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Tralopyril bioconcentration and effects on the gill proteome of the Mediterranean mussel *Mytilus galloprovincialis*

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Abstract

Antifouling (AF) systems are used worldwide as one of the most cost-effective ways of protecting submerged structures against heavy biofouling. The emergence of environment-friendly AF biocides requires knowledge on their environmental fate and toxicity. In this study we measured the bioconcentration of the emerging AF biocide tralopyril (TP) in the Mediterranean mussel *Mytilus galloprovincialis* and investigated the effects of TP on the mussel gill proteome following acute (2 days) and chronic (30 days) exposure, as well as after a 10-day depuration period. The experiments were carried out with 1 µg/L TP; blank and solvent (5x10⁻⁵ % DMSO) controls were also included. Proteomics analysis was performed by mass spectrometry-based

multidimensional protein identification technology (MudPIT). Differentially expressed proteins were identified using a label-free approach based on spectral counts and G-test. Our results show that TP is rapidly accumulated by mussels at concentrations up to 362 ng/g dw (whole tissues), reaching the steady-state condition within 13 days. Ten days of depuration resulted in 80% elimination of accumulated TP from the organism, suggesting that a complete elimination could be reached with longer depuration times. In total, 46 proteins were found to be regulated in the different exposure scenarios. Interestingly, not only TP but also DMSO alone significantly modulated the protein expression in mussel gills following acute and chronic exposure. Both compounds regulated proteins involved in bioenergetics, immune system, active efflux and oxidative stress, often in the opposite way. Alterations of several proteins, notably several cytoskeletal ones, were still observed after the depuration period. These may reflect either the continuing chemical effect due to incomplete elimination or an onset of recovery processes in the mussel gills. Our study shows that exposure of adult mussels to sublethal TP concentration results in the bioconcentration of this biocide in the tissues and modulates the expression of several proteins that may intervene in important metabolic pathways.

1. Introduction

Antifouling (AF) systems are used to prevent biofouling, a natural phenomenon characterized by the attachment and growth of living organisms on submerged surfaces. Biofouling may lead to elevated economic losses, not only due to the deterioration of submerged structures, but also, in the case of vessels, due to the increase of frictional drag resulting in the rise of fuel consumption, decrease of manoeuvrability and reduction of its operational time. In addition, biofouling has the potential to contribute to ecological problems as a consequence of the transport and dissemination of non-indigenous species (Dafforn et al., 2011). In turn, the leaching of biocides from AF systems has also been accountable for several adverse ecological impacts (Dafforn et al., 2011; Thomas and Brooks, 2010). Thus, a balance between effective AF properties and environmental protection is a major challenge.

Recently, new AF biocides claiming to be more environment-friendly have been introduced onto the AF paint market. Tralopyril (TP; 4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1*H*-pyrrole-3-carbonitrile) is the main biocide in

the non-metallic AF system ECONEA[®] (Janssen PMP, Belgium) used to control the incrustation of barnacles, hydroids, mussels, oysters and polychaetes (Thomas and Brooks, 2010). As TP was recently accepted for inclusion under the Biocide Products Regulation (BPR, EU Regulation No. 528/2012 (EU, 2012)) and therefore considered an ecologically safer alternative to previously used hazardous biocides (Mert et al., 2014), its use is likely to become widespread in Europe. TP is thought to act as an uncoupler, ultimately affecting ATP production (ECHA, 2014a). The exact mode of action of this compound is, however, not known since uncoupling refers to any energy-dissipating process that competes with routine mitochondrial functions, thus inducing a metabolically futile waste of energy (Wallace and Starkov, 2000). Substances classified as uncouplers have been shown to have the highest toxicity among other groups of chemicals such as organophosphates, carbamates or nonpolar narcotic chemicals (Hoff et al., 2010; Raimondo et al., 2007). Despite these indications, few studies on the toxicity of TP have been performed, especially in non-target species (Kempen, 2011; Martin-Rodriguez et al., 2014; Oliveira et al., 2014).

The toxicity of emerging AF biocides is required to be very specific: high to target organisms and low to non-target ones. However, target organisms are sometimes hard to define and mussels are a perfect example of such a dilemma. In the coastal shores, mussels represent a key species operating as ecosystem engineers by providing habitats for other organisms, filtering out sediments and pollutants and providing food for higher trophic levels (Carrington et al., 2015). On the other hand, especially during their larval stage, mussels can be considered target organisms as they belong to the fouling community attaching onto underwater structures. This conflict makes mussels an interesting and relevant species to study. Furthermore, their sessile and filter-feeding nature, plus the typically large and widely distributed population size make this species very attractive to biomonitoring and ecotoxicological studies. Besides, mussels are known to accumulate (and also tolerate) high concentrations of many organic and inorganic pollutants (Goldberg, 1975; Ogata et al., 2009) and for that reason are used in monitoring programs (commonly known as "Mussel Watch") to assess aquatic pollution (ICES, 2011).

In our previous study (Oliveira et al 2014), the effects of TP in the target-phase of mussels resulted in an acute EC₅₀ of 3.1 µg/L on the abnormal development of its D-veliger larvae, demonstrating the effectiveness of this biocide in the

protection against fouling by mussel larvae. The same study predicted a maximum environmental concentration of 0.7 µg/L in a seawater marina. In the present study we have focused on the non-target mussel adult stage and assessed the effects of exposure to sublethal TP concentrations close to those possibly occurring in a marina. Due to the high sensitivity of proteomic studies and because gills have a protective function as a first physical barrier against toxic contaminants present in the water, we chose to evaluate the TP effects by looking at the alterations in the mussel gill proteome. Environmental proteomics aims to identify protein variations induced by xenobiotics or any other biotic or abiotic factors in order to identify proteins that respond to these stressors (Campos et al., 2012; Nesatyy and Suter, 2008; Shepard et al., 2000). Although the molecular mechanisms underlying a toxic exposure are not totally understood, proteomics may still provide new insights into the mechanism of action of a specific compound (Monsinjon and Knigge, 2007).

In the present study we assessed TP bioconcentration in the whole mussel tissues and applied a Multidimensional Protein Identification Technology (MudPIT; Washburn et al, 2001) to inspect the TP-induced changes on the gill proteome of the adult Mediterranean mussel, *Mytilus galloprovincialis*. Proteomics analysis was performed for three different exposure scenarios: (i) after two days of exposure (T2; acute exposure) (ii) after thirty days of exposure (T30; chronic exposure) and (iii) after ten days of depuration following thirty days of exposure (T40; recovery).

2. Material and Methods

2.1. Chemicals

TP (PESTANAL®, analytical standard, Fluka), dimethyl sulfoxide (DMSO, > 99.5 % purity), tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), iodoacetamide (IAA), CHAPS, and Protease Inhibitor Cocktail were purchased from Sigma–Aldrich (Switzerland). Trypsin (sequencing grade) was obtained from Roche Applied Science and the dye reagent for the Bradford test from Bio-Rad. Other conventional chemicals were either from Sigma–Aldrich or Fluka (Switzerland). HPLC grade solvents were either from Acros Organics (Belgium) or Rathburns (Sigma-Aldrich, Germany).

2.2. Experimental procedure

M. galloprovincialis specimens 40-50 mm long were collected from the intertidal shores of a pristine site in the outer part of Ria de Vigo (NW Iberian coast, Spain) in February 2011 and acclimated in the laboratory for 7 days.

A TP stock solution was prepared by diluting the compound in DMSO, divided into 5 aliquots and kept at -20 °C until use. One aliquot was defrosted each week and kept at 4 °C to prepare the exposure solution in filtered natural seawater (FSW; 1 µm pore size Betafine XL 19PP010DG Filter Cartridge) collected at a pristine site from the local coast of Spain. In recent years, low levels of pollutants (Cu, Zn, Pb, Cd, PAHs and PCBs) have been measured in mussel tissue collected from this site (control site (V0) in Vidal-Liñán et al., 2010). Although the use of FSW simplifies the protocol and simulates the natural conditions, a disadvantage is the limited replication of results as seawater composition may vary geographically and temporally. Mussels were exposed to solvent control (5×10^{-5} % DMSO; this treatment is hereafter abbreviated as DMSO) or TP (1 µg/L; hereafter abbreviated as TP) during 30 days, and then transferred to clean FSW for depuration over 10 days. Clean FSW controls were also run in parallel (this treatment is hereafter abbreviated as CTRL). The experiment was performed in 30 L glass tanks in darkness, continuously aerated (0.20 µm pore size filters Midisart® 2000, Sartorius) and at a constant temperature of 16 °C. The ratio of one animal per 1.5 L of seawater was constantly maintained. Mussels were fed three times per week for one hour with a mixed diet of *Isochrysis galbana*, *Tetraselmis suecica* and *Chaetoceros gracilis*. Due to TP's short half-life (Kempen 2011; Oliveira et al., 2016), the exposure was performed under semi-static conditions where a complete water exchange and the addition of a new chemical dose (DMSO or TP) was done daily. However, it is to note that in a real situation, the biocide would be constantly released from the AF-paint surface and thus the TP concentrations might be more constant than those obtained in our study. Water exchange was always performed two hours after feeding to ensure not only sufficient time for filter-feeding but also that no algae were present that could interfere with the freshly spiked compound. This schedule was maintained throughout the 40 days of the experiment.

To assess the TP content in the exposure aquaria, water samples were collected within one water exchange cycle (24 h) on 3 different days: approximately 1 L of water was collected at 0 (immediately after the compound spiking), 0.5, 1, 2, 3,

6, 9, 12, 18 and 24 h, spiked with atrazine-d₅ (used as internal standard) and frozen at - 20 °C. For easier transportation, water samples were thawed and extracted as follows: each HLB solid phase extraction cartridge (Waters, Sweden) was conditioned with 2 ml methanol followed by 4 ml Milli-Q water (18 MΩ·cm). The sample was then applied and the cartridge was dried under vacuum, wrapped in aluminum foil and frozen at - 20 °C until chemical analysis.

Samples of 10 animals from the CTRL, DMSO and TP treatments were collected at the beginning of the experiment (T0), after 2 (T2), 5 (T5), 9 (T9), 15 (T15), 20 (T20) and 30 (T30) days of exposure and after 10 days of depuration (T40). For each treatment, the gills from 4 animals were individually excised, immediately frozen in Eppendorf tubes using liquid nitrogen and kept at - 80 °C until proteomic analysis. The whole tissues of the other 6 animals (without shells) were pooled, frozen, freeze-dried and kept frozen in glass vials at - 20 °C until chemical analysis.

2.3. TP chemical analysis

Water samples were eluted from the HLB cartridges with 2 ml hexane plus 4 ml ethyl acetate, evaporated under nitrogen to approximately 1 ml using a TurboVap (Zymark, USA, 40 °C) and analyzed by LC-MS/MS (UPLC-Quattro Premier XE, Waters, Sweden).

For the mussel samples, about 1 g of lyophilized mussel tissue was extracted with ethyl acetate (~15 ml), shaken for 30 min and centrifuged for 10 min. The procedure was repeated with the shaking time decreased to 10 min. Both extracts were combined, concentrated to 1 ml using a TurboVap and filtered with a 0.45 µm filter (Teknolab, Oslo, Norway). Extracts were cleaned up by gel permeation chromatography (GPC). GPC was carried out on an Alliance 2695 system (Waters, Milford MA, USA) with two sequential Envirogel GPC clean-up columns (19 x 300 mm and 19 x 150 mm) and dichloromethane as a mobile phase at a flow rate of 5 ml/min. The 12.35 – 20.3 min fraction was collected. The extract was evaporated to near dryness and solvent exchanged for methanol/water. TP was measured by a LC-MS/MS method reported previously (Oliveira et al, 2016). Briefly, the mass spectrometer was operated in negative ion mode and two transitions were monitored in multiple reaction monitoring. The transitions 348.9 → 80.7 and 346.9 → 78.7 were used for quantification and qualification, respectively. The internal standard, atrazine-

d₅, was determined in positive mode (m/z 221.2 → 179.1). The detection limit was 0.05 ng/L in water and 1 ng/g dry weight (dw) in mussels.

2.4. Modelling TP bioconcentration

One-compartment models were chosen to describe and predict internal concentrations of TP in the whole mussel as well as to determine its empirical uptake and elimination constant rates. This approach assumes that a chemical, which enters an organism, is distributed instantaneously and equally. This concept can be described with the following equation (Landrum et al., 1992; Stadnicka et al., 2012):

$$\frac{d}{dt}C_{\text{int}}(t) = k_{\text{in}} \cdot C_{\text{w}}(t) - k_{\text{out}} \cdot C_{\text{int}}(t) \quad (\text{eq. 1})$$

where $C_{\text{int}}(t)$ is the internal chemical concentration (amount $\times$ mass⁻¹), $C_{\text{w}}(t)$ is the chemical concentration in the water (amount $\times$ volume⁻¹), k_{in} is the uptake rate constant (volume $\times$ mass⁻¹ $\times$ time⁻¹) and k_{out} is the elimination rate constant (time⁻¹).

The time to steady-state condition was obtained using the equation: $-\ln(0.05)/k_{\text{out}}$ (Landrum et al., 1992; Stadnicka et al., 2012). Empirical uptake and elimination constant rates for TP were determined by calibrating respective parameters from eq. 1 based on measured data of TP concentrations in water and mussel over time, obtained in this study.

Measured internal concentrations of TP were compared with predicted values obtained by using a simple one-compartment toxicokinetic model (Hendriks et al., 2001). This approach describes the accumulation kinetics of organic chemicals as a function of the octanol-water partition coefficient, as well as the weight, lipid content and trophic level of the species. It was applied with the following model inputs: TP half-life in water: 6.1 h (Oliveira et al., 2016); TP pKa: 7.08 (ECHA, 2014a); water pH: 8.1 (this study); TP corrected log Kow: 3.54 (calculated based on pH and pKa); organism lipid content: 4.94% (from 24.7% of dry weight; Prato et al., 2010); organism water content = 20% (Sousa et al., 2009); mussel wet weight: 0.49 g (Sievers et al., 2013). All models were implemented and solved using ModelMaker (version 4.0, Cherwell Scientific Ltd., Oxford, UK).

2.5. Multidimensional Protein Identification Technology (MudPIT)

For the protein extraction, the gills of three mussels were pooled, placed in the lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% Triton X-100, 0.1 M Tris-HCl,

19 Protease Inhibitor Cocktail, pH 8) and homogenized with a grinder. The mixture was then sonicated on ice with three successive 10 s bursts (30 s pauses) and centrifuged at 13 000 rpm for 60 min at 4 °C. The supernatant was transferred to a clean tube and again centrifuged at 13 000 rpm for 60 min at 4 °C. The two latter steps were repeated twice. Subsequently, the proteins were precipitated from the supernatant using a conventional methanol/chloroform method. The pellet formed after the precipitation was air-dried for 5 min and redissolved in resolubilization buffer (9 M urea, 2 M thiourea, 0.1 M Tris-HCl, pH 8.5). To improve the solubility the pellet was wetted with 0.2 M NaOH before adding the resolubilization buffer. The protein concentration was measured by the Bradford method. To prepare the tryptic digests, 100 µg of proteins were reduced with TCEP, alkylated (carbox-amidomethylated) with IAA, and digested with trypsin at a ratio of 100:1 during incubation overnight (14 to 16 h) at 37 °C with shaking. The reaction was quenched by adding formic acid, filtered (0.45 µm, Durapore PVDF, Merck Millipore) and placed into glass vials. The peptide mixture was loaded onto the nano-HPLC device (Ultimate 3000, Dionex), by first trapping it on a commercial C18 column (Dionex, 5 mm, 300 µm ID, 5 µm, 100 Å, C18 Acclaim PepMap 100), then eluting it onto an in-house filled SCX column (Macherey Nagel AG, 3.5 cm, 100 µm ID, 5 µm, Nucleosil 100-5 SA), followed by an analytical in-house filled C18 column (Macherey Nagel AG, Switzerland; 4.5 cm, 100 µm ID, 3 µm, 100 Å, Nucleodur C18 Pyramid) linked in series. A 25 µm ID fused silica linked the SCX to the analytical column. Both in-house built columns were made from fused silica tubing (100µm ID, 363µm OD, BGB Analytik AG, Switzerland). The SCX was closed with a frit and pressure filled, while the analytical column was pulled to a needle using a needle puller (Sutter Instrument Co., Model P-2000, Science Products AG, Basel) before pressure filling. The SCX and analytical column were replaced after three analytical runs. The sample was directly sprayed from the analytical column into the LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany). The instrument was calibrated using a mixture from Thermo Scientific (CalMix, pos. ProteoMass LTQ/FT Hybrid, Supelco), tuned on the doubly charged angiotensin molecule ($m/z = 648.846$) and operated at 1.2 kV spray voltage in positive ion mode with the tube lens set to 135 V and the ion transfer capillary temperature to 200 °C. Peptides were sequentially eluted from the SCX resin to the reverse phase resin by a fully automated standard 11-step MudPIT protocol

with increasing salt concentration, followed by an organic gradient (Groh et al., 2011). One full scan FT mass spectrum (300–1,600 m/z, resolution of 60 000) was run in parallel to 7 data-dependent MS/MS scans acquired in the linear ion trap with normalized collision energy (setting of 35%). Precursor ions for MS/MS analysis were selected scan-dependently with dynamic exclusion set to 60 s and minimal signal intensity of the protonated molecular ion to 10^3 counts. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific). Two technical replicates of each sample were performed.

2.6. Analysis of LC-MS/MS data

Scripts developed in-house were used to generate peak lists by converting the raw data files into MASCOT generic format files (mgf). The data generated was searched for peptide hits using the Open Mass Spectrometry Search Algorithm (OMSSA; Geer et al., 2004) against an in-house curated protein database containing the available protein sequences for *M. galloprovincialis* downloaded from NCBI on October 21, 2013. Redundant protein entries were partially removed by the software CD-HIT (Li and Godzik, 2006) with the threshold set to 0.95. The database was supplemented with the sequences of common contaminants that could originate from sample processing, such as highly abundant human proteins (e.g. keratins) and trypsin. Equal numbers of randomized sequences produced by the decoy.pl PERL script freely available from MASCOT (Matrix Sciences, Boston, MA, USA) were also added to the database for calculating false discovery rates (FDR) based on the target-decoy database approach (Käll et al., 2008). During database search carbamidomethyl-cysteine was set as fixed modification while protein N-terminal acetylation, oxidation of methionine, deamidation of asparagine and glutamine and peptide N-terminal formation of pyroglutamic acid were set as variable modifications. Tryptic specificity was set to a maximum of two missed cleavages. The charge of precursors to be searched was +2 and +3, and minimum precursor charge to start considering multiply charged products was set to +2. Only discrete hits with FDR less than 1% were considered for further analysis. Label-free protein quantitation was performed using spectral counts for observed peptide hits. In this analysis the data from the two technical replicates were combined and analyzed by G-test (Zhang et al., 2006). For each protein and for all experimental conditions the normalized spectral

abundance factor was determined (NSAFs; Zybailov et al, 2006). The protein fold changes were estimated by dividing this value in the exposure conditions by the corresponding value in the control condition. Subsequently, count tables were created for the three different samples (CTRL, DMSO and TP) at the three time points (T2, T30 and T40). Proteins that did not have at least twenty counts in at least one of the conditions were removed from the final list.

A principal component analysis (PCA) was performed on the NSAFs from the 9 individual samples, using Mayday software (Battke et al., 2010). PCA reduces the large number of dimensions of a dataset describing data as linear combinations (principal components) of so-called scores and loadings that give a simplified and succinct description of the dataset variance (Verhoeckx et al., 2004).

3. Results

The response of the Mediterranean mussel to mechanical stimuli during the daily water exchange procedure appeared to be normal throughout the experiment duration. No malformations were observed at the different collection times and no mortality was recorded during the 30 days of exposure or the 10 days of depuration in any of the treatments.

3.1. TP content in water and mussel tissues

Since TP has a half-life of 6.1 h (Oliveira et al., 2016) and seawater spiked with TP was exchanged daily, the theoretical TP concentration in water, throughout the exposure time, is expected to follow the pattern presented in Figure 1A. However, the initial concentration measured after the spiking varied considerably and was always lower than the nominal concentration of 1 $\mu\text{g/L}$ (Figure 1B).

Figure 1 – Tralopyril (TP) content in exposure water: A. estimation for the whole experiment based on the 1 $\mu\text{g/L}$ dosed concentration and TP half-life of 6.1 h (Oliveira et al., 2016); B. measured

by LC-MS/MS at different time points (starting right after spiking) within one water exchange cycle (24 h). The experiment was repeated on three different days and values are presented as mean $\pm$ SD.

TP content in the mussel tissues was inspected for all samples in order to assess TP bioconcentration. Tissue samples from CTRL and DMSO treatments at all time points, as well as those sampled from the TP treatment at the beginning of the experiment (T0), were below the detection limit for TP (except for DMSO T5 where 6.4 ng/g dw of TP was measured, possibly due to a contamination during the sample collection). A simple one-compartment model was fitted to the concentrations measured in the TP-treated mussels (Figure 2A). According to the model, the uptake and elimination rates were $246 \text{ L} \cdot (\text{kg dw} \cdot \text{day})^{-1}$ and 0.23 day^{-1} , respectively. TP could already be detected in mussel tissues following 2 days of exposure (T2; 158.8 ng/g dw) and accumulated rapidly until 13 days where it appeared to plateau. After 10 days of depuration in clean seawater, TP content was reduced by 80 % but remained measurable (T40; 74.6 ng/g dw).

The predicted bioconcentration of TP in mussels (Figure 2B) was very similar to measured values (Figure 2) however two differences were noted: the depuration process was much slower than the modelled and the experimental time to steady state took longer to be reached. According to this mechanistic toxicokinetic model (Figure 2B), depuration should have occurred less than 5 days after placing the mussels in clean seawater and the time to steady-state conditions should have been reached within 3 days, which did not happened.

Figure 2 – Tralopyril (TP) content in mussel tissues: fitted (A) and predicted (B) by using one-compartment toxicokinetic models. Measured internal TP concentrations (◆) and model simulations (black lines) are plotted.

3.2. Effects of exposure on mussel gill proteome

Differential proteomics studies usually focus on the comparison of two proteomes, control vs. exposed. Here, changes in the protein expression in mussel gills after exposure to solvent control (DMSO) or to 1 µg/L TP were investigated. The solvent control had to be included in the experiment due to low solubility of TP in water. Protein expression profiles for each of the three applied treatments (CTRL, DMSO and TP) were assessed at three different times of exposure: T2 (after acute exposure), T30 (after chronic exposure) and T40 (after depuration period). At each time point, protein expression was compared for the following treatment pairs: DMSO vs. CTRL, TP vs. CTRL and TP vs. DMSO. In this way, comparing DMSO to CTRL would provide information on the effects of exposure to DMSO alone, while comparing TP to DMSO treatment would give an indication of additional TP-only effects. Such an approach has already been used in other studies that compared the tested compound to the solvent control instead of comparing to water controls only, in order to better understand specific effects of the chemical itself (Kamel et al., 2012; Lacaze et al., 2015). When comparing TP to CTRL, one must keep in mind that the changes observed would correspond to the effects of the mixture of both TP and DMSO. This mixture might behave in a complex and rather different way than just the sum of effects provoked by TP or DMSO individually.

An average of 298 ± 116 , 292 ± 105 and 275 ± 82 unique protein sequences were identified at three time points for CTRL, DMSO and TP treatments, respectively. Forty six proteins across the three treatments and three time points were found to fulfil the criteria of G-Test significance ($p < 0.01$) and to have more than 20 counts in at least one of the conditions (Figure 3).

Exposure not only to TP, but also to DMSO alone, affected cytoskeleton and chromatin structural proteins, as well as proteins involved in oxidative and general stress defence, immune response, active efflux, electron transfer, glycolysis, gluconeogenesis, apoptosis and steroidogenesis (Figure 3). The observed effects of DMSO on the mussel gill proteome were quite surprising, since several earlier studies had reported no significant effects of this solvent on the protein expression in mussel tissues. For example, no significant effects were detected in the protein expression profiles of mussel hepatopancreas exposed to 0.002% DMSO (Ji et al., 2014) and in mussel gills exposed to 0.002% DMSO (Ji et al., 2013) or 0.005% DMSO (Maria et

al., 2013). In our study, despite the even lower DMSO concentration used ($5 \times 10^{-5}\%$), the differences between water control and solvent control cannot be disregarded.

	Protein Name	T2			T30			T40		
		DMSO vs. CTRL	TP vs. CTRL	TP vs. DMSO	DMSO vs. CTRL	TP vs. CTRL	TP vs. DMSO	DMSO vs. CTRL	TP vs. CTRL	TP vs. DMSO
Electron Transfer Chain	cytochrome c oxidase subunit I	*				*	*			
	COX2 gene product (mitochondrion)	*		*						
	cytochrome c oxidase subunit III (mitochondrion)	*		*						
Glycolytic Process	fructose biphosphate aldolase	*		*		*	*			
Gluconeogenesis	phosphoenolpyruvate carboxykinase	*		*					*	
ABC Transporters	ATP-binding cassette sub-family G member 2-like	*		*		*	*	*	*	
	ABCB/P-glycoprotein-like protein	*	*	*				*		*
Apoptosis	caspase 2			*		*	*			
	putative caspase 3	*	*				*			
	caspase 3/7-2	*	*	*		*	*			*
Immune System	mytilin B antimicrobial peptide precursor					*	*			
	NF-kappaB transcription factor Rel	*		*		*	*			*
	myeloid differentiation factor 88a		*	*	*	*	*		*	*
	toll-like receptor I precursor	*	*	*				*	*	*
	lectin	*		*						
	C1q-domain-containing protein		*	*	*			*	*	*
	putative C1q domain containing protein MgC1q4	*		*						
	putative C1q domain containing protein MgC1q5	*	*	*						
	putative C1q domain containing protein MgC1q6	*	*	*			*			*
	putative C1q domain containing protein MgC1q8	*	*	*	*	*	*	*	*	*
	putative C1q domain containing protein MgC1q11	*	*	*	*	*	*	*	*	*
	putative C1q domain containing protein MgC1q33	*	*	*	*	*	*			
	putative C1q domain containing protein MgC1q46	*	*	*	*	*	*			
Steroidogenesis	17 β -hydroxysteroid dehydrogenase 10	*		*	*	*	*			
Oxidative Stress	glutathione S-transferase GSTpi1	*		*	*	*	*			*
	thioredoxin 1	*	*	*	*	*	*			*
	CuZn superoxide dismutase		*	*	*	*	*	*	*	*
	glutathione S-transferase σ 2	*		*	*	*	*	*	*	*
	glutathione S-transferase σ 3	*		*	*	*	*	*	*	*
	superoxide dismutase	*	*	*	*	*	*	*	*	*
General Stress Response	procollagen-proline dioxygenase β subunit	*	*	*	*	*	*	*	*	*
	heat shock protein 70	*		*	*	*	*	*	*	*
	heat shock protein 90	*		*	*	*	*	*	*	*
Chromatin	small heat shock protein 24.1	*	*	*	*	*	*	*	*	*
	histone H1	*		*	*	*	*	*	*	*
	histone H2B	*	*	*	*	*	*	*	*	*
Protein Biosynthesis	histone variant H2A.X	*	*	*	*	*	*	*	*	*
	ribosomal protein	*	*	*	*	*	*	*	*	*
	ribosomal protein	*	*	*	*	*	*	*	*	*
Cytoskeleton	ribosomal protein	*	*	*	*	*	*	*	*	*
	calponin-like protein	*	*	*	*	*	*	*	*	*
	myosin heavy chain	*	*	*	*	*	*	*	*	*
	paramyosin	*	*	*	*	*	*	*	*	*
	ras, partial	*	*	*	*	*	*	*	*	*
	α -tubulin	*	*	*	*	*	*	*	*	*
	tropomyosin	*	*	*	*	*	*	*	*	*
		*	*	*	*	*	*	*	*	*

Fold Induction: not detected <0.25 0.25-0.5 0.5-1 1 - 2 2 - 4 >4

Figure 3 - Protein regulation in *Mytilus galloprovincialis* gills in response to $5 \times 10^{-5}\%$ DMSO (DMSO vs. CTRL), 1 $\mu\text{g/L}$ tralopyril (TP vs. DMSO) and mixture of DMSO and TP (TP vs. CTRL) at three time points: T2 (after two days of exposure, acute) T30 (after 30 days of exposure, chronic) and T40 (after 10 days of depuration following 30 days of exposure). Please note that only the proteins marked with * are significantly regulated ($p < 0.01$). Non-significant regulations were included in order to have a global picture of regulation trends.

From the three time points tested (T2, T30 and T40), the highest number of significantly regulated proteins was observed after acute exposure (T2). Within T2,

the highest number of significantly regulated proteins was found in response to TP alone (TP vs. DMSO). Protein upregulation was the most frequent answer to TP alone while DMSO (DMSO vs. CTRL) produced more protein downregulations (Figure 4); the proteins commonly regulated by both compounds often answered in opposite ways (Figure 3). Compared to acute one, chronic exposure resulted in far less proteome changes (Figure 3 and 4). Similarly to what was observed at T2, protein upregulation was most frequent in response to TP alone while downregulation was associated with exposure to DMSO (Figure 4). A number of proteome changes were still observed after 10 days of depuration in clean water (T40) and their extent was comparable to that after chronic exposure. However, opposing trends induced by DMSO and TP treatments were not observed anymore (Figure 3, 4).

Figure 4 – Percentage of up- and downregulated proteins in relation to the total number of significantly regulated (G-test, $p < 0.01$) proteins within each comparison pair (DMSO vs. CTRL, TP vs. CTRL and TP vs. DMSO) at each time point (T2, T30 and T40). Abbreviations are the same as in Figure 3.

3.3. Principal component analysis

PCA was performed on NSAFs from 9 individual samples (CTRL, DMSO and TP at T2, T30 and T40). NSAFs indicate the relative proportion of each protein among all detected in a particular proteomic analysis. The first three components were considered for data classification as they explained most of the data variability: the first principal component (PC1) accounts for 35% of the data variability and the next two components, PC2 and PC3, explain 19 and 13 % of the remaining variability, respectively. Strikingly, the first PCA plot (PC2 against PC1; Figure 5 A) suggested

that protein expression patterns cluster more according to time points than to chemicals, as samples from three different treatments clustered together within each time point. Consequently, the variability observed between the 9 conditions is mostly explained by the time of sampling and less so by different chemical treatments.

Two further hints can be obtained from the second PCA plot (PC3 against PC1; Fig 5B): the first is that depuration indeed leads to a reduction of DMSO and TP effects as all treatments from T40 cluster clearly apart from those at the other two time points. The second is that for T2 and T30 samples, a more chemical-dependent clustering can be observed. Both T2 and T30 controls are located close together with T30 DMSO, the latter probably due to the mussel's adaptation to chronic DMSO exposure. On the other hand, the two TP-exposed samples (T2 TP and T30 TP) and T2 DMSO are positioned farther apart from respective controls, supporting the chemical-dependent clustering that differentiates these samples from all the others.

Figure 5 – Principal component analysis performed with the normalized spectral abundance factors (NSAFs) from each treatment (CTRL, DMSO and TP) at the three different time points (T2, T30 and T40). Abbreviations are the same as in Figure 3.

4. Discussion

The goal of our study was to investigate the changes in the protein expression in the gills of mussels exposed to the emergent AF biocide TP in order to better understand its effects and toxicity. The discovery of sensitive responders would allow getting early warnings of TP presence in the environment.

4.1. TP bioconcentration

In an earlier study, we predicted the TP environmental concentrations in a saltwater marina (PEC; 0.7 µg/L) and calculated the EC₅₀ of 3.1 µg/L for causing the abnormal development in the mussel's D-veliger larvae (Oliveira et al., 2014). The sublethal 1 µg/L concentration chosen here is less than half of the estimated EC₅₀ for larvae and relatively close to the PEC to be considered as an environmental relevant concentration.

Despite the short half-life of TP in water (Oliveira et al., 2016), this compound accumulated quickly in the mussel tissues (Section 3.1, Fig 2) reaching equilibrium within 13 days. TP bioconcentration in mussels during the uptake phase was very well described (Fig. 2A) and predicted (Fig. 2B) by the one-compartment toxicokinetic model. Our study confirms the potential of TP to bioconcentrate in the aquatic organisms, also predicted by the high logK_{ow} of TP (predicted: 4.7; corrected based on pKa and water pH: 3.54). According to the European Chemical Agency (ECHA), logK_{ow} ≥ 3 indicates a high bioconcentration potential and requires further assessment (ECHA, 2014b; Schäfer et al., 2015). However, opposite to the predictions, 10 days of depuration was not sufficiently long to entirely eliminate TP. The difference between measured and predicted elimination of TP may result from an inaccuracy of mussel wet weight which was taken from the literature to be used in the model. This value may influence the time to steady-state conditions but usually it does not interfere with the chemical bioconcentration at the steady-state conditions (Stadnicka et al. 2012). That is in agreement with our predictions presented in Figure 2B, according to which, the modelled time to steady-state was much shorter than that obtained empirically but the internal concentrations measured and modelled at steady-state conditions were similar. Nevertheless, the lower internal TP concentration at T40 points towards the possibility of complete elimination with longer time in clean water. Based on the calculated elimination rate, less than 5% of TP accumulated in mussels during the uptake phase should remain in the organism after 14 days of depuration. Overall, internal TP concentrations measured in our study indicate that the observed effects are due to the actual TP exposure and accumulation in mussels.

4.2. Effects of exposure on mussel gill proteome

For TP, like for many other emerging contaminants, information is lacking on several aspects of its toxicity including effects of exposure and exact mode of action.

Due to similarities to its parent compound – chlorfenapyr – it is thought that TP might dissipate the proton gradient necessary for ATP production as it may transport the protons back into the mitochondrial inner matrix (ECHA, 2014a).

Due to the low solubility of TP in water, DMSO was used as solvent. DMSO has been described as an intercellular electrical uncoupler (Santos et al., 2003) causing membrane loosening, pore formation and bilayer collapse (de Ménorval et al., 2012; Notman et al., 2006; Sum and de Pablo, 2003). These effects can enhance diffusion of molecules through lipid membranes. Exposure to DMSO has also been linked to an effective uncoupling of oxidative phosphorylation in rat liver mitochondria (Mhatre 1983) and to other significant alterations in gene expression, protein content and functionality of different kinds of cells (Pal et al., 2012). Although many effects have been described as a result of DMSO exposure, it was not expected that concentrations as low as those used in this study would result in significant proteome changes in the mussel gills. Interestingly, DMSO and TP were often found to affect the same proteins, therefore we hypothesized that both compounds might have similar modes of action.

4.2.1. Acute exposure

Cytoskeleton

In global proteomics, the modulation of cytoskeletal proteins is one of the most frequently detected effects, probably due to their high abundance in the organisms. Recently it has been suggested that most of these changes are responsive to the stress level rather than to specific stressors (Groh and Suter, 2015; Miao et al., 2015). Our data is in accordance with this statement since at T2, together with the regulation of cytoskeleton proteins, an upregulation of general and oxidative stress related proteins was also observed. For TP, the initial assault led to the upregulation of myosin heavy chain (fold induction of 2.9), paramyosin (2.1) and ras-partial. Myosin and paramyosin are involved in motility and contractile functions without expending too much energy (Yamada et al., 2000). However, the overexpression of ras-partial (33.5) has been linked to altered proliferation and/or differentiation giving rise to neoplastic processes (Di et al., 2011; Santos and Nebreda, 1989). On the other hand, exposure to DMSO alone caused a downregulation of the mentioned proteins.

Only α -tubulin, involved in cell transport, motility, division and intracellular positioning of organelles (Apraiz et al., 2006) was found to be upregulated by this treatment. Membrane disruption after DMSO exposure probably caused this upregulation and many other effects observed.

Electron transport chain

The coupling reaction between the electron transport chain (ETC) and oxidative phosphorylation (OxPhos) occurs due to the proton gradient generated across the inner mitochondrial membrane required for ATP synthesis. Cytochrome c oxidase (COX) is the terminal component of the electron transport chain. Whilst subunits I and II of COX have all the required components to complete its function, the role of subunit III remains unclear, however, it is known that it provides additional stability to the enzymatic activity especially at high pH (Sharma et al., 2015). In our study, DMSO induced a 14-fold upregulation of the subunit III while the subunits I and II were strongly downregulated. TP upregulated subunit II, while subunit III was downregulated and subunit I was not even detected. Cells are thought to regulate the COX activity levels in response to changes in energy demands (Ciocan et al., 2012). Some recent proteomic studies have suggested that the abundance, composition or post-translational modifications of both complex I and III of the ETC are potential regulatory sites for overall COX activity (reviewed in Tomanek, 2015). Our observations support this hypothesis.

Energy metabolism

Energy metabolism plays an essential role in organism survival and in its stress adaptation and tolerance. Therefore, maintaining energy levels is an important strategy to cope with toxic stress (Sokolova, 2013). For example, fructose biphosphate aldolase catalyses a key reaction in glycolysis that normally leads to a net energy production. This enzyme was found to be significantly downregulated by DMSO and upregulated by TP. The same was observed for phosphoenolpyruvate carboxykinase, involved in gluconeogenesis. While glycolysis uses glucose to generate ATP, gluconeogenesis represents almost the opposite pathway. Under normal conditions, a specialized enzymatic control ensures that both pathways are not active at the same time (Berg et al., 2002). However, under pathological conditions or

when there is a huge demand for energy, this control may be lost, resulting in their simultaneous activation (Berg et al., 2002). This seems to be the case after acute exposure to TP, where the expression of both proteins was upregulated. In the case of DMSO the downregulation of fructose biphosphate aldolase may be attributed to the lack of sufficient ATP resources needed in the first steps of glycolysis (Ramos-Martínez et al., 1993).

Oxidative stress defense and active efflux

A similarly contrasting response to TP and DMSO was observed in relation to the oxidative stress defense: depressed by DMSO, it was enhanced by TP. The upregulation of the oxidative stress defense is one of the most common answers observed in response to a wide range of stressors (Rodríguez-Ortega et al., 2003; Tomanek, 2015). It is believed that uncouplers enhance ROS production when disconnecting the ETC and OxPhos (Shabalina and Nedergaard, 2011). Effectively, TP induced the upregulation of superoxide dismutase (SOD) and the intermembrane CuZn SOD as a first line of defense that converts the superoxide anion to H_2O_2 . The phase II enzyme Glutathione S-transferase (GST) pi1 was also upregulated by acute TP exposure. On the other hand, DMSO exposure caused a depletion of the antioxidant protective mechanism reflected in the downregulation of GSTpi1 and thioredoxin I.

A third phase of the oxidative stress defense involves excretion of water-soluble conjugates through ATP-dependent efflux transporters (Luckenbach and Epel, 2008; Torre et al., 2014). ABC transporters are grouped into eight subfamilies termed ABCA to ABCH, of which members of ABCB (or P-glycoproteins), ABCC and ABCG subfamilies were identified as being capable of interacting with xenobiotics causing the so-called multixenobiotic resistance (MXR) (Epel et al., 2008; Rioult et al., 2014). MXR-related transporters are physiologically expressed in mussel gill cells, providing a protective barrier against the entrance of xenobiotics into the cytoplasm or nucleus by pumping the xenobiotics out of the cell (Lüdeking and Köhler, 2002; Luckenbach and Epel, 2008). Two efflux transporters (ABCB/P-glycoprotein-like protein and ATP-binding cassette sub-family G member 2-like) were found to be strongly upregulated (fold induction of 8) by TP exposure correlating with the upregulation of the oxidative stress related proteins. Although

uncouplers are thought to enhance ROS production and consequently the antioxidant defense, a depletion was observed instead, after DMSO exposure. This may be because two days after exposure, activation of the oxidative stress defense was no longer detectable.

General stress response and apoptosis

The heat shock protein (HSP) family belongs to the molecular chaperone group and plays an essential role in protein maintenance. HSPs are usually triggered by adverse environmental conditions and are part of the general stress response (Feder and Hofmann, 1999; Sørensen et al., 2003) conserved across species (Fabbri et al., 2008). Several studies have related upregulation of HSP70 to a protective role when exposed to different stressors such as thermal stress (Roberts et al., 1997), organic chemicals (Clayton et al., 2000) and combinations of stressors (Banni et al., 2015). The upregulation of HSP70 has also been linked with a protection against necrosis or excessive apoptosis functioning as endogenous modulators of cell death, inhibiting caspase-dependent events (Garrido et al., 2001; Kefaloyianni et al., 2005).

Apoptosis is a multifunctional process considered vital to cellular and tissue homeostasis, normal cell turnover and proper functioning of the immune system, embryonic development and chemical-induced cell death (Elmore, 2007). In bivalves, the apoptotic pathways usually involve the regulation of caspases (Sokolova, 2009), starting with the action of initiator caspases (e.g. caspase 2) that leads to the cleavage and subsequent activation of the effector caspases (including caspase 3). These in turn act upon irreparably damaged intracellular targets in order to eliminate them (Creagh et al., 2003; Sokolova, 2009).

In our study, DMSO exposure resulted in an upregulation of HSP70 and HSP24.1, together with the downregulation of caspase 3/7-2 and putative caspase 3. After TP exposure, HSP70 and small HSP24.1 were found to be significantly downregulated whilst HSP90 was upregulated. Since all HSP70 functions are performed through an ATP-regulated cycle of substrate binding and release in the presence of different cofactors (Fabbri et al., 2008), we think that the observed downregulation may be related to the decrease in the ATP availability. If this ability of the chaperone complex to bind and hydrolyze ATP is disrupted or if ATP is not

available, it may lead to inhibition of the catalytic cycle, consequently leading to protein degradation and eventual cell death. In fact, together with the downregulation of HSP70 there was also a significant upregulation of the apoptosis involved protein - caspase 3/7-2 - by TP.

Immune system

The invertebrate immune system is still a puzzling issue with several knowledge gaps. Currently, it is accepted that mollusks lack adaptive response but have an effective innate immune system composed of cellular and humoral components (Gestal et al., 2008; Allam and Raftos, 2015). Investigations in gill, digestive gland and hemocytes of mussels have revealed a high expression of several immune-related genes or proteins showing a complex collection of pattern recognition receptors (PRRs) and downstream pathway members such as effector molecules (Philipp et al., 2012; Rosani et al., 2013). PRRs are evolutionary conserved families central to the innate immune system and in bivalves comprise extracellular, membrane-bound or cytosolic molecules (reviewed by Gerdol and Venier, 2015). Our study suggests that both DMSO and TP cause immunomodulation in *M. galloprovincialis* through the regulation of several proteins belonging to extracellular PRRs (lectins and several C1q domain-containing (C1qDC) proteins), transcription factors NF- κ B and myeloid differentiation factor 88, involved in the expression of membrane-bound PRRs) and effector molecules (mytilin B antimicrobial peptide precursor). All immune related proteins from DMSO-exposed mussels were downregulated (except MgC1q6, which was upregulated). In accordance with our results, Varotto and co-workers (2013) have also reported downregulation of C1qDC proteins in the gills of mussels exposed to DMSO. Moreover, DMSO was also reported to suppress the immune response in mice (Lin et al., 2015) and salmon (Milston et al, 2003). In TP-exposed mussels, the opposite answer was observed: all immune-related proteins except MgC1q6 were found to be upregulated. Downregulation of proteins involved in the immune system (Galloway and Depledge, 2001; Gomes et al., 2014; Renault, 2015) has been described considerably more frequently than their upregulation (Maria and Bebianno, 2011). However, some studies have also described an initial period of immunostimulation followed by a period of immunosuppression, which occurs when the immune system can no longer

cope with the stress (Farcy et al., 2011). Accordingly, the duration and intensity of exposure seem to be determinant in the observed response.

Chromatin structure and protein biosynthesis

Proteins belonging to chromatin structure and protein biosynthesis are involved in several basic functions and can be found in all eukaryotic organisms. They are responsible for DNA packaging in the cell nucleus thus having a critical role on chromatin metabolism that among other processes includes DNA transcription, replication, recombination and repair (Eirín-López et al., 2004; González-Romero et al., 2012). Accordingly, histone upregulation in response to TP exposure can be understood as a natural process induced by the overexpression of numerous other proteins: the more proteins are produced due to their upregulation, the more histones are needed to control this processes. The enhancement of ribosomal protein expression would also be expectable since these proteins are constituents of the ribosome and play a crucial role in translation. However, they can also play other roles in cellular growth. As before, the opposite answer was found in DMSO-exposed mussels possibly reflecting a downregulation of the majority of the regulated proteins.

Steroidogenesis

The enzyme 17 β -hydroxysteroid dehydrogenase 10 (17 β -HSD10) was found to be down- and upregulated in response to DMSO and TP exposure, respectively. In vertebrates, this enzyme is located within the mitochondria and is involved in processing steroid hormones and fats. Although there are reports suggesting the presence of (vertebrate-type) sex steroids such as testosterone, 17 β -estradiol, and progesterone in molluscs including bivalves (Lafont and Mathieu, 2007; Porte et al., 2006), information regarding enzymatic pathways involved in steroid synthesis and catabolism in mussels is still fragmentary (Fernandes et al., 2011). Some studies have reinforced the role of 17 β -HSD in a hormone biosynthesis pathway in bivalves due to the observed increase of 17 β -HSD activity during sexual maturation and decline after spawning (Le Curieux-Belfond et al., 2001; Matsumoto et al., 1997). However, there is a lack of evidence that steroid hormones have an endogenous origin and it is unclear if these hormones play a physiological role in bivalves (Fernandes et al., 2011; Scott, 2012), making further studies on the endocrine system of invertebrates

necessary before possible effects of DMSO or TP on bivalve steroidogenesis can be interpreted.

4.2.2. Chronic exposure

Whilst acute exposure may reflect not only a specific chemical effect but also a general stress response arising in order to fight the initial assault, chronic exposure may provide insights into the regulation of more specifically responding proteins. If protein modulations were chemical-specific then one could expect that the permanent presence of a certain substance in the water would induce a stronger response with time. However, chronically exposed mussels did not show a regulation of protein biosynthesis, chromatin, cytoskeleton and general stress related proteins in response to DMSO or TP, except for the downregulation of HSP70 and one histone variant in DMSO exposure. This indicates that a certain adaptation to DMSO and/or TP presence in water has occurred during chronic exposure and the mussels were able to cope with the many consequences of the initially occurring stress. However, several significant protein alterations were still observable in the chronically exposed mussels, and these will be discussed next.

Electron transport chain

Due to the uncoupling effect of both DMSO and TP, regulation of the ETC- and/or OxPhos-related proteins was expected. Indeed, an upregulation of COX subunit I was observed after chronic exposure to both compounds. As stated previously, the regulation patterns of COX proteins are incompletely understood, but such changes are thought to respond to different energy demands (Ciocan et al., 2012).

Energy metabolism

In relation to energy metabolism, only fructose biphosphate aldolase was regulated by chronic TP exposure. We assumed that the upregulation of this protein at T2 reflected an initial promotion of glycolysis aimed at increasing the ATP production. However, after long-term exposure (T30), when ATP production is ceased (or decreased) due to the compound's uncoupling action, it could be expected

that the cell would activate some kind of negative feedback, since promoting glycolysis is not leading to the increase in the net energy. In fact, we observed a significant downregulation of fructose biphosphate aldolase at T30 while phosphoenolpyruvate carboxykinase was not affected significantly. Hence it seems that chronic TP exposure decreases the organism's ability to produce energy. In contrast, chronic exposure to DMSO alone does not appear to affect energy metabolism.

Oxidative stress defense and active efflux

Chronic DMSO exposure led to the upregulation of the first line of defense against ROS production - CuZn superoxide dismutase – however, phase II protein GSTpi1 continued to show a downregulation, same as during acute exposure. In the case of chronic TP exposure only thioredoxin I was found to be upregulated. This protein is one of the constituents of the thioredoxin-peroxiredoxin (Trx-Prx) system that is frequently recruited to scavenge H₂O₂ in replacement of the glutathione system. In accordance with this, thioredoxin I was downregulated after acute TP exposure probably because the scavenging was still being supported by the glutathione system especially by GSTpi1 (strongly upregulated at T2).

Concerning active efflux, the subfamilies of ABC transporters differ by the substrates used: while for ABCB the substrates are mostly unmodified xenobiotics (phase 0), the ABCGs recognize phase I and II metabolites (Sarkadi et al., 2006). This may explain why the ABCB/P glycoprotein was upregulated both by acute and chronic exposure when TP was still present in the water, while after 10 days of depuration, when no fresh compound was available anymore, this protein was significantly downregulated. The protein from the sub-family G (ATP-binding cassette sub-family G member 2-like) was upregulated even stronger after chronic TP exposure. We believe that at T30 more metabolites are likely to occur: while TP bioconcentrates in the mussel tissues, enhanced degradation might also take place inside the organism thus leading to the increasing expression of ABCBGs proteins at this time point. DMSO chronic exposure still showed a downregulation of ATP-binding cassette sub-family G member 2-like, already observed at T2, possibly reflecting a continuous effect of DMSO on the membrane organization.

Apoptosis

Chronic TP exposure led to the upregulation of caspase 2 ($p < 0.001$) and caspase 3/7-2 (not significant), demonstrating that this compound induces apoptosis. Chronic DMSO exposure showed a downregulation of caspase 2 indicating that caspase-dependent apoptosis is not triggered or even is suppressed by chronic exposure to DMSO.

Immune system

Less immune system-related proteins were significantly affected by chronic exposure. In mussels exposed to DMSO three C1qDC proteins and myeloid differentiation factor 88a were significantly downregulated and one more C1qDC protein (MgC1q8) was significantly upregulated. In contrast, TP caused significant upregulation of two C1qDC proteins (MgC1q11 and MgC1q46). C1q super-family members are known to be involved not only in pathogen recognition and host defense but also in inflammation, autoimmunity, apoptosis and cell differentiation (Kishore et al., 2004). Thus the upregulation of these proteins in response to chronic TP exposure may be related to the upregulation of caspases involved in apoptosis stimulation. The contrary (downregulation of caspases and many C1qDC proteins) was observed during DMSO chronic exposure.

4.2.3. Depuration

After 10 days of depuration following chronic chemical exposure, significant protein changes were still observed. Such alterations can indicate either the continuing effect of chemical, since the depuration time was not sufficient to fully eliminate the internal TP concentration, or an effort towards the recovery of mussel gills. Potentially, both occur simultaneously. The upregulation of heat shock proteins at this time point can be regarded as a consequence of finalizing the TP detoxification. On the other hand, PCA results (Section 3.3; Figure 5B) seem to point towards a (partial) recovery from the exposure. The upregulation of cytoskeletal proteins after depuration might be interpreted as a sign of energy balance re-establishment since some of the energy could now be engaged towards biomass restoration instead of

detoxification. This notion is also supported by the upregulation of histones and ribosomal proteins and the downregulation of caspase 3/7-2 and several immune system indicators. Furthermore, the significant downregulation of ABCB/P glycoprotein-like protein can also be explained by the absence of fresh TP in the water. We believe that this energy re-establishment might be the starting point for a possible gill recovery at this time point.

4.3. Study limitations and future directions

The PCA of protein expression patterns showed that sampling time explained more of the data variability than the chemical exposure conditions. The stronger influence of time compared to experimental treatments could have been caused by the season in which the exposures took place. On the Galician coast, mussels are reproductively active in the mid-spring (Villalba 1994). This period overlaps with the time when our study was performed (March and April, 2011). Spawning is known to be a large energy dispense for mussels. Hence, even though proteins were measured in mussel gills, a tissue that is not directly involved in reproduction, spawning may still have influenced the results.

Another reason for a high dissimilarity of controls sampled at different time points could be related to the fact that both genders were used randomly for proteomic analysis. Historically, gill tissue was not thought to respond in a sex-specific manner (Monsinjon et al. 2006). However, a recent study showed a significant gender difference in the altered proteome of mussel gills after exposure to dioxin-like PCBs (Riva and Binelli, 2014). In our study, animals were randomly picked without gender determination and the gills from three individuals were pooled. This could have resulted in different ratios of males/females between samples, possibly bearing a significant influence on the outcome of the proteomics analysis. Further studies should take into account not only the reproductive period of the population assessed but also the gender of the organisms used for proteomic analysis.

Other study limitations comprise the effects caused by DMSO exposure. Although we might have circumvent this problem by comparing TP with DMSO to assess the effects of TP alone, further experiments with grading DMSO solutions would be important to fully understand the extent of the solvent influence on the

effects caused by TP. Besides, additional solvents could be tested in order to understand if a safer option is available for further studies.

Another important factor to keep in mind refers to the variations inherent to the technique itself. We observed a 10% variation in the detected number of spectra among replicates, similar to one other study that used global proteomics (Cooper et al., 2010). Shotgun proteomics is a useful technique when little information is available on the effects of a certain compound. However, it has the disadvantage of excluding the proteins expressed at low copy counts, since these can be masked by highly abundant ones. Furthermore, the genomic and proteomic data currently available for mussels is rather incomplete, limiting the degree of proteome coverage that can be obtained (Campos et al., 2012; Suárez-Ulloa et al., 2013). In this study, a significant proportion of the collected spectral data could not be matched to any mussel peptides derived based on the protein databases available. This reinforces the need for further improvements in regard to the depth and breadth of mussel proteome coverage. Another limitation is brought in by the dearth of basic knowledge on some mussel systems, such as the endocrine and immune system, which makes data interpretation, a formidable task. The results of genomic and proteomic analyses should guide the design of functional studies to be undertaken in non-model organisms.

5. Conclusions

Our results show that TP rapidly bioconcentrates in whole mussel tissue. The proteome alterations observed in TP-exposed mussels are therefore caused by the internal TP exposure. However, not only TP but also DMSO alone caused proteome alterations in the gills of acutely and chronically exposed mussels. The fact that both compounds affected the same proteins, albeit almost always in the opposite way, led us to identify common targets affected by these compounds, such as biological membranes, ETC and sites of ATP production. Chronic exposure caused less proteome changes than the acute one, showing that many of the regulations observed after acute exposure probably represent a common stress response signature rather than a specific effect induced by the chemical. Overall, sublethal TP exposure affected several proteins involved in active efflux, immune response and bioenergetics. After the depuration period (T40), the expression of several proteins

was still altered. These changes may reflect either the initiation of recovery processes or the continuing effects of the chemical, as 10 days of depuration were not sufficient to fully remove TP from the tissues. Nevertheless, the lower internal TP concentration after the depuration period points towards the possibility of complete elimination with time in clean water.

We believe that TP may be a risk to mussel populations residing in locations where a continuous exposure to this biocide is likely to occur (e.g. seawater marinas). Indeed, we have shown that TP at concentrations close to those expected in such an environment not only bioconcentrates in tissues but is also able to affect proteins involved in important cellular pathways. On the other hand, we have also shown that TP rapidly degrades and that the mussels are able to depurate and show signs of recovery when placed in clean water. Hence, in places where TP exposure might not be constant (e.g. beaches and open sea), this biocide might represent a safer AF alternative compared to previously used AF compounds.

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