NET4mPLASTIC Project

Work Package 3.4

Deliverable 3.4.2

REPORT OF DATA COLLECTION ABOUT CO-PRESENCE OF CONTAMINANTS AND MPS IN BIVALVES

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SUMMARY

This document refers to Deliverable 3.4.2, which is related to edit "Report of data collection about co-presence of contaminants and MPs in bivalves". Scientific papers and reviews were analyzed in order to evaluate the exposure of the bivalves to both microplastics and other environmental contaminants.
The main aims of the Deliverable 3.4.2 were:

- Evaluation of the co-presence of MPs and environmental contaminants in bivalves.
- Experimental design to analyze and quantify both the MPs and the other contaminants in bivalves.

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1. Introduction

Bivalve mollusc shellfish are extensively studied in marine monitoring programmes. Among them, mussels recover an important role as referring organism for the environmental pollution. Indeed, as consequence of to their wide geographic distribution, easy sampling, tolerance to a considerable range of salinity, resistance to stress and high accumulation of a wide range of pollutants, they are the ideal test organisms for environmental monitoring [Tanabea S. et al.; 2000].

Mussels are benthic filter feeders with a selective mechanism of suspension feeding. They process relatively large amounts of water during the feeding, maximizing their exposure to any harmful material within the water column. This can result in the accumulation of Microplastics [MPs], chemical pollutants and microorganisms within their tissues [Barker Jørgensen; 1990].

Mussels are commonly used in monitoring the environmental pollution (bio-monitoring) due to their sedentary nature and capacity of bio-accumulating contaminants such as metals (e.g. Mercury [Hg], Vanadium [V], Nickel [Ni], Copper [Cu], Zinc [Zn], Cadmium [Cd], Lead [Pb], Chromium [Cr]), halogenated compounds (dioxins, Polychlorinated Biphenyls [PCBs], Polychlorinated Dibenzofurans [PBDEs]) or Polycyclic Aromatic Hydrocarbons [PAHs]. In particular, mussels of the genus *Mytilus* have been successfully used as environmental indicators, giving information about chemical contamination of the water.

Recently, mussels have been used to study the fate and toxic effects of MPs in laboratory experimental exposures. Consequently, MPs contamination in mussels has been proposed as a marine health status parameter [De Witte B. et al.; 2014], and added to the European database on environmental contaminants of emerging concern in seafood [Vandermeersch G. et al.; 2015].

Mussels are vulnerable to environmental pollutant, such as heavy metals, organic pollutants and MPs, but the relevance of contaminants accumulation in bivalves is related also to the human health. Actually, mussels act as vectors that transfer MPs and the associated contaminants within the human food chain [NET4mPLASTIC Deliverable 3.4.1; 2020 – Conti I. et al.; 2021].

2. Occurrence of Environmental Chemical Contaminants in Mussels from the Adriatic Sea

2.1 Occurrence of Heavy Metals Contamination in Mussels from the Adriatic Sea

Several works evaluated the heavy metals contamination in mussels from the Adriatic Sea.
Bajt et al. during the Project MYTIAD provided a set of data about the presence of some contaminants in Adriatic water, mapping their occurrence in different sites. The study is considered by the authors the first comprehensive overview of contamination in the Adriatic Sea with critical comparisons of related studies over the Mediterranean Sea. In the study, eight metals were analysed in mussels: Hg, Ni, Cu, Zn, Cd, Cr, Pb and V. Moreover, due to the high metal concentration, several hotspots were detected such as Taranto, which was defined as the most contaminated site for Hg [Bajt O. et al.; 2019].
Industrialization and urbanization influence the environmental contamination. Six trace metals (Cd, Cr, Cu, Hg, Pb and Zn) were analyzed in the waters of the eastern Adriatic coasts, that are characterized by the densely populated and industrialized areas. Elevated concentrations of all the six trace metals were evaluated in mussels from the areas with the higher input of industrial, harbour and urban wastes. However, the obtained data provided no proof of significant pollution of these coasts [Kljakovic´-Gašpic´ Z. et al.; 2010].
Moreover, the human activities determine the kind of environmental pollution affecting the contaminants accumulation within the marine organisms. Trace metals were generally higher than other organic pollutants in tissues of 5 species of bivalve molluscs, including *Mytilus galloprivincialis*, collected from 13 different locations along the eastern Adriatic coast. However, the levels of trace metals were lower than the maximum residue levels in seafood prescribed by the European Commission, indicating that there is not an evident health risk for consumers of harvested and cultured bivalves [Milun V. et al.; 2016].
Evaluation of the public health risk associated to the consumption of mussels was proposed by Mihajlo Jovic´ and Slavka Stankovic (2014). The study aimed to identify the levels of trace metals (Iron [Fe], Manganese [Mn], Cobalt [Co], Cu, Zn, Ni, Cd, Pb and Hg) in the mussels *Mytilus galloprovincialis* sampled along the south-eastern Adriatic Sea (Montenegro). The trace metals concentrations in mussels, at seven different locations and three seasons, did not exceed the European Union and US Food and Drug Administration requirements [Jovic M and Stankovic S; 2014].

Heavy metals detected in the bivalve molluscs from different locations of the Adriatic Sea are listed in Table 1.

Table 1: Heavy metals detected in the marine organisms collected from the Adriatic Sea.

Species	Chemicals detected	Sampling point	n. sampling sites	Sampling period	References
Mussels	Hg, Ni, Cu, Zn, Cd, Cr, Pb, V	Eastern and Western Adriatic Sea	38	2008 (from May to July)	Baji O. et al., 2019
Mussels	Cd, Cr, Cu, Hg, Pb, Zn	Eastern Adriatic coast	14	2006 (March)	Kljakovi-Gašpic Z. et al.; 2010
Bivalves, molluscs	Trace metals	Croatian Adriatic coast	11	2012 (May and November)	Milun V. et al.; 2016
Mussels	Fe, Mn, Cu, Zn, Co, Ni, Cd, Pb, Hg	Boka Kotorska Bay	7	2009 (winter, spring)	Jovic M, Stankovic´ S.; 2014

Cd] = Cadmium; [Co] = Cobalt; [Cu] = Copper; [Fe] = Iron; [Hg] = Mercury; [Mn] = Manganese; [Ni] = Nickel; [Pb] = Lead; [Zn] = Zinc.

2.2 Occurrence of Organic Contaminants in Mussels from the Adriatic Sea

Other than heavy metals, several studies evaluated the occurrence of Persistent Organic Pollutants [POPs] in mussels sampled from different locations in the Adriatic Sea. PAHs and PCBs have been the most studied organic contaminants since their toxicity and the related impact on both the environmental and the human health.

Organo-chlorinated pesticides [OCPs] and different PCBs (PCB-28, PCB-31, PCB-52, PCB-101, PCB-105, PCB-118, PCB-138, PCB-153) were analysed in the whole tissues of mussels from the eastern and western coasts of Adriatic Sea. 16 PAHs were determined and their higher concentrations were detected in Split, Trieste and Taranto [Baji O. et al.; 2019].

As for the heavy metals pollution, industrialization and urbanization influence the POPs contamination. 17 PCBs and 7 OCPs were monitored in the coastal waters of the eastern Adriatic, showing elevated concentrations of organic contaminants in the densely populated and industrialized areas. Among the analyzed POPs, PCB-138 and PCB-153 were identified as the major contaminants in the studied mussels [Kljakovic´-Gašpic´ Z. et al.; 2010].

Levels of OCPs and POPs were monitored biannually in bivalve molluscs from 13 different locations along the eastern Adriatic coast. Five species of molluscs were analyzed, including *Mytilus galloprovicnialis*, establishing PCBs as the major POPs. However, the found levels of POPs were lower than the maximum residue levels in seafood prescribed by the European Commission [Milun V. et al.; 2016].

PAHs were analyzed in *Mytilus galloprovincialis* and other three species of molluscs from the Central Adriatic Sea, since their wide distribution and common use within the Italian diet. The total PAHs concentration was of 34.73 ng/g of mollusc wet weight, and within the PAHs, benzo(a)pyrene was of 1.56 ng/g of mollusc wet weight [Perugini M. et al.; 2007].

Mussels were used as bio-indicators to evaluate the coastal water quality focusing on the levels and spatial distribution of POPs (PBDEs and PCBs) in the coastal marine environment. This study revealed a higher contamination by POPs in mussels sampled in the Ionian Sea compared to those collected from the Adriatic Sea. Indeed, PCBs levels were found up to seven times higher in mussels from Ionian than from the Adriatic Sea. Moreover, the PCBs levels were above the maximum values indicated by both European Community (EC) and National regulation in several sample sites, and their concentrations were particularly high in some stations, suggesting that these locations require a much specific attention [Giandomenico S. et al.; 2013]. Different edible marine species, including mussels, were analyzed for their content of POPs, such as Polychlorinated Dibenzodioxins [PCDDs], Polychlorinated Dibenzofurans [PCDFs] and PCBs. Evaluation of organic contamination in organisms collected form the northern, central and southern areas of the Adriatic Sea, showed the PCDDs and PCDFs as the lower pollutants [Bayarri S. et al.; 2001].

Organic contaminants detected in the bivalve molluscs and shellfish from different locations of the Adriatic Sea are listed in Table 2.

Table 2: POPs detected in the marine organisms collected from the Adriatic Sea.

Species	Chemicals detected	Sampling point	n. sampling sites	Sampling period	References
Mussels	OCPs, PCBs, PAHs	Eastern and Western Adriatic Sea	38	2008 (from May to July)	Baji O. et al., 2019
Mussels	OCPs, PCBs	Eastern Adriatic coast	14	2006 (March)	Kljakovi-Gašpic Z. et al.; 2010
Bivalves, molluscs	PCBs	Croatian Adriatic coast	11	2012 (May and November)	Milun V. et al.; 2016
Mussels	PCBs, PAHs	Boka Kotorska Bay	7	2009 (winter, spring)	Jovic M, Stankovic´ S.; 2014
Bivalves, cephalopods, crustaceans, fish	PAHs	Central Adriatic Sea (Abruzzo)	-	2004 (July and December)	Perugini M. et al.; 2007
Mussels	PBDEs, PCBs	Apulia region coast	32	2008 (September and October)	Giandomenico S. et al.; 2013
Molluscs, crustaceans, fish	PCDDs, PCDFs, PCBs	Adriatic Sea	19	1997-1998 (from April to June - from November to January)	Bayarri S. et al.; 2001

[OCPs] = Organo-Chlorinated Pesticides; [PAHs] = Polycyclic Aromatic Hydrocarbons; [PCBs] = Polychlorinated Biphenyls; [PCDDs] = Polychlorinated Dibenzodioxins; [PCDFs] = Polychlorinated Dibenzofurans; [PBDEs] = Polybrominated Diphenyl Ethers.

2.3 Toxicological Effects of Chemical Pollutants and Current Legislation

Several studies showed the toxic effects of both heavy metals and POPs on the environment and on the organisms, including the human health [NET4mPLASTIC Deliverable 3.4.1.; 2020]. International agencies have recognized some heavy metals and POPs as “dangerous” substances (Table 3). Moreover, national and International policies have been adopted to limit their use in order to reduce the environmental pollution The maximum limits of chemical contaminants in food are established by regulation (EC) n.1881/2006 (Tables 4, 5, 6) [EC; 2006].

Table 3: Chemical pollutants recognized as toxic by agencies and authorities.

Substances	Authors	Year	References
PCDDs, PCDFs, dl-PCBs	EFSA	2018	EFSA; 2018
PAH	EFSA	2008	EFSA; 2008
Cd	Agency for Toxic Substances and Disease Registry	2012	ATSDR; 2012
Pb	Agency for Toxic Substances and Disease Registry	2019	ATSDR; 2019
Hg	Agency for Toxic Substances and Disease Registry	1999	ATSDR; 1999

[PCDDs] = Polychlorinated Dibenzodioxins; [PCDFs] = Polychlorinated Dibenzofurans; [PCBs] = Polychlorinated Biphenyls; [PAHs] = Polycyclic Aromatic Hydrocarbons; [Cd] = Cadmium; [Pb] = Lead; [Hg] = Mercury; [EFSA] = European Food Safety Authority; [ATSDR] = Agency for Toxic Substances and Disease Registry.

Table 4: Current legislation about metals in mussels (*https://eur-lex.europa.eu*).

	Foodstuffs (1)	Maximum levels (mg/kg wet weight)
3.1	Lead	
3.1.11	Bivalve molluscs (26)	1,50
3.2	**Cadmium**	
3.2.17	Bivalve molluscs (26)	1,0
3.3	**Mercury**	
3.3.1	Fishery products (26) and muscle meat of fish (24) (25), excluding species listed in 3.3.2. The maximum level for crustaceans applies to muscle meat from appendages and abdomen (44). In case of crabs and crab-like crustaceans (*Brachyura and Anomura*) it applies to muscle meat from appendages.	0,50

Table 5: Current legislation about dioxins and PCBs in mussels (*https://eur-lex.europa.eu*).

	Foodstuffs	Maximum levels		
		Sum of dioxins (WHO-PCDD/F-TEQ) (32)	Sum of dioxins and dioxin-like PCBS (WHO-PCDD/F-PCB-TEQ) (32)	Sum of PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180 (ICES – 6) (32)
5.3	Muscle meat of fish and fishery products and products thereof (25) (34), with the exemption of: — wild caught eel — wild caught spiny dogfish (*Squalus acanthias*) — wild caught fresh water fish, with the exception of diadromous fish species caught in fresh water — fish liver and derived products — marine oils The maximum level for crustaceans applies to muscle meat from appendages and abdomen (44). In case of crabs and crab-like crustaceans (*Brachyura* and *Anomura*) it applies to muscle meat from appendages.	3,5 pg/g wet weight	6,5 pg/g wet weight	75 ng/g wet weight

Table 6: Current legislation about PAHs in mussels (*https://eur-lex.europa.eu*).

	Foodstuffs	Maximum levels (µg/kg)	
6.1	Benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene	Benzo(a)pyrene	Sum of benzo(a)-pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene (45)
6.1.6	Smoked sprats and canned smoked sprats (25) (47) (*Sprattus sprattus*); Smoked Baltic herring ≤ 14 cm length and canned smoked Baltic herring ≤ 14 cm length (25) (47) (*Clupea harengus membras*); Katsuobushi (dried bonito, *Katsuwonus pelamis*); bivalve molluscs (fresh, chilled or frozen) (26); heat treated meat and heat treated meat products (46) sold to the final consumer	5,0	30,0

3. Occurrence of MicroPlastics in Mussels

Mussels have been widely used in laboratory experiments to study the uptake, the accumulation, the clearance characteristics and the impact on the organism after exposure to MPs.
MPs uptake was showed in several experiments where mussels were exposed to different plastic concentrations [Li J. et al.; 2019].

Results of MPs uptake and accumulation in mussels are listed in Table 7.

Table 7: Uptake and accumulation of MPs by mussels in laboratory exposure.

Exposure microplastic			Exposure concentration	Exposure time	Uptake and accumulation organs	Reference
Types	Shapes	Sizes				
Mytilus edulis						
PS	spheres	3, 9.6 μm	42 particles/L	3 h-48 d clearance	gut, haemolymph	Browne et al., 2008
PS	particles, beads	100 nm, 10 μm	1.3×10^4 particles/ml and 1000 beads/ml	45 min-72 h clearance	digestive gland	Ward and Kach, 2009
HDPE	powders	0–80 μm	2.5 g/L	96 h	gill, stomach, digestive gland	Von Moos et al., 2012
PS	beads	30 nm	0.1,0.2,0.3 g/L	8 h	foot	Wegner et al., 2012
PS	spheres	10, 30, 90 μm	110 particles/ml	14 d-24 h clearance	whole soft tissue	Van Cauwenberghe et al., 2015
	beads, fragments and fibers		100,1000 particles/L	5 d	whole soft tissue	Qu et al., 2018
	fibers		2000 microfibers/L	48 h-48 h clearance	gill, intestine, foot, stomach, mantle, gonad, adductor visceral tissue	Kolandhasamy et al., 2018
PS, PE, PP	beads, fibers	7–30 μm (beads) or 23 × 3000 μm (fibers)	50 beads/ml or 0.1 fibers/ml	60 min	whole soft tissue	Porter et al., 2018
M. galloprovincialis						
PS, PE	powders	<100 μm	1.5 g/L	7 d	haemolymph, gill digestive gland	Avio et al., 2015
LDPE	particles	20–25 μm	2.34×10^7 particles/L	28 d	hemolymph, gills, digestive glands, intestine	Pittura et al., 2018
PE	fragments (derived from toothpaste)	50–590 μm	0.01 g/L	21 d	digestive tract, whole body	Bråte et al., 2018a
PS	spheres	3 μm	50-1 × 10^4 particles/ml	24 h-192 h clearance	gut of larva	Capolupo et al., 2018
Mytilus spp.						
PS	beads	2, 6 μm	32 μg/L/day = 2000 beads/ml/day	7 d-7 d clearance	digestive tract intestine, gills	Paul-Pont et al., 2016
Dreissena polymorpha						
PS	beads	1, 10 μm	1×10^6 or 4×10^6 particles/L	6 d	gut, digestive gland, haemolymph	Magni et al., 2018
Geukensia demissa						
PS, PE	spheres	5, 250–300 μm	3.467 g/L	2 h-24 h clearance	stomach, digestive tubules, intestine	Khan and Prezant, 2018
Perna perna						
PVC	spheres	0.1–1 μm	0.5 g/L	3 h-12 d clearance	gut, haemolymph	Santana et al., 2017

Abbreviations: PS, polystyrene; PE, polyethylene; HDPE, high-density polyethylene; LDPE, low-density polyethylene; PP, polypropylene; PVC, polyvinyl chloride.

During active feeding, mussels can continuously pump and filter seawater through their cilia localized at the gill epithelium surface. A coordinated action of cilia allows to pump the seawater at a rate of 50 mL per minute [Famme P. et al.; 1986].
Different pathways of MPs intake and accumulation have been hypothesized, according to mussel feeding strategies and laboratory exposure studies. When dispersed MPs encounter gill surfaces, they may be captured and trapped into mucus and subsequently assimilated over the gill epithelium or

transported into the mouth and the digestive system [von Moos N. et al.; 2012; Kolandhasamy P. et al., 2018].
However, not all the captured particles by gills are ingested. Actually, mussels are able to separate and reject non-nutritive particles as pseudo-faeces in order to defend themselves against the high quantities of suspended particulate matter [Santana M. F. et al., 2018].
Mussels can ingest and accumulate MPs (0-80 µm) in digestive system epithelial cells within hours [von Moos N. et al.; 2012]. However, smaller particles appeared to be ingested and retained in mussels more easily compared to the larger particles [Van Cauwenberghe L. et al., 2015]. Van Cauwenberghe L. et al. (2015) demonstrated that only MPs of the smallest size (10 µm) were detected in mussels exposed to three sizes (10 µm, 30 µm, 90 µm) of MPs. These findings indicate a mussel's selection for a specific size range of MPs during ingestion and egestion process.
In addition to size variation, environmentally aged MPs are differentially ingested. Pre- weathered MPs were more ingested by mussels, in comparison to virgin MPs [Bråte I. L. N. et al.; 2018].
However, it should be highlighted that in many laboratory studies, organisms were exposed to unrealistically high doses of virgin MPs, with uniform size or shape and for relatively short times. On the other end, environmentally exposed plastics are subjected to weathering, abrasion and photo-degradation processes that induce a broad size distribution and various shapes. Therefore, to mimic environmental weathering, some studies exposed organisms to MPs collected from beaches or deployed in a bay for a period time [Bråte I. L. N. et al.; 2018]. Field investigation on mussels showed fibres as the major accumulated MPs, while beads were most ingested by mussels after five-day indoor exposure [Qu X. et al.; 2018]. One possible explanation could be that the presence of fibres in mussels result from long-term accumulation in the marine environment, while beads are more easily ingested by mussels in short time periods. Moreover, the gills and hepato-pancreas tissues trapped fibres cannot be easily removed by individuals [Renzi M. et al., 2018].
Depuration mechanism has been demonstrated to reduce the MPs content in plastic-treated mussels identifying the digestive system as the probable deposit of the eliminated particles. Both wild and farmed mussels showed similarly reduction in MPs content (about the 33% of the total) when kept in an aquarium without feeding for stomach depuration [Birnstiel S. et al.; 2019]. However, many particles were still detected after depuration performed up to 7 days (i.e. 3 – 7 days), suggesting that MPs could have been translocated to other tissues or even to the circulatory system, or that the tested depuration times may not have been long enough to completely eliminate them [Van Cauwenberghe L., Janssen C. R.; 2014 – Paul-Pont I. et al.; 2016 - Browne et al.; 2008].

4. Co-presence of MPs and Environmental Contaminants in Bivalves

Plastic pollution is a recent emerging issue, therefore no literature data are available about the co-presence of MPs and the other environmental contaminants in bivalve molluscs. Moreover, the actual laboratory protocols do not allow the contemporary evaluation and quantification of MPs and the other contaminants. Different experiments provide separately data on the presence of MPs, heavy metals or organic pollutants in mussels, and only the merging of all these results could help to establish their association. A common protocol to evaluate the co-presence of the contaminants is not still developed.

Besides, the experimental design to MPs analysis is not officially defined and regulated by a Reference Regulation, unlike the other environment contaminants. Heavy metals and POPs analyses are standardized and validated according to the European Commission Regulation.

4.1 Collection and analysis of MPs in mussels

The actual protocol for the collection and analysis of MPs in mussels consists of the following steps:

I. Mussels collection at three different positions in the water column (at the surface, at the middle and at the bottom of the natural banks or mussel farms) and their mixture in order to obtain a global sample.

II. Excision of soft tissue from the shells and its digestion using 30% H_2O_2 to degrade the organic matter facilitating the detection of small MPs.

III. MPs analysis using stereomicroscope (visual identification) avoiding any synthetic materials and applying the "Hot Needle Test" to distinguish MPs from other organic debris [Marine and Environmental Research Institute (MERI); 2015].

5. Conclusions

Mussels can ingest environmental contaminants such as heavy metals, POPs and MPs as consequence of their filtering activity. These pollutants can be egested or accumulated in mussel's tissues and transfer to human through the food chain.

Actually, no officially methods have been developed to allow the contemporary analysis of MPs and the other environmental contaminants in the same organisms. Therefore, data about the co-presence of MPs, heavy metals and POPs results only from the merging of their different evaluations. Moreover, the protocols for MPs analysis is still under development.

Further studies will be necessary to define an official method for MPs analysis and to develop a common experimental protocols for the detection of the co-presence of MPs, heavy metals and POPs.

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