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Biological effects of diethylene glycol (DEG) and produced waters (PWs) released from offshore activities: A multi-biomarker approach with the sea bass *Dicentrarchus labrax*

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A biological risk for marine organisms can be excluded for DEG concentrations as those normally associated to produced waters discharged in the Adriatic Sea.

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ABSTRACT

Diethylene glycol (DEG) is largely used during oil and gas exploitation by offshore platforms. The aim of this work was to investigate if this compound induces direct molecular/cellular effects in marine organisms, or indirectly modulate those of produced waters (PWs). Sea bass (*Dicentrarchus labrax*) were exposed to DEG dosed alone or in combination with PWs from an Adriatic platform. A wide array of analysed biomarkers included cytochrome P450-dependent enzymatic activity, bile metabolites, glutathione S-transferases, acetylcholinesterase, peroxisomal proliferation, antioxidant defences (catalase, glutathione reductase, glutathione peroxidases, glutathione), total oxyradical scavenging capacity, malondialdehyde and DNA integrity (single strand breaks and frequency of micronuclei). Results did not reveal marked effects of DEG, while PWs influenced the biotransformation system, the oxidative status and the onset of genotoxic damages. Co-exposures caused only limited differences of biomarker responses at some experimental conditions, overall suggesting a limited biological impact of DEG at levels normally deriving from offshore activities.

1. Introduction

Produced waters (PWs) represent the major effluent discharged by offshore platforms, constituted by formation water (naturally present in oil and gas reservoirs) and injection water, forced into the well to improve recovery (Neff, 2002). The chemical composition of PWs can be very complex and variable, with trace metals, alkylphenols, volatile organic chemicals, polycyclic aromatic and aliphatic hydrocarbons dissolved from the fossil fuels, and other compounds added by Companies during phases of production (Neff, 2002). Among the additives, diethylene glycol (DEG; CAS Number 111-46-6) is largely used during the gas–water separation process to prevent hydrate formation and corrosion events (Ballantyne and Snellings, 2005). After the extraction, DEG is normally separated by hydrocarbons through a filtration system which, however, does not eliminate the potential occurrence of this compound in PWs. Since 2001 the open water discharge of produced waters is regulated in Italy by an authorization decree of the Ministry of Environment which indicate for DEG a maximum concentration of 3500 mg l^{-1}.

The knowledge of specific effects of DEG on aquatic species is limited to few experimental results principally obtained in freshwater organisms (Staples et al., 2001), while more studies evaluated toxicity of various ethylene glycol ethers (EGEs) on human or murine models (Mathews et al., 1991). Although these compounds can exhibit considerable differences on potential toxicological effects on living systems (Welsch, 2005), nonetheless chemical and physical similarities of these molecules reflect some general properties (Staples et al., 1998). Ethylene glycol ethers are biodegradable, not biomagnificated compounds and their biotransformation by the cytochrome P450 pathway has been suggested to modulate toxic consequences of EGEs on reproductive, nervous and immune systems (Ballantyne and Snellings, 2005; Staples et al., 1998, 2001; Welsch, 2005). Developmental toxicity of DEG has been recently shown in mice (Ballantyne and Snellings, 2005), while acute effects have been revealed also by a number of human poisoning episodes, mostly due to erroneous formulation of this molecule in medicinal products (Bowie and Mc Kenzie, 1972; O'Brien et al., 1998; Ferrari and Giannuzzi, 2005).

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Despite these studies, the total absence of data on marine species does not allow to define a safe threshold for DEG levels and the risk deriving from this compound released with produced waters. Considering the importance of monitoring the effects of offshore activities in the Adriatic Sea, the aim of this work was to investigate if DEG may induce direct sublethal effects at the molecular or cellular levels in marine organisms, or indirectly modulate responses caused by discharged PWs.

In this respect, specimens of sea bass *Dicentrarchus labrax* were exposed in laboratory conditions to a wide range of DEG concentrations, dosed alone or in combination with produced waters from an Adriatic platform (PW-G) to evaluate potential interactions between these classes of chemicals. An ecotoxicological approach was applied to reveal molecular and cellular alterations through a wide battery of biomarkers representing early warning signals of environmental disturbance, sensitive toward particular classes of contaminants and reflecting different levels of cellular unbalance and toxicity (Orbea et al., 2002; Regoli et al., 2002, 2003; Viarengo et al., 2007). The biotransformation pathway of organic xenobiotics was measured in fish to assess if this system is involved in metabolism of DEG as shown for other EGEs (Mathews et al., 1991), and to investigate if during co-exposures DEG modulate bioavailability or reactivity of PAHs potentially present in produced waters. Analysed parameters included the activity of phase I cytochrome P450 (EROD), phase II glutathione S-transferases (GST) and levels of aromatic metabolites in bile (Goksøyr and Förlin, 1992; Aas and Klungsøyr, 1998; Regoli et al., 2003, 2005; Gorbi et al., 2005).

Also peroxisomal proliferation appeared of particular interest in our study since several papers demonstrated the induction of peroxisomal enzyme Acyl CoA oxidase (AOX) by organic xenobiotics like water accommodated fraction of crude and lubrificant oils, PAHs, polychlorinated biphenyls (PCBs), phthalate ester plasticizers and alkylphenols (Orbea et al., 2002; Bocchetti and Regoli, 2006). The latter compounds have been suggested to cause deleterious effects on the reproductive fitness of fish exposed to produced waters (Sturve et al., 2006; Meier et al., 2006). On the other hand, inhibitory effects on AOX have also been suggested from complex mixtures of chemicals especially when containing trace metals (Zorita et al., 2007; Bocchetti et al., 2008).

Acetylcholinesterase (AChE) is a crucial enzyme in the nervous system of vertebrates and invertebrates, and its inhibition is traditionally associated to organophosphate or carbammate pesticides (Rickwood and Galloway, 2004). However, other chemicals have been recently shown to influence this enzymatic activity (Frasco et al., 2005), thus suggesting to include AChE in the multibiomarker battery analysed in exposed sea bass.

Oxidative status of marine organisms is altered by exposure to chemicals which can both enhance intracellular formation of reactive oxygen species (ROS), or modify the efficiency of antioxidant defences (Regoli, 2000). Prooxidant effects of DEG and/or produced waters on sea bass were evaluated by variations of the main antioxidants, including the activity of catalase, Se-dependent and Se-independent glutathione peroxidases, glutathione reductase and levels of total glutathione. These data were integrated with the measurement of total oxyradical scavenging capacity (TOSC) which quantifies the overall resistance toward different ROS like peroxyl radicals and hydroxyl radicals (Regoli and Winston, 1999; Gorbi and Regoli, 2003). Compared to individual antioxidants TOSC is less sensitive but has a greater prognostic value since an impaired capability to neutralize ROS has been associated with the onset of several oxidative damages (Regoli, 2000; Frenzilli et al., 2001; Regoli et al., 2004; Benedetti et al., 2007). In this respect, malondialdehyde (MDA) was analysed as one of the main products of lipid peroxidation (Regoli et al., 2004), while genotoxic alterations in exposed sea bass were evaluated as loss of DNA integrity by the Comet assay (Frenzilli et al., 2001) and frequency of micronuclei (MN) as a less reversible effect from chromosome breakage or aneuploidy during cell division (Bolognesi et al., 2004; Nigro et al., 2006).

From the overall results, the main objective of this study was to characterize whether DEG concentrations ranging from environmentally realistic values for offshore activities up to the actual normative limits, can directly induce molecular, cellular and genotoxic effects, or indirectly modulate those caused by produced waters. Such data will be important to better assess the potential risk arising from DEG discharges to marine organisms and to evaluate the opportunity to update the threshold of this compound in PWs imposed by Italian authorization decree.

2. Materials and methods

2.1. Organisms and laboratory exposure

Juvenile specimens of sea bass (*D. labrax* L. 1758) were obtained from a local farm and acclimatised for 10 days to laboratory conditions in flow-through aquaria with filtered seawater, 18 ± 1 °C and salinity at 28–30‰. During this period organisms were daily fed with a specific grower feed (by Aller-Aqua, Aller Thalassa 2 mm, crude protein 50%, crude fat 15%).

Two different experimental designs were carried out to test the effects of DEG dosed alone and in combination with produced waters. In the first experiment, a total of 60 specimens (weight = 30 ± 5 g) were exposed to increasing concentrations of DEG (0, 50, 100, 500, 1000, 5000 mg l^{-1}), directly dissolved in seawater. These concentrations were selected to include values comparable to DEG levels recently detected in some Adriatic produced waters (2–15 mg l^{-1}, Cianelli et al., 2008), up to the maximum limit allowed by authorization decree for open water discharge (3500 mg l^{-1}).

For the second experiment, produced waters were obtained from an Adriatic platform (PW-G) and analytically characterized (ICRAM, 2006a). The reported chemical composition (Table 1) revealed Ba, Fe and, to a lesser extent, Mn, Cu, Ni, Zn as typical metals associated to PW-G, although in lower concentrations compared to values reported for PWs of North Sea and Gulf of Mexico (Neff, 2002). Among hydrocarbons, one-ring volatile BTEX (Benzene, Toluene, Ethylbenzene, Xylene), which normally predominate in PWs from gas wells, showed higher concentrations than aliphatic and polycyclic aromatic hydrocarbons with values within the ranges reported for offshore platforms from North Sea, Gulf of Mexico and Indonesia (Neff, 2002). Levels of DEG in PW-G were 2.4 mg l^{-1}, a much lower value compared to the normative limit. A total 90 of specimens (weight = 25 ± 5 g) were exposed to PW-G diluted to 1% and 5%, and added with various concentrations of DEG (0, 1000, 5000, 10 000 mg l^{-1}). Levels of produced waters were considered environmentally relevant, since a 1:1000 dilution has been indicated to roughly approximate the

Table 1
Chemical characterization of the produced waters obtained from an Adriatic platform (PW-G) and used for the laboratory exposures with the sea bass *Dicentrarchus labrax*. More detailed results and methods are given in ICRAM (2006a).

Chemicals	(mg l^{-1})
As	<0.01
Ba	10.53
Cd	<0.0005
Cr	<0.01
Cu	0.02
Fe	25.48
Hg	<0.0005
Mn	0.42
Ni	0.02
Pb	<0.01
Zn	0.02
Benzene	0.256
Toluene	0.0506
Ethylbenzene	0.1152
Σo,m,p-Xylene	0.86
ΣBTEX	1.28
ΣAliphatic hydrocarbons C_7–C_{20}	0.034
ΣPolycyclic aromatic hydrocarbons	0.15
ΣChlorophenols	<0.002
ΣNitrophenols	<0.002
ΣAlkylphenols	0.078
Diethylene glycol (DEG)	2.4