Supplementary Materials

Diclofenac stress responses and biotransformation pathways in the marine Diatom Phaeodactylum Tricornutum

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Growth of *P.tricornutum* cultures

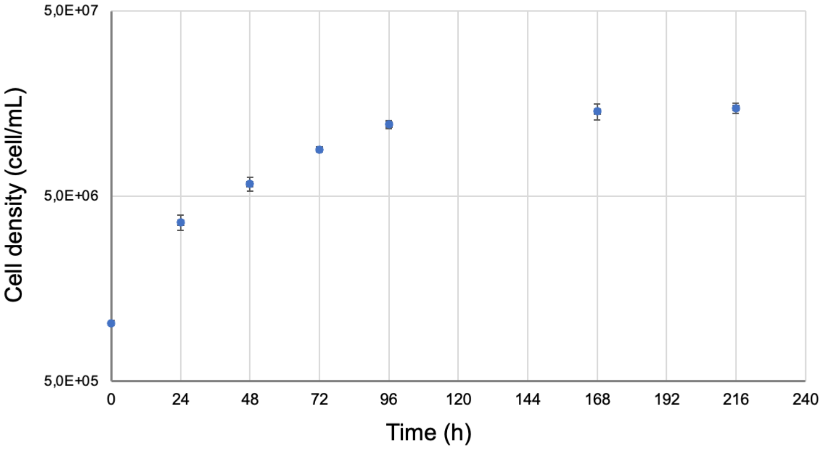
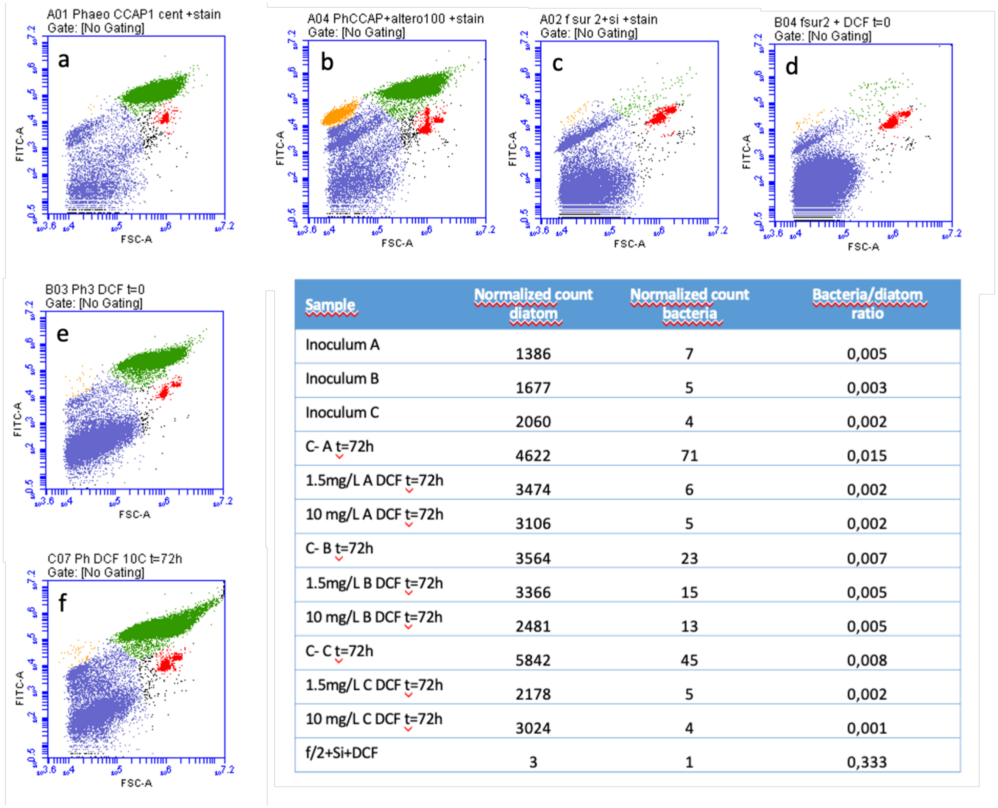


Figure S1: Growth curve of *P. tricornutum* CCAP1055/18 cultures grown in f/2 medium under controlled conditions (20°C, continuous agitation at120 rpm, warm white fluorescent lighting at 50 µE with a 16/8 light:dark cycle)

Axenicity of *P.tricornutum* cultures

P. tricornutum CCAP1055/18 cultures axenicity was investigated in the beginning and at the end of the diclofenac exposure tests. Samples of cultures were collected and stained with the SYTO BC bacteria stain (Bacteria counting kit, Molecular Probes, Invitrogen) following the manufacturer protocol. Samples were analyzed using the flow cytometer BD Accuri C6 plus (BD biosciences). Gating strategy adopted to quantify diatom cells and bacterial cells is described in Figure S2.



Sample	Normalized count diatom	Normalized count bacteria	Bacteria/diatom ratio
Inoculum A	1386	7	0,005
Inoculum B	1677	5	0,003
Inoculum C	2060	4	0,002
C- A t=72h	4622	71	0,015
1.5mg/L A DCF t=72h	3474	6	0,002
10 mg/L A DCF t=72h	3106	5	0,002
C- B t=72h	3564	23	0,007
1.5mg/L B DCF t=72h	3366	15	0,005
10 mg/L B DCF t=72h	2481	13	0,005
C- C t=72h	5842	45	0,008
1.5mg/L C DCF t=72h	2178	5	0,002
10 mg/L C DCF t=72h	3024	4	0,001
f/2+Si+DCF	3	1	0,333

Figure S2: Verification of algae axenicity before and during the exposure test. Gates for quantification of algal cells (green population), bacterial cells (yellow population) and inert particles (violet population) were defined analyzing axenic *P. tricornutum* cultures (a), *P. tricornutum* cultures inoculated with *Alteromonas* cells (b), f/2+Si medium (c) and f/2+Si medium with diclofenac at 10 mg L^{-1} concentration (d). E xamples of cytograms obtained for samples collected at the beginning of the test (t=0) and at the end of the test (t=72h) are reported in € and (f) respectively. The table displays the counts of diatom and bacterial cells determined in each sample and normalized with respect to the beads (red population). The number of bacterial cells measured in the test cultures was comparable to the noise measured in the sterile exposure medium and did not change during the test. Even assuming the presence of bacterial cells inside *P. tricornutum* cultures, their number would be more than two orders of magnitude lower than diatom cells and can be considered irrelevant with respect to the obtained results.

HPLC sequence and procedure used to measure DCF in exposure medium

Injection volume was set at 20 µL with a 1 mL/min flow rate. The working UV wavelength was 220 nm. For DCF samples concentration was quantified based on calibration curved that were obtained for different concentration points. Two different calibration curves were used. The curve for lower DCF concentrations included 5 calibration points (0.05, 0.1, 0.5, 1 and 2 mg/L). The curve for higher DCF concentrations included 5 additional calibration points 5 calibration points (1, 7, 15, 30 and 60 mg/L). Calibration standards were prepared from a stock solution of DCF dissolved in EtOH. During sample measurement, Instrument stability was checked by regularly injecting a concentration corresponding to one point on the calibration curve during the analysis sequence. Peak integration and data analyses were performed using the Chromoleon software. Measurements were performed in 10 × concentrated SPE extracts. The concentrations in f/2 medium samples were calculated taking the extraction recoveries into account.

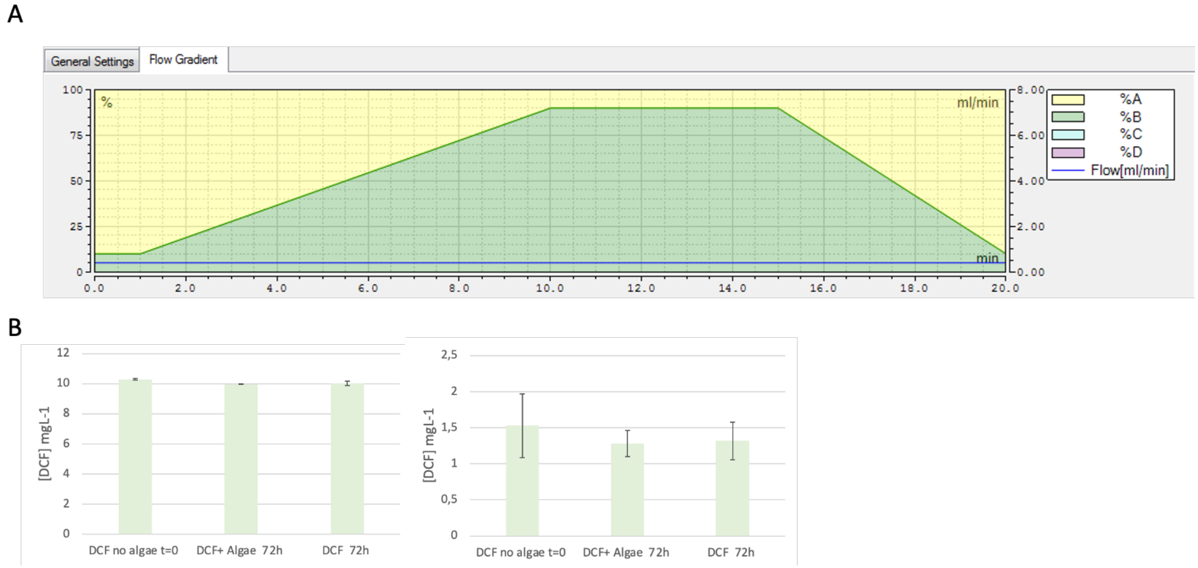
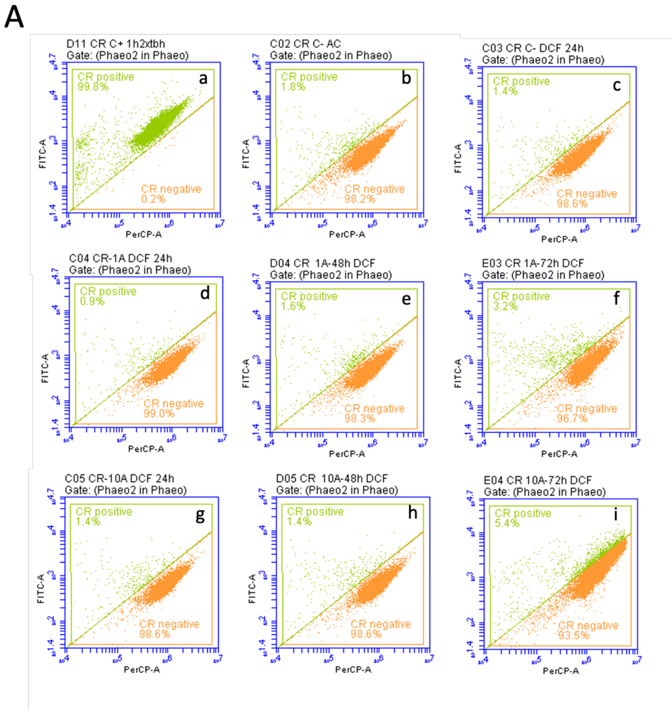


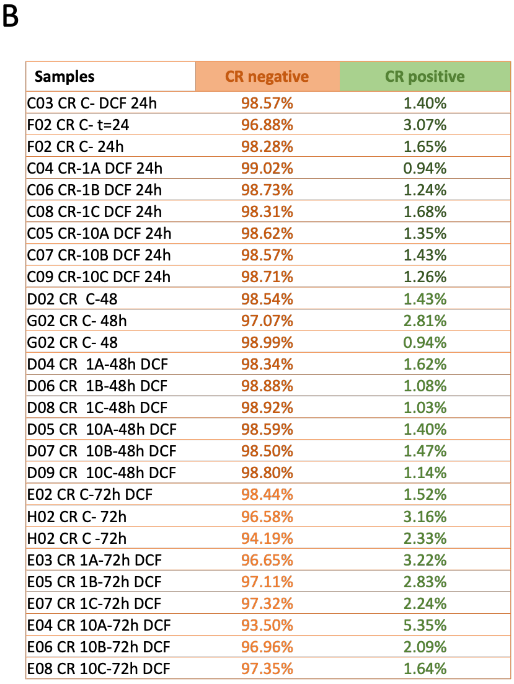
Figure S3: (A) HPLC-UV gradient used for DCF determination in exposure medium, phase A corresponds to water, phase B corresponds to acetonitrile. (B) DCF concentrations measured in exposure medium in the beginning and at the end of the test in presence and absence of algae.

Flow cytometry measurements of intracellular oxidative stress

Detection of reactive oxygen species (ROS) in living cells was performed via flow cytometry using the CellROX™ green flow cytometry assay kit (Molecular Probes, Life Technologies). Samples were collected after 24, 48 and 72 hours from control and exposed cultures (1.5 and 10 mg L^{-1} DCF) and stained following the manufacturer protocol. Samples were analyzed using the flow cytometer BD Accuri C6 plus (BD biosciences). Gating strategy was designed based on negative and positive controls. Two gates were identified. The Cell Rox (CR) positive gate was designed to detect populations of cells undergoing oxidative stress that was induced using the tert-butyl hydroperoxide solution (TBHP, an ROS inducer) provided in the kit. The Cell Rox (CR) negative gate was designed to detect populations of cells that were not affected by oxidative stress, these cells were treated with N-acetylcysteine (NAC) to increase the antioxidant capability of the cell before performing the tert-butyl hydroperoxide treatment. Detailed information about solutions concentrations and incubation time can be found in the manufacturer protocol.



B



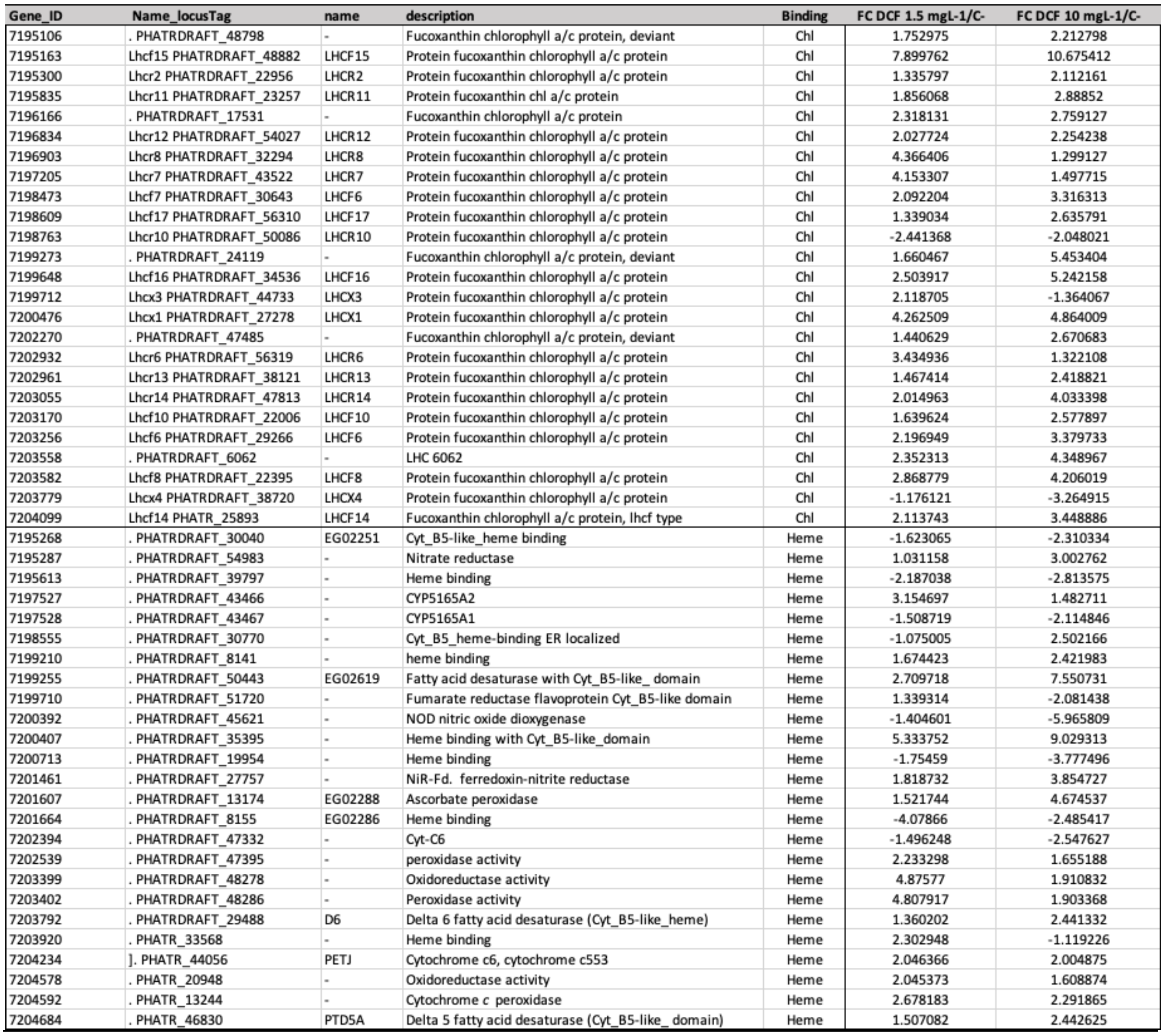
Samples	CR negative	CR positive
C03 CR C- DCF 24h	98.57%	1.40%
F02 CR C- t=24	96.88%	3.07%
F02 CR C- 24h	98.28%	1.65%
C04 CR-1A DCF 24h	99.02%	0.94%
C06 CR-1B DCF 24h	98.73%	1.24%
C08 CR-1C DCF 24h	98.31%	1.68%
C05 CR-10A DCF 24h	98.62%	1.35%
C07 CR-10B DCF 24h	98.57%	1.43%
C09 CR-10C DCF 24h	98.71%	1.26%
D02 CR C-48	98.54%	1.43%
G02 CR C- 48h	97.07%	2.81%
G02 CR C- 48	98.99%	0.94%
D04 CR 1A-48h DCF	98.34%	1.62%
D06 CR 1B-48h DCF	98.88%	1.08%
D08 CR 1C-48h DCF	98.92%	1.03%
D05 CR 10A-48h DCF	98.59%	1.40%
D07 CR 10B-48h DCF	98.50%	1.47%
D09 CR 10C-48h DCF	98.80%	1.14%
E02 CR C-72h DCF	98.44%	1.52%
H02 CR C- 72h	96.58%	3.16%
H02 CR C -72h	94.19%	2.33%
E03 CR 1A-72h DCF	96.65%	3.22%
E05 CR 1B-72h DCF	97.11%	2.83%
E07 CR 1C-72h DCF	97.32%	2.24%
E04 CR 10A-72h DCF	93.50%	5.35%
E06 CR 10B-72h DCF	96.96%	2.09%
E08 CR 10C-72h DCF	97.35%	1.64%

Figure S4: Effects of Diclofenac on intracellular oxidative stress. **A**) Examples of cytograms obtained for positive control (TBHP treatment) (a), negative control (NAC+TBHP treatment) (b) and not exposed control culture of the Diclofenac treatment experiment (c). ROS in cells treated with DCF 1.5 mgL^{-1} (d, e, f) and 10 mg L^{-1} (g, h, l) were measured after 24, 48 and 72 hours incubation. **B**) Table with measured percentages of not affected (CR negative) and affected (CR positive) cells obtained for the three treatments (control, DCF1.5 mgL^{-1} and 10 mg L^{-1}) after 24, 48 and 72 hours incubation.

Table S1: List of genes differentially expressed in 10 mg L^{-1} treatment with respect to 1.5 mg L^{-1} treatment that have a DNA binding function.

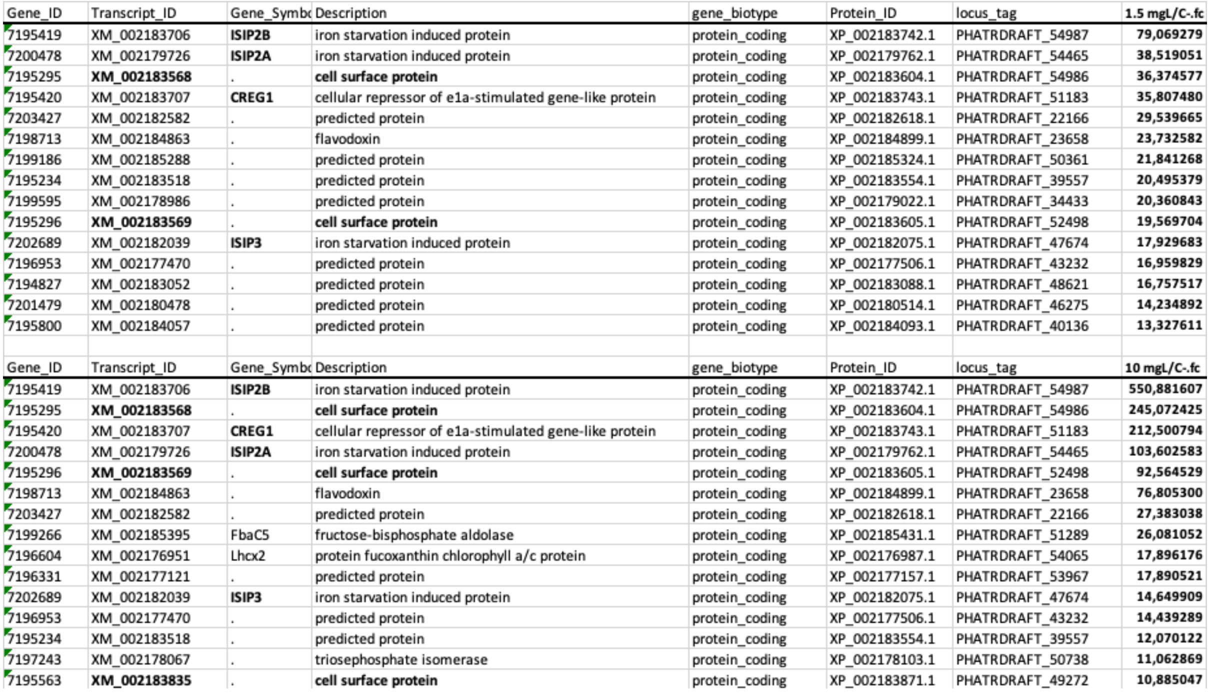
Term ID	Term name	Adjusted p value	intersection size	Gene ID	Transcript ID	Gene Symbol	**10 mg/1.5 mg**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7195220	XM_002183504	DEL	**5,027548**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7195452	XM_002183737	.	**-2,705821**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7195641	XM_002183760	.	**-3,462452**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7195642	XM_002183761	.	**-3,090048**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7195662	XM_002183781	.	**-3,569197**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7196246	XM_002177362	.	**-5,719785**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7196272	XM_002176556	.	**-4,526316**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7197371	XM_002177514	.	**-2,217568**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7197429	XM_002177573	.	**-2,163617**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7197512	XM_002178042	.	**-2,403998**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7198088	XM_002178576	.	**-2,046734**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7198226	XM_002184347	.	**-2,339921**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7198249	XM_002184368	.	**-4,903104**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7198250	XM_002184371	HSF2	**-4,430202**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7198282	XM_002184388	.	**-2,771365**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7198798	XM_002184883	.	**-2,566059**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7199608	XM_002178781	.	**-2,239250**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200092	XM_002179404	.	**-2,041418**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200184	XM_002179126	.	**-5,198998**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200308	XM_002179351	.	**-5,822985**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200327	XM_002179145	.	**-5,831303**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200452	XM_002179894	.	**-2,098044**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200486	XM_002179735	.	**-7,733135**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7200625	XM_002179829	.	**-7,329756**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7201958	XM_002181395	.	**4,692164**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7202319	XM_002181462	.	**-4,348011**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7202512	XM_002181681	.	**-5,261197**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7203194	XM_002182372	.	**-2,634361**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7203588	XM_002182779	.	**-3,276483**
GO:0003700	DNA-binding transcription factor activity	**0,019581495**	30	7204220	XM_002186084	.	**-2,060471**

Table S2: List of genes differentially expressed in 1.5 mg L^{-1} treatment and 10 mg L^{-1} treatment with respect to control and that bind a tetrapyrrole. Genes are grouped between Chloropyll binding (upper part of the table) and Heme binding (lower part of the table).



Gene_ID	Name_locusTag	name	description	Binding	FC DCF 1.5 mgL-1/C-	FC DCF 10 mgL-1/C-
7195106	. PHATRDRAFT_48798	-	Fucoxanthin chlorophyll a/c protein, deviant	Chl	1.752975	2.212798
7195163	Lhcf15 PHATRDRAFT_48882	LHCF15	Protein fucoxanthin chlorophyll a/c protein	Chl	7.899762	10.675412
7195300	Lhcr2 PHATRDRAFT_22956	LHCR2	Protein fucoxanthin chlorophyll a/c protein	Chl	1.335797	2.112161
7195835	Lhcr11 PHATRDRAFT_23257	LHCR11	Protein fucoxanthin chl a/c protein	Chl	1.856068	2.88852
7196166	. PHATRDRAFT_17531	-	Fucoxanthin chlorophyll a/c protein	Chl	2.318131	2.759127
7196834	Lhcr12 PHATRDRAFT_54027	LHCR12	Protein fucoxanthin chlorophyll a/c protein	Chl	2.027724	2.254238
7196903	Lhcr8 PHATRDRAFT_32294	LHCR8	Protein fucoxanthin chlorophyll a/c protein	Chl	4.366406	1.299127
7197205	Lhcr7 PHATRDRAFT_43522	LHCR7	Protein fucoxanthin chlorophyll a/c protein	Chl	4.153307	1.497715
7198473	Lhcf7 PHATRDRAFT_30643	LHCF6	Protein fucoxanthin chlorophyll a/c protein	Chl	2.092204	3.316313
7198609	Lhcf17 PHATRDRAFT_56310	LHCF17	Protein fucoxanthin chlorophyll a/c protein	Chl	1.339034	2.635791
7198763	Lhcr10 PHATRDRAFT_50086	LHCR10	Protein fucoxanthin chlorophyll a/c protein	Chl	-2.441368	-2.048021
7199273	. PHATRDRAFT_24119	-	Fucoxanthin chlorophyll a/c protein, deviant	Chl	1.660467	5.453404
7199648	Lhcf16 PHATRDRAFT_34536	LHCF16	Protein fucoxanthin chlorophyll a/c protein	Chl	2.503917	5.242158
7199712	Lhcx3 PHATRDRAFT_44733	LHCX3	Protein fucoxanthin chlorophyll a/c protein	Chl	2.118705	-1.364067
7200476	Lhcx1 PHATRDRAFT_27278	LHCX1	Protein fucoxanthin chlorophyll a/c protein	Chl	4.262509	4.864009
7202270	. PHATRDRAFT_47485	-	Fucoxanthin chlorophyll a/c protein, deviant	Chl	1.440629	2.670683
7202932	Lhcr6 PHATRDRAFT_56319	LHCR6	Protein fucoxanthin chlorophyll a/c protein	Chl	3.434936	1.322108
7202961	Lhcr13 PHATRDRAFT_38121	LHCR13	Protein fucoxanthin chlorophyll a/c protein	Chl	1.467414	2.418821
7203055	Lhcr14 PHATRDRAFT_47813	LHCR14	Protein fucoxanthin chlorophyll a/c protein	Chl	2.014963	4.033398
7203170	Lhcf10 PHATRDRAFT_22006	LHCF10	Protein fucoxanthin chlorophyll a/c protein	Chl	1.639624	2.577897
7203256	Lhcf6 PHATRDRAFT_29266	LHCF6	Protein fucoxanthin chlorophyll a/c protein	Chl	2.196949	3.379733
7203558	. PHATRDRAFT_6062	-	LHC 6062	Chl	2.352313	4.348967
7203582	Lhcf8 PHATRDRAFT_22395	LHCF8	Protein fucoxanthin chlorophyll a/c protein	Chl	2.868779	4.206019
7203779	Lhcx4 PHATRDRAFT_38720	LHCX4	Protein fucoxanthin chlorophyll a/c protein	Chl	-1.176121	-3.264915
7204099	Lhcf14 PHATR_25893	LHCF14	Fucoxanthin chlorophyll a/c protein, lhcf type	Chl	2.113743	3.448886
7195268	. PHATRDRAFT_30040	EG02251	Cyt_B5-like_heme binding	Heme	-1.623065	-2.310334
7195287	. PHATRDRAFT_54983	-	Nitrate reductase	Heme	1.031158	3.002762
7195613	. PHATRDRAFT_39797	-	Heme binding	Heme	-2.187038	-2.813575
7197527	. PHATRDRAFT_43466	-	CYP5165A2	Heme	3.154697	1.482711
7197528	. PHATRDRAFT_43467	-	CYP5165A1	Heme	-1.508719	-2.114846
7198555	. PHATRDRAFT_30770	-	Cyt_B5_heme-binding ER localized	Heme	-1.075005	2.502166
7199210	. PHATRDRAFT_8141	-	heme binding	Heme	1.674423	2.421983
7199255	. PHATRDRAFT_50443	EG02619	Fatty acid desaturase with Cyt_B5-like_ domain	Heme	2.709718	7.550731
7199710	. PHATRDRAFT_51720	-	Fumarate reductase flavoprotein Cyt_B5-like domain	Heme	1.339314	-2.081438
7200392	. PHATRDRAFT_45621	-	NOD nitric oxide dioxygenase	Heme	-1.404601	-5.965809
7200407	. PHATRDRAFT_35395	-	Heme binding with Cyt_B5-like_domain	Heme	5.333752	9.029313
7200713	. PHATRDRAFT_19954	-	Heme binding	Heme	-1.75459	-3.777496
7201461	. PHATRDRAFT_27757	-	NiR-Fd. ferredoxin-nitrite reductase	Heme	1.818732	3.854727
7201607	. PHATRDRAFT_13174	EG02288	Ascorbate peroxidase	Heme	1.521744	4.674537
7201664	. PHATRDRAFT_8155	EG02286	Heme binding	Heme	-4.07866	-2.485417
7202394	. PHATRDRAFT_47332	-	Cyt-C6	Heme	-1.496248	-2.547627
7202539	. PHATRDRAFT_47395	-	peroxidase activity	Heme	2.233298	1.655188
7203399	. PHATRDRAFT_48278	-	Oxidoreductase activity	Heme	4.87577	1.910832
7203402	. PHATRDRAFT_48286	-	Peroxidase activity	Heme	4.807917	1.903368
7203792	. PHATRDRAFT_29488	D6	Delta 6 fatty acid desaturase (Cyt_B5-like_heme)	Heme	1.360202	2.441332
7203920	. PHATR_33568	-	Heme binding	Heme	2.302948	-1.119226
7204234	]. PHATR_44056	PETJ	Cytochrome c6, cytochrome c553	Heme	2.046366	2.004875
7204578	. PHATR_20948	-	Oxidoreductase activity	Heme	2.045373	1.608874
7204592	. PHATR_13244	-	Cytochrome *c* peroxidase	Heme	2.678183	2.291865
7204684	. PHATR_46830	PTD5A	Delta 5 fatty acid desaturase (Cyt_B5-like_ domain)	Heme	1.507082	2.442625

Table S3: List of top 15 genes significantly up regulated in the two DCF treatments with respect to control



Gene_ID	Transcript_ID	Gene_Symb	Description	gene_biotype	Protein_ID	locus_tag	**1.5 mgL/C-.fc**
7195419	XM_002183706	**ISIP2B**	iron starvation induced protein	protein_coding	XP_002183742.1	PHATRDRAFT_54987	**79,069279**
7200478	XM_002179726	**ISIP2A**	iron starvation induced protein	protein_coding	XP_002179762.1	PHATRDRAFT_54465	**38,519051**
7195295	**XM_002183568**	.	**cell surface protein**	protein_coding	XP_002183604.1	PHATRDRAFT_54986	**36,374577**
7195420	XM_002183707	**CREG1**	cellular repressor of e1a-stimulated gene-like protein	protein_coding	XP_002183743.1	PHATRDRAFT_51183	**35,807480**
7203427	XM_002182582	.	predicted protein	protein_coding	XP_002182618.1	PHATRDRAFT_22166	**29,539665**
7198713	XM_002184863	.	flavodoxin	protein_coding	XP_002184899.1	PHATRDRAFT_23658	**23,732582**
7199186	XM_002185288	.	predicted protein	protein_coding	XP_002185324.1	PHATRDRAFT_50361	**21,841268**
7195234	XM_002183518	.	predicted protein	protein_coding	XP_002183554.1	PHATRDRAFT_39557	**20,495379**
7199595	XM_002178986	.	predicted protein	protein_coding	XP_002179022.1	PHATRDRAFT_34433	**20,360843**
7195296	**XM_002183569**	.	**cell surface protein**	protein_coding	XP_002183605.1	PHATRDRAFT_52498	**19,569704**
7202689	XM_002182039	**ISIP3**	iron starvation induced protein	protein_coding	XP_002182075.1	PHATRDRAFT_47674	**17,929683**
7196953	XM_002177470	.	predicted protein	protein_coding	XP_002177506.1	PHATRDRAFT_43232	**16,959829**
7194827	XM_002183052	.	predicted protein	protein_coding	XP_002183088.1	PHATRDRAFT_48621	**16,757517**
7201479	XM_002180478	.	predicted protein	protein_coding	XP_002180514.1	PHATRDRAFT_46275	**14,234892**
7195800	XM_002184057	.	predicted protein	protein_coding	XP_002184093.1	PHATRDRAFT_40136	**13,327611**

Gene_ID	Transcript_ID	Gene_Symb	Description	gene_biotype	Protein_ID	locus_tag	**10 mgL/C-.fc**
7195419	XM_002183706	**ISIP2B**	iron starvation induced protein	protein_coding	XP_002183742.1	PHATRDRAFT_54987	**550,881607**
7195295	**XM_002183568**	.	**cell surface protein**	protein_coding	XP_002183604.1	PHATRDRAFT_54986	**245,072425**
7195420	XM_002183707	**CREG1**	cellular repressor of e1a-stimulated gene-like protein	protein_coding	XP_002183743.1	PHATRDRAFT_51183	**212,500794**
7200478	XM_002179726	**ISIP2A**	iron starvation induced protein	protein_coding	XP_002179762.1	PHATRDRAFT_54465	**103,602583**
7195296	**XM_002183569**	.	**cell surface protein**	protein_coding	XP_002183605.1	PHATRDRAFT_52498	**92,564529**
7198713	XM_002184863	.	flavodoxin	protein_coding	XP_002184899.1	PHATRDRAFT_23658	**76,805300**
7203427	XM_002182582	.	predicted protein	protein_coding	XP_002182618.1	PHATRDRAFT_22166	**27,383038**
7199266	XM_002185395	FbaC5	fructose-bisphosphate aldolase	protein_coding	XP_002185431.1	PHATRDRAFT_51289	**26,081052**
7196604	XM_002176951	Lhcx2	protein fucoxanthin chlorophyll a/c protein	protein_coding	XP_002176987.1	PHATRDRAFT_54065	**17,896176**
7196331	XM_002177121	.	predicted protein	protein_coding	XP_002177157.1	PHATRDRAFT_53967	**17,890521**
7202689	XM_002182039	**ISIP3**	iron starvation induced protein	protein_coding	XP_002182075.1	PHATRDRAFT_47674	**14,649909**
7196953	XM_002177470	.	predicted protein	protein_coding	XP_002177506.1	PHATRDRAFT_43232	**14,439289**
7195234	XM_002183518	.	predicted protein	protein_coding	XP_002183554.1	PHATRDRAFT_39557	**12,070122**
7197243	XM_002178067	.	triosephosphate isomerase	protein_coding	XP_002178103.1	PHATRDRAFT_50738	**11,062869**
7195563	**XM_002183835**	.	**cell surface protein**	protein_coding	XP_002183871.1	PHATRDRAFT_49272	**10,885047**

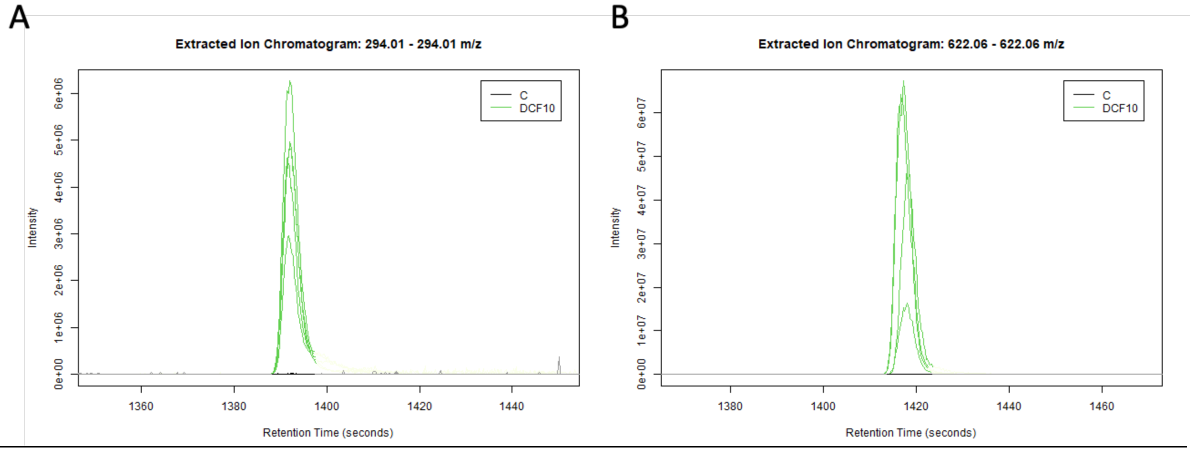


Figure S5: Peaks obtained with HPLC-Orbitrap MS analysis of cultures exposed to 10 mg L^{-1} DCF. (A) peak of DCF and (B) peak of metabolite M6

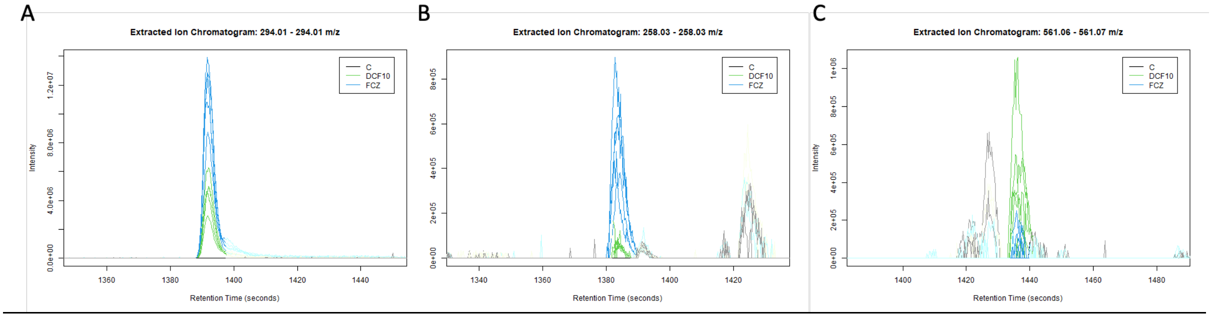


Figure S6: Peaks obtained with HPLC-Orbitrap MS analysis of cultures exposed to 10 mg L^{-1} DCF (green line) and to 10 mg L^{-1} DCF+Fluconazole (blue line) (A) peak of DCF, (B) peak of metabolite M3, (C) peak of metabolite M7.