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Unraveling the molecular effects of oxybenzone on the proteome of an environmentally relevant marine bacterium

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Abstract

The use of Benzophenone-3 (BP3), also known as oxybenzone, a common UV filter, is a growing environmental concern in regard to its toxicity on aquatic organisms. Our previous work stressed that BP3 is toxic to *Epibacterium mobile*, an environmentally relevant marine α–proteobacteria. In this study, we implemented a label-free quantitative proteomics workflow to decipher the effects of BP3 on the *E. mobile* proteome. Furthermore, the effect of DMSO, one of the most common solvents used to vehicle low concentrations of lipophilic chemicals, was assessed to emphasize the importance of limiting solvent concentration in ecotoxicological studies. Data-independent analysis proteomics highlighted that BP3 induced changes in the regulation of 56 proteins involved in xenobiotic export, detoxification, oxidative stress response, motility, and fatty acid, iron and amino acid metabolisms. Our results also outlined that the use of DMSO at 0.046% caused regulation changes in proteins related to transport, iron uptake and metabolism, and housekeeping functions, underlining the need to reduce the concentration of solvents in ecotoxicological studies.

Graphical abstract

[Graphical abstract here]

Keywords : Oxybenzone, ecotoxicology, UV-filters, proteomics, label-free

1. Introduction

The toxicity of organic UV filters in aquatic biota is well-documented[1]. Benzophenone-3, also known as oxybenzone, is one of the most controversial UV filters found to be toxic to freshwater[2–4] and marine[5–8] organisms. Its use has been prohibited in Hawaii and the Republic of Palau in 2020 because of its adverse effects on coral reefs. Oxybenzone was detected worldwide from the nanogram[9,10] to the milligram per liter range[11]. Oxybenzone toxicity toward marine organisms, in particular, has been described for mussels[6], algae[8], fish[5,12], corals[7,11,13], and more recently bacteria[14].

Marine bacteria prevail in the biomass[15] and the support of major functions in marine ecosystems[16]. Because they are the heart of biogeochemical cycles, it is essential to comprehend the effect of emerging pollutants on these microorganisms. Several studies addressed – through proteomic based approach – the response of marine bacteria exposed to changing salt concentrations and culture media components[17], cold stress[18], copper[19], UV radiation[20], heat shock, and variation in metal concentration[21]. Bacteria displayed a core proteome and an accessory proteome involved in the response to environmental and anthropogenic changes. This approach provides comprehensive information on cellular physiological adaptations toward bactericidal agents, and their mode of action[22] on environmentally relevant species. Studies evaluating the effect of emerging pollutants (manufactured nanoparticles, personal care products) on axenic culture through proteomic analysis are scarce[23,24].

Epibacterium mobile, formerly known as *Ruegeria mobilis*[25], and *R. pelagia*[26,27] was isolated from the Sargasso Sea in the Atlantic Ocean. *E. mobile* is a member of the *Roseobacter* clade, that belongs to the *Alphaproteobacteria*, the most abundant phylum in marine biota[16]. It was shown to be sensitive

to oxybenzone from 100 µg L^{-1}, with an EC_{50} of 364 µg L^{-1} [14]. *E. mobile* BBC367 strain showed high tolerance to UV radiation[28] alone and combined with UV filters[14]. A proteogenomic analysis of *E. mobile* BBCC367 cultured under 16 conditions, such as thermal and oxidative stresses, revealed that 81% of proteins belonged to the adaptative proteome, allowing the bacterium to effectively cope with its environment[21].

In the present study, we investigated, through label-free quantitative proteomics, the response of the marine *Roseobacter*, *Epibacterium mobile* exposed to oxybenzone in order to decipher the molecular adaptation mechanisms. Quantification, using mass spectrometry in data-independent analysis (DIA), was conducted twice on five biological replicates, to ensure results robustness. To our knowledge, this is the first UV filter toxicity investigation involving quantitative proteomics. Organic solvents are common in ecotoxicology studies when assessing highly hydrophobic compounds such as UV filters. The use of solvents in stock solutions is mandatory to vehicle a low concentration of the compound of interest. However, the solvent impact on the studied organism is rarely questioned. DMSO is often utilized with concentrations ranging from 0.1 to 4 %[29–31]. Therefore, DMSO effect was evaluated alone, aiming to provide a comprehensive overview of the proteome response of *E. mobile*.

2. Materials and methods

2.1 Chemicals and bacterial cultures

Benzophenone-3 (CAS-No. 131-57-7) was purchased from Sigma-Aldrich (Steinheim, Germany). The stock solution of Benzophenone-3 was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, purity >99%) at a concentration of 750 mg L^{-1} and stored in the dark at room temperature.

Table 1. Physicochemical properties of benzophenone-3.

[Table 1 here]

Bacterial strains were kept at -80°C in marine broth 2216 (DIFCO, United States) with 35% glycerol. Bacteria were grown on marine agar plates. After 24-48 hours of incubation, colonies were suspended and grown aerobically on a rotary shaker (110 rpm) at 25ºC in artificial seawater with 3

mM D-glucose, vitamins, and trace elements (ASW-G) (Eguchi et al., 1996). Bacterial cultures of 70 ml were prepared in Erlenmeyer flasks under three conditions: (1) seawater control (2) the solvent control including 0.0467% DMSO and (3) 350 µg L^{-1} BP3 + 0.0467% DMSO. Five replicates per condition were performed. When an optical density (620 nm) of 0.15 was reached, cultures were centrifuged at 8,000 g for 15 min, and cell pellets were stored at -80 °C until processing.

2.2 Assessment of BP3 toxicity

Bacterial cultures of *E. mobile* were prepared as described above under the same conditions, namely control, DMSO, and BP3. Optical density (620 nm) was monitored for 67 h. Three replicates per condition were performed and growth curves were generated using Excel (Microsoft Office).

2.3 Protein extraction

Protein extraction was performed as described in Matallana-Surget et al. (2018). Briefly, the cell pellet was re-suspended in one pellet volume of lysis buffer (6 M guanidine chloride), and cells were sonicated on ice (5 cycles of 1 min with tubes on ice, amplitude 30%, 0.5 pulse rate). Sonicated cells were centrifuged at 16,000 g at 4 °C for 15 min. Protein reduction was conducted with 25 mM dithiothreitol (DTT) at 56 °C for 30 min and alkylated with 50 mM iodoacetamide at room temperature for 30 min. Proteins were precipitated with cold acetone overnight at -80 °C, with an acetone/aqueous protein solution ratio of 4:1. The protein pellet was dissolved in 100 mM phosphate buffer (pH 8) containing 2 M urea. For LC-MS/MS analysis, tryptic digestion (sequencing grade modified trypsin, Promega) was performed overnight at 37 °C, with an enzyme/substrate ratio of 1:25.

2.4 LC-MS analysis and data processing

Protein samples were analyzed on an ultra-high-performance liquid chromatography–high-resolution tandem mass spectroscopy (UHPLC-HRMS/MS) Eksigent 2D Ultra and AB Sciex TripleTOF 5600 system. Two micrograms of peptides were analyzed using acquisition parameters previously reported[32]. In order to generate the spectral library, analyses were conducted with the

instrument operating in data-dependent acquisition (DDA). MS/MS spectra were acquired in the 100 – 1,800 m/z range. Mass spectrometry runs were conducted twice: in micro (40 min LC separation) and nano injection (120 min LC separation) modes in order to increase the number of quantified and identified proteins.

Protein searches were performed with ProteinPilot (ProteinPilotSoftware 5.0.1; Revision: 4895; Paragon Algorithm: 5.0.1.0.4874;AB SCIEX, Framingham, MA, United States) (Matrix Science,London, United Kingdom; v. 2.2). Paragon searches were conducted using LC MS/MS Triple TOF 5600 System instrument settings. Other parameters used for the search were as follows: Sample Type: Identification, Cys alkylation: Iodoacetamide,Digestion: Trypsin, ID Focus: Biological Modifications andAmino acid substitutions, Search effort: Thorough ID, DetectedProtein Threshold [Unused ProtScore (Conf)]>: 0.05. Proteins identified at a false-discovery rate of below 1% were used as the reference spectral library for subsequent data-independent acquisition (DIA). Resulting .wiff files were processed using the AB Sciex PeakView 2.1 software and the SWATH™ Acquisition MicroApp considering up to 6 peptides (with at least 99% confidence) and 6 transitions per peptide. The XIC peak area was extracted and exported in AB Sciex MarkerView™ 1.2 software for normalization using Total Area Sums. Principal component analyses (PCA) were performed on normalized data using MarkerView™. The significance of the relative abundance (fold change) observed between our experimental conditions was determined through Student's t-test with a statistical threshold set at 5% (P-value < 0.05). The cut-off for significant differential regulation (fold change) was set to 2 for upregulated proteins or below 0.5 for downregulated proteins.

The list of differentially regulated proteins was obtained by combining the quantification results from nano and micro injections: quantified proteins that met the significance criteria present in the macro injection and absent from the nano injection were added in order to increase the number of quantified proteins. Subsequently, protein functions were manually annotated. The protein list including fold change and p-values for each protein is provided in Supplementary file S7. Raw data files for both identification (exported from ProteinPilot) and quantification (exported from

MarkerView) were provided in Supplementary files S1 - S6 and S8 – S9 respectively. Mass spectrometry data were submitted to the iProx repository (project ID: IPX0002836000).

3. Results and discussion

3.2 Effect of DMSO and benzophenone-3 on the bacterial growth

The effect of BP3 was assessed by culturing *E. mobile* under three conditions: control, DMSO, and BP3 (Figure 1). No difference was observed between the control and the DMSO conditions. BP3 delayed the bacterial growth, the exponential phase starting after 39h of culture versus 15 hours in the DMSO and control conditions. Stationary phase was reached after 39h of culture in control and DMSO conditions, while 63h were needed under BP3 exposure. Noteworthy, the slope in the exponential phase and the optical density reached in stationary phase were similar in all conditions, meaning that *E. mobile* metabolism was notably delayed by BP3 but not fully inhibited at 350 µg L^{-1}. Previous work highlighted that BP3 concentrations from 2000 µg L^{-1} were necessary to show bactericidal effects[14].

[Figure 1 here; 1.5-column fitting image; no color needed]

Figure 1. Growth curves of *Epibacterium mobile* in artificial seawater only (dark circle), with 0.047 % DMSO (white circle), or with 350 µg L^{-1} BP3 and 0.046 % DMSO (square) (average ± standard deviation, n =3)

3.3 Protein identification

DDA dataset was used to generate a protein list per condition. An average of 901, 887, and 890 proteins were identified in the Control, DMSO, and BP3 conditions, respectively (Supplementary file S10). The number of identified proteins was not significantly different between conditions. Overall, 1182 proteins were identified, representing a proteome coverage of 27.4% of its theoretical proteome.

3.4 Label-free protein quantification

Label-free data-independent acquisition (DIA) proteomics was performed to compare the relative concentrations of proteins within the proteome of bacteria cultured under BP3 and solvent control conditions. Principal component analyses (PCA) including the data of all replicates, in micro injection (Figure 2A) and nano injection mode (Figure 2B) were conducted. All replicates clustered by conditions, meaning there is a statistical difference among treatment groups, and proteome consistency throughout the replicates, regardless of the injection mode. PCA revealed that the control and solvent control conditions were closer compared to the BP3 condition, mainly differentiated through the PC1 axis. In addition, these results indicate that the three treatments led to significantly different proteomes, suggesting phenotypic adaptations to DMSO and BP3, to a greater extent.

[Figure 2 here; 2-column fitting image; color needed]

Figure 2. Principal component analysis of *E.mobile* proteomes.

3.5 Effect of DMSO on protein regulation

In our study, DMSO concentration was reduced to a minimal level, i.e. 0.046 %, in order to evaluate cells in optimal physiological fitness. Hence, the proteomes cultured in artificial seawater with DMSO (solvent control) and without (control) were compared.

When comparing solvent control and control conditions, downregulated and upregulated proteins accounted for 12 and 11, respectively. Transporter was the most upregulated functional category, with 4 proteins, followed by general metabolism with 3 upregulated proteins (Figure 3B). Among the downregulated proteins, Energy and DNA processing were the most affected functional category, with 5 and 4 downregulated functions, respectively (Figure 3B).

Table 2. Identification, function, and fold change of proteins differentially regulated between *E. mobile* exposed to 0.046% DMSO and artificial seawater only. Proteins are classified from the most downregulated to the most upregulated. Color code match the function group highlighted in Figure 3. Proteins included displayed a p-value < 0.05, bold in the fold change column indicate p-value <0.01

[Table 2 here; color needed]

RdgB/HAM1 family non-canonical purine NTP pyrophosphatase was the most upregulated protein (Table 2). Involved in nucleotide metabolism, RdgB was shown to prevent modified purine dNTPs from incorporating DNA, hence protecting the cell from mutation[33]. Interestingly, the iron-related proteins downregulated in presence of BP3, were upregulated with DMSO, when compared to the control condition (Figure 4). The Fe^{3+} ABC transporter substrate-binding protein displayed a fold change of 2.43 and the hemin uptake protein (HemP) was second most upregulated (5.68 fold). DMSO was reported to form complexes with cations, including Fe^{3+}[34,35]. The upregulation of proteins involved in iron uptake might be a cell response to counteract the iron starvation induced by the formation of DMSO-Fe^{3+} complexes. Other proteins, involved in general metabolism and housekeeping functions such as oxidoreduction, peptidase activity, and phosphorylation displayed dysregulation (Figure 4)

Overall, our work highlighted that even at a low concentration, DMSO did not alter bacterial growth (Figure 1), but induced a differential protein regulation at the molecular level (Figure 2). Since the solvent concentration is rarely debated, our results corroborate that reducing the concentration to a minimum is the best strategy to adopt in ecotoxicological studies.

3.6 Effect of benzophenone-3 on protein regulation

Downregulated and upregulated proteins accounted for 13 and 43, respectively, with protein level variation ranging from 0.2 to 8.38 fold between BP3 and solvent control conditions (Table 3). Among the upregulated proteins, the most represented function groups were transporters (13 proteins), followed by putative & unknown functions (9 proteins, Figure 3A). Interestingly, transporter was also the most downregulated functional category with 10 proteins (Figure 3A). Other functions involved in general metabolism, energy, motility and oxidative stress response were affected.

Table 3. Identification, function, and fold change of proteins differentially regulated between *E. mobile* exposed to 350 µg/L BP3 + 0.046% DMSO and 0.046% DMSO only. Proteins are classified from the most downregulated to the most upregulated. Color code matching the functional category highlighted in Figure 3. Proteins included **displayed a p-value < 0.05, bold** in the fold change column indicate p-value < 0.01.

[Table 3 here; color needed]

[Figure 3 here; 2-column fitting image; color needed]

Figure 3. Number of upregulated (green bars) and downregulated (red bars) proteins induced by the presence of BP3 (A) and DMSO (B), classified by functional category.

ABC transporters, the largest family of ATP-dependent transporters, were upregulated and, to a lesser extent, downregulated in bacteria cultured with BP3 (fold change up to 7.14). ABC transporters play a crucial role in the import and export of numerous compounds, hence participating in cellular integrity and homeostasis. Previous studies reported that ABC transporters conferred xenobiotic and antibiotic resistance through their export[36–38].

The TMAO reductase system periplasmic protein TorT was the most upregulated protein (8.38 fold) in presence of BP3. TorT is a periplasmic molecular sensor, able to bind trimethylamine N-oxide (TMAO), and subsequently, overexpress the torCAD operon[39,40]. In anaerobic conditions, TMAO can be used as a final electron acceptor. Upregulation of the torT transcript was described in anaerobic conditions[40]. This up-regulation could be explained by a BP3 driven sequestration of the aerobic respiration pathways, or an energy deficit, forcing the cell to trigger all respiration routes available.

A total of 4 proteins involved in the response and signaling of oxidative stress were upregulated (Figure 4). Peroxiredoxin – displaying a 3.11 fold change (Table 3) – is a core member of the antioxidant system in both eukaryotic and prokaryotic organisms. This thiol specific peroxidase can reduce various substrates such as H_2O_2, alkyl hydrogen peroxides, and peroxynitrite[41]. 5-oxoprolinase hydrolyzes 5-oxoproline – a toxic intermediate of the glutathione metabolism[42] – to form glutamate. This enzyme was upregulated (3.68 fold) along with allophanate (5.40 fold). Genes coding for these proteins are known to cluster in the genome (pxpABC genes cluster) suggesting a functional

222 relationship[42]. Such variation might be resulting from an increased activity of the glutathione
223 detoxification system, to counteract BP3 induced oxidative stress. PrkA family serine protein kinase –
224 a widely distributed kinase – displayed a 2.55 fold change. PrkA assumes diverse functions in signal
225 transduction and regulation through phosphorylation of multiple substrates[43]. Lima et al. (2020)
226 revealed, through the investigation of the *Listeria monocytogenes* proteome, that PrkA interacted with
227 62 proteins involved in 19 functional categories[44]. Previous studies showed PrkA upregulation in the
228 presence of low oxygen, high heat, and two microbicides: chlorhexidine gluconate and benzalkonium
229 chloride[45]. Hydroperoxidase I (HPI) – another important enzyme for cell homeostasis, produced by
230 bacteria under peroxide stress[46] – displayed a 2-fold change, in response to BP3. Lastly, the
231 transcriptional repressor LexA, involved in DNA damage response, showed a 3.10 fold change. This
232 upregulation demonstrates that BP3 induced DNA damage through oxidative stress. Two proteins
233 involved in oxidative stress response were downregulated. Pyridoxine 5'-phosphate synthase (0.49
234 fold), involved in the synthesis of vitamin B6, is known to provide resistance against oxidative stress
235 by quenching ROS[47]. A previous study showed that a *Mycobacterium tuberculosis* pyridoxine 5'-
236 phosphate synthase mutant showed a decreased viability due to the subsequent vitamin B6
237 auxotrophy[47]. Furthermore, the YchF ATPase, known to mitigate oxidative stress through the
238 regulation of ATPase activity under stress condition[48], was downregulated (0.49 fold).

239 Upregulation of a biotin-dependent carboxyltransferase family protein was characterized (6.39 fold).
240 This enzyme is part of a multisubunit enzyme such as Acyl-CoA carboxylases and known to play key
241 roles in fatty acid synthesis and polyketide synthesis pathway[49]. The upregulation of this enzyme –
242 along with the upregulation of an acyl-CoA dehydrogenase family protein – could be a response from
243 the cell to restore damaged membranes.

244 Motility was one of the most represented functions in upregulated proteins with 3 flagellins
245 and 1 flagellar hook-basal body complex protein that displayed fold change ranging from 2.24 to 2.58.
246 Du and coworkers (2011) demonstrated that flagellar gene expression was induced in hyperosmotic
247 stress in *Salmonella enterica* to survive in the human intestine[50]. To the best of our knowledge, the

upregulation of flagellin in the presence of xenobiotics in marine bacteria has never been reported. Known as a part of the chemotaxis component, the upregulation of these proteins could be linked to a defense mechanism, such as negative chemotaxis, towards benzophenone-3.

Changes in the regulation of proteins involved in amino acid metabolism were reported. Succinyl-diaminopimelate desuccinylase (DapE) was the most downregulated protein (0.2 fold) in BP3-exposed *E.mobile*. DapE is a major component of lysine biosynthesis through the succinylase pathway, the only one in most bacterial species[51]. Because lysine, an essential amino acid, is only available through succinylase pathway, antimicrobial compounds have been designed to target DapE[52], hence inhibiting protein synthesis. Among the most downregulated proteins, citramalate synthase and the RidA family protein displayed a 0.29 and 0.34 fold change, respectively. Both proteins are involved in L-isoleucine biosynthesis. Conversely, the 2-isopropylmalate synthase, involved in L-Leucine biosynthesis was upregulated. A previous study demonstrated that leucine containing peptides inhibited the growth of *E. coli*, while the addition of isoleucine reversed toxicity[53]. The authors postulate that leucine accumulation impaired isoleucine biosynthesis. This imbalance in isoleucine/leucine ratios could therefore be a side-effect of BP3 toxicity. Furthermore, RidA was shown to protect cells from damaging reactive intermediate such as 2-aminoacrylate[54], reinforcing that its downregulation could be detrimental for cell integrity.

Interestingly, three proteins involved in iron uptake and metabolism, namely the iron-sulfur cluster assembly accessory protein (0.41 fold), the Fe^{3+} ABC transporter substrate-binding protein (0.47 fold), and the hemin uptake protein (HemP) (0.32 fold), were downregulated. Since Iron is a cofactor of multiple enzymes[55], dysregulation in its provisioning could severely hamper vital cellular processes.

[Figure 4 here; 2-column fitting image; color needed]

Figure 4. Schematic representation of up and downregulated proteins within the proteome of benzophenone 3 exposed *E. mobile* (350 µg/L), compared to the solvent control condition.

4. Conclusion

Our study deciphered for the first time how UV filter toxicity affected marine bacteria at a proteomic level. We demonstrated that *E. mobile* responded to BP3 by upregulating proteins involved in export, detoxification, cell motility and fatty acid synthesis. We also reported the downregulation of proteins involved in amino acid metabolism and iron uptake. Our research stressed that BP3 toxicity implied complex processes, altering multiple cellular functions, with an emphasis on oxidative stress and amino acid synthesis. Lower proteomic variations were found between the control and solvent control groups. (23 vs 56 dysregulated proteins). Our work suggested that DMSO induced disequilibrium in iron homeostasis, emphasizing the importance of reducing solvent concentration in ecotoxicological studies.

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Notes

The authors declare no competing financial interest.

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