 to 1:5000 dilution range.

3.2.1.1.1. IC50 values

From figure 3.19. we can observe that *C. racemosa* C fraction actually showed the most potent inhibition potential, because there was an effect determined for both uptake transporters with low IC50 values: only 8.3 μg/mL and 44.05 μg/mL for DrOatp1d1 and DrOct1, respectively. Nevertheless, all three fractions of *C. taxifolia* showed similarly low IC50 values for DrOatp1d1 transporter. No effect was observed for *C. taxifolia* ABC fractions to the DrOct1 transporter, unlike the DrOatp1d1 transporter where the A fraction resulted in the lowest IC50 value out of all *Caulerpa* fractions (4.84 μg/mL; Fig. 3.19.).

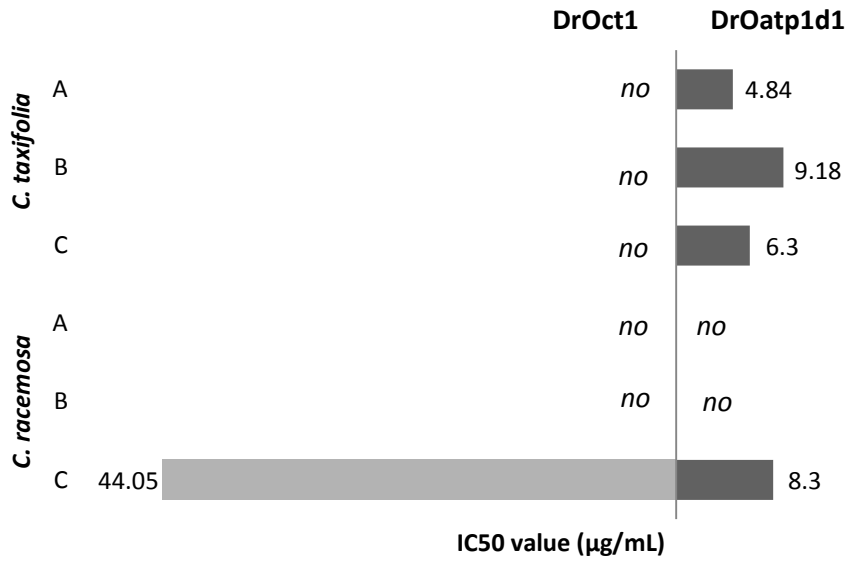
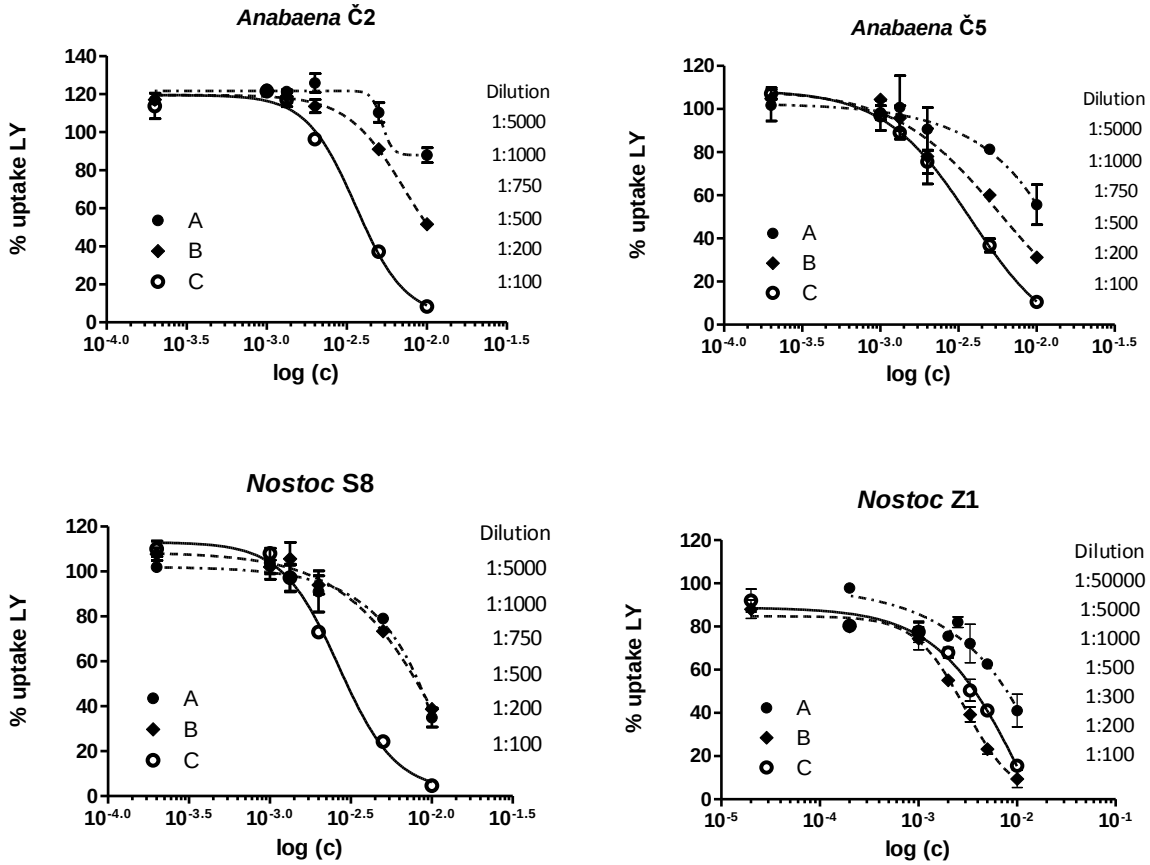


Figure 3.19. DrOatp1d1 and DrOct1 inhibitory potential determined and calculated for *C. racemosa* and *C. taxifolia* ABC fractions. Values are expressed as IC50 and expressed in μg/mL. *no* – not obtained.

3.2.1.2. Cyanobacterial fractions

A range of 6 to 7 concentrations was used for testing uptake activity of DrOatp1d1 and DrOct1. All cyanobacterial strains demonstrated the weakest inhibition of DrOatp1d1 transporter in the A fraction (Fig. 3.20.). A fraction of *Anabaena* Č2 showed no effect on the DrOatp1d1 transporter activity while DrOatp1d1 uptake decreased to 40-50% in HEK293 cells exposed to A fraction of all other strains. The strongest inhibition percentage was observed at the maximal concentration of C fraction for all cyanobacterial strains, which on average resulted in 10% (or below) uptake of model substrate. B fractions of *Nostoc* Z1, *Phormidium* Z2 and *Oscillatoria* K3 showed similar inhibition effect as C fractions. A 30-50% decrease in uptake was observed at maximal concentrations of B fractions of *Anabaena* Č2 and Č5 and *Nostoc* S8 (Fig. 3.20).



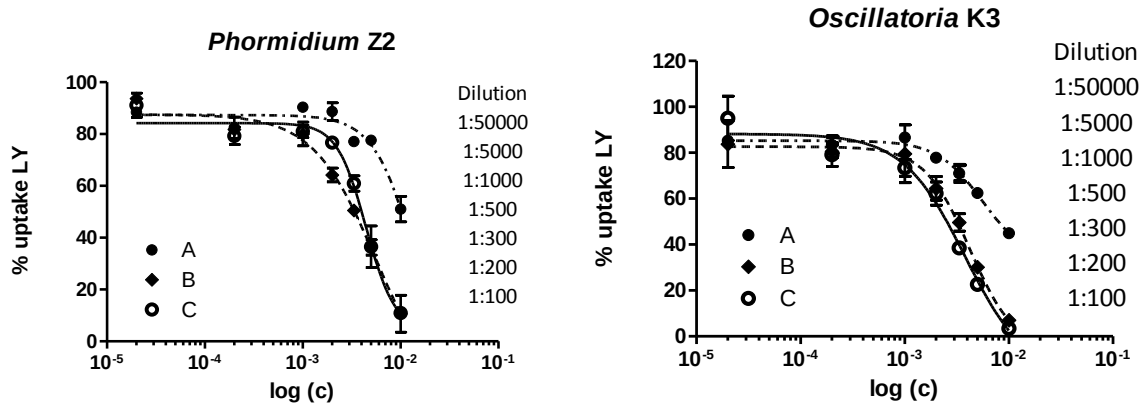
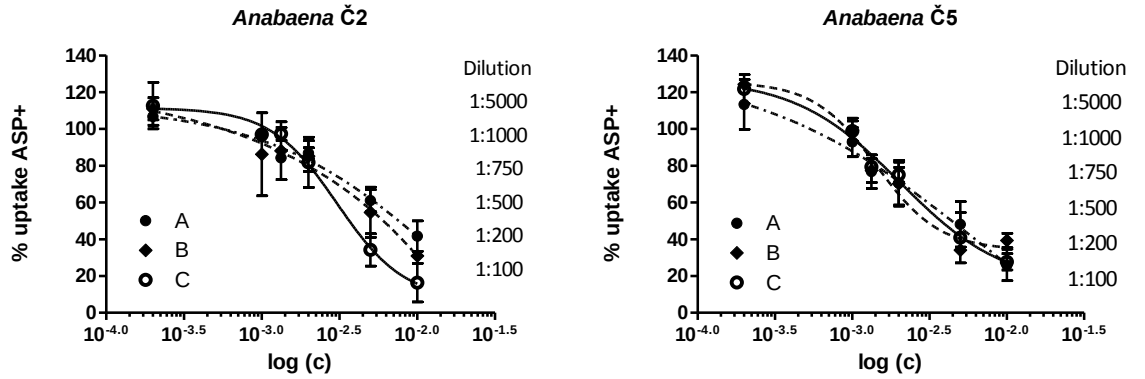


Figure 3.20. Inhibition of DrOatp1d1 transporter by ABC fractions of cyanobacterial strains. Results are shown as percentages of LY substrate uptake in HEK293 cells. Mean ± SD values with 95% CI are shown, as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

The effect of cyanobacterial strains on DrOct1 activity was lower than for DrOatp1d1 inhibition, as was the case with *Caulerpa* species. Certain strains such as *Phormidium* Z2 and *Anabaena* Č5 had very similar inhibition of DrOct1 transporter in all three fractions. For most of the cyanobacterial strains their ABC fractions reduced DrOct1 uptake activity to 40-60%. B fraction of *Oscillatoria* K3 showed the highest inhibition of uptake activity which was around 90% at fraction maximum concentration. Strong inhibition was also observed in C fraction of *Anabaena* Č2, with 16% uptake activity determined at the highest concentration (Fig. 3.21.).



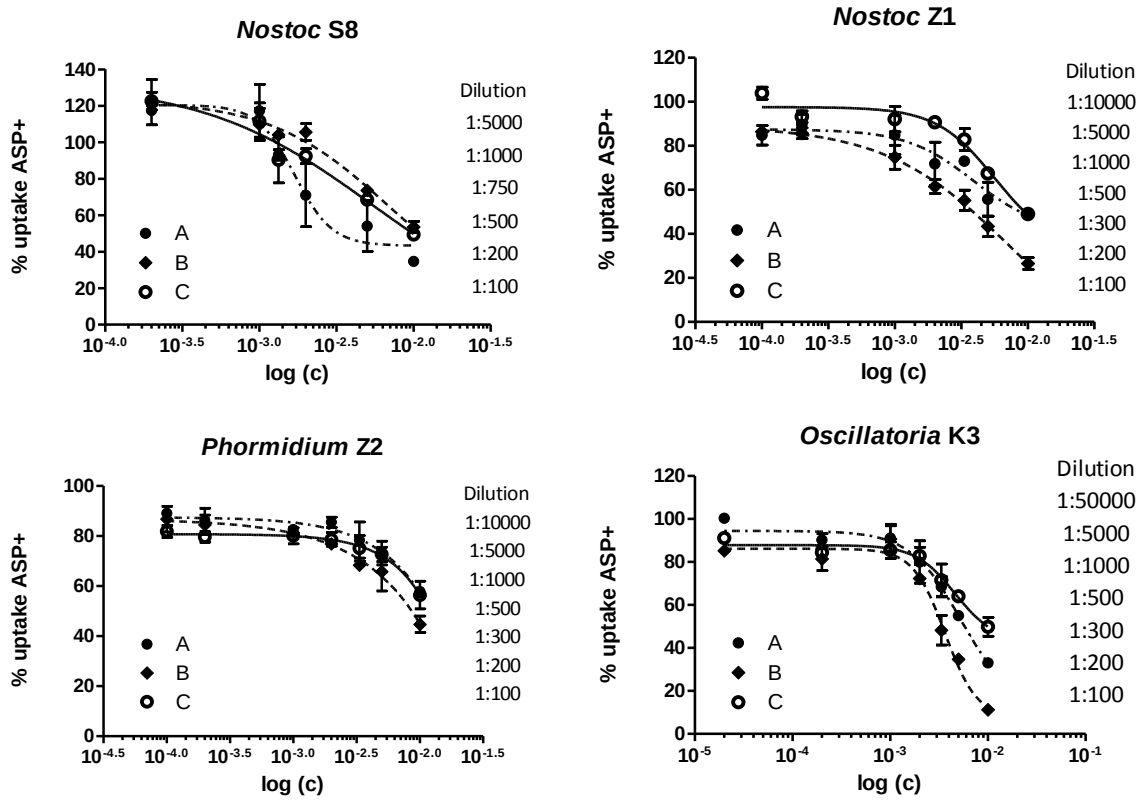


Figure 3.21. Inhibition of DrOct1 transporter by ABC fractions of cyanobacterial strains. Results are shown as percentage of ASP+ substrate uptake in HEK293 cells. Mean ± SD values with 95% CI are shown, as calculated from duplicates. Dilution ratios are shown on the right side of each panel.

3.2.1.2.1. IC50 values

Significant IC50 values were obtained mostly for B and C fractions of cyanobacterial strains. The lowest IC50 values for DrOatp1d1 inhibition was determined and calculated for C and B fraction of *Nostoc* S8 and Z1, respectively, while *Anabaena* Č5 B fraction showed the lowest IC50 value for DrOct1 (Fig. 3.22.). In general, aquatic strains obtained lower IC50 values for DrOatp1d1 inhibition, between 25 and 68 μg/mL, while IC50 values of terrestrial strains were between 26 and 147 μg/mL (Fig. 3.22.). Similar IC50 values for both organic transporters were obtained only for the *Oscillatoria* K3 strain. Furthermore, all three fractions of *Oscillatoria* K3 strain obtained IC50 values for DrOatp1d1 and DrOct1 inhibition (Fig. 3.22.). Terrestrial strains demonstrated the lowest IC50 values for DrOct1 inhibition, mostly *Anabaena* Č2 and Č5 whose all three fractions obtained IC50 values between 17 and 175 μg/mL (Fig. 3.22.). Apart from A, B and C fractions of *Oscillatoria* K3, IC50 values for

DrOct1 inhibition were obtained in only one other aquatic strain, B fraction of *Nostoc* Z1 (Fig. 3.22.).

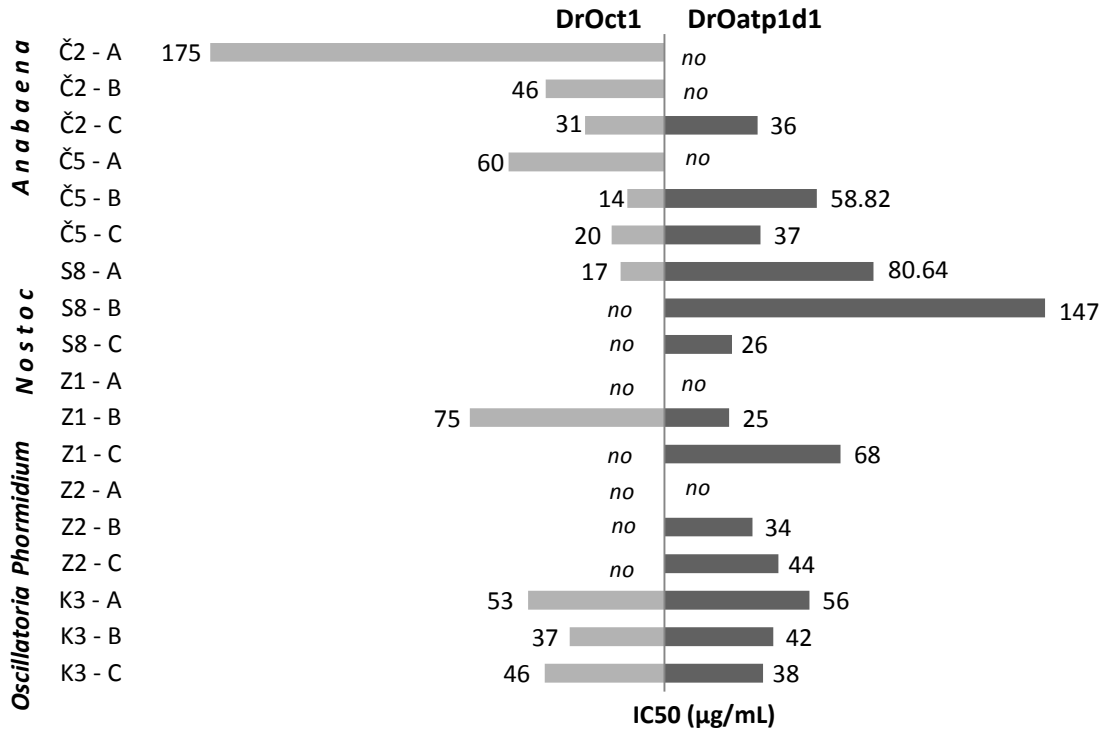
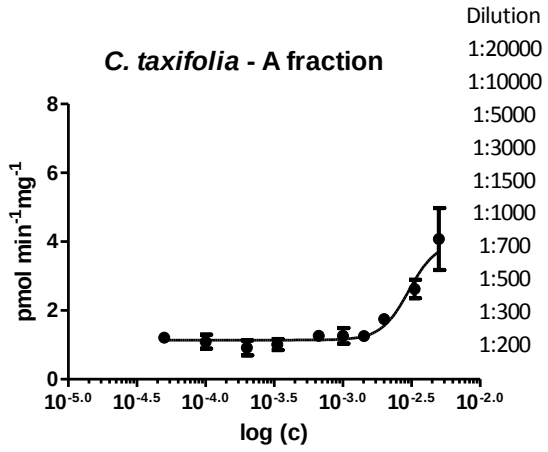


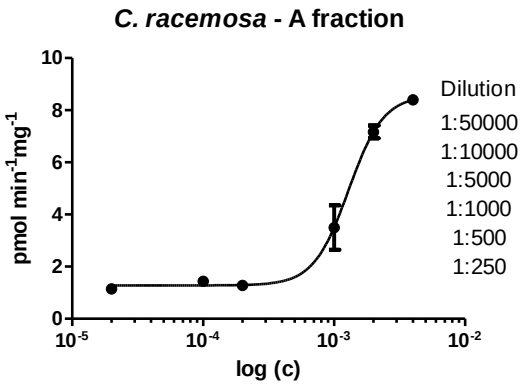
Figure 3.22. DrOatp1d1 and DrOct1 inhibitory potential of the ABC fractions of cyanobacterial strains. Values are expressed as IC50 and expressed in µg/mL. *no* – not obtained.

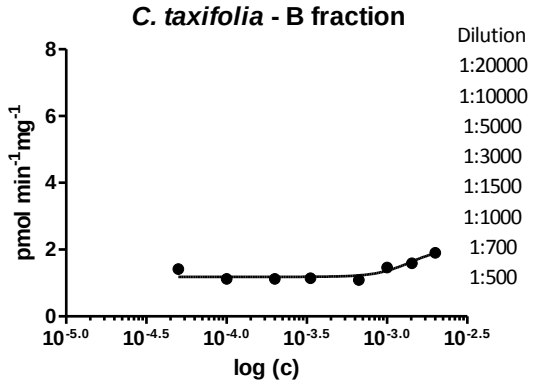
3.2.2. CYP1A1 induction

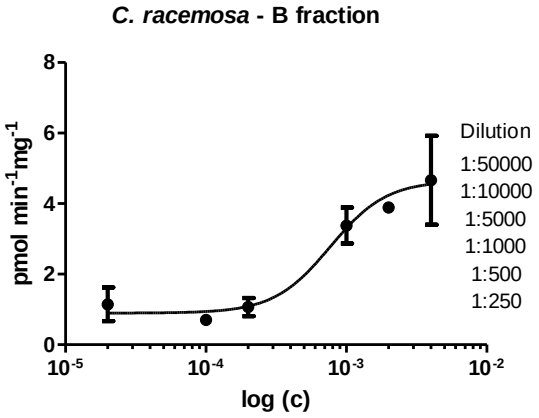
3.2.2.1. *C. racemosa* and *C. taxifolia* fractions

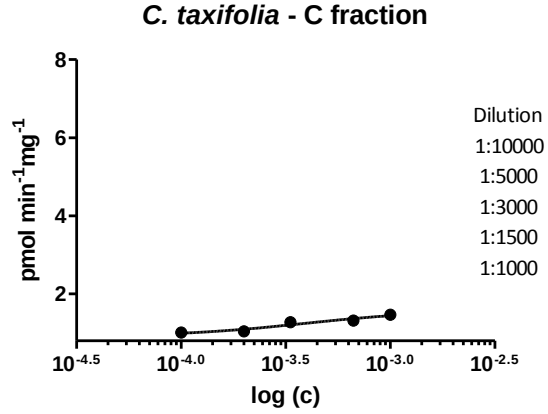
A range of 5 to 10 concentrations of fractions were tested for EROD activity within dilution range from 1:100 to 50000. A fractions of both *Caulerpa* species demonstrated the strongest effect on AhR activation and CYP1A1 activity induction. In general, *C. taxifolia* had weaker effect on CYP1A activity in PLHC-1/wt cells in all three fractions compared to *C. racemosa*. The highest induction was observed for *C. racemosa* A fraction resulting in 8.4 pmol/min/mg at the maximal concentration (Fig. 3.23.). Exposure of cells to B and C fractions also induced CYP1A1 to high levels, 4.66 pmol/min/mg and 5.35 pmol/min/mg, respectively. Except for the A fraction, B and C fractions of *C. taxifolia* resulted in EROD values lower than 2 pmol/min/mg (Fig. 3.23.).

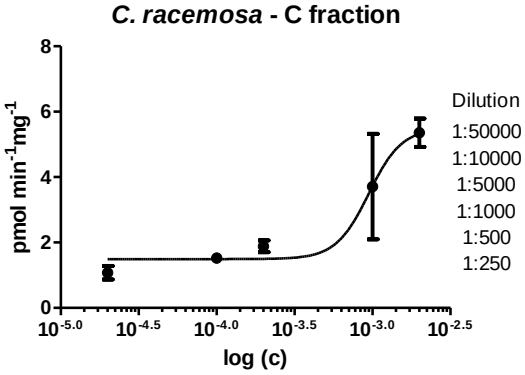

C. taxifolia - A fraction
Dilution
1:20000
1:10000
1:5000
1:3000
1:1500
1:1000
1:700
1:500
1:300
1:200
pmol min-1mg-1
log (c)


C. racemosa - A fraction
Dilution
1:50000
1:10000
1:5000
1:1000
1:500
1:250
pmol min-1mg-1
log (c)


C. taxifolia - B fraction
Dilution
1:20000
1:10000
1:5000
1:3000
1:1500
1:1000
1:700
1:500
pmol min-1mg-1
log (c)


C. racemosa - B fraction
Dilution
1:50000
1:10000
1:5000
1:1000
1:500
1:250
pmol min-1mg-1
log (c)


C. taxifolia - C fraction
Dilution
1:10000
1:5000
1:3000
1:1500
1:1000
pmol min-1mg-1
log (c)


C. racemosa - C fraction
Dilution
1:50000
1:10000
1:5000
1:1000
1:500
1:250
pmol min-1mg-1
log (c)

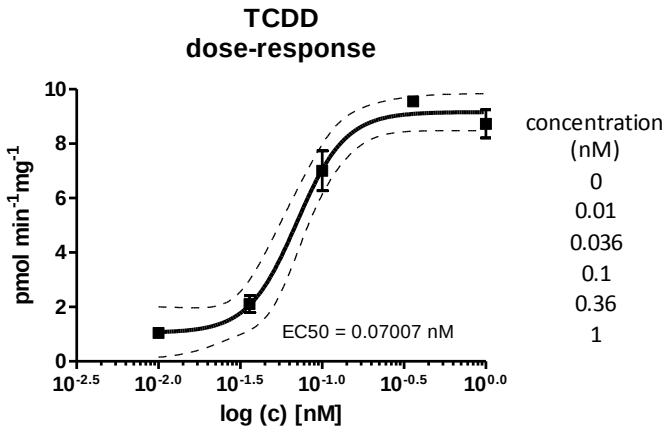


Figure 3.23. Induction of the CYP1A1 (EROD) activity in PLHC-1/wt cells exposed to *C. racemosa* (right) and *C. taxifolia* (left) ABC fractions, respectively. Positive control (TCDD) dose-response is shown at the bottom panel. Results are expressed as pmol/min/mg with mean ± SD values calculated from duplicates. Dilution ratios are shown on the right side of each panel.

3.2.2.1.1. TCDD equivalents

Sigmoidal dose-response parameters obtained for *Caulerpa* ABC fractions were adapted to EC5 value of the TCDD dose-response curve and are presented on Fig. 3.24. The highest TCDD Eq values are observed for *C. racemosa* B and A fraction, with 296 and 245 pg TCDD eq/mg, respectively. Other fractions did not significantly differ in obtained TCDD Eq values that were on average around 100 pg TCDD eq/mg (Fig. 3.24.).

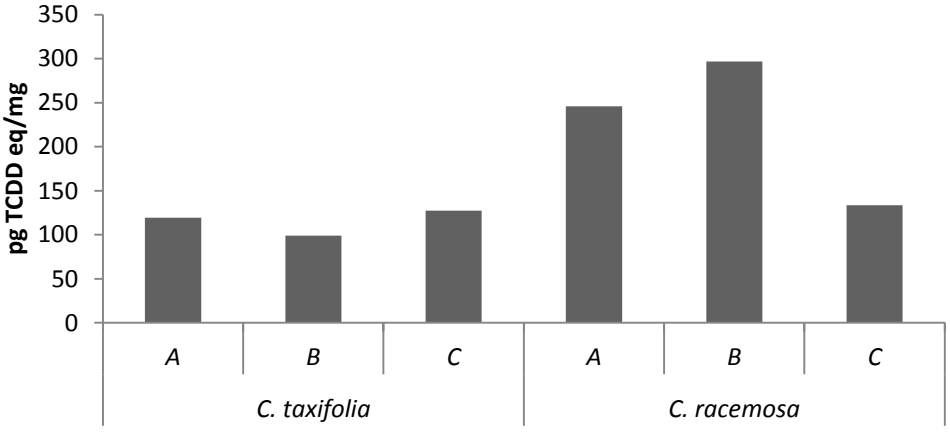


Figure 3.24. TCDD Eq determined and calculated for *Caulerpa* ABC fractions and expressed in pg TCDD eq/mg.

3.2.2.1.2. EC50 values

All three fractions of *C. racemosa* had stronger EROD induction potential compared to *C. taxifolia*. The most potent fractions of *C. racemosa* are A and B, with 12.9 and 7.66 μg/mL EC50 value, respectively (Fig. 3.25.). Obtained EC50 values for A and B fractions of *C. taxifolia* were around 30 μg/mL (Fig. 3.25.).

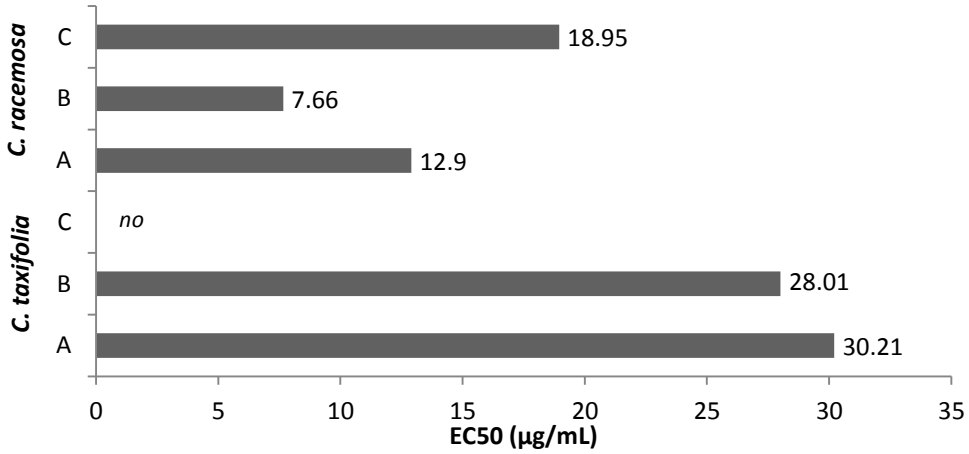
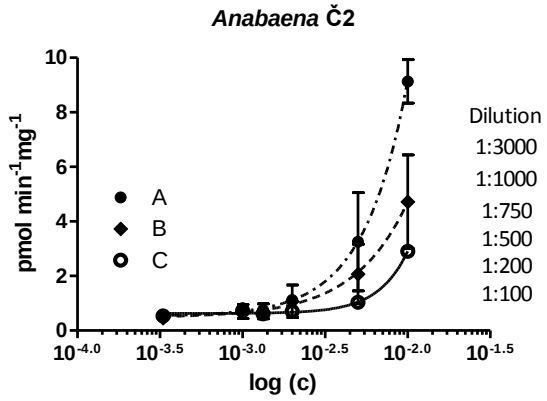
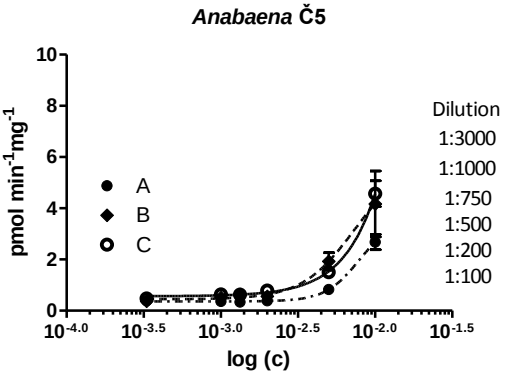


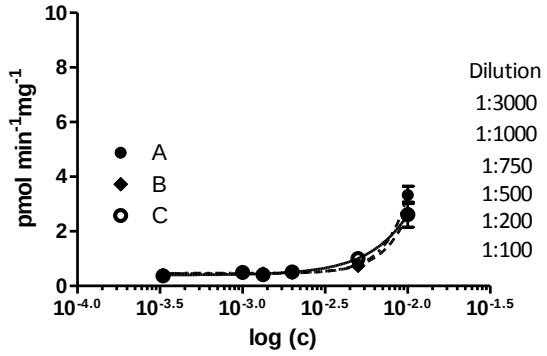
Figure 3.25. CYP1A1 induction potential of *Caulerpa* ABC fractions calculated and expressed as EC50 values (μg/mL). *no* – not obtained.

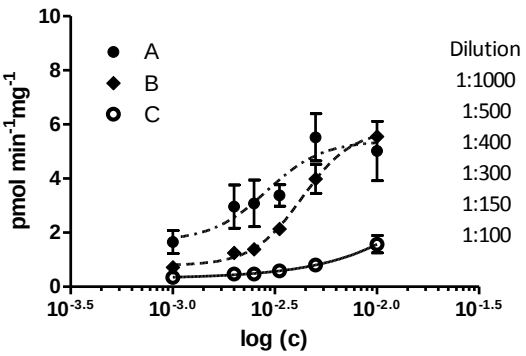
3.2.2.2. Cyanobacterial fractions

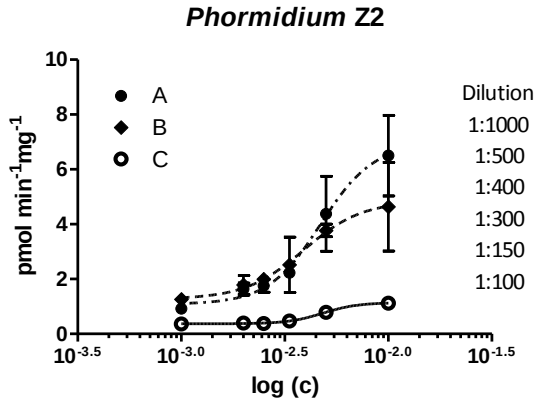
A range of 6 to 10 fraction concentrations were tested for EROD activity within dilution range from 1:100 to 5000. For most cyanobacterial strains the A fraction was the one with the strongest effect and the C fraction with the weakest effect on CYP1A1 activity (Fig. 3.26.). B fraction for most strains had slightly weaker induction values compared to the A fraction. C fractions for *Phormidium* Z2 and *Nostoc* Z1 had the lowest observed induction rates below 2 pmol/min/mg, while only Anabaena Č5 showed the highest induction in C fraction. We can distinguish *Anabaena* Č2 and *Oscillatoria* K3 A fraction that showed the highest induction values of 9.1 and 7.8 pmol/min/mg, respectively (Fig. 3.24.). All three fractions of *Nostoc* S8 strain showed similar influence on CYP1A1 activity whose values were around 3 pmol/min/mg (Fig. 3.26.).

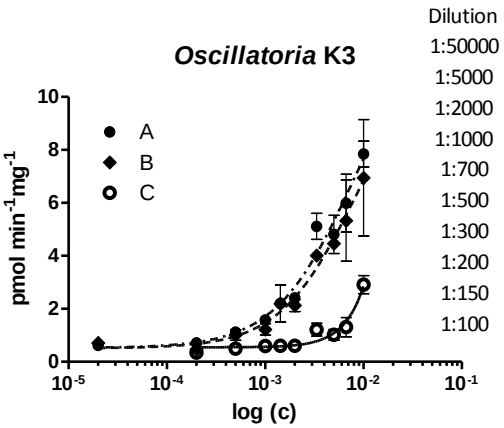

Anabaena Č2
pmol min-1mg-1
log (c)
A
B
C
Dilution
1:3000
1:1000
1:750
1:500
1:200
1:100


Anabaena Č5
pmol min-1mg-1
log (c)
A
B
C
Dilution
1:3000
1:1000
1:750
1:500
1:200
1:100


Nostoc S8
pmol min-1mg-1
log (c)
A
B
C
Dilution
1:3000
1:1000
1:750
1:500
1:200
1:100


Nostoc Z1
pmol min-1mg-1
log (c)
A
B
C
Dilution
1:1000
1:500
1:400
1:300
1:150
1:100


Phormidium Z2
pmol min-1mg-1
log (c)
A
B
C
Dilution
1:1000
1:500
1:400
1:300
1:150
1:100


Oscillatoria K3
pmol min-1mg-1
log (c)
A
B
C
Dilution
1:50000
1:5000
1:2000
1:1000
1:700
1:500
1:300
1:200
1:150
1:100

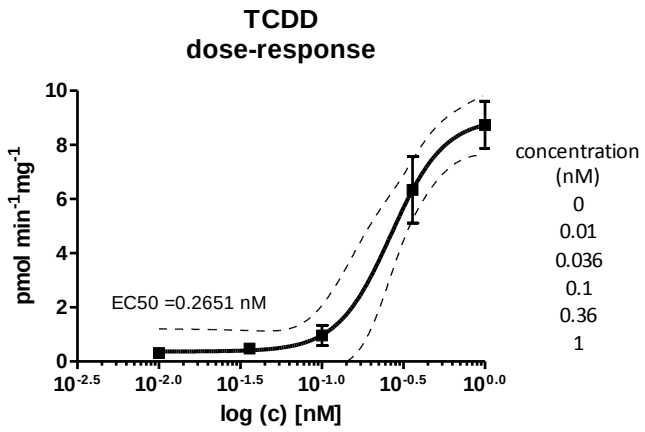


Figure 3.26. Induction of CYP1A1 (EROD) activity in PLHC-1/wt cells exposed to cyanobacterial ABC fractions. TCDD dose-response (positive control) is shown at the bottom panel. Results are expressed as pmol/min/mg with mean ± SD values calculated from duplicates. Dilution ratios are shown on the right side of each panel.

3.2.2.2.1. TCDD equivalents

Sigmoidal dose-response parameters obtained for cyanobacterial ABC fractions were adapted to EC5, EC10 and EC20 values of TCDD. From Fig. 3.27. we can see that the most potent induction potential was observed for A and B fractions of two aquatic strains, with *Nostoc* Z1 being the first and *Phormidium* Z2 the second in order of potency. *Nostoc* Z1 A fractions demonstrated the highest TCDD Eq value of 3383 pg TCDD eq/mg while A and B fractions of *Phormidium* Z2 had 2286 pg TCDD eq/mg and 1916 pg TCDD eq/mg, respectively. The minimal TCDD Eq values were observed for C fractions of all aquatic strains. Terrestrial strains obtained similar values for most of their ABC fractions, on average around or below 1000 pg TCDD eq/mg, except B fraction of *Anabaena* Č5 that had 1418 pg TCDD eq/mg, same as *Oscillatoria* K3 A fraction (Fig. 3.27.).

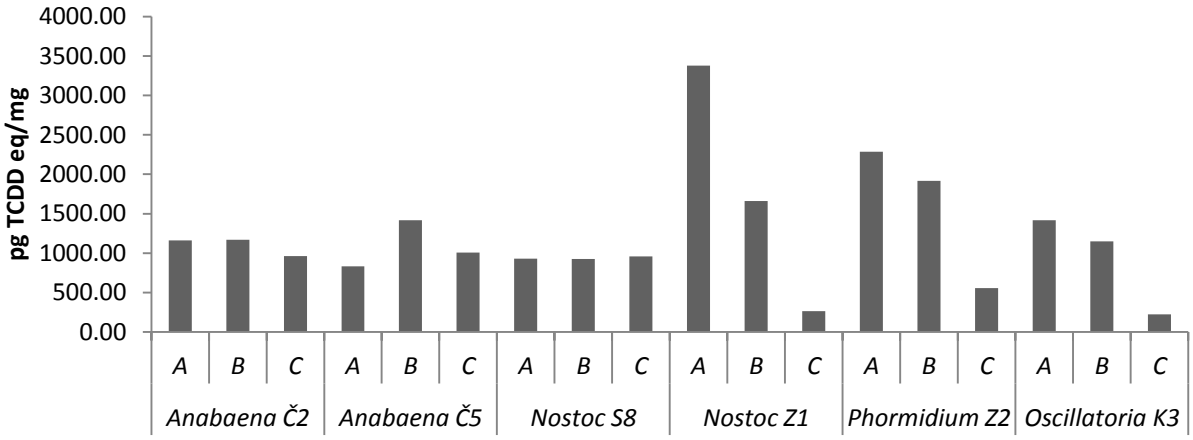


Figure 3.27. TCDD equivalents of cyanobacterial ABC fractions expressed in pg TCDD eq/mg.

3.2.2.2.2. EC50 values

In general, the obtained EC50 values for cyanobacterial strains did not significantly differ among fractions, except for *Oscillatoria* K3 C fraction with 2777 μg/mL EC50 value. Most ABC fractions of terrestrial strains have approximate EC50 values that are around 50 μg/mL (Fig. 3.28.). The lowest EC50 values were obtained for aquatic strains where *Nostoc* Z1 A fraction obtained the lowest 28.9 μg/mL EC50 value. All three fractions of *Phormidium* Z2 showed similar EC50 values that were around 40 μg/mL. *Oscillatoria* K3 and *Anabaena* Č5 A and B fractions obtained similar but higher EC50 values around 70-80 μg/mL (Fig. 3.27.).

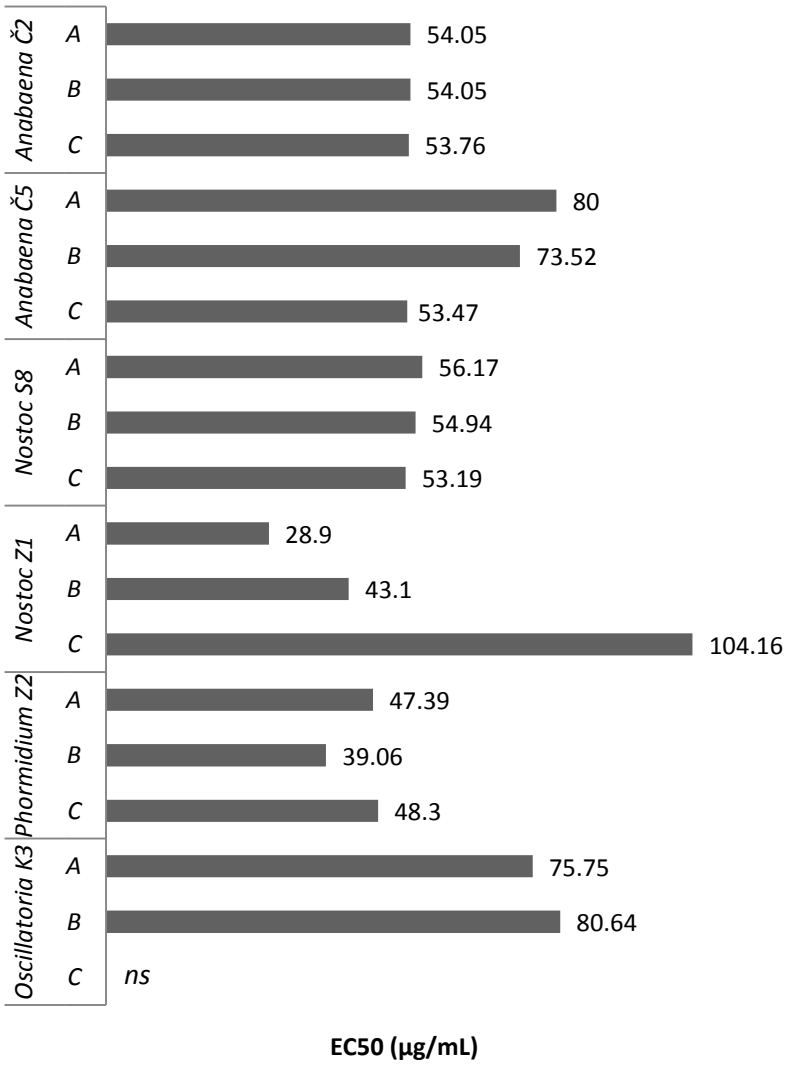
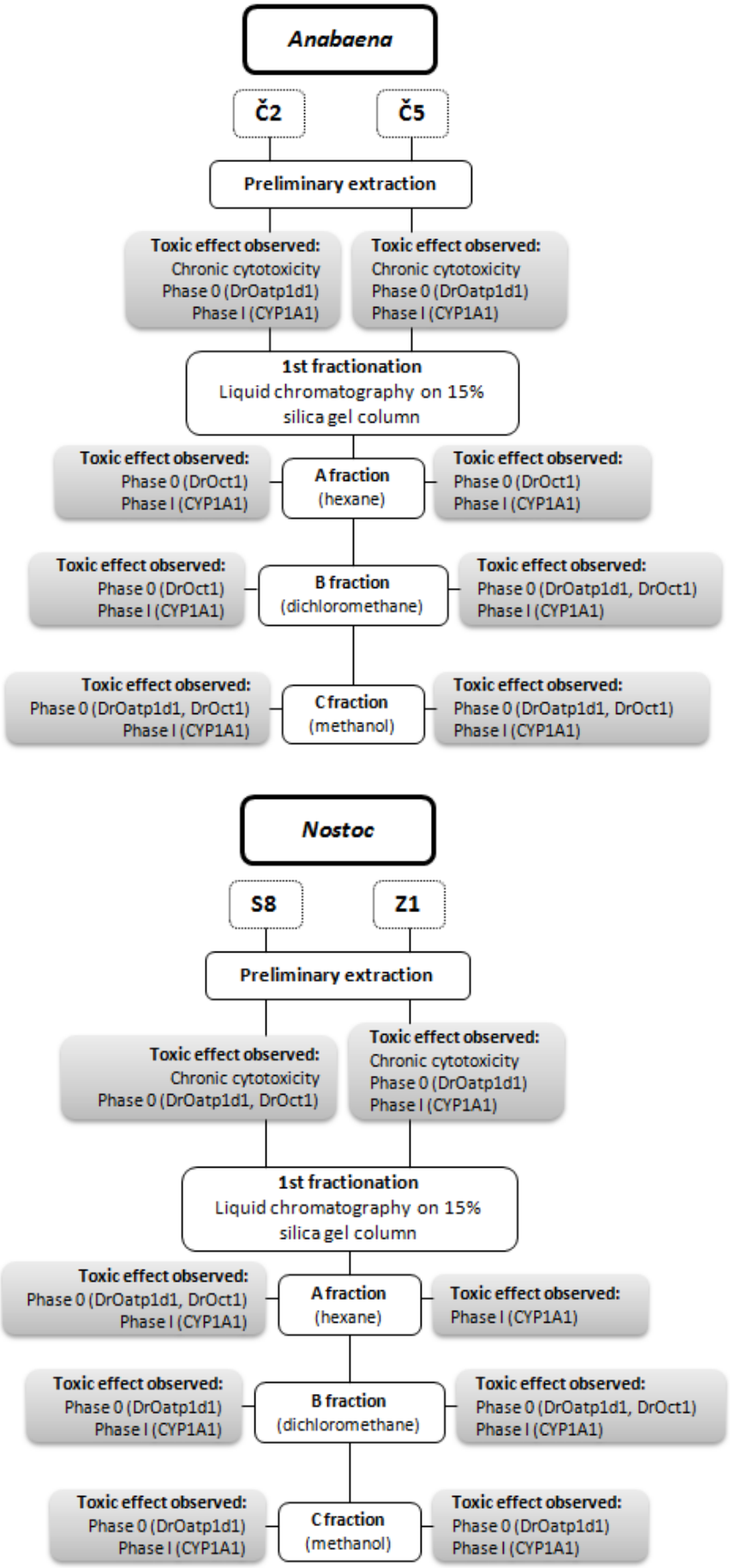


Figure 3.28. CYP1A1 induction potential of cyanobacterial ABC fractions expressed as EC50 values (µg/mL). *ns* – not shown.

Detected toxic effects of cyanobacterial strains in EDA workflow are displayed on Fig. 3.29.


Anabaena
Č2
Č5
Preliminary extraction
Toxic effect observed: Chronic cytotoxicity Phase 0 (DrOatp1d1) Phase I (CYP1A1)
Toxic effect observed: Chronic cytotoxicity Phase 0 (DrOatp1d1) Phase I (CYP1A1)
1st fractionation Liquid chromatography on 15% silica gel column
Toxic effect observed: Phase 0 (DrOct1) Phase I (CYP1A1)
A fraction (hexane)
Toxic effect observed: Phase 0 (DrOct1) Phase I (CYP1A1)
Toxic effect observed: Phase 0 (DrOct1) Phase I (CYP1A1)
B fraction (dichloromethane)
Toxic effect observed: Phase 0 (DrOatp1d1, DrOct1) Phase I (CYP1A1)
Toxic effect observed: Phase 0 (DrOatp1d1, DrOct1) Phase I (CYP1A1)
C fraction (methanol)
Toxic effect observed: Phase 0 (DrOatp1d1, DrOct1) Phase I (CYP1A1)
Nostoc
S8
Z1
Preliminary extraction
Toxic effect observed: Chronic cytotoxicity Phase 0 (DrOatp1d1, DrOct1)
Toxic effect observed: Chronic cytotoxicity Phase 0 (DrOatp1d1) Phase I (CYP1A1)
1st fractionation Liquid chromatography on 15% silica gel column
Toxic effect observed: Phase 0 (DrOatp1d1, DrOct1) Phase I (CYP1A1)
A fraction (hexane)
Toxic effect observed: Phase I (CYP1A1)
Toxic effect observed: Phase 0 (DrOatp1d1) Phase I (CYP1A1)
B fraction (dichloromethane)
Toxic effect observed: Phase 0 (DrOatp1d1, DrOct1) Phase I (CYP1A1)
Toxic effect observed: Phase 0 (DrOatp1d1) Phase I (CYP1A1)
C fraction (methanol)
Toxic effect observed: Phase 0 (DrOatp1d1) Phase I (CYP1A1)

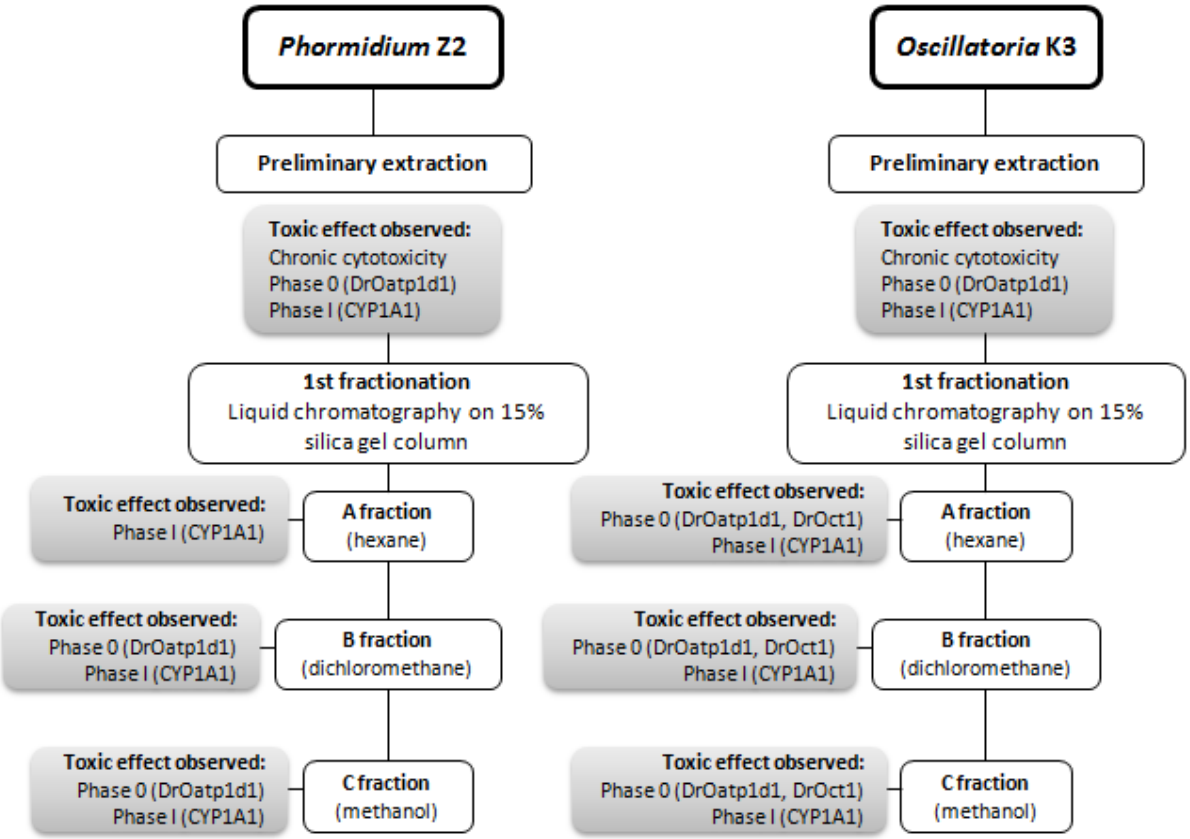


Figure 3.29. Schematic presentations of detected toxic effects of cyanobacterial strains in modified EDA study.

3.3. TOXICOLOGICAL CHARACTERIZATION OF HPLC SUB-FRACTIONS

Concentrations of HPLC sub-fractions of *C. taxifolia* and *C. racemosa* were 5 mg/mL, and the obtained volumes of all sub-fractions are listed in Table 2.7. in the Materials and Methods section. Maximal solvent (DMSO) concentration in all HPLC sub-fractions tested never exceeded 1%.

3.3.1. *C. racemosa* and *C. taxifolia* tested sub-fractions

Selected HPLC sub-fractions obtained from B and C fractions of both *Caulerpa* species were tested with *in vitro* biological assays. Table 3.1. shows which HPLC sub-fractions were screened for DrOatp1d1 and DrOct1 inhibition. That included 15 and 16 sub-fractions of *C. taxifolia* B and C fractions, respectively, and 19 and 21 sub-fractions of *C.*

racemosa B and C fractions, respectively. The selection was carried out according to the availability of the sub-fractions material. Other fractions were not tested due to small volume obtained after dissolution with DMSO (<20 μL) or low amount of substances as read chromatographically (Table 2.7. in the Materials and Methods section). Furthermore, additional volume of DMSO solvent was added to certain fractions to completely dissolve the dry residue.

Table 3.1. HPLC sub-fractions of the *Caulerpa* species B and C fractions selected for *in vitro* testing.

HPLC fraction number	*C. taxifolia*		*C. racemosa*	
	B	**C**	**B**	**C**
1			+	
2	+	+		+
3	+	+	+	+
4			+	
5		+	+	+
6	+	+		
7	+		+	
8	+		+	+
9	+		+	
10			+	+
11				
12	+			+
13			+	
14			+	
15	+			+
16	+	+		+
17		+		
18	+	+	+	
19				+
20		+		+
21		+	+	
22	+	+		
23		+		+
24			+	+
25	+	+	+	
26		+		+
27	+	+	+	+
28		+		+
29			+	+
30	+		+	+
31		+		
32				+
33				+
34		+		
35				+
36				
37				+
38	+	+	+	
39				
40			+	

3.3.2. Inhibition of phase 0 transporters

3.3.2.1. DrOatp1d1

Clear dose-response curves were obtained for 3 out of 19 tested *C. racemosa* B sub-fractions: B27, B29 and B30. Other sub-fraction had no effect on DrOatp1d1 uptake activity and these data are not shown. All three sub-fractions caused strong, 99-100% inhibition of DrOatp1d1 transporter activity at the highest concentrations (Fig. 3.30.). Dilution range that showed response in these sub-fractions was between 1:100 and 1:10000. These fractions were obtained during 100% B (ACN) HPLC gradient elution mode.

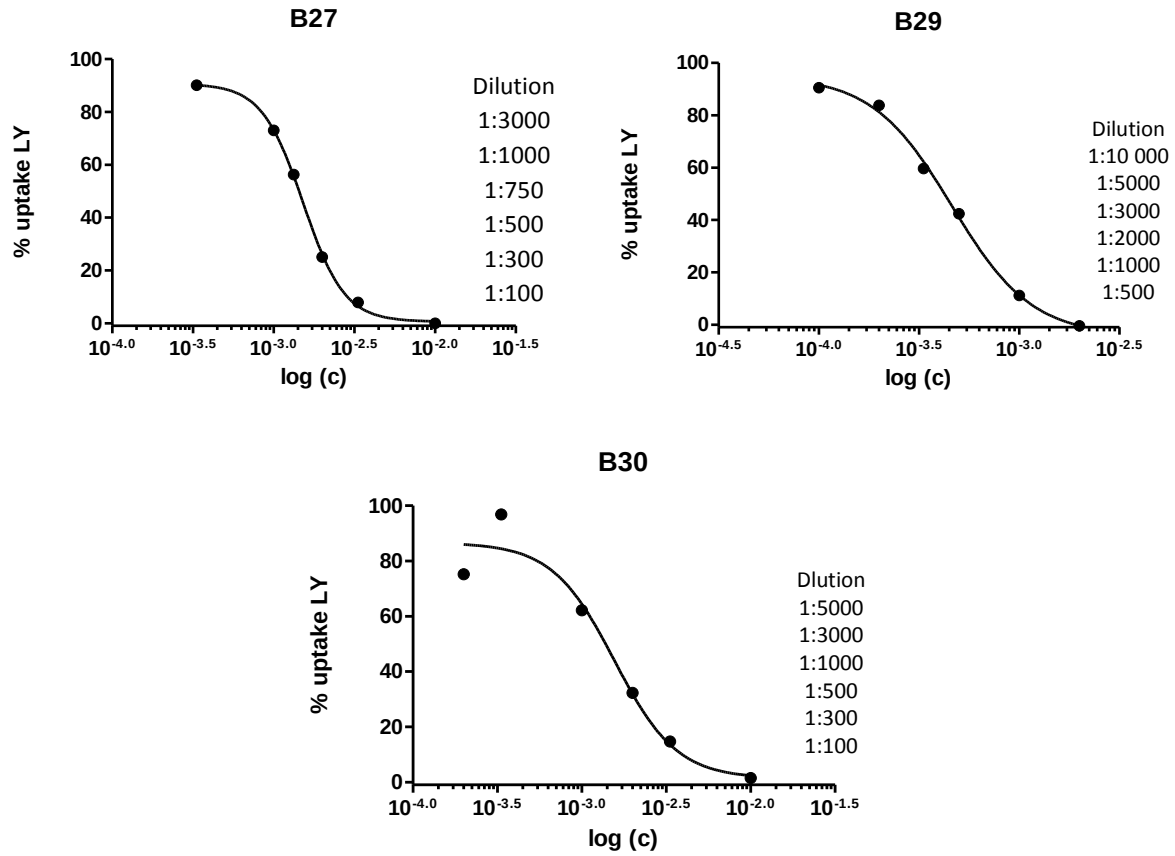
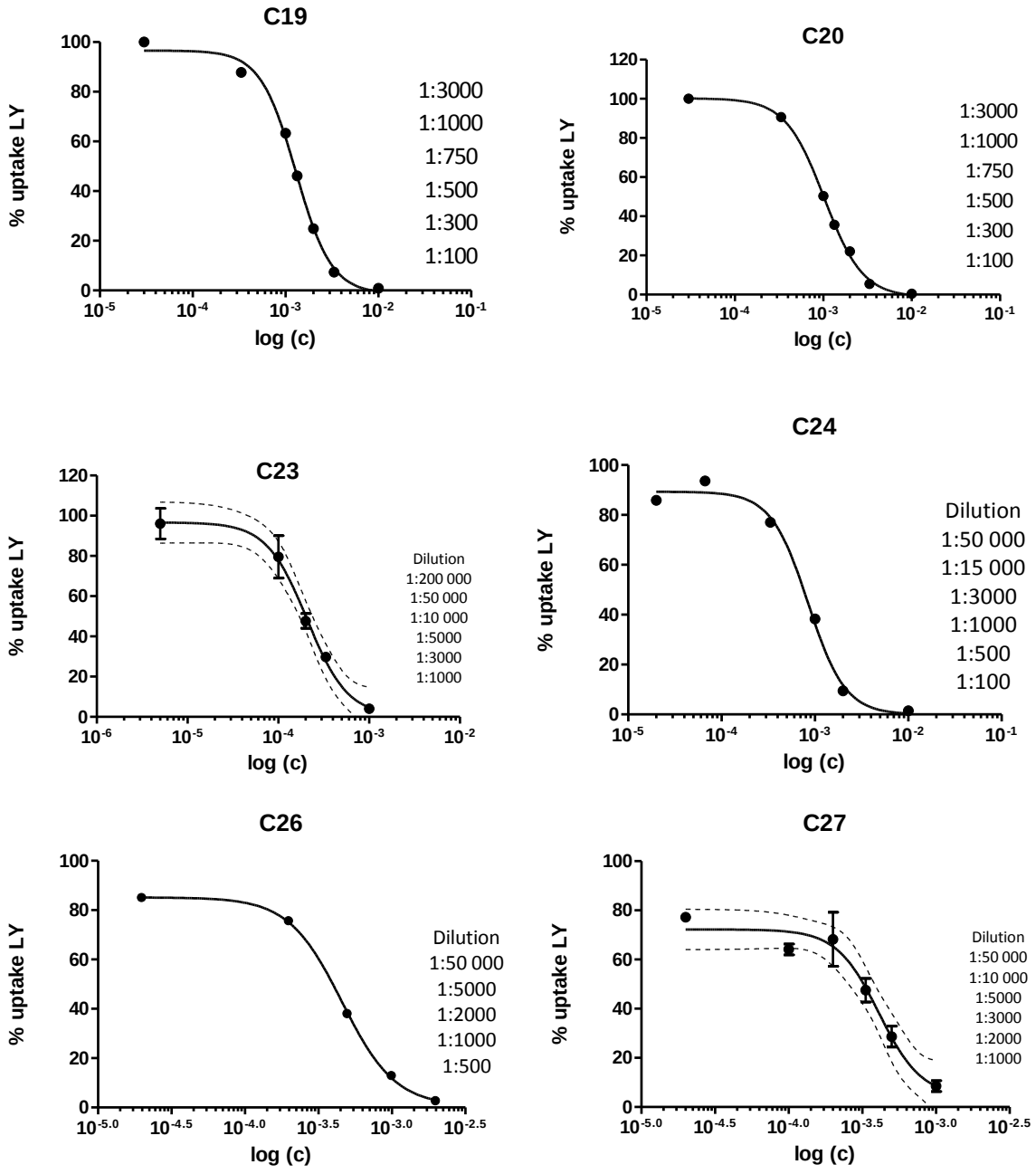


Figure 3.30. Dose-response curves of *C. racemosa* B sub-fractions that resulted in significant inhibition of DrOatp1d1 transport. Results are expressed as percentage of LY model substrate uptake. Sub-fractions were tested in 3 - 4 independent experiments and the most representative curve of each sub-fraction is displayed. Dilution ratios are shown on the right side of each panel.

C. racemosa C sub-fractions were more effective in inhibiting DrOatp1d1 uptake activity compared to B sub-fractions. More than 50% of all screened sub-fractions showed very strong inhibition, most of which belong to B gradient elution phase with 100% pure ACN. Sub-fractions from C19 to C37 resulted in clear dose-response curves but the most

potent effect on DrOatp1d1 uptake was recorded for C23 to C30 sub-fractions whose response was observed at higher dilution ranges from 1:1000 (or 1:300) to 1:200000. Dose-response curves obtained for *C. racemosa* C sub-fractions are shown on Fig. 3.31. Six most potent *C. racemosa* sub-fractions included C23, C26, C27, C28, C29, and C30 (Fig. 3.31.). Maximal concentration of most of positive sub-fractions resulted in almost complete inhibition of the LY uptake (remaining active uptake less than 5%) (Fig. 3.31.). The results of sub-fractions that showed no response are not shown.



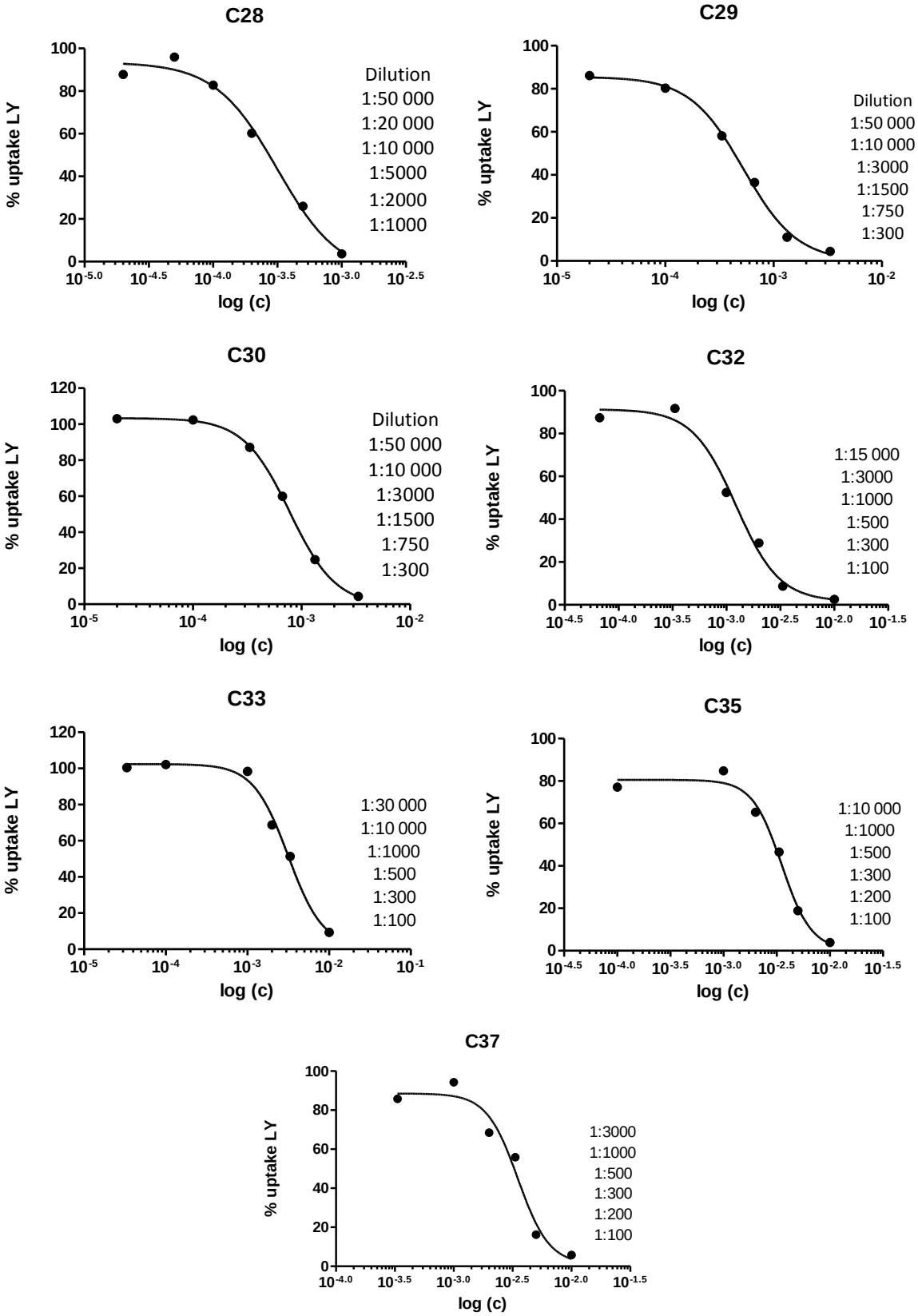


Figure 3.31. Dose-response curves of *C. racemosa* C sub-fractions that resulted in significant inhibition of DrOatp1d1 transport. Results are expressed as percentage of LY model substrate uptake. Sub-fractions were tested in 3 - 4 independent experiments and the most representative curve of each sub-fraction is displayed. Dilution ratios are shown on the right side of each panel.

Only 4 out of 15 tested B sub-fractions of *C. taxifolia* demonstrated inhibitory effect on anionic transporter activity and their dose-response curves are presented on Fig. 3.32. At their minimal dilution, sub-fractions B22, B25, B27 and B30 reduced DrOatp1d1 uptake activity to 2% or below (Fig. 3.32.). Again, the sub-fractions that were obtained during 100% B HPLC gradient phase are the ones that showed the effect. Dilution range within which response was observed was 1:100 and 1:10000. Results are not shown for sub-fractions that did not show influence on anionic transporter.

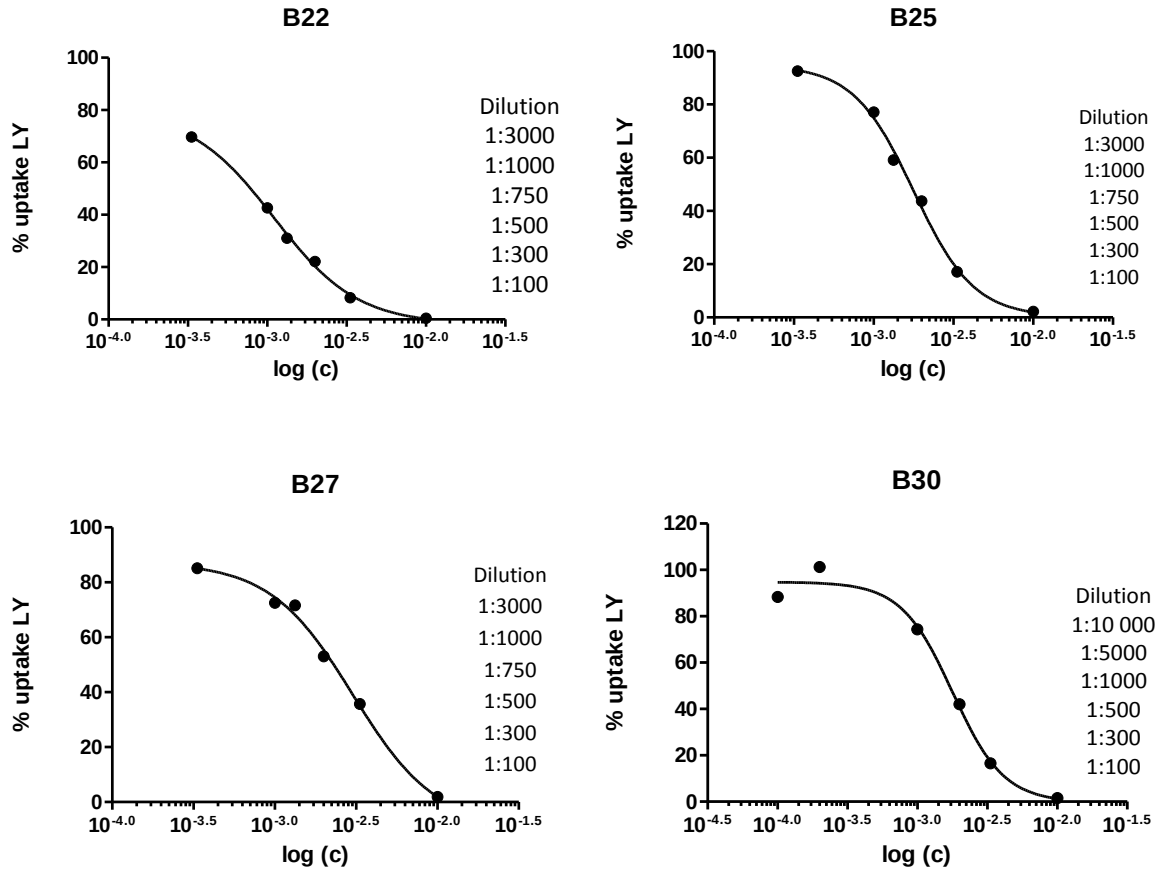
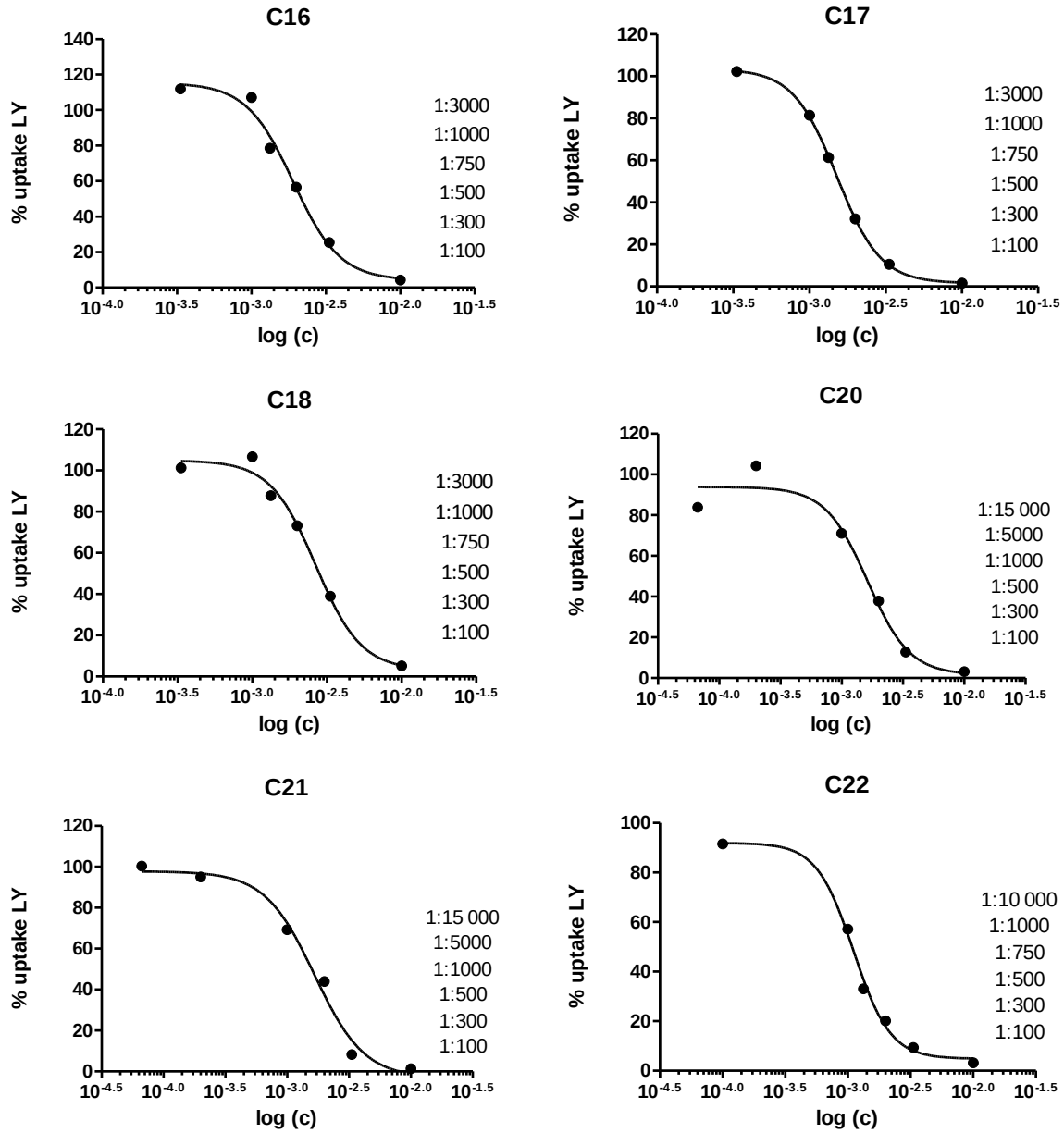


Figure 3.32. Dose-response curves of *C. taxifolia* B sub-fractions that resulted in significant inhibition of DrOatp1d1 transport. Results are expressed as percentage of LY model substrate uptake. Sub-fractions were tested in 3 - 4 independent experiments and the most representative curve of each sub-fraction is displayed. Dilution ratios are shown on the right side of each panel.

As for *C. racemosa*, more potent inhibition was observed in C compared to B sub-fractions of *C. taxifolia*. Inhibition of DrOatp1d1 activity was determined for 60% of all tested C sub-fractions of *C. taxifolia*. These 11 sub-fractions obtained throughout the whole 100% B elution phase were positive for DrOatp1d1 inhibition. Response in the highest dilution range from 1:500 (or 1:300) to 1:10000 was observed in 2 sub-fractions, C23 and

C27 (Fig. 3.33.). The highest determined inhibitory effects resulted in 5% or below uptake of LY for C23 and C27 fractions, respectively (Fig. 3.33.). Dose-response curves were obtained also for C16, C17, C18, C20, C21, C22, C25, C26 and C28 sub-fractions (Fig. 3.33.). Obtained response was in a lower dilution range from 1:100 to 1:3000, and all sub-fractions caused 95-99% inhibition of the anion transporter activity. Results of other 7 fractions with no effect are not shown.



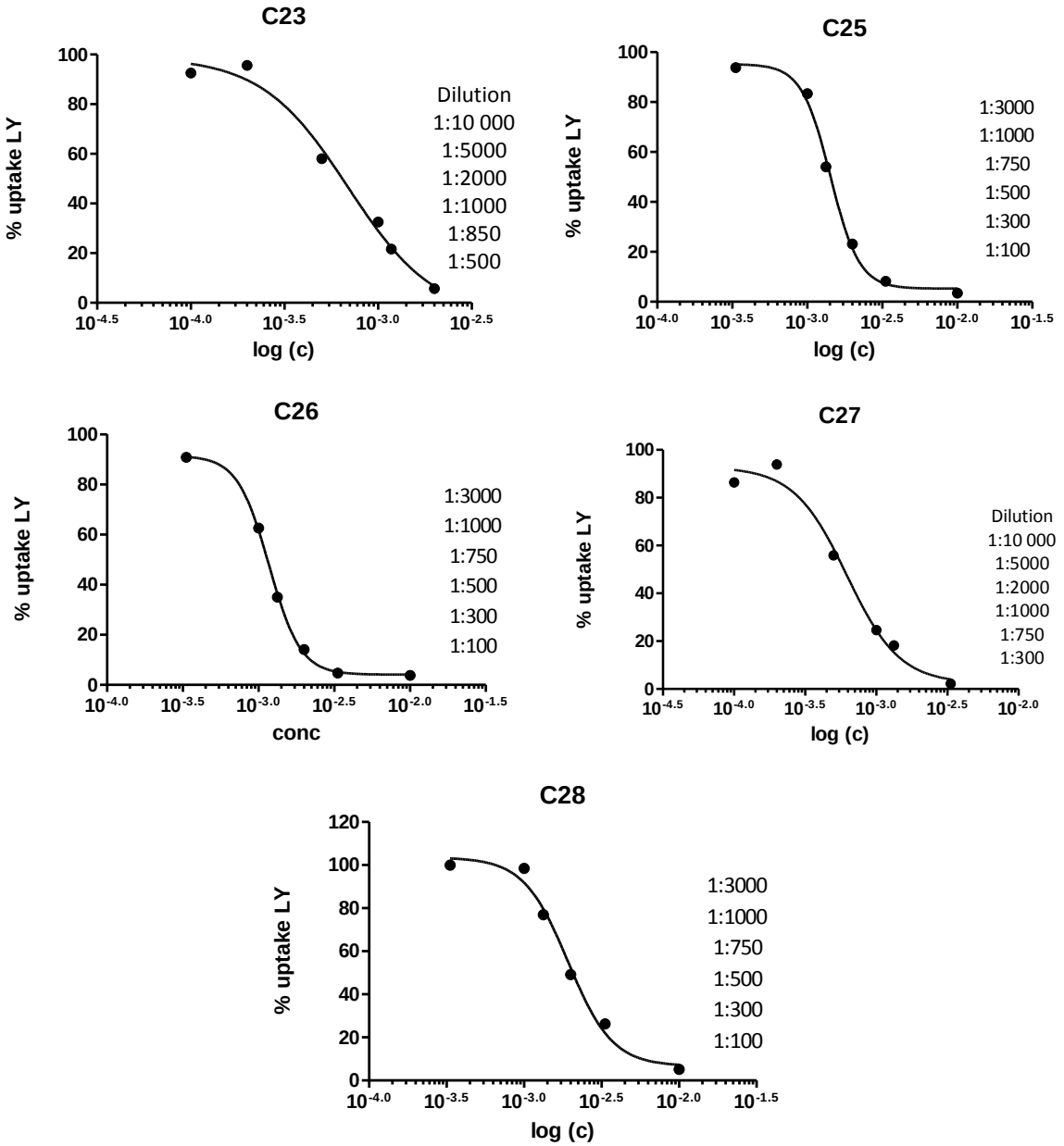


Figure 3.33. Dose-response curves of *C. taxifolia* C sub-fractions that resulted in significant inhibition of DrOatp1d1 transport. Results are expressed as percentage of LY model substrate uptake. Sub-fractions were tested in 3 - 4 independent experiments and the most representative curve of each sub-fraction is displayed. Dilution ratios are shown on the right side of each panel.

3.3.2.2. DrOct1

Compared to DrOatp1d1 effect, B and C sub-fractions of *C. racemosa* caused weaker inhibition of cation transporter DrOct1, and in most cases no inhibitory response was observed at all. Of all tested *C. racemosa* B sub-fractions only 3 of them reduced uptake activity to below 50%: B27, B29 and B30. The strongest effect was observed for B27 where uptake activity dropped to 6% at its maximal concentration (Fig. 3.34.). Inhibition of DrOct1

transporter that was over 50% was observed for 6 C sub-fractions of *C. racemosa*: C23, C24, C26, C27, C28 and C29.

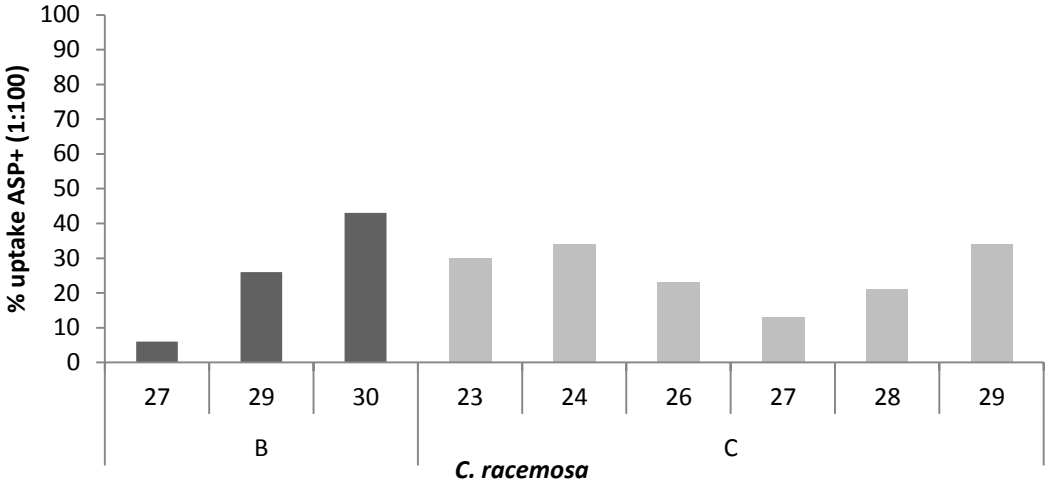


Figure 3.34. DrOct1 inhibition by *C. racemosa* B and C sub-fractions as determined at the highest sample concentration (1:100 or 1%). Results are expressed as percentages of ASP+ model substrate uptake. Sub-fractions were tested in 2 - 3 independent experiments and the most representative values are displayed.

This sub-fractions reduced DrOct1 uptake activity to 20-30% while C27 showed the highest inhibition (87%), as it was the case with B27 (Fig. 3.34). B and C sub-fractions that demonstrated effect on DrOct1 transporter are the ones that were included in 100% B HPLC gradient elution phase, same as it was for DrOatp1d1 transporter. Other B and C sub-fractions of *C. racemosa* showed no significant effect (not shown).

Compared to *C. racemosa*, more *C. taxifolia* sub-fractions have had an effect on the cation transporter. Six B and eight C sub-fractions of *C. taxifolia* caused more than 50% inhibition of DrOct1 transport activity. The response was observed mostly in sub-fractions ranged from 16 to 34, and the corresponding fractions resulted in reduction of DrOct1 uptake activity to 30 – 40% of the remaining uptake of model substrate ASP+ (Fig. 3.35.). Here again, B27 sub-fraction indicated the lowest observed uptake, only 19%, while B30 was close with 20% of active uptake remaining (Fig. 3.35.). Most of these fractions are also obtained during pure ACN elution phase, except for fractions B7, B8, C31 and C34 that were obtained during 100% of the A phase.

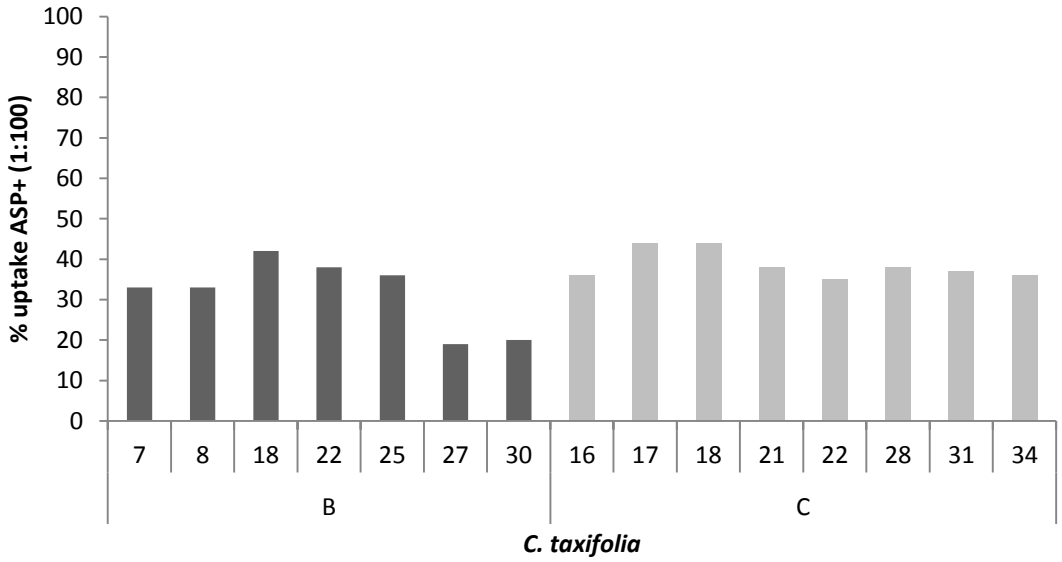


Figure 3.35. DrOct1 inhibition by *C. taxifolia* B and C sub-fractions as determined at the highest sample concentration (1:100 or 1%). Results are expressed as percentages of ASP+ model substrate uptake. Sub-fractions were tested in 2 - 3 independent experiments and the most representative values are displayed.

3.2.2.3. IC50 values

For both *Caulerpa* species, C sub-fractions were more potent in DrOatp1d1 inhibition then B sub-fractions. As presented on Fig. 3.36., the strongest DrOatp1d1 inhibitory potential was observed for *C. racemosa* C sub-fractions, with IC50 values below 1 μg/mL for 2 sub-fractions – C27 and C28 – while value for C23 sub-fraction was 1 μg/mL. Other C and B sub-fractions resulted in IC50 values around or below 6 μg/mL, except C33 – C37 sub-fractions. DrOatp1d1 inhibitory potential for most of *C. taxifolia* B and C sub-fractions was between 3 and 9 μg/mL (IC50 values). Same as for *C. racemosa*, C27 and C23 sub-fraction had the lowest IC50 value observed, only 3.1 μg/mL and 3.45 μg/mL, respectively (Fig. 3.36.). Since most B and C sub-fractions of *Caulerpa* species had no effect on DrOct1 transporter activity, IC50 values could not be calculated and therefore are not shown. *C. racemosa* sub-fractions C27, C28 and B27 that exhibited effect to DrOct1 transporter activity obtained following IC50 values: 4.81, 5.81 and 9.31 μg/mL, respectively. Only 2 sub-fractions of *C. taxifolia*, B27 and B30, obtained IC50 values (8.88 and 15.72 μg/mL, respectively).

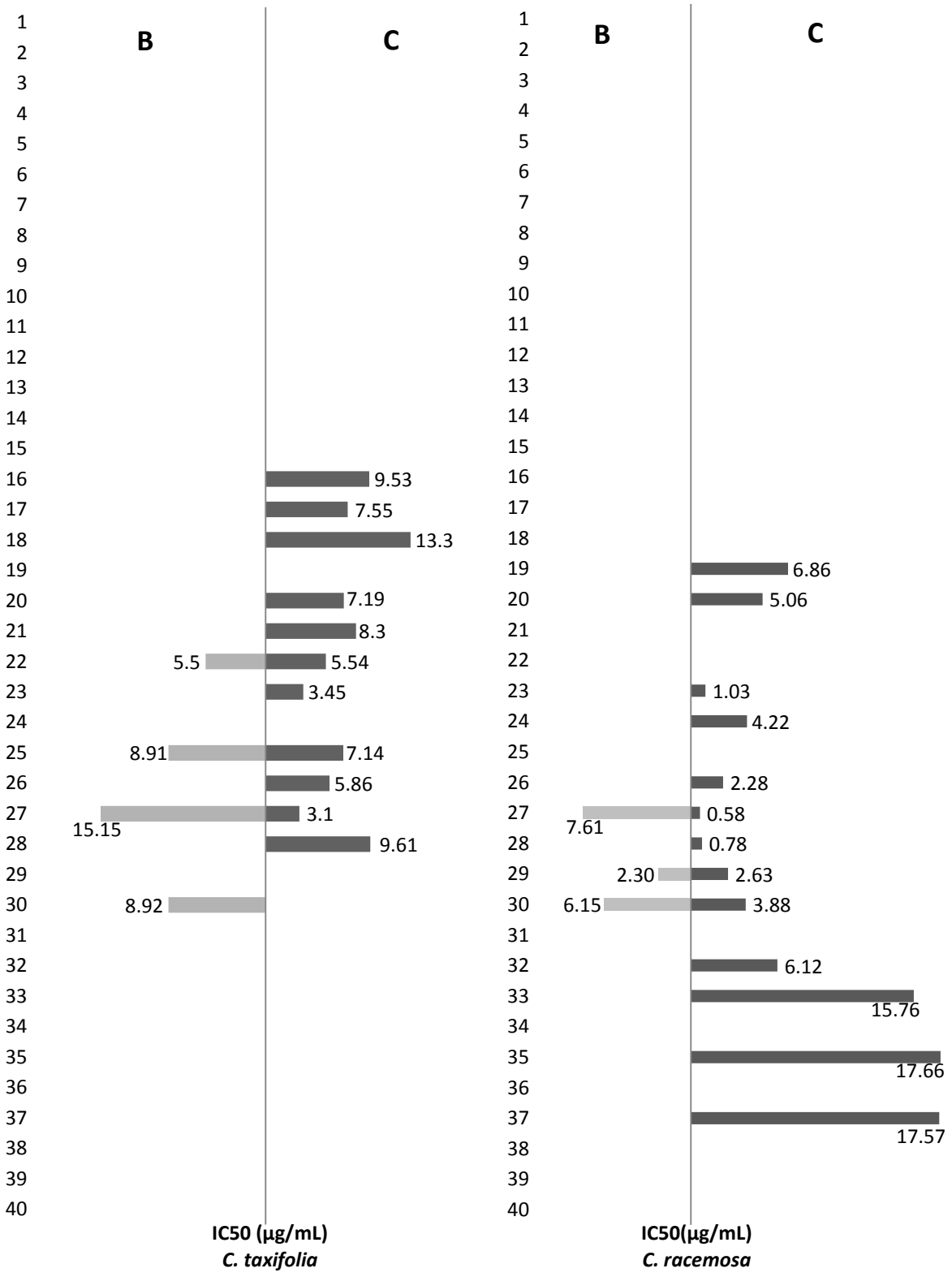


Figure 3.36. DrOatp1d1 inhibitory potential of *C. racemosa* (right) and *C. taxifolia* (left) B and C sub-fractions. Results are expressed as IC50 values (µg/mL).

3.3.3. HPLC chromatograms

3.3.3.1. *C. racemosa*

From previous results we can observe that B sub-fractions of *C. racemosa* that showed the effect on DrOatp1d1 are the same ones that showed the effect on DrOct1 transporter: B27 – B30. These sub-fractions match the peaks on the obtained initial HPLC chromatogram (Fig. 3.37.). Peak values of these sub-fractions were around or below 0.5 AU (arbitrary units) (Fig. 3.37.). The highest peak was observed in the fraction B23 (2.0 AU). The strongest effects of DrOatp1d1 and DrOct1 inhibition are obtained for B29 and B27 sub-fractions, with detected peaks on chromatogram around 0.6 AU (Fig. 3.37.). Therefore, the effects on biological assays are consistent with chemical HPLC analysis for *C. racemosa* B sub-fractions.

C. racemosa C sub-fractions that showed the most potent inhibitory potential for anionic transporter also correspond to the ones that showed the highest DrOct1 inhibition percentage. C sub-fractions with the strongest response to DrOatp1d1 and DrOct1 inhibition include sub-fractions 19 – 32, and are clearly detected on the initial HPLC chromatogram as a series of peaks (Fig. 3.38.). These peaks have high AU values between 1.5 and 2.0. Sub-fractions that demonstrated weaker biological effect responded to chromatogram peaks detected between 32 and 40 minutes, with lower AU values around 0.1 – 0.2 (Fig. 3.38.). C27 and C28 had the most potent inhibitory effect on DrOatp1d1 and DrOct1, with detected peaks at around 1 AU (Fig. 3.38.). High peaks were also detected at the beginning of the chromatogram. Consequently, these sub-fractions were also screened for DrOatp1d1 and DrOct1 uptake inhibition but did not show any effect.

3.3.3.2. *C. taxifolia*

The observed effects of *C. taxifolia* B sub-fractions to DrOatp1d1 and DrOct1 inhibition are overlapping and are most pronounced from B22 to B30 sub-fractions. For all these sub-fractions, significant peaks were detected on the initial HPLC chromatogram that correspond to the positive biological response, with AU values from 0.5 up to 2 (Fig. 3.39.). The highest peak was observed during the 25th minute of elution, but the strongest DrOatp1d1 and DrOct1 inhibition was observed in B22 and B27 sub-fractions, respectively, with peaks detected at 0.7 and 1.3 AU (Fig. 3.39.). Several higher peaks were also detected

during the first 2-3 min of elution. Again, corresponding sub-fractions were tested but that did not show any effect on organic anion and cation transporter. For B7 and B8 sub-fractions weaker effect on DrOct1 was observed but no peaks were detected on chromatogram.

HPLC chromatogram of *C. taxifolia* C sub-fractions detected high amounts of different chemical compounds during 14 - 35 min time interval, whose values for some sub-fractions were much above 2.0 AU (Fig. 3.40.). Again, effects of DrOatp1d1 and DrOct1 inhibition are observed within the same time range and are determined from C16 – C34 sub-fractions. The strongest effects were observed in C23 and C27 for DrOatp1d1 inhibition and C22 for DrOct1 inhibition. These peaks are over 2.0 AU (Fig. 3.40.). Like for *C. racemosa*, the observed biological effects are consistent with the chemical analysis in B and C sub-fractions of *C. taxifolia*.

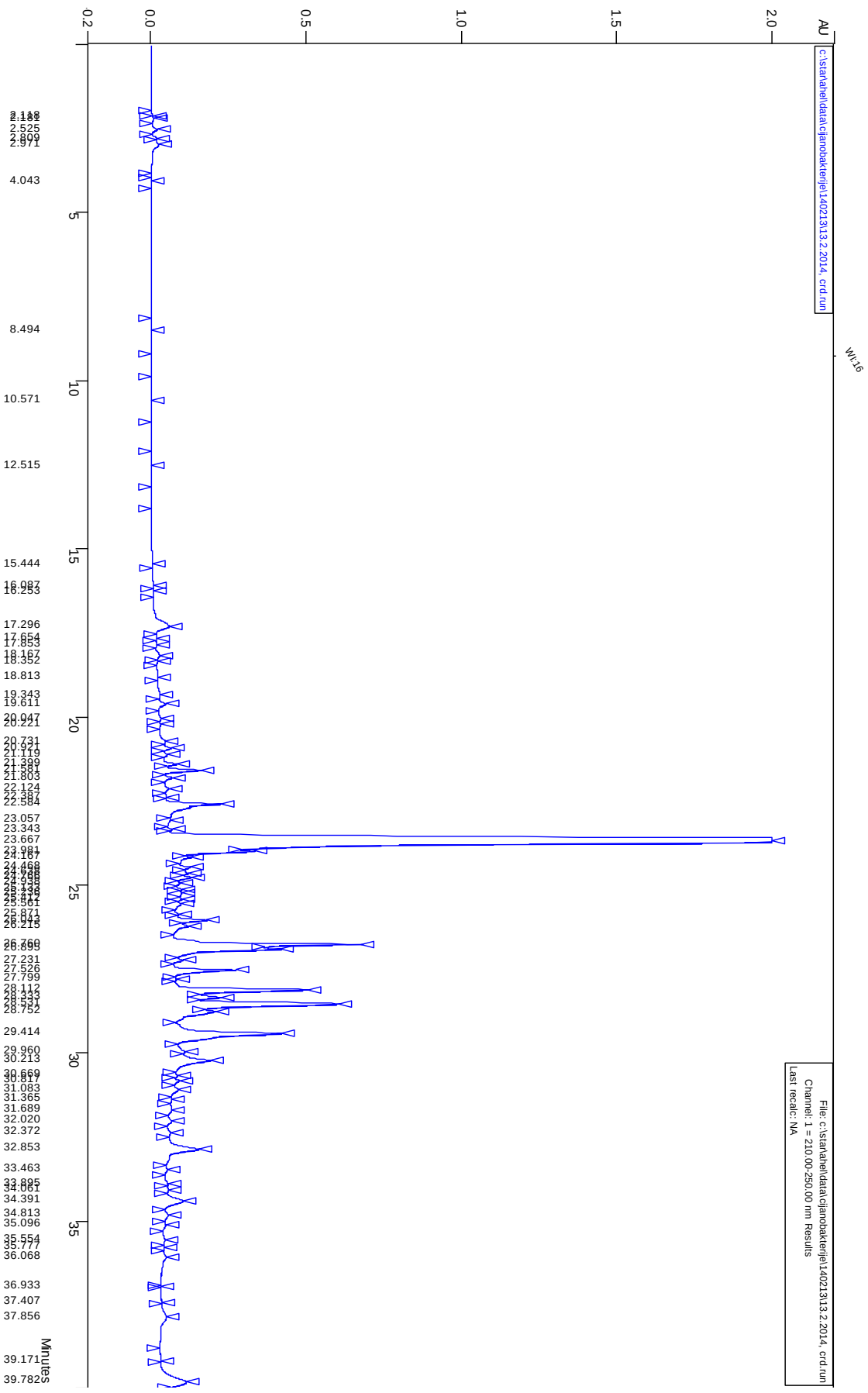


Figure 3.37. HPLC chromatogram of *C. racemosa* B sub-fractions. Results are expressed as arbitrary units during 40 min gradient phase elution.

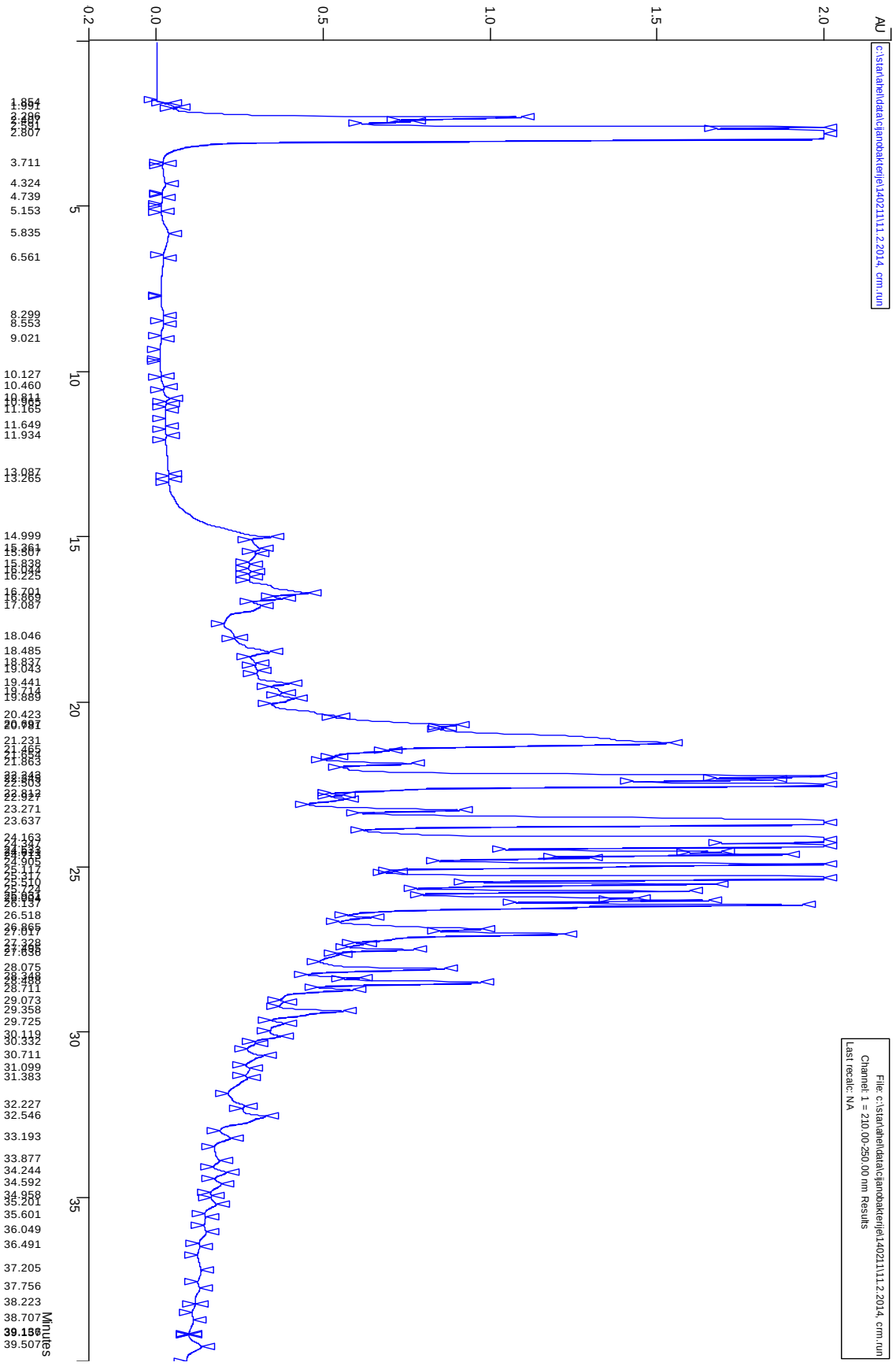


Figure 3.38. HPLC chromatogram of *C. racemosa* C sub-fractions. Results are expressed as arbitrary units during 40 min gradient phase elution.

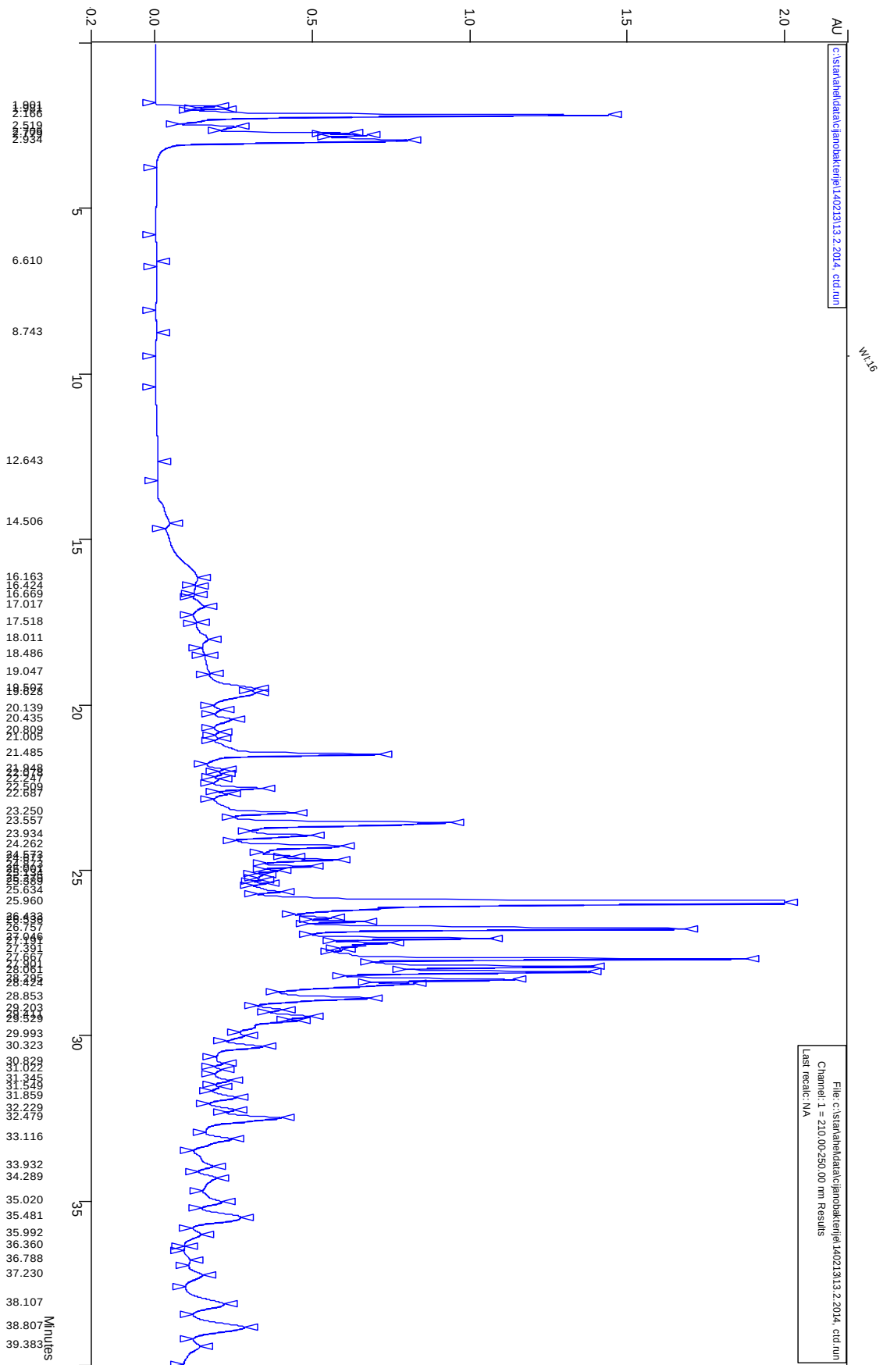


Figure 3.39. HPLC chromatogram of *C. taxifolia* B sub-fractions. Results are expressed as arbitrary units during 40 min gradient phase elution.

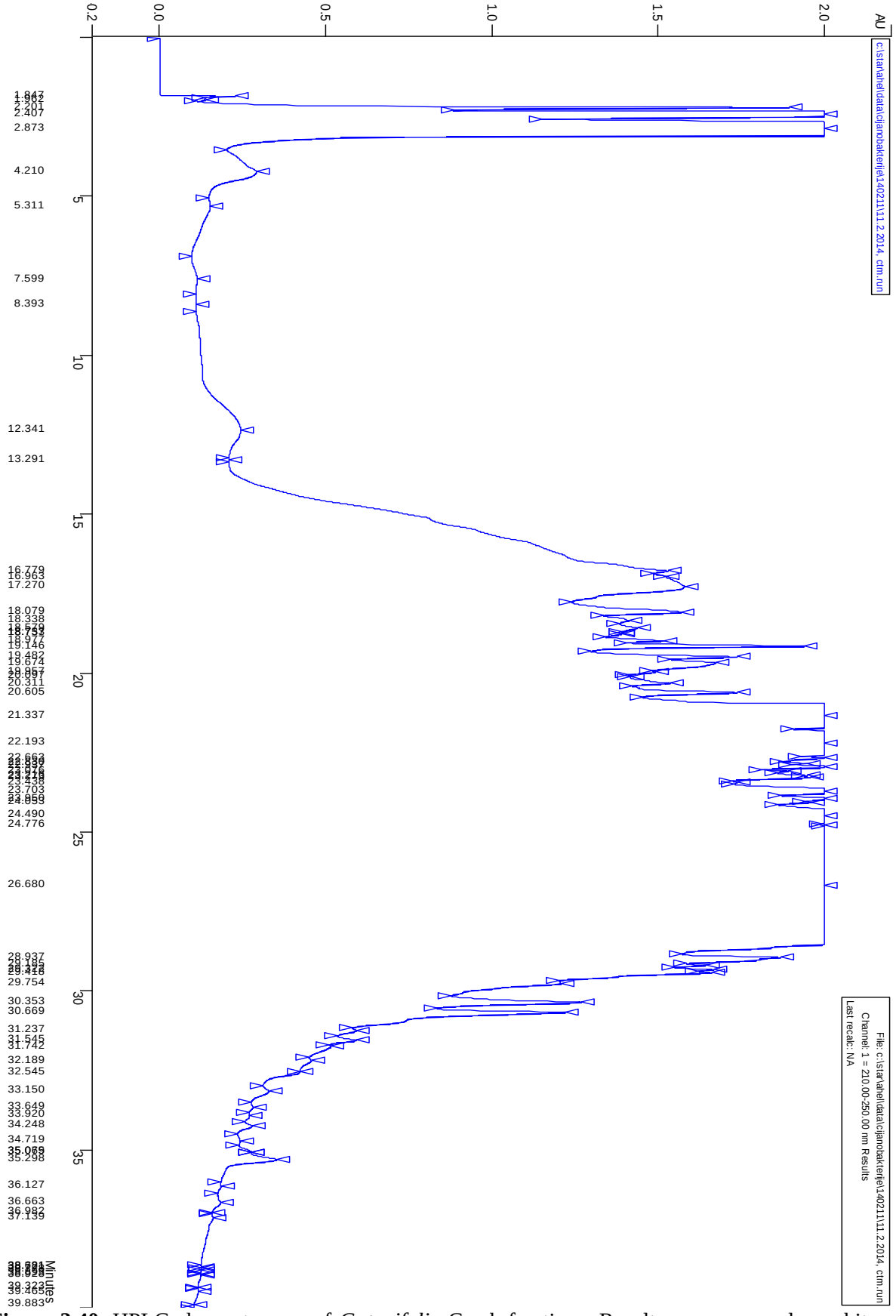


Figure 3.40. HPLC chromatogram of *C. taxifolia* C sub-fractions. Results are expressed as arbitrary units during 40 min gradient phase elution.

3.4. LC-MS ANALYSES

3.4.1. Caulerpenyne standard

The TIC LC-MS chromatogram of the purified (standard) CYN solution shows dominant CYN peak at 10.47 min (Fig. 3.41.). Positive electrospray ionization (ESI) MS provided ionization by sodium adduct ion formation, producing ion at m/z 397.162 that is exclusive of CYN (Fig. 3.41.).

3.4.2. *C. racemosa*

Results of LC-MS analyses conducted on ABC fractions of *C. racemosa* did not show characteristic peaks of CYN or CLP in the fraction A (Fig. 3.42.). The major metabolite in *C. racemosa* was found to be CLP, with dominant peak at 8.93 min detected in the LC-MS chromatogram of the fraction B (Fig. 3.42.). The formation of CLP producing ion on the mass spectrum was at m/z 399.132 in the positive ion mode (Fig. 3.42.). Presence of CLP was found also in the C fraction where peaks of numerous other chemical components were detected as well.

Seven B and 12 C sub-fractions of *C. racemosa* obtained by HPLC were analyzed by LC-MS (Table 2.8. in the Materials and Methods section). CLP was further confirmed as dominant compound in *C. racemosa* B and C sub-fractions (Figs. 3.43. and 3.44.). Approximately 80% of CLP was found in B sub-fractions while C sub-fractions contained about 20% of CYN (Figs. 3.43. and 3.44.). The dominant peak of CLP at 8.94 min in the TIC LC-MS chromatogram was detected in sub-fraction B24 where around 90% of the total CLP was eluted, followed by a decreasing trend from B25 to B28 sub-fractions (Fig. 3.43.). Traces of CLP were also recorded in B29 while B30 had almost no CLP detected (Fig. 3.43.). Therefore, CLP characteristic peak was detected in 6 out of 7 tested B sub-fractions. CYN was detected in B sub-fraction but with lower levels compared to CLP. Only sub-fraction B26 contained CYN metabolite. CLP metabolite was again detected in C24 sub-fraction where it was the most abundant, around 90% (Fig. 3.44.). The dominant peak of CLP was at 8.86 min in the TIC LC-MS chromatogram (Fig. 3.44.). CLP was also present from C25 up to C29 sub-fraction, being detected in 6 out of 12 tested C sub-fractions (Fig. 3.44.). As expected, CYN was not detected in C sub-fractions. Numerous chromatographic peaks other than CLP and

CYN were also detected in HPLC sub-fractions of *C. racemosa* indicating a presence of various chemical compounds.

3.4.3. *C. taxifolia*

As it was the case with *C. racemosa*, LC-MS analyses on ABC fractions of *C. taxifolia* did not detect characteristic peaks of both CYN and CLP in the fraction A (Fig. 3.45.). Dominant CYN peak was detected at 10.49 min in the TIC LC-MS chromatogram of the fraction B of *C. taxifolia* (Fig. 3.45.). The formation of CYN producing ion on the mass spectrum was at m/z 397.162 in the positive ion mode responding to the obtained mass spectrum values for purified CYN (Fig. 3.45.). Numerous other chromatographic peaks were detected in the fraction C but there was no characteristic peak of CYN.

Nine B and 13 C sub-fractions of *C. taxifolia* obtained by HPLC were analyzed by LC-MS (Table 2.8. in the Materials and Methods section). LC-MS analyses on HPLC sub-fractions further confirmed that CYN was the dominant compound in *C. taxifolia* detected in B sub-fractions (Fig. 3.46.). The dominant peak of CYN was detected at 10.47 min in the TIC LC-MS chromatogram in the sub-fraction B26 (Fig. 3.46.). The following sub-fraction B27 had about 10 times less CYN than B26, detected at 10.52 min while other sub-fractions did not contain characteristic CYN signal (Fig. 3.46.). Therefore, 2 out of 9 tested B sub-fractions of *C. taxifolia* contained CYN. CLP characteristic peak was detected only in sub-fraction B24 of *C. taxifolia*, and its level was approximately 3 - 4 times less than CYN in B26. As expected, CYN was not detected in C sub-fractions of *C. taxifolia* but neither was CLP. Numerous chromatographic peaks other than CLP and CYN were also detected in HPLC sub-fractions of *C. taxifolia* indicating presence of various chemical compounds.

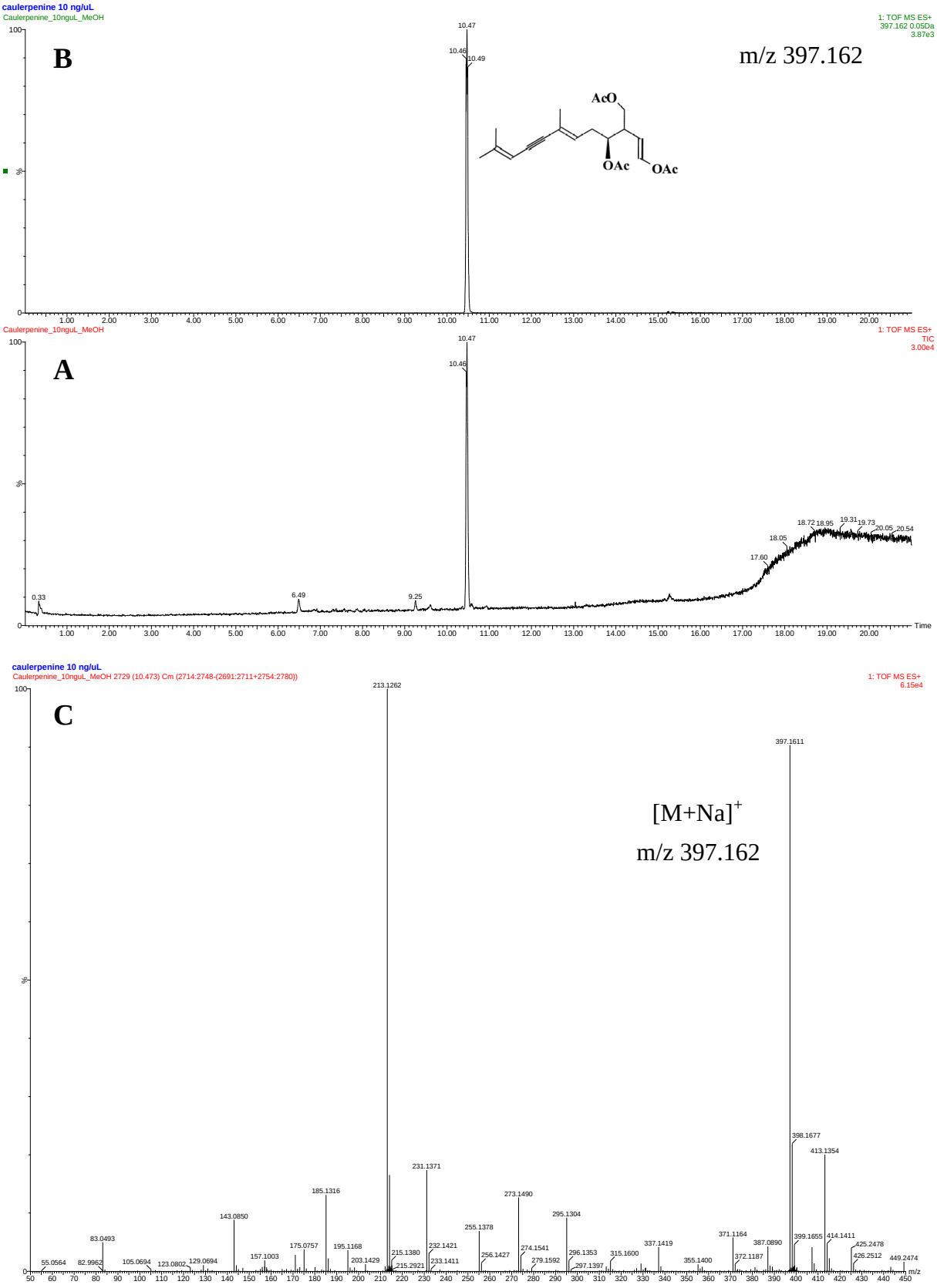


Figure 3.41. LC/MS chromatogram of the standard caulerpenyne (CYN) solution, showing total ion current chromatogram (**A**) and extracted ion chromatogram reconstructed using accurate mass of the CYN adduct with sodium (**B**), and mass spectrum of CYN using positive electrospray ionization (**C**).

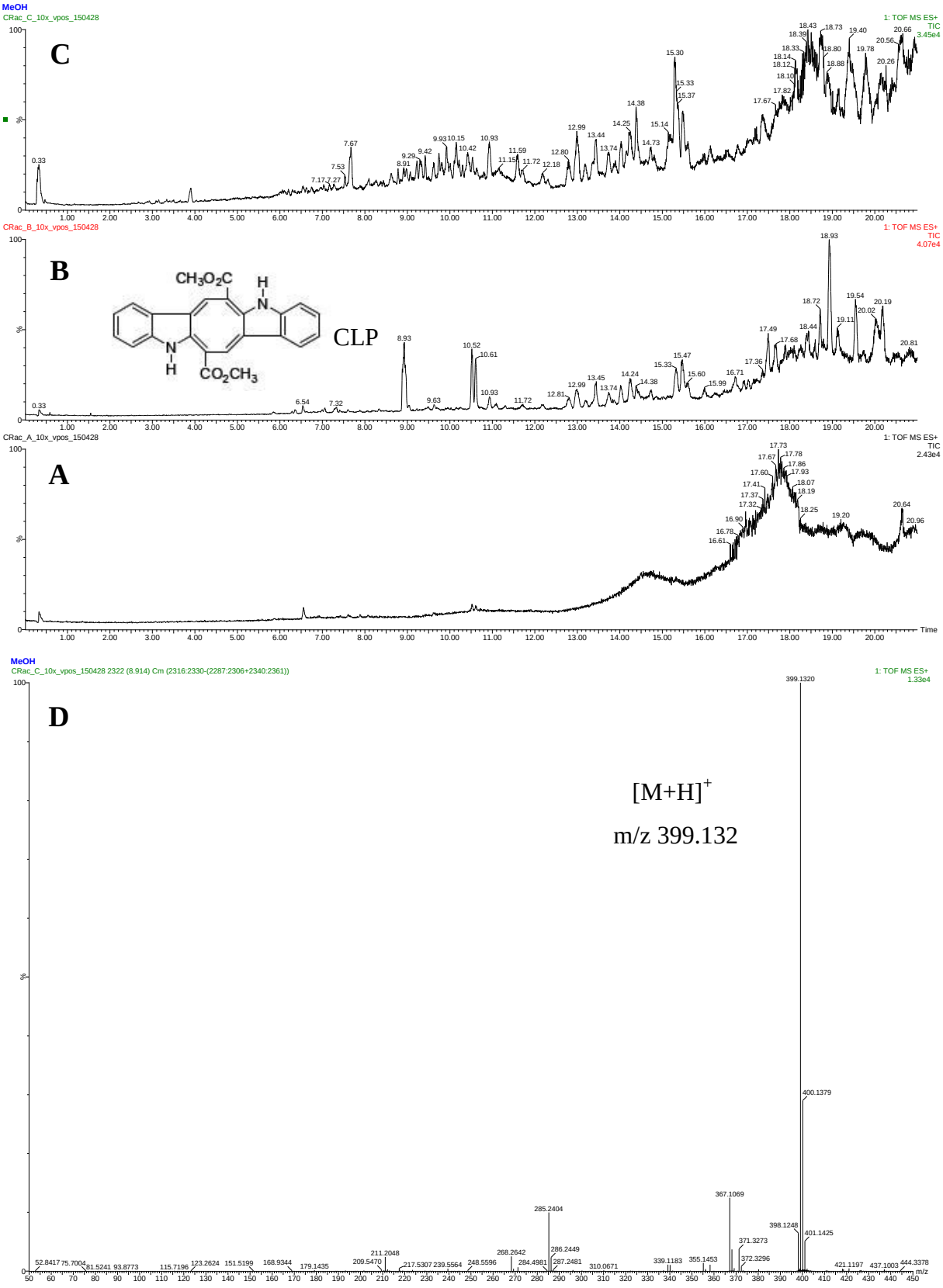


Figure 3.42. LC/MS chromatograms of hexane (**A**), dichloromethane (**B**) and methanol (**C**) fractions of the total extract of *Caulerpa racemosa* (see the experimental section for details). The mass spectrum of the dominant peak at 8.93 min in the chromatogram B, attributed to caulerpin (CLP), is shown in Fig. 3.42. **D**.

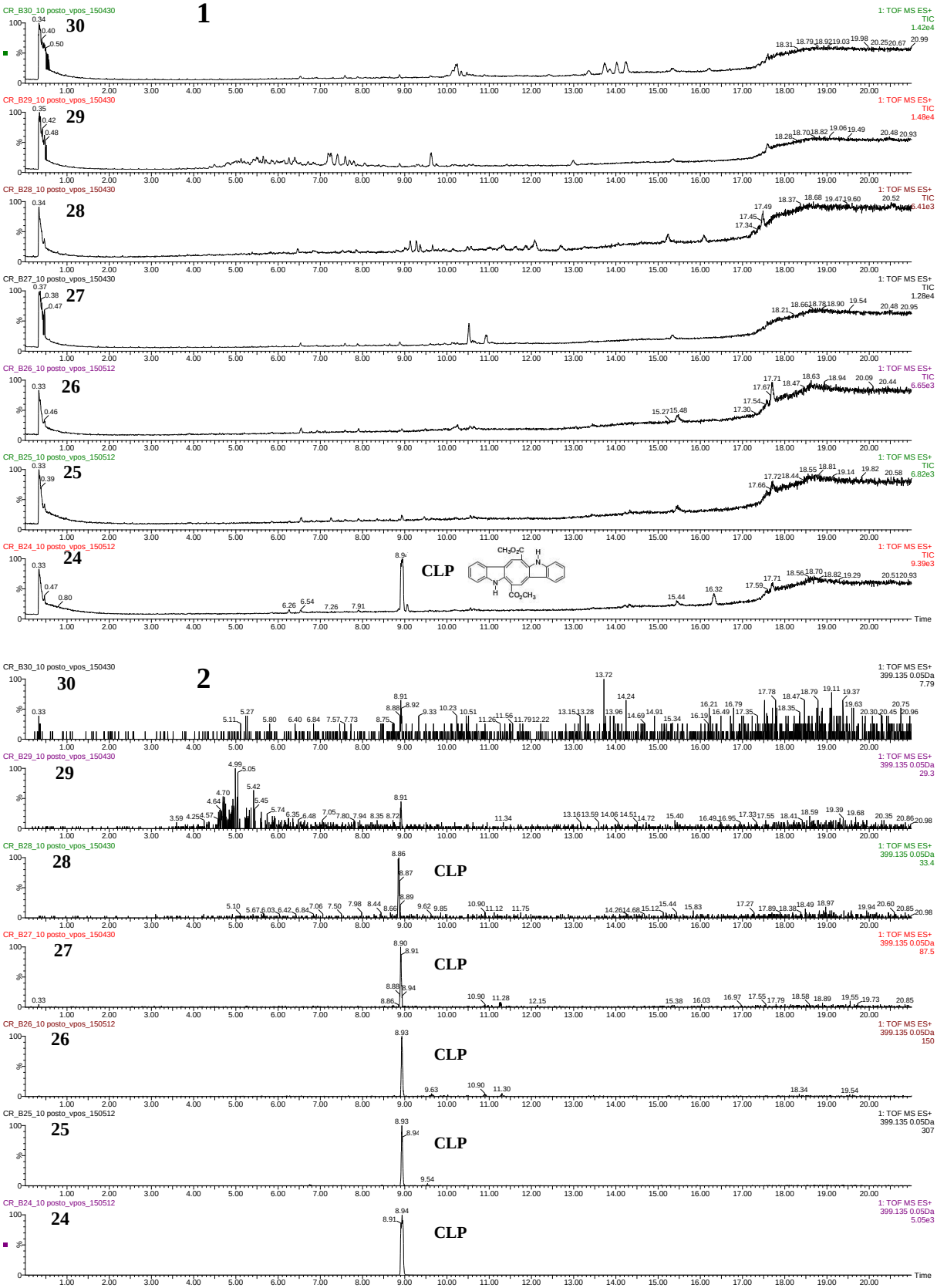


Figure 3.43. LC/MS chromatograms of detailed HPLC fractions of the sub-fraction B from *Caulerpa racemosa* showing total ion current chromatograms (**1**) and extracted ion chromatograms reconstructed on the basis of accurate mass of caulerpin (CLP) m/z 399.136 (**2**). CLP is predominantly eluted in fraction B24 with some minor percentages in fractions B25-B28 and the least in B29.

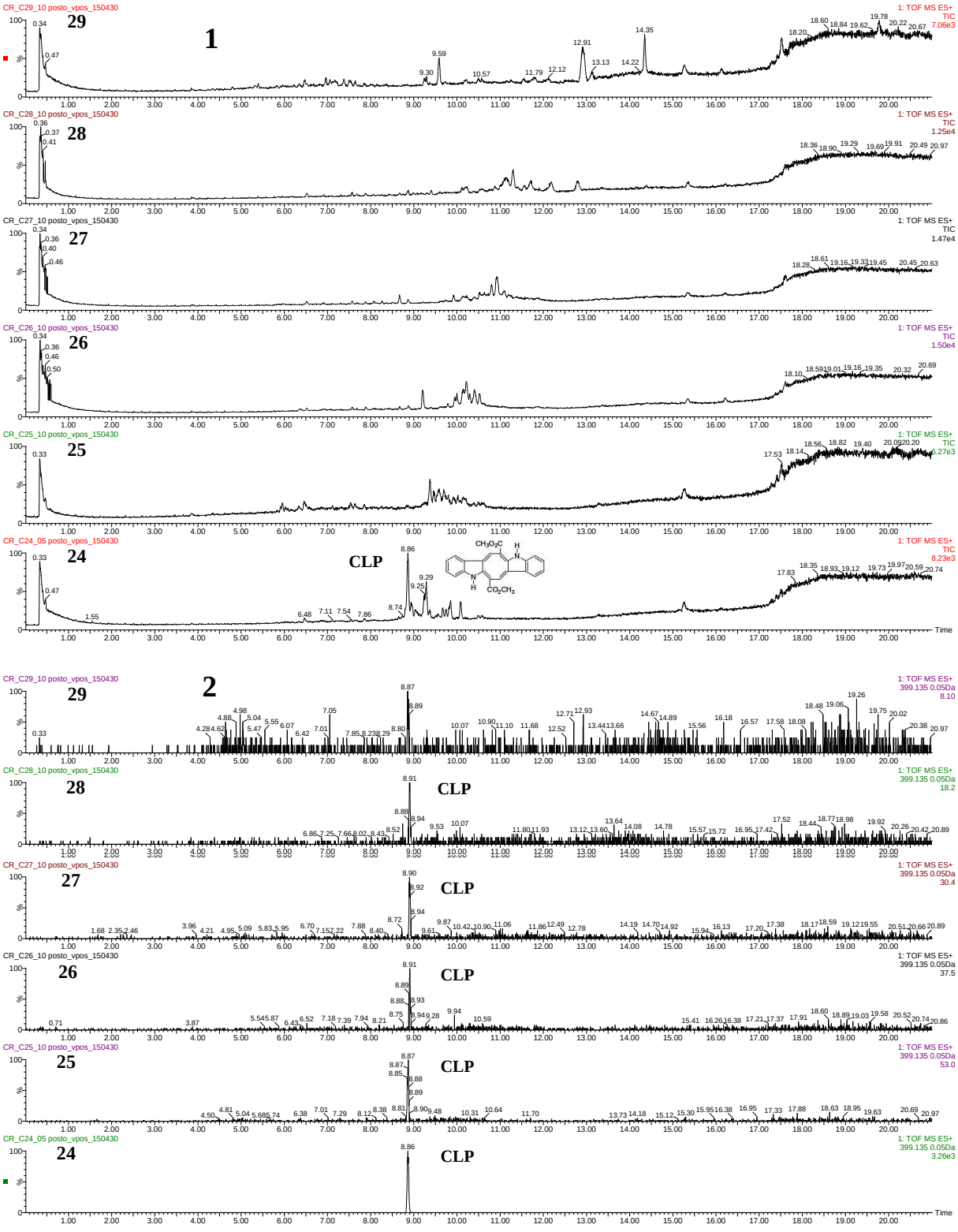


Figure 3.44. LC/MS chromatograms of detailed HPLC fractions of the sub-fraction C from *Caulerpa racemosa* showing total ion current chromatograms (**1**) and extracted ion chromatograms reconstructed on the basis of accurate mass of caulerpin (CLP) m/z 399.136 (**2**). CLP is predominantly eluted in fraction C24 with some minor percentages in fractions C25-C28 and the least in C29.

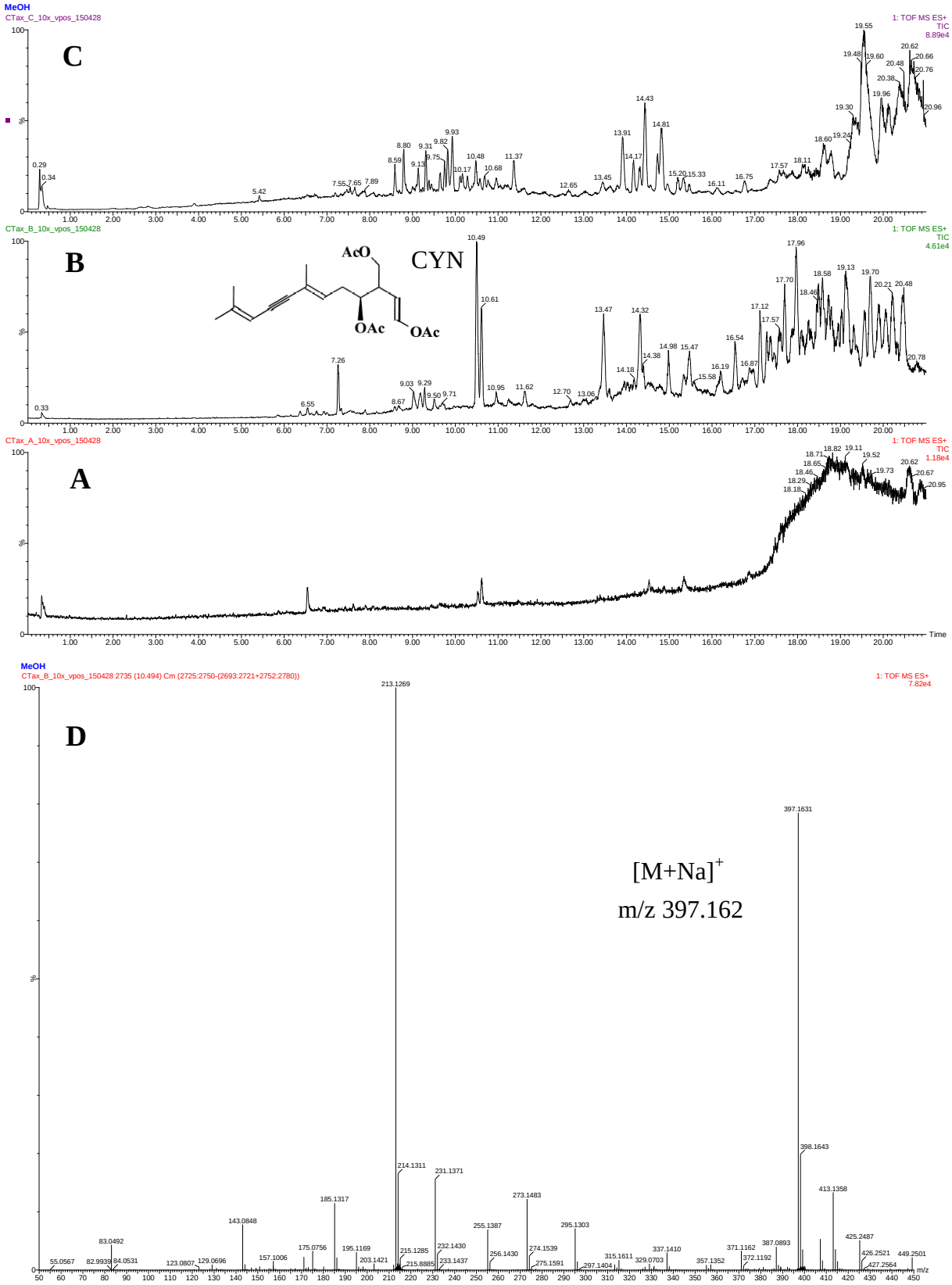


Figure 3.45. LC/MS chromatograms of hexane (**A**), dichloromethane (**B**) and methanol (**C**) fractions of the total extract of *Caulerpa taxifolia* (see the experimental section for details). The mass spectrum of the dominant peak at 10.49 min in the chromatogram B, attributed to caulerpenyne (CYN), is shown in Fig. 3.45. **D**.

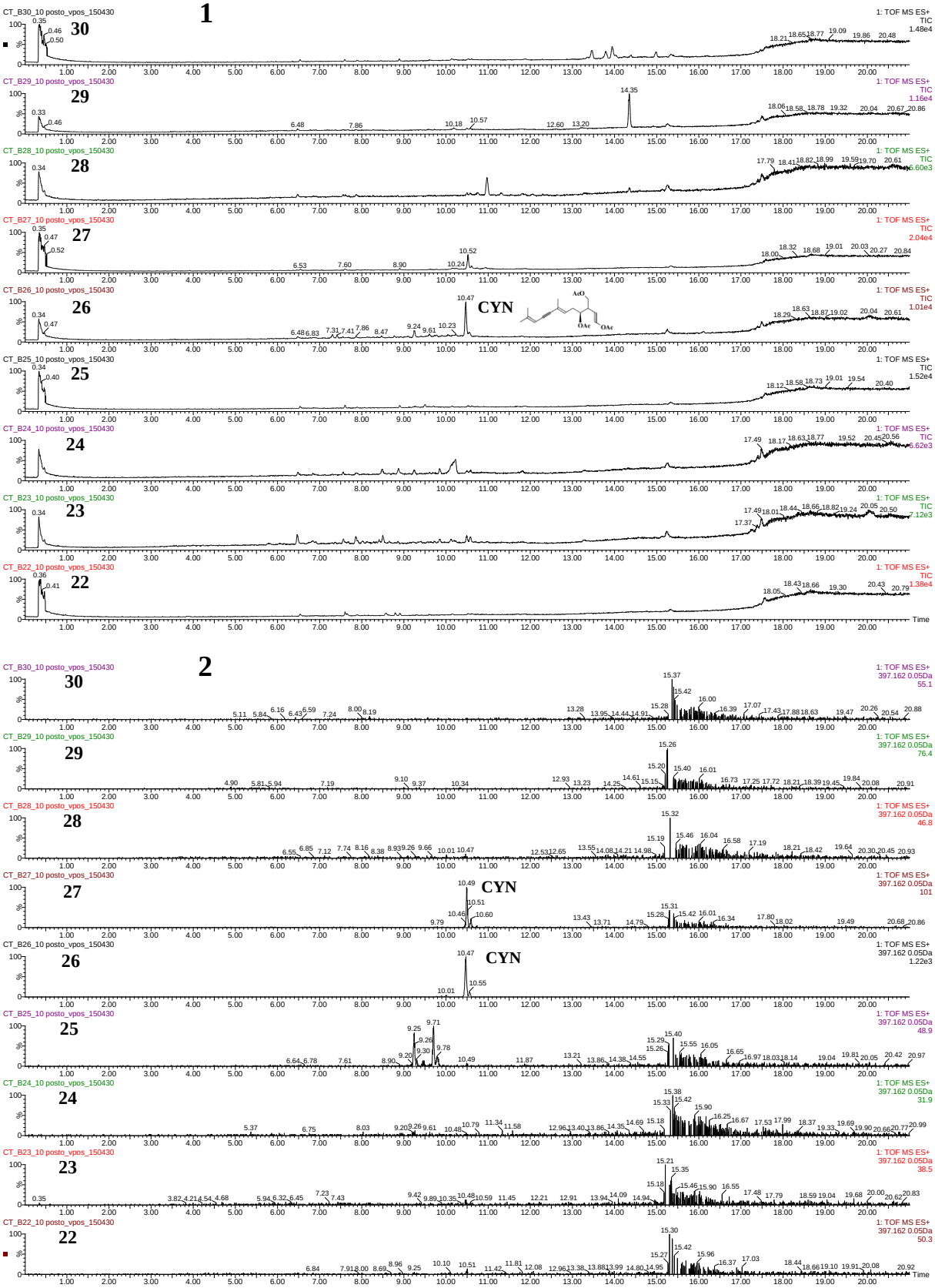


Figure 3.46. LC/MS chromatograms of detailed HPLC fractions of the sub-fraction B from *Caulerpa taxifolia* showing total ion current chromatograms (**A**) and extracted ion chromatograms reconstructed on the basis of accurate mass of caulerpenyne (CYN) adduct with sodium m/z 397.162 (**B**). CYN is predominantly eluted in fraction B26 with some minor percentage in fraction B27.

3.5. TOXICOLOGICAL EFFECTS OF PURE CAULERPENYNE ON UPTAKE TRANSPORTERS

The activity of uptake transporter DrOatp1d1 present in zebrafish has been inhibited by pure CYN. DrOatp1d1 uptake dose-response curve was obtained within the CYN concentration range from 267 (or dilution 1:100) to 1.335 µM (or dilution 1:20000) (Fig. 3.47.). A decrease in uptake of model substrate LY was observed, resulting in 25% of uptake determined in non-treated cells observed at the maximum concentration of CYN (Fig. 3.47.). DrOatp1d1 inhibitory potential of CYN standard was 17.97 µM (IC50 value).

On the contrary, pure CYN demonstrated a weak interaction with DrOct1 uptake transporter. Any significant dose-response curve was not obtained because at the highest concentration of CYN (267 µM or dilution 1:100) the uptake of ASP+ substrate decreased only to 74% in comparison to non-treated cells (Fig. 3.47.). IC50 value is not shown since it could not be obtained.

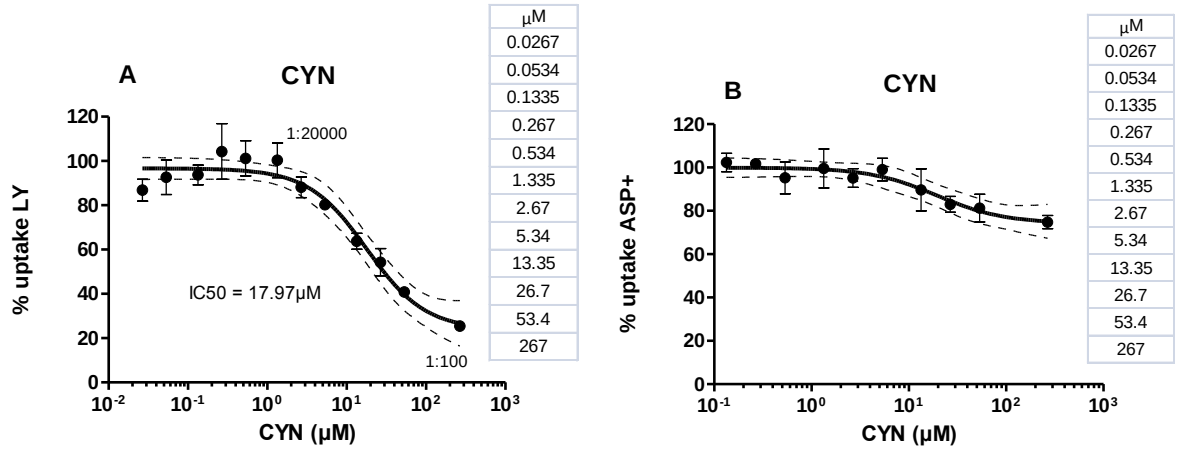


Figure 3.47. Concentration-dependent inhibition of DrOatp1d1 (**A**) and DrOct1 (**B**) transport activities by pure caulerpenyne (CYN) standard. Results are shown as percentages of LY and ASP+ uptake in HEK293 cells. Mean ± SD values with 95% CI are shown as calculated from duplicates. CYN standard was tested in 2 independent experiments. Concentrations of CYN standard expressed in µM are shown on the right side of each panel.

The entire EDA workflow with detected toxic effects of *C. taxifolia* and *C. racemosa* samples and identified secondary metabolites are presented in Figs. 3.48. and 3.49., respectively.

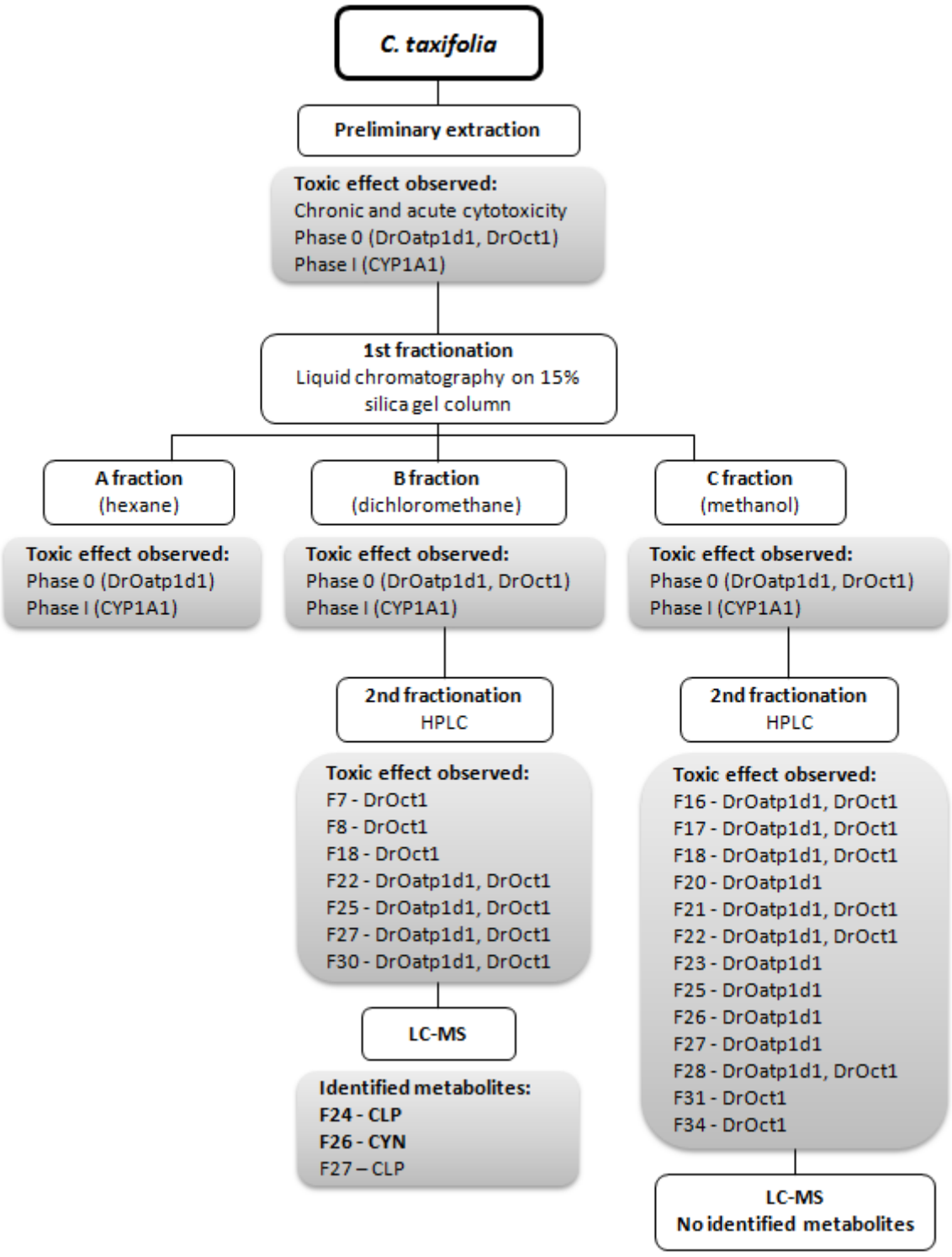


Figure 3.48. Schematic presentation of the workflow used for determination of *C. taxifolia* toxic effects in our modified EDA study, along with the identified secondary metabolites (CYN - caulerpenyne, CLP - caulerpin).

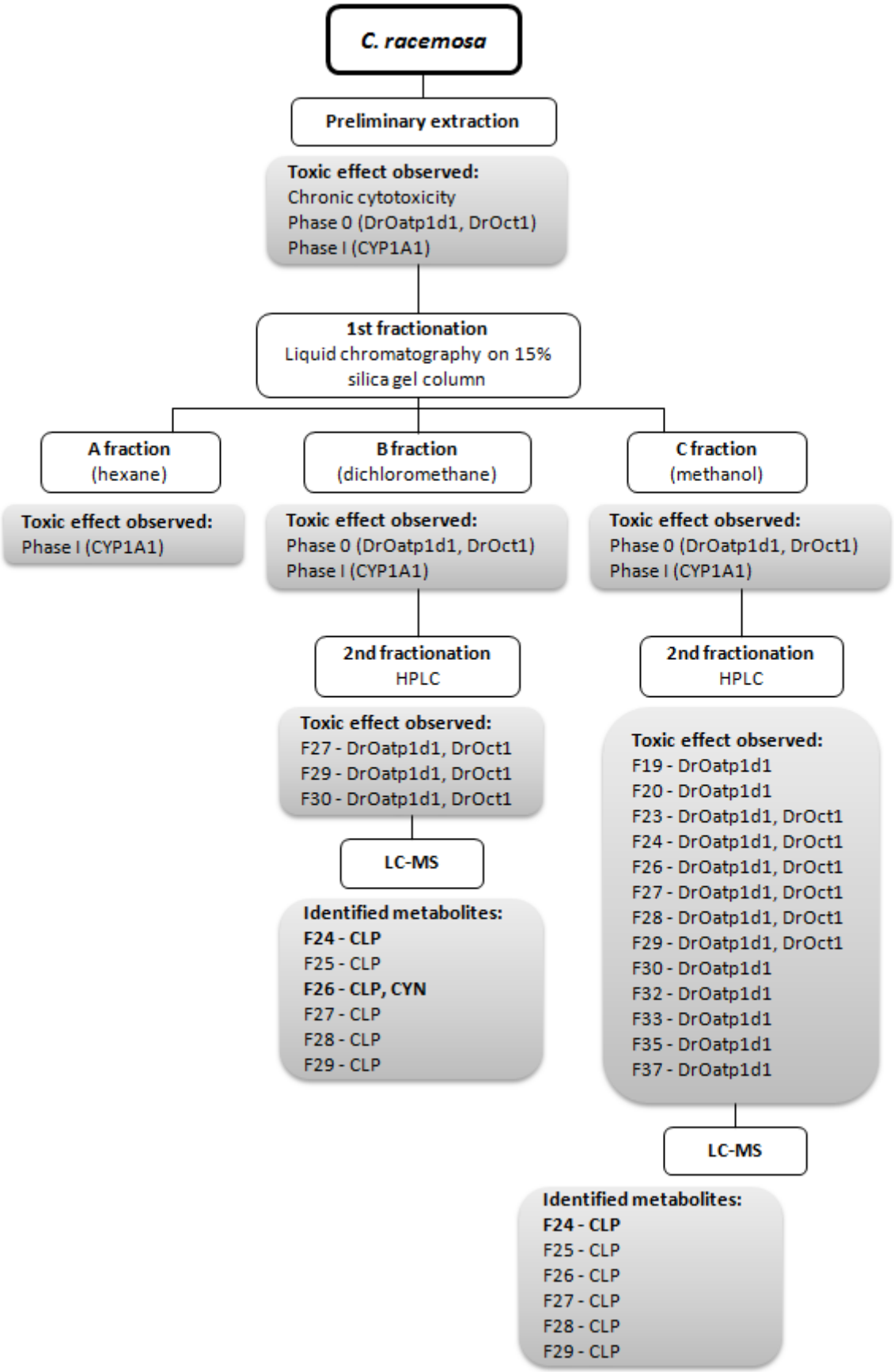


Figure 3.49. Schematic presentation of the workflow used for determination *C. racemosa* toxic effects observed in our modified EDA study, along with the identified secondary metabolites (CYN - caulerpenyne, CLP - caulerpin).

4. DISCUSSION

In this research the EDA concept was applied for the first time for a detailed ecotoxicological characterization of complex biological samples. In addition, we modified the traditional EDA approach by developing and using protocol based on a series of *in vitro* or small-scale bioassays rather than 1-2 biotests. The tests used in our approach were both high-throughput screening techniques that utilize minimal amounts of a test sample, and methods that address toxicologically relevant endpoints ranging from acute and chronic toxicity to interaction with critical phases of cellular detoxification. Finally, although the EDA was originally developed as a research tool for prioritization of environmental contaminants present in complex environmental samples highly affected by human activity, in this study we demonstrate a new potential of this concept that may be applied for research focused on identification of new and unknown toxic compounds of potential biomedical and/or pharmacological relevance. As biological effects monitored in our EDA study were the most pronounced for *Caulerpa* samples, resulting in the most powerful biological responses in respect to several toxicological endpoints, our EDA research at this stage focuses more on *Caulerpa* species.

4.1. Ecotoxicological characterization of invasive green algae *C. racemosa* and *C. taxifolia*

Secondary metabolites synthetized by *Caulerpa* species play a major role as evolutionary developed chemical defense mechanisms against various herbivores, epiphytes and other competitors, and most often cause mortality to the cells of the surrounding organisms (Paul and Fenical, 1986; Pohnert and Jung, 2003; Erickson et al., 2006). That was also demonstrated in our study were both *Caulerpa* species were shown to be cytotoxic exhibiting acute and/or chronic toxicity, especially *C. taxifolia*. Results of the initial toxicity testing of preliminary extracts indicated that *C. racemosa* and *C. taxifolia* caused chronic toxicity with similar responses (LC50 of 23 and 39 μg/mL, respectively; Fig. 3.3.). *C. taxifolia* was shown to cause strong acute toxicity as well (LC50 47 μg/mL; Fig. 3.4.). On the other hand no acute toxicity was observed for *C. racemosa* preliminary extract. These data are in agreement with the previous study on the acute toxicity of CLP and caulerpicin isolated from *C. racemosa* var. *clavifera* which also reported negative results (Vidal et al., 1984). Another study on a human melanoma cancer cell line demonstrated low cytotoxic effect of the crude extract of *C. racemosa* to C32 cell line, while pure CLP had no acute toxicity (Rocha et

al., 2007). Cytotoxic effects of CYN from *C. taxifolia* have been also demonstrated on mammalian and fish cells, mouse, mollusks and sea urchin eggs in previous studies (Paul and Fenical, 1986; Lemee et al., 1996; Parent-Massin et al., 1996; Pesando et al., 1996; Barbier et al., 2001). CYN and CYN-derived metabolites are considered to be strong cytotoxic agents, as was also demonstrated in our study.

Further results showed that both *Caulerpa* species interact with phase 0 of the cellular detoxification mechanism. This is the first report related to this mechanism of toxic action of *Caulerpa* species, as no previous studies on the effects of *Caulerpa* metabolites on the activity of the uptake transmembrane transporters have been made. In particular, both *Caulerpa* preliminary extracts showed the highest impact on DrOatp1d1 uptake transporter, with IC50 values of 3.5 and 27.15 μg/mL for *C. taxifolia* and *C. racemosa*, respectively (Fig. 3.9.). Transport activity of DrOct1 transporter was weakly inhibited by preliminary extracts of *C. racemosa*, while *C. taxifolia* demonstrated relevant interaction with DrOct1 transporter, although its inhibitory potential was lower compared to DrOatp1d1 (IC50 44 μg/mL; Fig. 3.9.).

After obtaining the results from the preliminary phase of our EDA study we were able to characterize the toxicant (or a group of toxicants) of concern as polar anionic compound(s). In the case of *C. racemosa* that was more evident, while interaction with anion and cation transporter indicates the presence of multiple bioactive compounds in *C. taxifolia*. The ABC fractions further confirmed the more complex composition of *C. taxifolia* secondary metabolites, as a significant DrOatp1d1 inhibitory potential was determined in all three fractions with similar effects; IC50 values obtained for A, B and C fraction were 4.84, 9.18, and 6.3 μg/mL, while interaction with DrOct1 was not observed in any of the fractions (Fig. 3.19.). This indicates that more different types of chemical compounds with distinct polarity features, that can exert the same or similar toxic effect on uptake transporters, are present in *C. taxifolia*. Nevertheless, we observed loss of the inhibitory effect of *C. taxifolia* ABC fraction to DrOct1 transport compared to moderate toxic response determined in preliminary extracts. It can be attributed to the losses of the sample material that arise when extracts are being passed through silica gel column that can leave behind the cationic compounds. On the contrary, the interaction with DrOatp1d1 transporter was observed only in C fraction of *C. racemosa* (IC50 8.3 μg/mL; Fig. 3.19.) indicating that the toxicant causing the adverse effect is of hydrophilic nature. Furthermore, weaker but relevant inhibition of DrOct1 transporter was also recorded only in the C fraction of *C. racemosa*. Therefore, regarding *C. racemosa*

metabolites we can say that a more specific group of compounds causes adverse effects on the uptake transporters. The involvement of membrane uptake proteins in the transport of metabolites from the genus *Caulerpa* suggests that these compounds are probably of higher molecular weights, implying that they cannot enter the cell passively, i.e. without interaction with uptake transporters.

Toxic effects of *Caulerpa* species have been also observed in their interaction with the phase I detoxification enzymes in our EDA study. This is in accordance with the well-known activation step of the cellular detoxification and suggests that once the causable toxicant or a group of toxicants enter the cell, they are being metabolized by the activation of biotransformation enzymes. Induction of the AhR and subsequently the CYP1A1 activity was caused by *C. racemosa* and *C. taxifolia* preliminary extracts with high enzymatic activities obtained: 1.6 pmol/min/mg and 2.4 pmol/min/mg, respectively (Fig. 3.10.). Here again, stronger CYP1A1 induction capacity was observed for *C. taxifolia*, with the lowest EC50 (4.9 µg/mL; Fig. 3.13.) and the highest TCDD Eq (900 pg TCDD eq/mg; Fig. 3.12.) values. After obtaining and testing the ABC fractions, the induction capacity of *C. taxifolia* fraction weakened, while *C. racemosa* A, B and C fractions showed the highest CYP1A1 activity of 8.4, 4.66 and 5.35 pmol/min/mg, respectively (Fig. 3.23.). Nevertheless, for both *Caulerpa* species the strongest toxic effects were observed in the A fraction that contains only lipophilic compounds. Biotransformation CYP enzymes primarily act on nonpolar and lipophilic substances, metabolizing them by addition of functional (polar) groups which consequently increases their solubility. This indicates the presence of lipophilic compounds from *C. racemosa* and *C. taxifolia* that are being transformed by phase I enzymes. These data are in accordance with the previous studies that showed the activation of CYP enzymes by *Caulerpa* toxic metabolites. A study by Feline et al. (2012) that investigated the molecular effects of CLP in white seabream (*Diplodus sargus*) demonstrated activation of CYP enzymatic pathway. Furthermore, in a recent study that monitored cellular responses upon CLP exposure, the authors concluded that *C. racemosa* can modulate biotransformation in white seabeam (Gorbi et al., 2014). Previous study on effects of CYN on scorpio fish (*Scorpaena porcus*) by Uchimura et al. (1999a) demonstrated that it modulates CYP activity. However, it was shown by Uchimura et al. (1999b) that CYN acts as a CYP inhibitor. Indeed, CYN is a terpenoid, a lipophilic compound that can easily enter through biological membrane thereby becoming available to interact with CYP enzymes on the endoplazmatic reticulum. CLP is a bis-indol alkaloid that is a phytoconstituent and red pigment in *C. racemosa*, and also a

lipohilic compound. Therefore, CLP appears to be a reliable candidate for the observed effects of CYP induction while CYN is probably not.

In disagreement with previous research, our EDA study showed no effect of *Caulerpa* species to the phase II of the detoxification mechanism. Purified cytosolic GST enzymes from zebrafish revealed no change in the activity while exposed to *C. racemosa* and *C. taxifolia* preliminary extracts. The involvement of CYP enzymes in metabolization of *Caulerpa* complex metabolites implies the subsequent activation of GST enzymes. Earlier studies on *Caulerpa* metabolites demonstrated the activation of phase II enzymatic pathway in various organisms. A gastropod *Bittium reticulatum*, scorpio fish, teleost *Coris julis*, sea urchin *Paracentrodus lividus* and white seabeam were all exhibiting higher GST after being exposed to CYN or CLP from *Caulerpa* species (Uchimura et al., 1999; Sureda et al., 2006; Sureda et al., 2009; Tejada et al., 2013; Gorbi et al., 2014). The observed negative result in our EDA study may be attributed to the fact that GST enzymes are responsive to phase I metabolites of the biotransformation pathway and that for activation of GST enzymes the potential GST metabolites have to be pre-activated by reactions of oxidation, hydrolysis or reduction during the first phase of the detoxification. Since all of the biotransformation phases were tested separately in our study, a further modification of the phase II (GST) biotesting protocol may be needed, e.g., by addition of the phase I enzymatic activation (microsomal S9 fraction of fish liver) in the reaction mixture in order to pre-activate potential GST interactors. Secondly, previous studies addressed GST activity mostly using CDNB as a nonspecific model GST substrate which does not enable distinction among interactions of tested compounds/samples with multiple GSTs typically present in biological systems used. In our study, however, we tested interactions of tested samples with single, specific zebrafish cytosolic GSTs highly and controllably expressed in suitable expression system.

Similarly, no interactions with phase III efflux transporters were determined for *Caulerpa* species. *C. racemosa* and *C. taxifolia* preliminary extracts showed no change in the efflux activity of P-gp, while previous study on the marine sponge *Geodia cydonium* showed that multixenobiotic membrane P-gp pump is negatively affected by both *C. taxifolia* extract and pure CLP (Schröder et al., 1998). So far, this is the only study that monitored the effects of *Caulerpa* metabolites on any efflux transmembrane transporter. Again, the specificity of the system (sponge) probably characterized by a series of transporters present and functional in sponges, with overlapping substrate specificities, is much lower in comparison with our *in vitro* system characterized by high overexpression of a single transporter. Therefore, those

data are difficult to compare, and it is clear that interaction of either single substances or complex samples with MXR transporters should be performed using specific experimental systems with single transporters expressed in appropriate heterologous expression systems.

Strong toxic effects of *Caulerpa* ABC fractions on phase 0 and I detoxification mechanism led to selection of B and C fractions of *C. racemosa* and *C. taxifolia* for the second fractionation step, as these fractions were recognized as the most responsive ones, i.e. positive toxic effects were observed in more than one biological assay. HPLC sub-fractions of B and C fraction of *Caulerpa* species demonstrated interaction with DrOatp1d1 uptake transporter while DrOct1 transporter was less responsive to *Caulerpa* sub-fractions, as it was mostly the case with previous extracts and fractions (Figs. 3.30. - 3.33.). *C. racemosa* and *C. taxifolia* C sub-fractions exhibited highly toxic effects to DrOatp1d1 uptake activity where inhibition was detected in a larger number of C sub-fractions (13 and 11, respectively) than B sub-fractions (3 and 4, respectively). This confirms the putative toxicant(s) characteristics observed from previous phases of the study as polar and anionic compound(s). Furthermore, the strongest interactions with DrOatp1d1 transporter were observed in *C. racemosa* C sub-fractions during the high elution strength phase of the gradient HPLC (100% B). *C. racemosa* C sub-fractions C27 and C28 exhibited the strongest inhibitor potential (IC50 0.58 μg/mL and 0.78 μg/mL) but C23 also showed similar effect (IC50 1.03 μg/mL; Fig. 3.36.). The strongest inhibitory potential of *C. racemosa* B sub-fractions was detected in B29 (2.30 μg/mL IC50; Fig. 3.36.). ACN (B) is a polar aprotic solvent with large dielectric constant (37) and dipole moment (3.92 D) characterized by high polarity that efficiently dissolves charged species such as various anions used as nucleophiles (e.g. CN(-), HO(-), etc.). Since ACN is aprotic, meaning it lacks the hydrogen bonding (no O-H or N-H), these nucleophiles are relatively "free" in solution and thereby more reactive. Hence, we can say that the most reactive anionic compounds are present in *C. racemosa* C27 and C28 sub-fractions. C27, as well as B27 were the only two sub-fractions that demonstrated relevant interaction with DrOct1 transporter with 94 and 87% inhibition of cation transporter activity, respectively (Fig. 3.34.). Therefore, the sub-fraction C27 gave the most potent responses for both transporters, indicating that the toxicant(s) of concern is/are in this fraction. Indeed, chemical compounds are observed in C27, C28 and C23 sub-fractions of *C. racemosa* that are indicated as peaks on the initial HPLC chromatogram (Fig. 3.38.). Other higher peaks have been detected on *C. racemosa* C fraction chromatogram as well, and the range of C sub-fractions that exerted responses to DrOatp1d1 and DrOct1 correspond to the detected peaks on the chromatogram. The matching

of the detected chemical compounds and biological effects confirms the main principle of cause-effect relationship in our EDA study. Chromatogram of detected compound from B sub-fractions of *C. racemosa* also corresponds to the observed effects on both uptake transporters (Fig. 3.37.).

Sub-fractions C23 and C27 were the most responsive to DrOatp1d1 activity in *C. taxifolia* C fraction similarly to *C. racemosa* (IC50 3.45 μg/mL and 3.1 μg/mL; Fig. 3.36.). B22 sub-fraction on the other hand demonstrated the strongest DrOatp1d1 inhibitory potential (IC50 5.5 μg/mL; Fig. 3.36.). As it was the case for *C. racemosa*, B27 fraction of *C. taxifolia* displayed the most relevant interaction with DrOct1 transporter (Fig. 3.35.). All sub-fractions mentioned above are obtained also in the high elution strength phase of the gradient HPLC (100% B). Initial chromatogram for the *C. taxifolia* C sub-fractions detected a series of numerous overlapping peaks displaying a very complex nature of chemical compounds present in this fraction and thereby confirming the previously observed pattern for *C. taxifolia* (Fig. 3.40.). C fraction possesses a more complex composition of metabolites and certainly demands further chemical analysis. Chemical properties of the *C. taxifolia* toxicant(s) that is(are) responsible for the adverse effect on cellular uptake transporters is(are) similar to those toxicants observed for *C. racemosa*; polar and mainly anionic. Furthermore, the presence of chemical compounds in the most responsive sub-fractions of *C. taxifolia* (C23, C27, B22, and B27) was detected on chromatograms (Figs. 3.39. and 3.40.). Biological effects observed for other B and C sub-fractions also corresponded to the detected chemical compounds on the associated chromatograms.

Further step in our EDA study included initial attempts directed to identification of chemical compound(s) in the active HPLC sub-fractions of both *Caulerpa* species by performing UPLC coupled with QTOFMS, a method that provides superior chromatographic efficiency and separation power (Terzic and Ahel, 2011). The distribution of two secondary metabolites in *Caulerpa* species, CYN and CLP, was monitored in the active HPLC sub-fractions of *Caulerpa*. Obtained LC-MS chromatograms and mass spectrum of CYN producing ion at m/z 397.162 shown in Figure 3.41. was in accordance with previous data detecting CYN at m/z 392 and 397 by ionspray ionization mass spectrometry method (Raffaelli et al., 1997). Further LC-MS analyses on ABC fractions of the *Caulerpa* species total extract identified two very distinctive chemical structures both present as major metabolites in these species. While CYN is the dominant compound in *C. taxifolia*, CLP signal is more abundant in *C. racemosa* (Figs. 3.42. and 3.45.). This data confirms the results

from previous studies (Schwede et al., 1986; Guerriero et al., 1992; Terlizzi et al., 2011). CYN was eluted only in B fraction of *C. taxifolia* while CLP was present both in B and C fractions of *C. racemosa*, indicating that CLP is more polar compound than CYN (Figs. 3.42. and 3.45.).

Bioassays on ABC fractions of both *Caulerpa* species demonstrated the most relevant toxicological effects on phase 0 and I of detoxification pathway exactly in fractions B and C (Fig. 3.48. and 3.49.). TIC LC-MS chromatograms of fraction A of both *Caulerpa* species did not detect any dominant peaks nor the characteristic peaks of CYN and CLP (Figs. 3.42. and 3.45.). Consequently, this fraction was considered of minor toxicological importance in our EDA workflow (Fig. 3.48. and 3.49.). Considering numerous other chemical compounds that have been detected by LC-MS, there are indications that CYN and CLP metabolites are also present in ABC extracts of *Caulerpa* species, such as epoxides and alcoholic compounds as metabolites of CYN, and caulerpinic acid in *C. racemosa* that is an alkaline hydrolysis product of CLP (Alarif et al., 2010). Nevertheless, further analytical analyses and confirmation studies with corresponding standards are needed to test these observations.

Detailed LC-MS analyses of HPLC sub-fractions of *C. racemosa* revealed that CLP is dominant compound in B24 and C24 sub-fractions, with minor presence from B25 to B29 or C25 to C29 sub-fractions (Figs. 3.43. and 3.44.). However, no toxic effects were observed in sub-fraction B24 or B25 of *C. racemosa*. The most potent DrOatp1d1 inhibitory potential (IC50 2.3 µg/mL; Fig. 3.36.) was observed in sub-fraction B29 where almost no CLP was found (Fig. 3.43.). DrOct1 activity was the most inhibited in B27 sub-fraction (Fig. 3.34.). On the other hand, in CLP dominant sub-fraction C24 toxic effects were observed where inhibitory potential for DrOatp1d1 was 4.22 µg/mL and DrOct1 uptake activity was reduced to 30% at maximum sample concentration (Figs. 3.34. and 3.36.). However, toxicologically most relevant C sub-fractions of *C. racemosa* were C27 and C28 where minor percentages of CLP were found (Fig. 3.44.). Overall, this suggests that CLP is not the main culprit responsible for the toxic effects observed in B and C sub-fractions of *C. racemosa*. Polar chemical structure(s) other than CLP are probably more relevant for the observed effects.

Opposite to CLP in *C. racemosa*, detailed LC-MS analyses of HPLC sub-fractions of *C. taxifolia* revealed that CYN is dominant compound in B26 with minor presence in B27 (Fig. 3.46.). Unlike CLP that disperses in 6 B or C sub-fractions of *C. racemosa*, CYN is eluted in one or two B or C sub-fractions of *C. taxifolia* further contributing to the observation

that CLP is more polar chemical structure compared to CYN. So far, sub-fraction B26 was not evaluated by toxicological bioassays but DrOatp1d1 inhibitory potential was observed in B27 (IC50 15.15 μg/mL; Fig. 3.36.) where the strongest DrOct1 inhibition was determined as well (only 19% of ASP+ uptake) (Figs. 3.35. and 3.36.). However, the most toxic sub-fractions to DrOatp1d1 activity were B22 and B25 where no presence of CYN was detected (Fig. 3.46.). Nevertheless, toxicological effects of pure CYN standard showed that CYN is a potent DrOatp1d1 inhibitor with low IC50 value of 17.97 μM (Fig. 3.47.). This indeed suggests that CYN-like chemical structures could be responsible for the toxic effects observed in B fraction of *C. taxifolia*. Weaker inhibitory effect of pure CYN to DrOct1 transporter is similar to the previous observation of DrOct1 toxic effects of HPLC sub-fractions.

4.2. Ecotoxicological characterization of cyanobacterial strains

Cyanobacteria are producers of a vast number of secondary metabolites that can manifest all kinds of biological activities to other living organisms, one of which is cytotoxicity. Our EDA study demonstrated cytotoxic effects of six terrestrial and aquatic cyanobacterial strains whose preliminary extracts caused chronic toxicity to freshwater algae. The most cytotoxic strain was *Nostoc* that obtained the most potent chronic inhibitory potential; LC50 values obtained for *Nostoc* Z1 and *Nostoc* S8 were 163 μg/mL and 196 μg/mL, respectively (Fig. 3.3.). The only identified cyanotoxin in the *Nostoc* genera is microcystin whose acute and chronic cytotoxic effects to animals and humans have been already observed (WHO, 1998; Duy et al., 2000; Dietrich and Hoeger, 2005). The main mechanism of microcystin toxicity involves inhibition of PP1/2a as the key regulatory enzymes in catalyzing dephosphorylation of serine/threonine residues in various phosphoproteins (structural proteins, enzymes, regulators) that leads to the loss of cytoskeletal integrity and subsequent cell apoptosis (Dietrich and Hoeger, 2005). *Anabaena*, *Oscillatoria* and *Phormidium* strains are also microcystin producers that showed cytotoxic effects, though the lowest chronic toxicity was observed in *Oscillatoria* K3 extract (LC50 738 μg/mL; Fig. 3.3.). Nevertheless, cyanobacterial strains possess highly complex ecotoxic potential that can be caused by other cyanobacterial components such as lipopolysaccharides, various peptides or other unidentified metabolites. Despite the previous observations, our EDA study did not detect significant acute toxicity of cyanobacterial strains which can be attributed to the fact that acute cytotoxicity was determined on cyanobacterial extracts and not the pure cyanotoxins.

All six preliminary extracts of cyanobacterial strains demonstrated interaction with the phase 0 of the detoxification pathway, primarily with DrOatp1d1 transporter. Low or no inhibition of DrOct1 was recorded for all tested cyanobacterial strains. Only *Nostoc* S8 exhibited 70% inhibition of DrOct1 uptake (Fig. 3.8.). This indicates that charged anion toxicant(s) is(are) present in preliminary extracts of cyanobacterial strains that cannot enter the cell by passive diffusion. The most responsive strain to DrOatp1d1 inhibition was aquatic strain *Oscillatoria* K3 with inhibitory potential of 263 μg/mL (IC50) (Fig. 3.9.). Moreover, aquatic *Nostoc* Z1 also exhibited similar toxic effects with IC50 value of 314 μg/mL. The weaker inhibitory potential was observed for terrestrial *Anabaena* Č5 strain with IC50 value of 810 μg/mL. We can observe that aquatic strains contain chemical compounds that are more reactive to anion uptake transporter. Microcystin is the major cyanotoxin in both aquatic cyanobacterial genera, *Oscillatoria* and *Nostoc*. Previous studies on microcystin showed that this cyanotoxin is a high molecular weight compound (909 to 1115 Da) that enters the cell by active transport (Kurmayer and Christiansen, 2009). So far the identified transporters for uptake of microcystins include Oatp1d1 from the liver of little skate *Leucoraja erinacea* (Meier-Abt et al., 2007), rat Oatp1b2 and human OATP1B1, OATP1B3 and OATP1A2 transporter from *Xenopus levis* expression system (Fischer et al., 2005), OATP3A1 and OATP4A1 from human epithelial colorectal adenocarcinoma cells (Zeller et al., 2011). Microcystins can act as anionic compounds due to functional groups present in their cyclic heptapeptid structure that enables them to form charged molecules. Thereby, it seems clear that microcystins can be transported into the cell by anion uptake transporters. Furthermore, freshwater cyanobacteria are characterized by the presence of acidic (aspartate and glutamate) and basic (arginine, lysine, histidine) amino acids in their secondary metabolites that are hydrophilic.

The ABC fractions of cyanobacterial strains further confirmed the *Oscillatoria* K3 strain as the most responsive strain to phase 0 transporters. *Oscillatoria* K3 strain was the only strain that exhibited toxic effects to both DrOatp1d1 and DrOct1 in all three fractions although the most relevant effects were obtained for B and C fraction (Fig. 3.22.). The inhibitory potential obtained in A, B and C fractions of *Oscillatoria* K3 were similar for both organic transporters; DrOatp1d1 inhibitory concentrations of A, B and C fraction were 56, 42 and 38 μg/mL, respectively, and DrOct1 inhibitory concentrations were 53, 37 and 46 μg/mL, respectively (Fig. 3.22.). This indicates that *Oscillatoria* K3 possesses a diverse group of metabolites with different polarity affinities that are able to cause toxic effects to phase 0

transporters. Based on chemical properties of microcystins we can assume that they are extracted in B and most likely in C fraction. Genus *Oscillatoria* also produces anatoxin-a, which is characterized by high polarity like all neurotoxins, and can also be extracted in B and C fractions. Although preliminary extract of *Oscillatoria* K3 showed weaker DrOct1 uptake inhibition, a group of medium polarity compounds was extracted after the separation on the column that demonstrated stronger response to DrOct1 uptake. Furthermore, B fraction of *Nostoc* Z1 exerted the most potent DrOatp1d1 inhibitory potential (IC50 25 μg/mL; Fig. 3.22.). This is in accordance with the results obtained from the *Nostoc* Z1 initial extracts where we also observed relevant response to anion uptake transporter. Again, we see that the toxic effects of biological extract observed in the preliminary phase of EDA are further present in associated fractions. Preliminary extracts of other cyanobacterial strains also demonstrated inhibition effect on DrOatp1d1 and this effect is later present in C fractions of every cyanobacterial strain (Fig. 3.20.). B fraction of most of the strains also contains compounds that can interact with phase I transporters, indicating that all these strains possess similar type of toxicant(s) responsible for the observed effect. C fraction of all terrestrial strains showed toxic effect to DrOatp1d1, while B and C fraction of all aquatic strains exhibited toxic effects to DrOatp1d1 with similar obtained values. Therefore, we can observe that cyanobacterial strains contain a group of chemical compounds with similar chemical properties that are able to inhibit DrOatp1d1 activity. A fraction in all cyanobacterial strains remained the least responsive to the activity of DrOatp1d1 uptake. So far we know that microcystin is present in all these strains. Overall, aquatic strains contain toxicant(s) of concern that express(es) relevant interaction with uptake transporters. The difference between aquatic and terrestrial strains is apparent in their interaction with DrOct1 transporter. Except *Oscillatoria* K3, other aquatic strains did not have any interaction with cationic transporter or the interaction was very weak. B and C fractions of *Oscillatoria* K3 were the most responsive to DrOct1 activity. This is in accordance with the results obtained on preliminary extracts that also demonstrated low or no toxic effect of aquatic strains to DrOct1 activity. On the other hand, fractions of terrestrial strains are more responsive to the activity of cation transporter. All three fractions of both *Anabaena* strains demonstrated toxic effects, but the most relevant inhibitory potential was observed in B and C fraction (Fig. 3.21.). The strongest inhibitory potential was observed in C fraction of *Anabaena* Č5 (IC50 14 μg/mL) and A fraction of *Nostoc* S8 (IC50 17 μg/mL; Fig. 3.22.). Although the preliminary extracts of the two *Anabaena* strains did not exert DrOct1 toxic effect, in the subsequent fractions the effect appeared. This can be attributed to the advantageous separation techniques and different

solvent use that enables the extraction of a wider range of chemical compounds. From the results observed after ABC fractionation of cyanobacterial strains we can imply that potential toxicant(s) in terrestrial strains is(are) cation(s), and in aquatic strains is(are) rather anion(s).

Toxic effects of cyanobacterial strains were also observed in their interaction with phase I enzymes where all six strains activated AhR and induced transcription of CYP enzymes. As in the uptake phase, the obtained responses of aquatic strains dominated with the highest CYP1A1 enzyme activity observed in *Oscillatoria* K3 preliminary extract (8.2 pmol/min/mg) and in *Nostoc* Z1 extract (4.2 pmol/min/mg) (Fig. 3.11.). *Oscillatoria* K3 metabolites showed to be the most reactive to phase 0 and I of the cellular detoxification pathway, indicating that this strain contains different groups of toxicants that exhibit multiple mechanisms of toxicity. *Anabaena* Č5 extracts also exhibited high CYP1A1 activity with 3.5 pmol/min/mg. Although *Anabaena* Č5 is terrestrial and *Oscillatoria* K3 is aquatic, both of these strains possess neurotoxin anatoxin-a, that was shown to be metabolized by CYP enzymes (Osswald et al., 2013). As mentioned before, *Oscillatoria* genus, as well as *Nostoc*, produce microcystins that have also demonstrated the induction of CYP enzymes in the heart and brain of the bird Japanease quail *Coturnix japonica* (Skocovska et al., 2007; Paskova et al., 2008). The combined effect of microcystin and anatoxin-a in *Oscillatoria* K3 can contribute to the toxic effects obtained for phase I detoxification pathway. Other cyanobacterial strains also possess microcystin and other neurotoxins such as anatoxin-a(S), homoanatoxin-a, that are also metabolized by CYP enzymes (Wiegand and Pflugmacher, 2005). Terrestrial strain *Nostoc* S8, although it does possess microcystin, did not show toxic effects towards phase I enzymes. ABC fractions of cyanobacterial strains further confirmed the results obtained on the preliminary extracts that distinguish *Oscillatoria* K3, *Nostoc* Z1 and *Phormidium* Z2 as more potent CYP inducers (Fig. 3.26.). A and B fractions of aquatic strains demonstrated higher activity of CYP1A1 enzyme, especially in *Oscillatoria* K3 where the activity of CYP1A1 enzyme was 7.8 pmol/min/mg and 6.9 pmol/min/mg for A and B fraction, respectively (Fig. 3.26.). The induction potential of A and B fractions of *Nostoc* Z1 and *Phormidium* Z2 were the most significant, indicating that nonpolar and lipophilic toxicant(s) is(are) present in the aquatic strains that is(are) responsible for the observed effect. Nevertheless, all three fractions of terrestrial strains also exhibited toxic effects towards phase I enzymes. *Nostoc* S8 preliminary extract demonstrated no effect to CYP enzymes while all three fraction exhibited lower interaction with phase I enzymes that was similar in all three fractions. Furthermore, the toxic effect observed for preliminary extracts was also present in

the fraction of both *Anabaena* strains. C fraction of all cyanobacterial strains was the least responsive to CYP activity indicating that hydrophilic substances extracted in this fraction are weak targets for biotransformation pathway.

The obtained results on the phase 0 and I of cellular detoxification mechanism indicated more pronounced (eco)toxic response of aquatic cyanobacterial strains. Since cyanobacterial blooms occur primarily in the aquatic ecosystems it is more likely that secondary metabolites produced by aquatic cyanobacteria express diverse and numerous toxicity mechanisms with powerful toxic potential. Furthermore, the observed aquatic toxicity could be more relevant to humans and animals since the exposure routes to cyanotoxins include water consumption or dermal exposure.

As it was the case for *Caulerpa* species, toxic effect on the phase II of the detoxification metabolism was lacking in preliminary extracts of all tested cyanobacterial strains. No change in the activity of six purified cytosolic GST enzymes was observed after exposure to six cyanobacterial strains. On the contrary, previous studies on cyanotoxins showed that phase II enzymes are included in the metabolization of microcystin, anatoxin-a, anatoxin-a(S), homoanatoxin-a, saxitoxin (Pflugmacher et al., 1998; Takenaka, 2001). The negative result obtained is probably because of the same reason as described for *Caulerpa*.

Likewise, cyanobacterial strains had no interaction with phase III efflux transporter, same as *Caulerpa* species. The efflux activity of P-gp did not change in the presence of cyanobacterial strains. The knowledge of the cellular efflux transporters role in the transport of cyanotoxins is still scarce. So far, the only two identified efflux transporters that interact with cyanotoxins are recorded for microcystin that include P-gp and the recent Abcb4 (Contardo-Jara et al., 2008; Amé et al., 2009; Lu et al., 2015). Although previous studies on P-gp demonstrated its involvement in microcystin transport, our EDA study on the cyanobacterial extracts did not show any influence of cyanobacterial secondary metabolites to P-gp.

Therefore, using the described modified EDA protocol we were for the first time able to detect relevant toxicological responses in environmentally highly relevant biological samples. Furthermore, we determined mechanisms of toxic action of tested *Caulerpa* species and various strains of aquatic and terrestrial cyanobacteria, and obtained first reliable indications of chemical characteristics of substances that may be responsible for the observed toxic effects. In the case of *Caulerpa* species we were able to identify the chemistry of CYN-

like chemical structures as the most plausible candidates for toxicological effects observed. Nevertheless, a more detailed chemical analytical studies directed to identification and confirmation of specific (eco)toxic chemical entities in investigated complex biological samples are clearly needed as a critical step in the second stage of the full-scale EDA process. This highly complex, time and resources demanding task is currently ongoing in our research group.

5. CONCLUSIONS

Implementation of the modified EDA approach in this study resulted in several relevant research conclusions:

1. Invasive green algae from the genus *Caulerpa* are ecotoxicologically relevant organisms. *C. racemosa* and *C. taxifolia* extracts are cytotoxic, acutely and/or chronically. Dominant mechanisms of cellular toxicity for both *Caulerpa* species include phase 0 and phase I of the cellular detoxification pathway. Specifically:
    a. Active substances from *C. racemosa* can inhibit zebrafish anion transporter DrOatp1d1 and are characterized as hydrophilic and anionic compounds. The most reactive anionic compounds are present in *C. racemosa*. Other lipophilic substances that are inducers of CYP1A1 enzyme are also present in *C. racemosa*.
    b. *C. taxifolia* contains complex composition of secondary metabolites. Bioactive compounds present in *C. taxifolia* are inhibitory to both tested anion (DrOatp1d1) and cation (DrOct1) transporters and are characterized as hydrophilic, cationic and anionic compounds. CYP1A1 reactive lipohilic substances are also present in *C. taxifolia*.
    c. The major metabolite in *C. racemosa* is CLP while CYN is the dominant compound in *C. taxifolia*. CYN, the major metabolite in *C. taxifolia* is inhibitor of the DrOatp1d1 anion transporter.

2. Cyanobacteria are ecotoxicologically relevant organisms. Terrestrial (*Anabaena* Č2, *Anabaena* Č5, *Nostoc* S8) and aquatic (*Nostoc* Z1, *Phormidium* Z2, *Oscillatoria* K3) cyanobacterial strains are chronically cytotoxic. *Nostoc* strains S8 and Z1 are the most cytotoxic. More specifically:
    a. Relevant mechanisms of toxicity for all cyanobacterial strains include phase 0 and phase I of the cellular detoxification pathway. Active substances from terrestrial strains are cations, inhibitors of the DrOct1 transport, and in aquatic strains are rather anions inhibitory to DrOatp1d1 transport;
    b. All cyanobacterial strains possess another type of biologically active substances – lipophilic CYP1A1 inducers;

c. The (eco)toxicity of aquatic strains to phase 0 and phase I is more prominent. *Oscillatoria* K3 is inhibitory towards anion (DrOatp1d1) and cation (DrOct1) transporters, and does possess cytochrome P-450 (CYP1A1) inducers.

3. Our modified EDA concept enables detailed ecotoxicological characterization of investigated *Caulerpa* species and cyanobacterial strains. Cause-effect relationships were confirmed, biologically relevant mechanisms of toxicity revealed and compounds with certain chemical characteristics responsible for the observed toxic effects have been preliminary detected. Therefore, the modified EDA protocol developed in this study proved to be useful diagnostic tool for ecotoxicological characterization of complex biological samples.

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7. SUMMARY

Effects-directed analyses (EDA) is a powerful multidisciplinary diagnostic tool developed in the field of environmental science that combines the use of advanced chemical and biological methods in order to identify, characterize and prioritize toxicants present in complex environmental samples. So far the EDA approach has been applied for identification and evaluation of environmental contaminants present in non-biological (sediment, surface water, wastewater, soil) complex environmental samples. In this study the EDA approach has been for the first time applied for a detailed ecotoxicological characterization of two types of complex biological samples including eukaryotic and prokaryotic organisms. Invasive tropical green algae from the genus *Caulerpa*, as well as various cyanobacterial species, produce toxic secondary metabolites that represent a significant threat to the environment and human health, especially during periods of intensive blooms. In order to improve the present portfolio of EDA methodology we applied a modified EDA procedure based on a series of *in vitro* or small-scale bioassays in combination with advanced chemical analytical protocols. Main goal of this research was to perform a preliminary identification of biologically active substances that cause observed toxic effects and reveal dominant mechanisms of toxicity in these complex biological samples.

Non-selective and non-target preparation techniques were used for extraction of a broad range of known and unknown chemical compounds present in both *Caulerpa* and cyanobacteria. Toxicological endpoints that were monitored in this study included acute and chronic toxicity, as well as interaction with basic phases of cellular detoxification. *C. racemosa* and *C. taxifolia* are invasive green algae present in the Adriatic Sea that were used in our EDA study for detailed ecotoxicological characterization. Three terrestrial (*Anabaena* Č2, *Anabaena* Č5, *Nostoc* S8) and three aquatic (*Nostoc* Z1, *Phormidium* Z2, *Oscillatoria* K3) cyanobacterial strains are a part of NSCCC and were used in our modified EDA study as well.

The observed toxic effects were the most prominent for both *Caulerpa* species that exhibited powerful cytotoxicity, both acutely and chronically, and interaction with phase 0 and I of cellular detoxification. *Caulerpa* species, especially *C. taxifolia* possess diverse and complex composition of secondary metabolites. Chemical compounds of *Caulerpa* species responsible for the observed effects are characterized as both polar and lipophilic. CLP was identified as a major metabolite in *C. racemosa* while CYN was identified as the dominant compound in *C. taxifolia*. Furthermore, toxic inhibitory effect of CYN compound to anionic uptake transporter (DrOatp1d1) was determined.

Cyanobacterial strains also demonstrated strong cytotoxic effects and interaction with phase 0 and I of the detoxification pathway. Of all tested cyanobacteria, aquatic strains obtained the most relevant biological responses to both detoxification phases and are considered to be of greater ecotoxicological importance. *Oscillatoria* K3 showed to be the most the interesting aquatic cyanobacterial strain because it was the most responsive strain to several observed biological effects. Causable toxicants present in cyanobacterial strains are also characterized as polar and lipophilic.

In conclusion, the modified EDA protocol developed and used in this study proved to be useful diagnostic tool for ecotoxicological characterization of complex biological samples.

8. SAŽETAK

Učincima usmjerena analiza (engl. *Effects-Directed Analyses – EDA*) predstavlja suvremeni multidisciplinarni dijagnostički alat znanosti o okolišu, koji kombiniranjem kemijskih i bioloških tehnika omogućava identifikaciju, karakterizaciju i prioritizaciju toksikanata prisutnih u složenim okolišnim uzorcima. U dosadašnjim istraživanjima EDA koncept se primjenjivao najčešće za identifikaciju i evaluaciju prvenstveno okolišnih zagađivala prisutnih u ne-biološkim okolišnim uzorcima (uzorci sedimenta, površinskih ili otpadnih voda, tla). U našem istraživanju prvi puta se koristi EDA koncept u svrhu provedbe detaljne ekotoksikološke karakterizacije dvaju tipova različitih bioloških uzoraka koji uključuju pripadnike prokariota i eukariota. Invazivne tropske zelene alge roda *Caulerpa,* kao i različite vrste cijanobakterija, stvaraju toksične sekundarne metabolite koji mogu značajno narušavati homeostazu ekosistema i/ili zdravlje ljudi, osobito u razdobljima intenzivnog rasta. Za svrhu unapređenja dosadašnje metodologije korištene u EDA studijama mi smo upotrijebili modificirani EDA postupak koji uključuje primjenu serije *in vitro* ili „small-scale" biotestova u kombinaciji s naprednim kemijsko analitičkim postupcima. Cilj ovog istraživanja bio je izvršiti preliminarnu identifikaciju biološki aktivnih supstanci koje uzrokuju opažene toksične učinke te otkriti relevantne mehanizme toksičnosti ovih kompleksnih bioloških uzoraka.

Neselektivne i ne-ciljane preparativne tehnike su se upotrijebile za ekstrakciju što šireg spektra poznatih i nepoznatih kemijskih komponenata prisutnih u obje vrste roda *Caulerpa*. Toksikološki učinci koji su bili praćeni uključivali su akutnu i kroničnu toksičnost, kao i interakciju s temeljnim fazama detoksikacijskog mehanizma. *C. racemosa* i *C. taxifolia* su invazivne zelene alge prisutne u Jadranskom moru, a koje smo u ovom radu pokušali detaljno ekotoksikološki karakterizirati korištenjem modificiranog EDA pristupa. Tri terestrička (*Anabaena* Č2, *Anabaena* Č5, *Nostoc* S8) i tri vodena (*Nostoc* Z1, *Phormidium* Z2, *Oscillatoria* K3) cijanobakterijska soja koja su se koristila za provedbu naše modificirane EDA studije pripadaju kolekciji cijanobakterijskih kultura Sveučilišta u Novom Sadu, Srbija.

Opaženi toksični učinci su bili najizraženiji za obje vrste roda *Caulerpa* koje su pokazale snažne učinke akutne i kronične citotoksičnosti, te interakcije s fazom 0. i I. stanične detoksikacije. Vrste *Caulerpa,* posebice *C. taxifolia* sadrže raznolik i kompleksan sastav sekundarnih metabolita. Kemijske komponente vrsti roda *Caulerpa* odgovorne za opažene učinke se karakteriziraju kao polarne i lipofilne supstance. Kaulerpin je identificiran kao glavni metabolit u vrsti *C. racemosa* dok je kaulerpenin bio dominantni spoj u vrsti *C. taxifolia*. Nadalje, ustanovljen je značajan inhibitorni učinak kaulerpenina na anionski

transporter (DrOatp1d1). Cijanobakterijski sojevi su također pokazali jake citotoksične učinke i interakciju s fazom 0. i I. stanične detoksikacije. Najznačajniji biološki odgovori na obje faze detoksikacije dobiveni su testiranjem vodenih sojeva cijanobakterija, zbog čega se smatraju od većeg ekotoksikološkog značaja. *Oscillatoria* K3 se izdvaja kao najzanimljiviji vodeni cijanobakterijski soj budući da je pokazao najviši ekotoksični potencijal u pogledu nekoliko opažanih bioloških učinaka. Toksikanti koji su uzročnici opaženih učinaka u cijanobakterijskim sojevima se preliminarno identificirani kao polarni i lipofilni spjevi.

Zaključno, modificirani EDA pristup razvijen u okviru ove disertacije pokazao se kao vjerodostojan metodološki pristup za karakterizaciju i evaluaciju kompleksnih bioloških uzoraka pružajući važne informacije o kemijskim osobinama detektiranih toksikanata i njihovim relevantnim mehanizmima toksičnosti.

9. ABBREVIATIONS

7-ER - 7-etoxiresorufine

ABC - ATP-binding cassette

ACN - acetonitrile

Adda - 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

ADHs - alcohol dehydrogenases

AhR - aryl hydrocarbon receptor

ALDHs - aldehyde dehydrogenases

AR - androgen receptor

ASP+ - 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide

ATCC - American Type Culture Collection

ATP - adenosine triphosphate

AU - arbitrary units

BSA - bovine serum albumin

C32 - unpigmented melanoma cell line

Ca-AM - calcein acetoxymethyl ester

CALUX - chemical activated luciferase gene expression

CDNB - 1-chloro-2,4-dinitrobenzene

CI - confidence interval

CLP - caulerpin

CYN - caulerpenyne

CYC - cyclosporin A

CYP - cytochrome P-450

D-Masp - D-erythro-β-methylaspartic acid

DCM - dichloromethane

DNA - deoxyribonucleic acid

DMEM - Dulbecco's modified Eagle medium

DMEM F12 - Dulbecco's modified Eagle medium/nutrient mixture F-12 HAM

DMSO - dimethyl sulfoxide

dox - doxorubicin

Dr - *Danio rerio*

DRE - extended dynamic range

DT40 - chicken DT40 B-lymphocyte cell line

EC50 - effective concentration that causes 50% of the maximal observed effect

EDA - effects-directed analyses

EPHs - epoxide hydrolases

ER - estrogen receptor

ERA - environmental risk assessment

EROD - 7-etoxyresorufin *O*-deethylase

FBS - fetal bovine serum

FMOs - flavin-containing monooxigenases

GC-MS - gass chromatography-mass spectrometry

GP - gluthatione peroxidase

GR - glutathione reductase

GSH - glutathione

GSM - geosmin

GSTs - glutathione S-transferases

H4IIE-Luc - rat hepatoma cell line

H295R - human adrenal cancer cells

HABs - harmful algal blooms

HEK293 - human embryonic kidney cell line

HEX - n-hexane

HIF-1 - hypoxia-inducible factor-1

HPLC - high performance liquid chromatography

IC50 - inhibitory concentration that causes 50% of the maximal inhibition

ITN - initial training networks

LC - liquid chromatography

LC50 - lethal concentration that causes 50% of the maximal lethal effect

LC-MS - liquid chromatography-mass spectrometry

LY - lucifer yellow

MAOs - monoamine oxidases

MAPK - mitogen-activated protein kinase

MATE - multidrug and toxic compound extrusion family

MCF-7 - invasive ductal breast cancer cell line

MDA - invasive ductal breast cancer cell line

MDA-KB - transformed MDA human breast cancer cell line

Mdha - N-methyldehydroalanine

Mdhb - N-methyldehydrobutyrine

MDR - multidrug resistance

MeOH - methanol

MEP - methyl-erythritol-4-phosphate

MIB - 2-methylisoborneol

mQH_2O - milli-Q H_2O

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MVLN - transformed MCF-7 human breast cancer cell line

MXR - multixenobiotic resistance

NAD - nicotinamide adenine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

NADPH-CPR - NADPH-dependent cytochrome P450 reductase

NETs - N-acetyltransferases

NIH 3T3-R/dox - doxorubicin resistant mouse embryonic fibroblast cell line

NMO - NAD(P)H-menadione oxidoreductase

NSCCC - Novi Sad Cyanobacterial Culture Collection

OATs - organic anion transporters

OATPs/Oatp - organic anion transporting polypeptide(s)

OCTs/Oct - organic cation transporter(s)

P-gp - P-glycoprotein

PAHs - polycyclic aromatic hydrocarbons

PBS - phosphate-buffered saline

PCB - polychlorinated biphenyles

PCDD/Fs - polychlorinated dibenzo-p-dioxins and dibenzofurans

PEI - polyethyleneimine

PI - positive ionization mode

PLHC-1/wt - *Poeciliopsis lucida* hepatoma cell line wild type

PP1/2a - protein phosphatases 1 and 2a

PrOH - 2-propanol

PSC 833 - valspodar

PSP - paralytic shellfish poisoning

QTOFMS - quadrupole-time-of-flight mass spectrometry

RLT-W1 - rainbow trout liver cells

ROS - reactive oxygen species

SAG - Culture Collection of Algae (Sammlung von Algenkulturen)

SCD - Serbian Cyano Database

SD - standard deviation

SDS - sodium dodecyl sulfate

SULT - sulfotransferase

T-47D - human ductal breast epithelial tumor cell line

T47DKBluc - transformed T-47D human breast cancer cell line

TCDD - 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TCDD Eq - TCDD equivalents

TIC - total ion current

TIE - toxicity identification evaluation

TOF - time-of-flight

UDP-GT - uridine 5'-diphospho-glucuronosyltransferase

UK - United Kingdom

UNS - University of Novi Sad

UPLC - ultra-performance liquid chromatography

USA - United States of America

US EPA - United States Environmental Protection Agency

UV - ultraviolet light

VER - verapamil

WHO - World Health Organization

wt - wild type

XICs - extracted ion chromatograms

YES - yeast estrogen screen

YAS - yeast androgen screen

10. CURRICULUM VITAE

Education

BSc degree in biology – ecology, 2009

University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia.

Work experience

2012 - **Junior research assistant**

Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Molecular Ecotoxicology, Zagreb, Croatia.

2009 – 2012 – **Junior research assistant**

Zagreb University, School of Medicine, University Hospital for Tumors, Department of Radiation Oncology, Zagreb, Croatia.

Publications

1. Beketić-Orešković, L., **Marić, P.**, Ozretić, P., Orešković, D., Ajduković, M., Levanat, S., 2012. Assessing the clinical significance of tumor markers in common neoplasms. Front. Biosci. (Elite Ed. E4) 7, 2558-2578.

2. **Marić, P.**, Ozretić, P., Levanat, S., Orešković, S., Antunac, K., Beketić-Orešković, L., 2011. Tumor markers in breast cancer - evaluation of their clinical usefulness. Coll. Antropol. 35, 241-247.

3. Beketić-Orešković, L., Ozretić, P., Rabbani, Z.N., Jackson, I.L., Šarčević, B., Levanat, S., **Marić, P.**, Babić, I., Vujasković, Ž., 2011. Prognostic significance of carbonic anhydrase IX (CA-IX), endoglin (CD105) and 8-hydroxy-2'- deoxyguanosine (8-OHdG) in breast cancer patients. Pathol. Oncol. Res. 17, 593-603.

Congres abstract in CC journals

Beketić-Oresković, L., Ozretić, P., Rabbani, Z., Jackson, I., Šarčević, B., Levanat, S., **Marić, P.**, Vujašković, Ž., 2011. Prognostic significance of carbonic anhydrase IX (CA-IX), endoglin (CD105) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in breast cancer patients. Radiother. Oncol. S303-S304 (poster, international review, abstract)

Study visit

2014 – 3 month stay in Laboratory for Ecotoxicology (Prof. Karl Fent), University of Applied Sciences and Arts Northwestern Switrezland (FHNW), Basel, Switzerland. Collaboration on a joint research project „Identification and characterization of cyanobacterial toxins based on their interaction with basic cellular detoxification systems in zebrafish (*Danio rerio*) and zooplankton *Daphnia magna*" funded by Swiss National Science foundation (SNSF) Grant SCOPES-IZ73ZO_152274/1.

Active participation in conferences

1. **Marić, P.**, Mihaljević, I., Popović, M., Smital, T. Characterization and toxicological significance of microsomal glutathione S-transferases in zebrafish. Zebrafish Meeting: Heart of Europe, 17-19 September 2014, Warsaw, Poland.
2. Smital, T., Popovic, M., Loncar, J., Mihaljevic, I., Zaja, R., **Maric, P**. Polyspecific membrane transport proteins as integral elements of the cellular detoxification and environmental stress response in zebrafish. Zebrafish Meeting: Heart of Europe, 17-19 September 2014, Warsaw, Poland.

Science projects

Present

1. project supported by Swiss National Science Foundation (SNSF) - "Identification and characterization of cyanobacterial toxins based on their interaction with basic cellular detoxification systems in zebrafish (*Danio rerio*) and zooplankton Daphnia magna", SCOPES 2014-2016 Joint Research Project, Grant SCOPES-IZ73ZO_152274/1 - member of the project team
2. project supported by Croatian Science Foundation (HRZZ) – „Identification and functional characterization of (eco)toxicologically relevant polyspecific membrane transport proteins in zebrafish (*Danio rerio*)", Project No 4806 (Call 2013-11) – member of the project team
3. project supported by Ministry of science, education and sports of the Republic of Croatia - „Ecotoxicological relevance of the ABC transport proteins in aquatic organism", Project No 098-0982934-2745 – member of the project team

Past

4. project supported by Ministry of science, education and sports of the Republic of Croatia - „Clinical significance of tumor hypoxia in patients treated with irradiation“, Project No 074-0982464-1187 - member of the project team
5. project supported by the Croatian companies „APO d.o.o.“ and „Hrvatske vode“ - „Examination of pollution in stream Gorjak sediments“ - participation for Bachelor's thesis work

Acknowledgments

Belong to 10% of the most successful graduates according to academic records in the year 2008/2009

Memberships

European Association for Cancer Research (EACR)
Croatian Society for Cancer Research (CACR)

Scholarships

State scholarship supported by Ministry of science, education and sports of the Republic of Croatia