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Maria Kazour

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Maria Kazour. Active and passive biomonitoring tools for microplastics assessment in two highly polluted aquatic environments : case study of the Seine estuary and the Lebanese coast. Earth Sciences. Université du Littoral Côte d'Opale, 2019. English. NNT : 2019DUNK0544 . tel-02484873v1

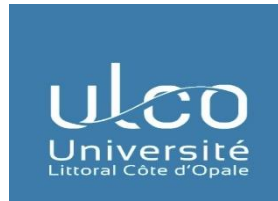
HAL Id: tel-02484873

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Submitted on 19 Feb 2020 (v1), last revised 28 Feb 2020 (v2)

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Thèse de doctorat de l'Université du Littoral

École Doctorale 104 *"Sciences de la matière, du rayonnement et de l'environnement"*
Spécialité: Géosciences, Ecologie, Paléontologie, Océanographie

Discipline: Agronomie, Productions animale et végétale, Agroalimentaire : Biologie de l'environnement, des populations, écologie

Active and passive biomonitoring tools for microplastics assessment in two highly polluted aquatic environments: case study of the Seine estuary and the Lebanese coast

La biosurveillance active et passive pour l'étude des microplastiques dans deux environnements aquatiques pollués : l'estuaire de la Seine et le littoral libanais

Thèse présentée par

Maria Kazour

Pour obtenir le grade de Docteur de l'Université du Littoral Côte d'Opale

Le 17 Décembre 2019

Jury:

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Sharif JEMAA	Docteur, CNRS-L	Invité

“The aim of argument, or of discussion, should not be victory, but progress.” –

Joseph Joubert

In the loving memory of my father, Afif, who always believed in my ability to be successful and taught me never to give up. You are gone but your belief in me has made this journey possible. This thesis is dedicated to you.

Acknowledgments

Foremost, I would like to thank the jury members: Professors Catherine MOUNEYRAC, Helene THOMAS, Johnny GASPERI and Guillaume DUFLOS for accepting to judge this work.

My sincere thanks to both of my directors, Professors Gaby Khalaf and Rachid Amara for their presence and their full trust of this work. A particular thanks to Rachid, for his heartwarming welcome and his continuous support of this research, for his patience, motivation, and immense knowledge. His guidance helped me throughout these three years and encouraged me in difficult moments. Also, I am indebted to my co-director Prof. Gaby Khalaf and my supervisor Sharif Jemaa for pushing me forward since my master 2 to this day; your perseverance made this PhD possible.

This thesis work has been realized in the projects Plastic-Seine and HQFISH funded by GIP Seine A thanks to everyone in these projects who contributed to this work. Also, I would like to express my gratitude to Maria El Rakwe at IRLD- IFREMER- Brest for training me on the Raman spectroscopy (project Plastic-Seine). I couldn't have asked for a better person to explain to me how Raman spectroscopy works!! Thanks for your patience during analysis and your heartwarming welcomes during my stays in Brest.

I would like to thank my colleagues in the Lebanese National Center for Marine Sciences (NCMS-L) – Batroun who provided a pleasant and supportive atmosphere for me during my stays in Lebanon. A special thanks to Roula who was my moral support way before my master 2 and yet continued to be by my side during my PhD journey.

Arriving to a new country (20 degrees colder than what I was accustomed!!) with barely being able to speak its language is not that easy. For that, I would like to appreciate single person who works/worked in our team: Khalef Rabhi, Marie-Anais Lepretre, Dimitri Collard, Mamadou Diop, Jeremy Denis, Sara Rachik and Rym Bouaziz. A big thank you for creating a rewarding work environment. I appreciated every moment we spent laughing during our lab and field work; these were the best memories. Also, my deepest gratitude for: Morgane Effner, Sarah Terki, Christelle Issa, and Konstantin Galin who helped this work to be done and taught me how hard the educational supervision is.

A big thanks to Alexandre Dehaut, Ludovic Hermabessiere and Charlotte Himber and everyone who works at ANSES- Boulogne-sur-mer. You guys welcomed me in the “microplastics world”.

Thank you for making me feel a part of your team when I first arrived; thank you for your advices, continuous motivation and support.

I could not have imagined France without you Nathalie. You were my PhD mate: We went through ups and downs together! Thank you for supporting me in my difficult moments and most importantly thank you for your presence and your dedication to hearing me nagging! (Yes, I do consider it a dedication). I will never forget our crazy Fridays and weekend laughs that made all the week's stress disappear.

To my friends back home: Batoul, Rida, Mohammed, Liliane, and Rosy; Thank you for always being on the other end of the phone telling me what I was missing. You were my invisible support. Also, I would like to thank two of my best friends who went abroad during my stay in France: Chantal, a big thanks to you. We both left the country months apart heading in different directions. We never let the time difference distance us. Your support has always been constant. Cheers to our 8 minutes long voice notes! Bahaa, you were always there cheering me up, you were always the one person who understood everything without explaining anything. I hope you reach success in your PhD (trust me, it is worth it). Thank you for being the best friend a person could ever ask for.

A special thanks to Ramy. You were the one who dealt with me during the ups and downs of my thesis and were always next to me no matter what (which I will never forget). Thanks for your continuous encouragements. I know I don't express it much but thank you for every time you yelled at me to get back to writing this thesis and stop wasting time. You were my true visible support in France.

To everyone in my family: the big and happy familia (naming everyone would need a full page!), I would like to thank you for your support and encouragement. Most importantly for always being there next to my mother. Thank you for never making her feel lonely during the time I was in France; knowing that I had a loving, protective and caring family back home made my stay easier. The biggest and most sincere thanks go to my mother (Georgette) and brother (Bernard) who were both my backbone during this research, they were always there to pick up the phone and encourage me; thank you for always finding the right words to motivate me.

TABLE OF CONTENTS

Funding.....	1
List of Figures (excluding publications)	3
List of Tables (excluding publications).....	7
Scientific Contributions.....	9
Abstract	13
Resume	15
CHAPTER 1: STATE OF THE ART	17
1. General overview: Plastics pollution.....	17
2. The new emerging pollutant ‘Microplastics’	19
2.1 History and definition	19
2.2 Origin of microplastics	20
2.3 Sources of microplastics in the aquatic environment	22
3. Occurrence of microplastics in the aquatic environment	29
3.1 Microplastics abundance in the water	30
3.2 Microplastics in sediments	33
3.3 Microplastics in biota	34
4. Microplastics biomonitoring	35
5. Objectives of the thesis	39
CHAPTER 2: STUDY SITES, METHODOLOGIES AND ANALYSIS PROTOCOLS.....	43
1. Study sites	45
1.1 Estuaries: the Seine, the Canche and the Liane.....	46
1.2 Coastal systems: Sainte-Adresse and the Lebanese coast.....	50
1.3 Wastewater treatment plant: Edelweiss.....	54

2. Sampling techniques of all three matrices (water, sediments and native biota)	56
2.1 Water surface.....	56
2.2 Sediment samples	59
2.3 Native biota	60
3. Caging experiment	63
3.1 Juvenile flounder caging experiment	64
3.2 Farmed blue mussels caging experiments	65
3.2 Caging model comparaisn	68
4. Laboratory analyses and samples preparation.....	69
4.1 Contamination prevention	69
4.2 Water samples	70
4.3 Sediment samples	71
4.4 Biota samples	73
5. Microplastics analyses.....	77
5.1 Visual observation.....	77
5.2 Micro-Raman spectroscopy analysis and polymer identification	79
CHAPTER 3: SOURCES OF MICROPLASTICS AND PASSIVE BIOMONITORING	86
A. Synthesis of Article 1:.....	88
I. Article 1: Sources of microplastics pollution in the marine environment: importance of wastewater treatment plant and coastal landfill	90
Abstract	91
1. Introduction	92
2. Material and methods	93
3. Results	97
4. Discussion	103

5. Conclusion.....	109
Supplementary files.....	110
B. Synthesis of Article 2.....	111
II. Article 2: Microplastics pollution along the Lebanese coast (Eastern Mediterranean Basin): Occurrence in surface water, sediments and biota samples.....	113
Graphical abstract.....	113
Abstract	114
1. Introduction	115
2. Materials and methods	116
3. Results and discussion.....	119
4. Conclusion.....	133
Supplementary files.....	135
CHAPTER 4: ACTIVE BIOMONITORING AS A TOOL FOR MICROPLASTICS ASSESSMENT	142
A. Synthesis of Article 3.....	144
III. Article 3: Juvenile fish caging as a tool for assessing microplastics contamination in estuarine fish nursery grounds.....	146
Abstract	147
1. Introduction	148
2. Material and methods	149
3. Results	151
4. Discussion	153
5. Conclusion.....	159
Supplementary files.....	160
B. Synthesis of Article 4:.....	164

IV. Article 4: Effect of exposure period on caged blue mussels (<i>Mytilus edulis</i>) microplastics bioaccumulation	166
Abstract	167
1. Introduction	168
2. Material and Methods.....	169
3. Results	172
4. Discussion	180
5. Conclusion.....	182
Supplementary files.....	183
C. Synthesis of Article 5.....	187
V. Article 5: Is blue mussel caging an efficient method for monitoring environmental microplastics pollution?	189
Graphical abstract.....	189
Abstract	190
1. Introduction	191
2. Material and methods	193
3. Results	200
4. Discussion	204
5. Conclusion.....	213
Supplementary files.....	215
CHAPTER 5: GENERAL DISCUSSION AND PERSPECTIVES	217
1. Is achieving a standard sampling and preparation protocol far from reality?	219
2. Microplastics sources in the aquatic environment	222
3. Microplastics occurrence in biota and the potential use of Biomonitoring as a tool for MPs monitoring	226
Perspectives.....	231

References	236
Résumé détaillée de la thèse en français	281
Annexes	295
Annex 1: Studies on microplastics occurrence in the surface water worldwide. PES: Polyester, PA: Polyamide, PUR: Polyurethane, PE: Polyethylene, PP: Polypropylene, PET: Polyethylene terephthalate, PS: Polystyrene	296
Annex 2: Microplastics contamination in different mollusks collected in different regions. ...	300

Funding

This thesis has been realized in Laboratoire d’Océanologie et de Géosciences – Wimereux and in the National Center for Marine Sciences – Batroun and was funded by a PhD fellowship from the Lebanese National Council for Scientific Research (CNRS-L – Lebanon) and Université du Littoral Côte d’Opale (ULCO – France). The studies conducted in this work were financially supported by the European Union (ERDF), the French Government, the Région Hauts-de-France and IFREMER, in the framework of the project CPER MARCO 2015- 2020. Part of the study was financially supported by the in the projects Plastic-Seine and HQFISH funded by GIP Seine Aval.

List of Figures (excluding publications)

Figure 1: Different types of polymers used in various European economical segments (taken from Plastics Europe, 2018).....	18
Figure 2: A) Microbeads of three different colors observed in Saint-Adresse coastal water, B) Blue microbead in the Lebanese surface water observed on a GF/A filter, and C) plastic pellets of various colors observed in the Lebanese sediment samples.	20
Figure 3: Microplastics affected by photo-degradation, mechanical and biological mechanisms found near the Dollemard abandoned coastal landfill.....	22
Figure 4: Different macroplastics observed in the sediment samples taken during spring and summer season from the Lebanese coasts. Cigarette buds and plastic bottles are abundantly observed.	23
Figure 5: Saint-Adresse abandoned coastal landfill. The wastes (plastic bags, bottles, metals...) are located directly along the coast.	29
Figure 6: Simplified schematic representation of different sampling sites as well as the different collected matrices done in this thesis.	45
Figure 7: Map of the Seine estuary (Northwestern of France, Normandy) with its three parts: downstream, "in-between" and upstream. Sampling sites along the Seine estuary are indicated as the species' symbol.	47
Figure 8: Map of Le Havre harbor with its various sampling sites chosen for this study.....	48
Figure 9: Map of the Canche and the Liane estuaries (Pas-de-Calais).	49
Figure 10: Different wastes from the Dollemard discharge along Sainte-Adresse coastal zone ..	51
Figure 11: Sampling sites located along the Lebanese coast (Tripoli, Beirut and Sidon).	52
Figure 12: Tripoli's coastal landfill with its protective peripheral wall	53
Figure 13: Different landfills surrounding Beirut region	53
Figure 14: Aerial view of Edelweiss WWTP with its various treatment steps (adapted from the report of CODAH, 2013).	54
Figure 15: Wastewater treatment steps inside Edelweiss WWTP with the different treatment phases and characteristics the water undergoes. Also, the three sampling points are indicated in the diagram (adapted from Edelweiss, 2016).....	55
Figure 16: Water filtration method used (water pump, flowmeter) and the sieves of different mesh sizes used.	57

Figure 17: Manta trawl used in collecting the water samples off the Lebanese coast.58

Figure 18: Core sampler used in collecting sediment samples in Le Havre and Sainte-Adresse. 59

Figure 19: Steel ring with two stainless-steel plates used in collecting sediments along the Lebanese coast.....60

Figure 20: A) Wild juvenile European flounder caught in the Canche estuary and B) its dissected digestive tract that is later digested.61

Figure 21: Individuals of native hybrid mussels collected inside Le Havre harbor. The right photo indicates the wide distribution of these mussels along the shore.....62

Figure 22: A) European anchovies and B) *Spondylus spinosus* caught in the Lebanese coastal waters.63

Figure 23: Procedure of implanting the Alpha tags on European flounders individuals prior to their caging deployment.64

Figure 24: Caging model and real photos of cages constructed for *Platichthys flesus* deployed in three different estuaries along the Eastern English Channel.....65

Figure 25: Farmed mussels depurated inside 160 L aquarium equipped with several airstones to ensure constant oxygenation of the individuals.67

Figure 26: Caging model and real photos of cages constructed for *Mytilus edulis* deployed in Le Havre harbor and Sainte-Adresse.....68

Figure 27: Density separation protocol adapted for the collected water samples.....70

Figure 28: Summary of studies that used different solutions to extract microplastics from sediments. (H_2O_2 : hydrogen peroxide, $ZnCl_2$: zinc chloride, H_2O : water, NaI: sodium iodide, $CaCl_2$: Calcium chloride, Na_2WO_4 : sodium tungstate).72

Figure 29: Sieved sediment on a 2 mm mesh size and visually sorted on a microscopic slide for Raman analysis.....73

Figure 30: Digestion protocol used for collected marine organisms.74

Figure 31: Ten Erlenmeyers containing digestive tracts and KOH 10% solution after 24 hours of digestion (including one blank with filtered KOH 10% only) and the obtained filter after filtration.74

Figure 32: Spiny oysters dissected and put inside clean Erlenmeyer filled with a volume of 400 mL of KOH 10%.75

Figure 33: Hybrid blue mussels (*Mytilus* sp.) on the left and blue mussels (*Mytilus edulis*) on the right.75

Figure 34: Samples observed using Leica stereomicroscope with several examples of observed microplastics (microbeads, fibers and fragments).....78

Figure 35: Observation direction used to observe filters under the stereomicroscope.78

Figure 36: Micro-Raman Xplora Plus (Horiba Scientific, France) used for microplastics identifying throughout this thesis. The open door (on the left) is closed (on the right) during samples analyses (limiting airborne contamination to filters).....80

Figure 37: Baseline correction for a polyethylene spectrum.....82

Figure 38: Polypropylene characteristic peak observed in an obtained dye spectrum.....83

Figure 39: Mixture analysis realized on a dye spectrum (Hostasol Green G-K): A) represent the obtained composite spectrum, B) represent the pigment spectrum and C) represents the polymer (PE) spectrum.84

Figure 40: Lifecycle of plastic and plastics products (source Boucher and Friot, 2017).....223

Figure 41:Estimated microplastics particle flow in wastewater treatment plant with primary, secondary and tertiary treatment process (source: Sun et al., 2019)225

List of Tables (excluding publications)

Table 1: Categories and densities of plastics found in the marine environment (adapted from Andrady, 2011 and GESAMP, 2015).....	19
Table 2: Types and sources of plastics and microplastics in the aquatic environment (adapted from GESAMP, 2017).....	24
Table 3: Microplastics found in the influent, effluent and the removal rate in different WWTP types and locations (p.e.: population equivalents).	27
Table 4: Studies on microplastics occurrence in the sea surface water in different regions worldwide (see Annex 1 for full details). PES: Polyester, PA: Polyamide, PUR: Polyurethane, PE: Polyethylene, PP: Polypropylene, PET: Polyethylene terephthalate, PS: Polystyrene	31
Table 5: Studies done on microplastics caging using bivalves with their different characteristics and results.....	38
Table 6: Length and width of the manta net, the distance and the volume of water sampled along the Lebanese coast.....	58
Table 7: Differences between the two conducted caging experiments using <i>Mytilus edulis</i>	66
Table 8: Summary of different characteristics for caging models used for fish and mussels.....	68
Table 9: Calculated digestion efficiency (%) for farmed mussels (F1 to F9) and native hybrid mussels (W1 to W9).....	76
Table 10: Advantages and disadvantages of using micro-Raman spectroscopy during microplastics analysis (adapted from K��ppler et al. 2016)	81
Table 11: Advantages and disadvantages of using water pump or manta trawl during water collection.	220

Scientific Contributions

Published articles:

Kazour, M., Amara, R. (2019) Is blue mussel caging an efficient method for monitoring environmental microplastics pollution? *Science of the Total Environment*, 135649, *in press*

Kazour, M., Jemaa, S., Issa, C., Khalaf, G., Amara, R. (2019). Microplastics pollution along the Lebanese coast (Eastern Mediterranean Basin): Occurrence in surface water, sediments and biota samples. *Science of the Total environment*. 696, 133933

Kazour, M., Terki, S., Rabhi, K., Jemaa, S., Khalaf, G., Amara, R. (2019). Sources of microplastics pollution in the marine environment: Importance of wastewater treatment plant and coastal landfill. *Marine Pollution Bulletin*, 146, 608-618.

Kazour, M., Jemaa, S., El Rakwe, M., Duflos, G., Hermabassiere, L., Dehaut, A., Le Bihanic, F., Cachot, J., Cornille, V., Rabhi, K., Khalaf, G., Amara, R. (2018). Juvenile fish caging as a tool for assessing microplastics contamination in estuarine fish nursery grounds. *Environmental Science and Pollution Research*, 1-12.

Hermabessiere, L., Himber, C., Boricaud, B., **Kazour, M.,** Amara, R., Cassone, A. L., Laurentie, M., Paul-Pont, I., Soudant, P., Dehaut, A., Duflos, G. (2018). Optimization, performance, and application of a pyrolysis-GC/MS method for the identification of microplastics. *Analytical and bioanalytical chemistry*, 410(25), 6663-6676.

Article in preparation:

Kazour, M., Amara, R. Effect of exposure period on caged blue mussels (*Mytilus edulis*) microplastics bioaccumulation.

Oral Presentations:

Kazour, M., Jemaa, S., Khalaf, G., Amara, R. “Microplastics contamination of the Lebanese coast: ingestion by commercial seafood species” in Fish Forum 2018 – Rome, Italy – 10-14 December 2018

Kazour, M., Terki, S., Rabhi, K., Jemaa, S., Khalaf, G., Amara, R. “Sources of microplastics pollution into the marine environment: importance of wastewater treatment plant and coastal dump” in MICRO 2018 – Arrecife, Lanzarote (Spain) – 19-23 November 2018

Kazour, M., Jemaa, S., El Rakwe, M., Duflos, G., Hermabassiere, L., Dehaut, A., Le Bihanic, F., Cachot, J., Cornille, V., Rabhi, K., Khalaf, G., Amara, R. “Nouvelle approche pour l’étude in situ de la contamination des poissons par les microplastiques: engagement de juvéniles de flets

(Platichthys flesus) dans des nourriceries estuariennes” in “14ème colloque international francophone en écotoxicologie aquatique EcoBIM 2018” – Bordeaux (France) – 22-25 May 2018

Poster presentations:

Kazour, M., Terki, S., Rabhi, K., Jemaa, S., Khalaf, G., Amara, R. “Sources of microplastics pollution into the marine environment: Importance of wastewater treatment plant and coastal landfill” in “EurOCEAN 2019”, UNESCO, Paris (France) – 11-12 June 2019 (the submitted abstract is accepted and is selected as one of the best 10 submissions)

Kazour, M., Terki, S., Rabhi, K., Amara, R. “Développement d'approches de biomonitoring actif (encagement d'organismes) pour l'étude in situ de la contamination des organismes marins par les microplastiques” in “Journées de l'ARCUS-E2D2” – Rabat (Maroc)- 9-11 April 2018

Kazour, M., Jemaa, S., El Rakwe, M., Duflos, G., Hermabassiere, L., Dehaut, A., Le Bihanic, F., Cachot, J., Cornille, V., Rabhi, K., Khalaf, G., Amara, R. “Nouvelle approche pour l'étude in situ de la contamination des poissons par les microplastiques” in “3ème colloque international Francophone en environnement et santé” – Dunkerque (France) – 23-25 October 2017

Awards:

- Best poster presentation in ARCUS-E2D2
- Best poster presentation in EurOcean 2019

Scientific outreach:

Kazour, M., “Etude de la cinétique d'ingestion des microplastiques dans les moules: approche par encagement en milieu naturel” in “Journée des doctorant LOG”- Wimereux (France)- 24 June 2019

Kazour, M., “Les plastiques dans le milieu aquatique” présentation pour des élèves de l'école primaire de Wimereux – Wimereux (France) – 26 April 2019

Kazour, M., “Nouvelle approche pour l'étude in situ de la contamination des poissons par les microplastiques : encagement de juvéniles de flets (*Platichthys flesus*) dans des nourriceries estuariennes” in “Journée des doctorant LOG”- Wimereux (France) - 22 June 2018

Kazour, M., “Sources de pollution de l'environnement marin par les microplastiques: Rôle des stations d'épuration et des décharges côtières” in “Journée MARCO” Nausicaa – Boulogne-sur-mer (France) – 11 October 2018

Kazour, M., “Développement d'approches de biomonitoring actif (encagement d'organismes) pour l'étude in situ de la contamination des organismes marins par les microplastiques” in “Journée scientifique du Pôle EMLM” – Calais (France) - 19 February 2018

Internship co-supervision:

Terki, S., 2018. Etude de la contamination de l'estuaire de la Seine par les microplastiques : utilisation de la biosurveillance passive et active avec la moule *Mytilus edulis*. Master 2, Lille University

Issa, C., 2018. Etude de la pollution par les microplastiques dans les sédiments, la colonne d'eau et les espèces *Spondylus spinosus* et *Engraulis encrasicolus* au niveau du littoral Libanais. Master 2, Lebanese university.

Galin, K., 2019. Etude de la cinétique d'ingestion de microplastiques dans les moules : approche par encagement en milieu naturel. Master 1, University of Littoral.

Abstract

Plastic fabrication is increasing worldwide in response to daily human demands. This mass production is linked to the immense plastic marine litter found all around the world: each synthetic material is meant to find its way back into the aquatic systems. Anthropogenic pressure and the immense human population, the lack of appropriate plastic treatment process and the growing industrial activities advocate their presence in the aquatic environments. These plastics are then found in the form of microplastics (microscopic particle with a size < 5 mm) observed in the water, in the sediments and are prone to be ingested by various marine organisms along the trophic chain. This thesis focuses on (1) assessing microplastics sources and input into the aquatic environment and their occurrence in biota, and (2) to test the feasibility of using transplanted organisms (caging) for monitoring microplastics pollution in the marine coastal environment.

Two coastal areas highly impacted by anthropogenic pressures were studied: Le Havre in France and the Lebanese coast. For the former, the role of a municipal wastewater treatment plant (WWTP) effluent and an abandoned coastal landfill as pathways for microplastics (MPs) input into the marine environment was assessed. MPs were first analyzed in raw sewage influent, sludge and effluent samples, and their fate was studied along a distance gradient from the WWTP in three matrices: surface water, sediments and wild mussels (*Mytilus* spp). MPs were found in all matrices with a decreasing abundance from the effluent. Strong MPs abundances (higher than those found near the WWTP effluent) were observed in the vicinity of the coastal landfill suggesting its importance as a MPs entry route into the marine coastal environment.

Whereas for the Lebanese coast, we evaluated for the first time the MPs pollution in the seawater, sediments and two important seafood species (one pelagic fish: *Engraulis encrasicolus* and one bivalve: *Spondylus spinosus*). Results showed different patterns of MPs concentration in the analyzed matrices. The occurrence of MPs in the biota was high (83.4% and 86.3% in anchovies and spiny oysters, respectively). These results highlighted the high MPs pollution found in the Levantine Basin in comparison to other Western Mediterranean regions. In addition, the obtained results indicate the potential contribution of coastal landfills to this pollution.

Most often microplastics studies involve collection of organisms' samples from natural populations. In this thesis, we tested the feasibility of using transplanted organisms (caging) for monitoring microplastics' pollution in the marine coastal environment. We developed caging experiments with juvenile European Flounder, *Platichthys flesus*, in estuarine nursery grounds and blue mussels, *Mytilus edulis*, in coastal marine environment. For each species, the abundance and characteristics (shape, size, color and type of polymers) of MPs ingested by caged individuals are compared with those ingested by wild individuals collected at the same site and with those found in their surrounding environment (surface water and sediments). Our results suggest that transplanted organisms (caging) may be a promising tool for MPs biomonitoring making monitoring more reliable with an accurate assessment of the biological effects of MPs over a predetermined exposure period.

Keywords: Microplastics, sources, caging, micro-Raman, Seine estuary, Lebanon, mussels, fish

Resume

La production de plastique est en augmentation continue pour répondre à la forte demande mondiale. Cette production massive est la source d'importantes quantités de plastiques que l'on retrouve dans les milieux aquatiques. Parmi ces plastiques, les microplastiques (MP) (particules microscopiques d'une taille < 5 mm) se retrouvent dans l'eau, les sédiments et sont susceptibles d'être ingérés par divers organismes marins. Cette thèse se focalise sur (1) l'évaluation des sources et des apports de microplastiques au milieu aquatique et de leur présence dans les organismes, et (2) de tester la faisabilité d'utiliser des organismes vivants pour la surveillance de la pollution de l'environnement par les microplastiques.

Deux zones côtières affectées par des pressions anthropogéniques ont été étudiées : Le Havre et le littoral libanais. Dans le premier cas, le rôle d'une station de traitement des eaux usées (STEP) et d'une décharge côtière dans l'introduction des microplastiques dans l'environnement a été étudié. Les MPs ont d'abord été analysés dans l'influent, la boue et l'effluent de la STEP. Leur devenir a été suivi selon un gradient de distance de la STEP au niveau de trois matrices : eau de surface, sédiments et moules sauvages (*Mytilus* spp.). Les MPs ont été trouvés au niveau de toutes les matrices avec une concentration qui diminue en s'éloignant de l'effluent. Des taux de microplastiques élevés (plus élevés que ceux trouvés à côté de la STEP) ont été observés à proximité de la décharge côtière, suggérant son rôle important comme voie d'entrée des MPs dans les eaux côtières.

Le long de la côte libanaise, nous avons évalué pour la première fois la pollution par les MPs dans l'eau de mer, les sédiments et dans deux espèces marines importantes en terme de consommation humaine (un poisson pélagique: *Engraulis encrasicolus* et un bivalve: *Spondylus spinosus*). Les résultats ont montré différents patterns de concentration des MPs dans les matrices analysées. La présence de MPs dans les organismes était élevée (83.4% et 86.3% dans les anchois et les huîtres, respectivement). Ces résultats ont mis en évidence la pollution élevée liée aux MPs au niveau du bassin Levantin par rapport aux autres régions de la Méditerranée occidentale. De plus, les résultats obtenus indiquent une contribution potentielle des décharges côtières à cette pollution.

Le plus souvent, les études sur les microplastiques impliquent l'échantillonnage d'organismes indigènes. Au cours de cette thèse, nous avons testé la faisabilité d'utiliser des organismes transplantés (encagement) pour évaluer la pollution par les microplastiques dans l'environnement marin côtier. Nous avons mis au point des expériences d'encagement avec des juvéniles de flets, *Platichthys flesus*, en estuaires et des moules bleues, *Mytilus edulis*, en zone côtière. Pour chaque espèce, l'abondance et les caractéristiques (forme, taille, couleur et type de polymères) des MPs ingérés par les individus encagés ont été comparées avec les MPs ingérés par des individus sauvages capturés sur le même site et avec ceux trouvés dans leur environnement (eaux de surface et sédiments). Nos résultats suggèrent que la technique d'encagement d'organismes peut constituer un outil prometteur pour la biosurveillance des MPs la rendant plus fiable et permettant une étude précise des effets biologiques des MPs sur une période d'exposition prédéterminée.

Mots clés: Microplastiques, sources, caging, micro-Raman, l'estuaire de la Seine, Liban, moules, poissons

CHAPTER 1: STATE OF THE ART

1. GENERAL OVERVIEW: PLASTICS POLLUTION

In 1907, the breakthrough of plastics production began by finding the composite “Bakelite” (a mixture of formaldehyde and phenol). From that year, diverse polymers were progressively discovered and produced. It was not until the 1950’s when plastics manufacturing started increasing creating a wide family of materials that are daily used due to their wide usage in various applications and sectors. Worldwide plastics production reached 348 million tons in 2017 with high economic importance in the market value. The usage of plastic has become essential in daily activities yet raising awareness on its fate has led to an increase in plastic recycling and a decrease in its landfilling.

This first part highlights the most important concepts of plastics and their tremendous economic importance of the world daily life.

Plastics production increased tremendously since the year 1970. They are composed of a variety of materials with different properties that could meet the characteristics of numerous products. The daily dependence of plastics products leads to approximately a global of 8 billion tons of plastics accumulated till 2015 (Geyer et al., 2017) and a production of 348 million tons of plastics worldwide in 2017 (PlasticsEurope, 2018).

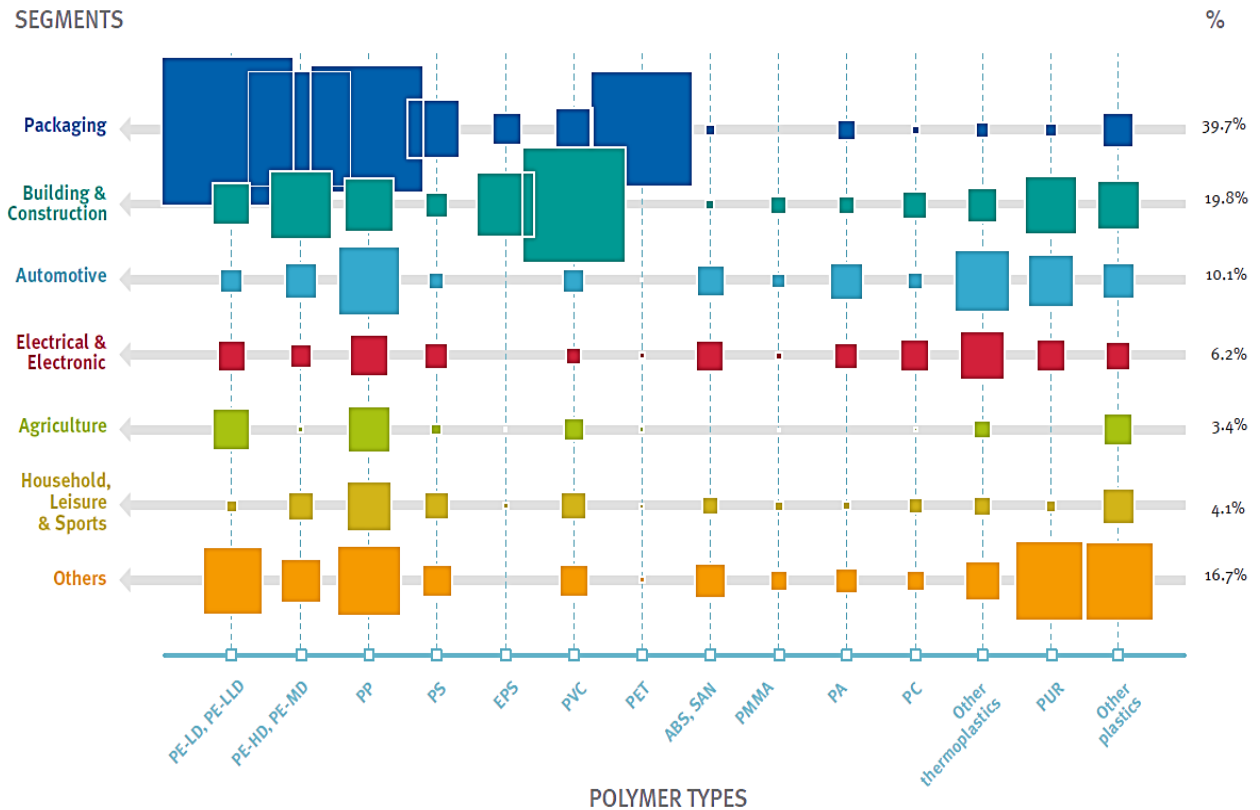


Figure 1: Different types of polymers used in various European economical segments (taken from Plastics Europe, 2018).

Most of the plastics produced are used in packaging (39.7%), in building and construction (19.8%), in automotive (10.1%) and in other applications (PlasticsEurope, 2018). Nine big plastics families are widely used in different sectors: polypropylene (PP), polyethylene (PE; low and high density: PELD and PEHD), polystyrene (PS), polyvinylchloride (PVC), polyethylene terephthalate (PET), acrylonitrile butadiene (ABS), polyurethane (PUR), polyamide (PA) and polycarbonate (PC) (see Figure 1). The disposal of these plastics products is the key problem that led to their abundant existence in the environment. Even though the recycling percentages increased throughout the last years, landfills keep on receiving the highest amount of wastes (Ellen MacArthur Foundation, 2016; O'Connor et al., 2016). These plastics will eventually end up in the ocean and accumulate in the aquatic environments (Thompson et al., 2009; UNEP, 2014).

2. THE NEW EMERGING POLLUTANT ‘MICROPLASTICS’

Large plastic items have been already recorded in turtles (Tomás et al., 2002), cetaceans (De Stephanis et al., 2013; Jacobsen et al., 2010) and seabirds (Rodríguez et al., 2012; Tanaka et al., 2013). But what about smaller plastic items?

2.1 HISTORY AND DEFINITION

In the last decade, more attention has been highlighted on smaller microscopic plastic items called “microplastics”. With the study done by Thompson et al. (2004), microplastics have been the “new emerging pollutant” of the decade. Within ten years, the number of researches done on microplastics increased by 3300% (from 18 articles published in 2009 to around 700 published in 2019, and a total of around 2400 articles). Several definitions of microplastics exist that differ by their limit size. In most of the published studies, as well as in this thesis, microplastics are defined as plastic items with a size below 5 mm (GESAMP, 2015). Yet, other studies still define microplastics as particles with a size below 1 mm (Browne et al., 2011; Claessens et al., 2011; Karami et al., 2017; Wright and Kelly, 2017). These microplastics have two lower size limit 0.1 (Lusher et al., 2017) or 1 μm (GESAMP, 2017). This limit actually differs between different authors, and it is usually related to the sampling or identifying method used. Generally, particles are measured to their longest length even though differences might exist between their weight and volume, and in their densities (Table 1).

Table 1: Categories and densities of plastics found in the marine environment (adapted from Andrady, 2011 and GESAMP, 2015).

	Polymer types	Density (g/cm^3)
Thermoplastic (can be reheated, reshaped and frozen repeatedly)	Polyethylene (PE)	0.91 – 0.95
	Polypropylene (PP)	0.90 – 0.92
	Polystyrene expanded (ePS)	0.01 – 1.05
	Polystyrene (PS)	1.04 – 1.09
	Polyvinyl chloride (PVC)	1.16 – 1.30
	Polyamide or Nylon (PA)	1.13 – 1.15
	Polyethylene terephthalate (PET)	1.34 – 1.39
Thermoset (cannot be re-melted and reformed)	Polyurethane (PUR)	0.018 – 0.05

In addition, microplastics have different shapes: fragments, fibers, pellets, films, foams and microbeads. Microplastics also have several bands of colors. Both of these observed characteristics vary according to different studies. Yet, what about their origin? How and why are they found in the environment?

2.2 ORIGIN OF MICROPLASTICS

Microplastics originates as primary or secondary microplastics sources.

A. Primary microplastics

Microplastics that are considered as “primary” are intentionally produced in a small size and are widely used. They can be found in different forms (Figure 2) such as plastic pellets or granules used for the production of bigger plastics materials, or as microbeads used in cosmetic, hygienic products, in the industrial abrasives and powders (Cole et al., 2011; Lusher et al., 2017). Even though small microscopic synthetic fibers result from the degradation of textile wear, some studies consider them as primary microplastics while other as secondary microplastics (Salvador Cesa et al., 2017).

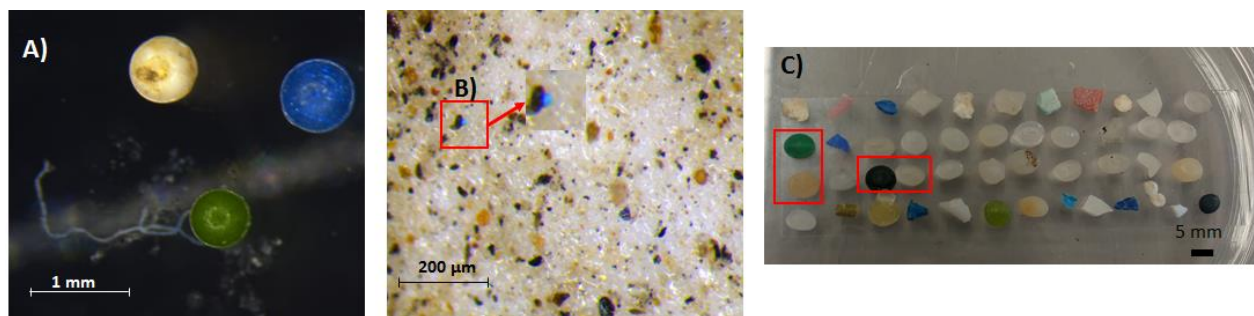


Figure 2: A) Microbeads of three different colors observed in Saint-Adresse coastal water, B) Blue microbead in the Lebanese surface water observed on a GF/A filter, and C) plastic pellets of various colors observed in the Lebanese sediment samples.

Plastic pellets can be present in sediments and in the aquatic environments due to mishandling or direct waste discharge of plastic industries (Shiber, 1979) and are abundantly observed near industrial zones (Norèn, 2007). High number of microbeads exist in liquid products; their abrasives properties lead to their high existence in cosmetic products (Fendall and Sewell, 2009). Other important propriety exist for these small microplastics, is that they have

adsorption/desorption capacities that identify them as a threat to the marine and coastal environment (Fendall and Sewell, 2009). They are susceptible to adsorb pollutants and release toxic molecules in the natural environment. Upon one usage (around 5 mL), thousands of microbeads would end up in the aquatic systems making them a potential danger to the surrounding environment (Napper et al., 2015). These microbeads are progressively being banned worldwide: in the US (2017), France and United Kingdom (2018) and other European Union regions are to ban microbead usage in 2020.

Thousands of textile fibers might be released in the aquatic environment after using one washing machine (Browne et al., 2011). Napper and Thompson (2016) were able to indicate that around 700 000 fibers can be released from 6 KG of washed textiles. These fibers could either be sent to wastewater treatment plants (WWTPs) or they can directly be released into the aquatic environment. In the former case, WWTPs don't always have the capacity to eliminate these fibers (Leslie, 2014) indicating that most of the observed fibers in the environment could have come from washing textiles (Browne et al., 2011).

B. Secondary microplastics

These microplastics are obtained after fragmentation of larger plastic particles. Physical, chemical or biological mechanisms can lead to an alteration of polymers' properties (Singh and Sharma, 2008). Plastics that are left in the environment are prone to be affected by several factors leading to their degradation (Figure 3). For example, ultra-violet (UV) radiations that affect plastics are the principal cause of their fragmentation in the marine environment (Barnes et al., 2009). This photo-degradation will lead to an oxidation of the polymer and, hence, the plastics' fragmentation. The speed of this degradation process depends on the (1) polymer's nature and the (2) presence of the plastics in the environment. Plastics found on beaches are more degraded than those found on the surface water or at the bottom (GESAMP, 2017); solar UV radiations along with heat and mechanical friction can lead to a higher photo-degradation. Mechanical processes such as the abrasions from waves and wind result to the fragmentation of larger plastics into microplastics (Andrady, 2011). Also, micro-organisms (fungi and bacteria) have been linked to degrading synthetic plastics (Shah et al., 2008; Yoshida et al., 2016) with several factors

influencing their degradation process (polymer type and characteristic, organism's type and the conditions to which the organism is submitted).

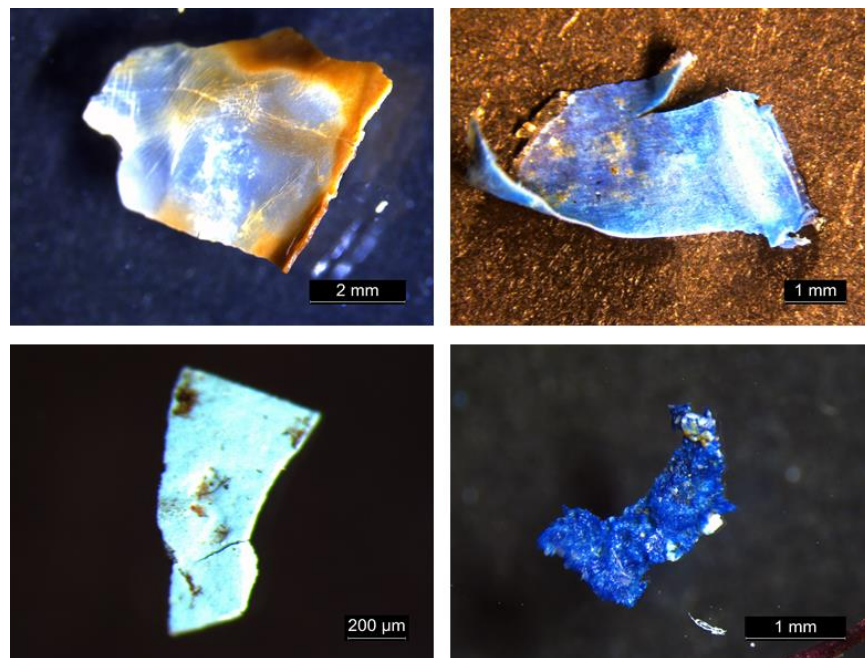


Figure 3: *Microplastics affected by photo-degradation, mechanical and biological mechanisms found near the Dollemard abandoned coastal landfill*

2.3 SOURCES OF MICROPLASTICS IN THE AQUATIC ENVIRONMENT

If microplastics are found either in their primary or secondary type, what are the sources that leads to their entry into the aquatic environment? Plastics from terrestrial activities consist the majority (80%) of the observed plastics in the marine environment (Andrady, 2011). Various sectors play a role as microplastics sources and lead to their increase in the aquatic systems. Table 2 indicates the sources of plastics as described by GESAMP (2017) as well the type of microplastics it forms. These sources come from four big categories:

- Producers
- Sectoral consumers
- Individual consumers
- Waste management

Among these sources, tourism and human population play an important source of macroplastics. Almost one quarter of the world population occupy the coastal zones (Small and Nicholls, 2003).

These zones are highly affected by plastics usage and favor plastics production that are likely mismanaged due to the lack of appropriate infrastructure (Figure 4). Cole et al. (2011) indicated that seasonal tourism activities can lead to high amount of wastes in a short period of time. The abundance of these macro wastes on the beaches would rapidly lead to their degradation and are more susceptible into forming secondary microplastics.



Figure 4: Different macroplastics observed in the sediment samples taken during spring and summer season from the Lebanese coasts. Cigarette buds and plastic bottles are abundantly observed.

Even if most of the sources are considered terrestrial (Jambeck et al., 2015), many marine sources play a role in plastic marine pollution. Fisheries, aquaculture and shipping industries are major sources for marine macroplastics pollution (Andrady, 2011). Fishermen usually abandon or lose fishing nets and lines in the seawater. Aquaculture also generates plastics wastes (Cole et al., 2011) and are locally significant (wastes resulting from shellfish farms for examples). This maritime pollution is highly contributed by maritime traffic: during the 70's, about 23 thousand tons of packaging were thrown in the sea (Cole et al., 2011). Later that decade, in 1978, international convention (MARPOL) has been held in order to limit waste disposal in the sea, by prohibiting the uncontrolled disposal of plastics and other synthetic materials (cords, nets, plastic bags) (Derraik, 2002). The Mediterranean Sea and the Northwestern France are two zones that are highly affected by maritime traffic. Cole et al. (2011) indicated that even though international agreements exist, the maritime traffic is one of the principal sources of marine pollution because of the lack of control, education and reluctances concerning practical modifications,.

Table 2: Types and sources of plastics and microplastics in the aquatic environment (adapted from GESAMP, 2017).

Source	Description	Entry points	Type
Plastics producers, fabricators	Pellets and fragments	River, coastline, atmosphere	Primary
Agriculture	Greenhouse-sheets, pots, pipes, nutrient pills	River, coastline, atmosphere	Secondary
Fisheries	Fishing gear, packaging	River, coastline, marine	Secondary
Aquaculture	Buoys, lines, nets, PVC pipes	River, coastline, marine	Secondary
Construction	EPS, packaging	River, coastline, atmosphere	Secondary
Terrestrial transportation	Pellets, tires, tire dust	River, coastline, atmosphere	Primary and secondary
Shipping/offshore industry	Paints, pipes, clothes, cargo, plastic-blasting	River, marine	Secondary
Tourism industry	Consumer goods, packaging, microbeads, textile fibers	River, coastline, marine	Primary and secondary
Textiles	Fibers	River, coastline, atmosphere, marine	Primary and secondary
Sport	Synthetic turf	River, coastline, atmosphere	Secondary
Food and drink single use packaging	Containers, plastic bags, bottles, caps, cups, etc.	River, coastline	Secondary
Cosmetics and personal care products	Microbeads, packaging, etc	River, coastline, marine	Primary and secondary
Solid waste (landfill)	Unmanaged or poorly managed waste disposal	River, coastline, atmosphere	Secondary
Water and wastewater	Microbeads, fragments, fibers	River, coastline	Primary and secondary

The knowledge about most of these stated sources above (11 out of 14) is considered low. The route of microplastics entry and their fate are not well-known, for there are several factors affecting their transportation such as wind, sea currents, river systems and runoff.

Some of these sources are also considered as a route for microplastics entry into the aquatic environment such as the role of wastewater treatment plants (Talvitie et al., 2015). In the last couple of years, the number of studies done on wastewater treatment plant role as microplastics entry sources have increased. But a lot of information are still missing concerning the role of WWTP and solid wastes (landfills) in microplastics entry in the aquatic environment.

A. Wastewater Treatment Plants

Wastewater treatment plants are able to treat both industrial and domestic water before discharging it into the aquatic environment (Zbyszewski et al., 2014). Whether it is a secondary or a tertiary plant, WWTP is not made for eliminating microplastics and particles will be released via the discharging water (Browne et al., 2011; Mathalon and Hill, 2014; Murphy et al., 2016; Roex et al., 2013). Yet, normal treatment steps are capable of removing microplastics. These different processes differ from a WWTP to another. Primary treatment processes can remove 63% to 98% (Sun et al., 2019); and an additional 7 to 20% are captured during secondary treatment (Gies et al., 2018; Ziajahromi et al., 2017). This retention during early stages suggest that MPs are removed during grit and grease screening and sludge formation (Lares et al., 2018; Leslie et al., 2017; Murphy et al., 2016). Other factors might increase the MPs in WWTP such as the plant's equipment of advanced treatment technologies (Lares et al., 2018; Magni et al., 2019; Michielssen et al., 2016; Ziajahromi et al., 2017). Among these techniques, membrane bioreactor sludge (MBR) (Lares et al., 2018), dissolved air flotation (Talvitie et al., 2017a) and reverse osmosis and decarbonation (Ziajahromi et al., 2017) showed high microplastics removal efficacy. Other studies showed that advanced technologies do not enhance microplastics retention (Carr et al., 2016; Leslie et al., 2017). This retention percentage generally ranges between 72 and 99.4% (Table 3) (Reviewed by Gatidou et al., 2019; Sun et al., 2019) but remains limited by current detection techniques. It is speculated that during grease and grit removal steps, most of the microplastics are retained (Magni et al., 2019; Murphy et al., 2016). Only half of the published articles analyzed the sludge inside WWTPs and each study analyzed their sludge samples using

different protocols. Fragments, fibers and microbeads have been observed during grit and grease removal as well as sludge samples representing various types of polymers. Even though important amounts of microplastics are retained, but the microplastics particles still reach the effluent water. The amount of discharged particles differs from one study to another (Table 3) and several shapes are observed: microbeads, fibers, fragments and films (Blair et al., 2019; Carr et al., 2016; Dris et al., 2015; Mason et al., 2016). Released volumes of treated water vary from one plant to another, and from one day to another. Studies indicated that up to 6.5×10^7 and 5×10^4 MPs/day are released in Scotland (UK) (Murphy et al., 2016) and San Francisco (USA) (Mason et al., 2016), respectively. These differences are influenced by the lack of a homogenous protocol: different sampling and analyses methodologies, diverse mesh sizes and various observation and identifying techniques. Whether using steel buckets, filtration pump, sieves and automatic sampler, or by samples filtration or density separation; this disparity between studies lead to an unreliable data comparison. Also, low identification percentage of polymers can be observed in WWTP: most of the studies identified polymers using FTIR spectroscopy whereas a couple studies used Raman microscopy. A small percentage of the total observed items were identified and were ranged from 1.3% (Lares et al., 2018) to 8% (Simon et al., 2018) and 16% (Murphy et al., 2016). Thus, there is a need of a standardized sampling and analyses protocol to have a more reliable comparison between studies.

Table 3: Microplastics found in the influent, effluent and the removal rate in different WWTP types and locations (p.e.: population equivalents).

Location	WWTP size (p.e.)	Treatment type	Influent volume (L)	Items found in the influent (items/L)	Effluent Volume (L)	Items found in the effluent (items/L)	Removal rate (%)	Reference
Sweden	1.4×10^4	Secondary	2	15	1000	0.008	99.9	(Magnusson and Norén, 2014)
France	-	Secondary	0.05	260-320	0.05	14-50	83-95	(Dris et al., 2015)
USA	-	Secondary and Tertiary	5	27	$1.89 \times 10^5 - 2.32 \times 10^5$	8×10^{-4}	99.99	(Carr et al., 2016)
USA	-	Secondary and Tertiary	1 – 2	133.0 ± 35.6	34 – 38	5.9	95.6-99.4	(Michielssen et al., 2016)
Scotland	6.5×10^5	Secondary	30	15.70 ± 5.23	50	0.25 ± 0.04	98.41	(Murphy et al., 2016)
Finland	$5 \times 10^4 - 8 \times 10^5$	Tertiary	0.4	0.5 ± 2.0	1000	0.03 ± 0.3	40 – 99.8	(Talvitie et al., 2017a)
Finland	8×10^5	Tertiary	0.1	636.7 ± 38.8	1000	3.2 ± 0.7	>99	(Talvitie et al., 2017b)
Canada	1.3×10^6	Secondary	1	31.1 ± 6.7	30	0.5 ± 0.2	99	(Gies et al., 2018)
Finland	-	Secondary	4	57.6 ± 12.4	30	1.0 ± 0.4	98.3	(Lares et al., 2018)
Denmark	-	Secondary and Tertiary	1	7216	81.5	54	99.3	(Simon et al., 2018)
Turkey	1.5×10^6	Secondary	5	23 – 26	5	7 – 4	73 – 79	(Gündoğdu et al., 2018)
USA	$3.2 \times 10^4 - 1.8 \times 10^5$	Secondary	0.5	100 – 250	1.5 – 15.5	2 – 30	74.8 – 98.1	(Conley et al., 2019)
Italy	1.2×10^6	Tertiary	30	2.5 ± 0.3	30	0.4 ± 0.1	84	(Magni et al., 2019)
Scotland	1.84×10^5	Tertiary	25	3 – 10	25	1	96%	(Blair et al., 2019)

B. Landfills

Landfills are sites for a large variety of wastes: municipal, commercial, industrial, agricultural and constructional. Plastics are among these deposited dry wastes. Plastics are durable and, once buried, persist in the environment. Several types of plastics can be observed: plastic bottles, carrier bags, packaging sheets, single films, polyvinyl chloride (PVC) pipes and large plastics. Inappropriate management would lead to major plastics release into the surrounding environment. Whereas in a properly managed landfill, wastes are daily covered with soil or synthetic material and fences, with an appropriate recycling program that would decrease 20 to 40% of the wastes composition (Barnes et al., 2009). From 2006 to 2016, landfilling has decreased by 43% in Europe while recycling increased by 79% (PlasticsEurope, 2018). Landfills are a potential source of microplastics, several factors affecting landfills (high temperature, pH and physical compacting) as well as anaerobic circumstances increase plastics degradation (Mahon et al., 2017; Sundt et al., 2014). These degraded microplastics enter the surrounding environments via air-borne pathway (Rillig, 2012) and leachates (He et al., 2019; Praagh et al., 2018; Su et al., 2019). Leachates are “the fluid percolating through the landfills and are generated from liquids present in the waste and from outside water, including rainwater, percolating through the waste” (Jayawardhana et al., 2016). Not only do untreated leachates contain higher microplastics than treated leachates (Praagh et al., 2018), but they are also vectors of heavy metals and organic contaminants (Sui et al., 2017). The role as a microplastics entry source will increase if the landfill is poorly managed or abandoned, or if its leachates are not treated. What about abandoned coastal landfills for example? As Figure 5 shows, abandoned coastal landfills with their waste directly along the coast would increase their role in macro and microplastics entry to the marine environment. With no proper management, wastes (including plastics) are in direct contact with the surrounding seawater and enter the aquatic environment via waves, high tides and winds. If landfills were considered as a primary disposal for wastes, they should be regarded as important sources of microplastics (Su et al., 2019).



Figure 5: Saint-Adresse abandoned coastal landfill. The wastes (plastic bags, bottles, metals...) are located directly along the coast.

3. OCCURRENCE OF MICROPLASTICS IN THE AQUATIC ENVIRONMENT

Once microplastics find their way in the aquatic environment, they are either observed in the surface water, sediments or biota. Several factors influence their distribution and abundance: items size, shape, density and biofouling (Andrady, 2011). Density plays an important role on the presence and distribution of plastics: low density ($<1.02 \text{ g/cm}^3$) polymers (PUR, PE and PS) tend to float on the seawater, whereas high density polymers (PVC, PET) tend to sink to the bottom. Yet, this density is influenced by biofouling, as well as by currents and resuspension. In the latter, the biofouling phenomenon affects low density polymers in the surface water: the items are covered by a colony of biofilms that will increase the density and lead to their sinkage to the bottom (Andrady, 2011). Resuspension phenomena have been already observed when high-density polymers were detected on the surface water but not fully understood (Enders et al., 2015; Suaria et al., 2016).

3.1 MICROPLASTICS ABUNDANCE IN THE WATER

A. Coastal marine systems

Scientific research has mainly focused on the abundance of microplastics in the marine environment with oceans considered as the final sink for these pollutants (Horton and Dixon, 2018). Microplastics distribution in marine environment is affected by both temporal and spatial variability due to seasonal patterns in oceanic currents (Cole et al., 2011; Ryan et al., 2009). The majority of studies on microplastics occurrence were reported in the surface and subsurface water of several areas worldwide (reviewed by Akdogan and Guven, 2019). Most of them have taken into consideration the spatio-temporal variation of MPs abundance, including their shape, size, color and polymer type. Microplastics have been reported in the water of Pacific Ocean (Desforges et al., 2014; Díaz-Torres et al., 2017; Lebreton et al., 2018; Rios Mendoza and Jones, 2015), the North Sea (Liebezeit and Dubaish, 2012; Lorenz et al., 2019), the Atlantic ocean (Kanhai et al., 2017; Lusher et al., 2014; Reisser et al., 2015), Bohai and South China Seas (Cai et al., 2018; Zhang et al., 2017), Arctic polar waters (Lusher et al., 2015), and the Mediterranean Sea (Baini et al., 2018; Cózar et al., 2015; Gündoğdu and Çevik, 2017; Lefebvre et al., 2019) (see Annex 1). Microplastics in coastal waters are affected by strong hydrodynamic factors (tides, wind, waves and salinity gradients), and undergo beaching, drifting and settling, with only a small fraction being carried into the open ocean (Zhang, 2017). Various sampling methods were used along with several mesh sizes (Table 4). Different analyses protocols were used in various studies: for clean water samples, no digestion method was used and they were directly filtered. If this was not the case, organic material destruction was used (enzymatic or mechanical) and samples underwent density separation (various solution were used between studies) (Reviewed by Stock et al. (2019)). Recovered items in the coastal water samples, were made of all kind of shapes: fibers, fragments, films and microbeads. The dominant shape varied from one study to another and from one region to another (Lusher et al., 2015, 2014; Pedrotti et al., 2016; Suaria et al., 2016): Fibers were abundant in the Western Mediterranean surface water (Lefebvre et al., 2019), whereas fragments were dominant in the Eastern Mediterranean (Gündoğdu, 2017; van der Hal et al., 2017). The dominant polymer type differed with the dominant shape: PE, PP and PS were dominant in the areas with high abundance of fragments (Pedrotti et al., 2016; Zhang et al., 2017); Polyester (PET) and PA were highly dominant in samples with important fibers occurrence (Lefebvre et al., 2019; Lusher et al., 2015)

Table 4: Studies on microplastics occurrence in the sea surface water in different regions worldwide (see Annex 1 for full details). PES: Polyester, PA: Polyamide, PUR: Polyurethane, PE: Polyethylene, PP: Polypropylene, PET: Polyethylene terephthalate, PS: Polystyrene

Sampling method	Number of conducted studies	Mesh sizes (μm)	Studied regions	Concentrations (average min-max)	Polymers found	Reference
Manta net	18	100 – 200 – 330 – 333 – 335 – 500 - 505	Arctic, Antarctic, Pacific, China Seas Mediterranean	0.004 to 245.4 items/ m^3 80 to 1067120 items/ km^2	PES, PA, PUR, PE, PET, PP, Copolymers	[1],[2],[3],[4], [5],[6],[7],[8], [9],[10],[11], [12],[13],[14], [15], [16],[17],[18]
Plankton net	5	53 – 80 – 120 – 300 – 330	China Seas, Qatar sea, Atlantic Ocean, Sri Lanka, Brazil	0 to 29 items/ m^3	PE, PP, PS, PES	[19],[20],[21], [22],[23]
Neuston net	4	200 – 333	Gulf of Oman, Mediterranean	0.31 to 1.25 items/ m^3	PS, PP, PE	[24],[25],[26], [27]
WP2 net	4	200	Mediterranean	0.17 to 0.62 items/ m^3 0.062 items/ m^2	PET	[28],[29],[30], [31]
Water pump	3	40 – 44 – 250	North Sea, China, Atlantic Ocean	2.46 to 194 000 items/ m^3	Alkyd resin, PES, PA	[31],[32],[33]
Bongo net	1	500	Mediterranean	16 items	PE	[34]
Bottle	1	5	U.K coastal waters	3500 items/ m^3	PES	[35]

[1] (Lusher et al., 2015) ; [2] (Lacerda et al., 2019) ; [3] (Zhang et al., 2017) ; [4] (Doyle et al., 2011) ; [5] (Mu et al., 2019) ; [6] (Lorenz et al., 2019) ; [7] (Collignon et al., 2012) ; [8] (Pedrotti et al., 2016) ; [9] (Schmidt et al., 2018) ; [10] (Constant et al., 2018) ; [11] (Ruiz-Orejón et al., 2016) ; [12] (de Lucia et al., 2014) ; [13] (Baini et al., 2018) ; [14] (de Haan et al., 2019) ; [15] (Güven et al., 2017) ; [16] (Gündoğdu, 2017) ; [17] (Gündoğdu and Çevik, 2017) ; [18] (van der Hal et al., 2017) ; [19] (Chen et al., 2018) ; [20] (Castillo et al., 2016) ; [21] (Bimali Koongolla et al., 2018) ; [22] (Scott et al., 2019) ; [23] (Olivatto et al., 2019) ; [24] (Aliabad et al., 2019) ; [25] (Suaria et al., 2016) ; [26] (Cózar et al., 2015) ; [27] (Fossi et al., 2016) ; [28] (Fossi et al., 2012) ; [29] (Lefebvre et al., 2019) ; [30] (Collignon et al., 2014) ; [31] (Panti et al., 2015) ; [31] (Lusher et al., 2014) ; [32] (Cai et al., 2018) ; [33] (Dubai and Liebezeit, 2013) ; [34] (Savoca et al., 2019) ; [35] (Li et al., 2018)

B. Estuaries

Estuaries are ecologically important habitats and are considered transitional ecosystems between the ocean and rivers (McLusky and Elliott, 2004). They constitute important fish habitats for juvenile to which they act as nursery grounds providing refuge and food for both estuarine and marine species (Selleslagh and Amara, 2008). These systems are exposed to important anthropogenic pressure (industry, cities, tourism and agriculture) and are among the most threatened aquatic environments (Halpern et al., 2008; Hidalgo-Ruz et al., 2012). Microplastics increased with increasing distance near estuaries (Luo et al., 2019). High levels of MPs were observed in different estuaries worldwide: 100 to 4100 items/m³ (Jiaojiang, Oujiang and Minjiang estuaries China) (Zhao et al., 2015), 4137.3 ± 2461.5 items/m³ (Yangtze estuary, China) (Zhao et al., 2014), 98, 246 and 1032 particles/m³ (Clyde, Bega and Hunter estuaries, Australia) (Hitchcock and Mitrovic, 2019). Several MPs shapes were observed in estuaries: fibers, fragments, pellets and films with important abundances of fibers and fragments (Hitchcock and Mitrovic, 2019; Luo et al., 2019). In estuarine waters where fragments were mostly abundant, the dominant size classes were < 200 μm (Hitchcock and Mitrovic, 2019), increasing their chances to be likely mistaken as food by biota. There is a lot of missing information concerning polymer identification in estuaries: The studies that identified polymers indicated the dominance of polyester and polypropylene (Luo et al., 2019; Zhao et al., 2015).

3.2 MICROPLASTICS IN SEDIMENTS

Microplastics in the surface water can be either washed up on the coast or sunk at the bottom. Deep sea sediments have been recognized to potentially accumulate microplastics and are considered as major sink of MPs (Martellini et al., 2018). Microplastics on the shore are affected by important anthropogenic pressure (tourism) whereas the offshore sediments are influenced by environmental conditions such as tides, wind speed and direction, resuspension, current and biofouling (Wang et al., 2018; Zhang, 2017). The abundance of microplastics in sediments vary worldwide. There is no standard sampling and extraction protocol: some digested their samples before using a density separation method, whereas others skipped digestion and went directly to density separation, and some even visualized their samples without treatment. Even different solutions were used as a density separation medium with sodium chloride (NaCl) the most used solution (see Figure 28). Microplastics observed after extraction from sediments are expressed in different units (items/kg; items/m²) complicating the comparison between various studies. Extreme high values were observed in sediments of Venice Lagoon reaching 2175 MPs/Kg d.w (Vianello et al., 2013) and in the Xiangshan Bay (China) 1739 ± 2153 items/Kg (Chen et al., 2018). Whereas other regions had lower number: 67 ± 76 items/Kg d.w in the French Atlantic Ocean (Phuong et al., 2018a); 85 ± 141 items/kg d.w in the North of Crete (Piperagkas et al., 2019), 45.9 ± 23.9 items/Kg in the Spanish Mediterranean coast (Filgueiras et al., 2019) and 60–240 items/Kg in the Yellow Sea and East China Sea (Zhang et al., 2019). All kinds of shapes can be observed: fragments, films, fibers and pellets with abundant shapes and polymers different between studies (Reviewed by Yao et al., 2019). Several studies indicated the high significance of pellets in the sediment samples (Fanini and Bozzeda, 2018; Karkanorachaki et al., 2018; Turner and Holmes, 2011; Turra et al., 2014). A wide range of polymers were detected in sediments: PE, PP, PS, PES, PA, PVC and rayon, and their distribution varied with different studies.

3.3 MICROPLASTICS IN BIOTA

Due to the size of the MP items and characteristics, they can be mistaken as food for various animals and may, therefore, be ingested and integrated within the trophic chain. Once microplastics have reached the different environmental aquatic environment (water and sediments), they are more or less available to the aquatic biota depending on the items shape and size (Cole et al., 2011; Thompson et al., 2009). The ingestion of microplastics has been observed in invertebrates, mollusks, fish, seabirds and big predatory mammals. The MPs items characteristics lead to their uptake by aquatic species. Evidence of microplastics ingestion has been observed in several marine species: crustaceans (Desforges et al., 2014), pelagic fish (Lefebvre et al., 2019), demersal fish (Neves et al., 2015), sea turtles (Duncan et al., 2018; Tomás et al., 2002), seabirds (Rodríguez et al., 2012; Tanaka et al., 2013) and mammals (De Stephanis et al., 2013). Also, these MPs were observed in invertebrates, mollusks and fish existing in the freshwater systems (reviewed by O'Connor et al., 2019). The same as in the other matrices, no standard protocol exist and the comparison between studies would not be accurate even if the MPs concentration were expressed in the same units.

Mussels and oysters are fixed animals, they obtain their food through filter-feeding and are, therefore, prone to ingesting microplastics. As Annex 2 shows, more studies were conducted on mussels rather than on oysters. Microplastics ingestion by wild and caged blue mussels (*Mytilus edulis*) varied from one study to another ranging from 0.2 ± 0.3 items/g in wild mussels collected from the French, Belgian and Dutch North Sea (Van Cauwenberghe et al., 2015) and 1.43 ± 0.30 items/g in wild mussels collected from South West coast of U.K. (Scott et al., 2019). Most of the studies realized indicated that fibers were the most abundant shape found in *Mytilus* sp. (Hermabessiere et al., 2019; Van Cauwenberghe and Janssen, 2014), whereas a couple of studies indicated the abundance of fragments (Digka et al., 2018; Gomiero et al., 2019).

Other than mollusks, microplastics ingestion in fish from different seas and regions have been observed. This ingestion was observed in pelagic, meso-pelagic, demersal and benthic species (Bellas et al., 2016; Güven et al., 2017; Neves et al., 2015; Rios-Fuster et al., 2019). Among these fish, flatfish as well as forage fish have been analyzed for microplastics ingestion. For example, European flounder, *Platichthys flesus*, and European anchovies, *Engraulis encrasicolus*, are two different species occupying different habitats and regions. Both species showed different microplastics ingestion occurrence from one study to another.

4. MICROPLASTICS BIOMONITORING

Living organisms (plants, microbes and animals) that provide information on the quality of the environment (e.g marine environment) are called “Bioindicators” (Burger, 2016). Their usage relies on their ability to accumulate pollutants existing in their environment (Bartell, 2016; Zukal et al., 2015). Numerous species have been used as bioindicators for various marine pollutants: mollusks (Cunha et al., 2017; Dirrigl et al., 2018; Viñas et al., 2018), turtles (Santos et al., 2018), sponges (Orani et al., 2018) and fish (Caçador et al., 2012; Smalling et al., 2016). Suitable organisms that reflect the contamination of their environment should be chosen. For a species to be considered as sentinel species for chemical contaminants, it has to fulfill several criteria:

- A wide geographical abundance for it to be present throughout the study area (for inter-sites comparisons)
- Capacity to accumulate contaminants without being affected by the encountered concentrations nor by the environmental stress.
- Sufficient amount of tissue for laboratory analyses
- Be easy to samples *in situ* and easy to manipulate in laboratory
- Capacity to integrate temporal fluctuations of a specific environment that cannot be followed in the water column

Two types of biomonitoring exist: passive and active. The former consists of choosing indigenous organisms as representatives of the pollution of a specific area. Mussels and oysters are sedentary animals capable of accumulating pollutants and have been widely used as indicators of metals contamination in different biomonitoring programs (Beyer et al., 2017). The “Mussel Watch” program was first introduced in 1976 and it aimed to be a monitoring tool in marine waters

(Goldberg, 1975). This approach has proven to be valid for monitoring metals and various organic contaminants by comparing them to a “reference” clean site. Also, fish have important structural and functional importance in aquatic trophic chain and have been considered as chemical pollutant indicators (Miramand et al., 1998). Passive monitoring is confronted to several factors such as species’ genetic differences, age, size, growth, nutrition status, gender, sexual maturity and spawning. In some cases, reference site is not present or is not under the same environmental conditions as the studied sites.

Whereas active approaches consist of caging experiments. Selected individuals are acclimated for a period of time before putting inside cages and transplanted in the study sites (Beyer et al., 2017; Oikari, 2006). The advantage of caging technique is the feasibility of using organisms to monitor a surrounding environment, and can also be used in ecotoxicology and population biology studies (Henry and Jenkins, 1995). Other advantages transplantation experiments have (Oikari, 2006):

- Knowledge of the caging and the duration of exposure
- Similarity in species age, size and physiological characteristics
- Can be conducted at any desired time depending on the species
- Transplantation depth can be controlled
- Evaluation of a specific site by limiting species’ area
- Prone to various modification in order to be standardized

Some species cannot tolerate caging conditions (deterioration of nutritional status, solitary living, cannibalism, unknown stress) (Oikari, 2006) and an acclimation period is recommended (e.g. maintaining the organism up to 1 week in laboratory conditions).

Till present, most biomonitoring techniques were used to evaluate chemical pollutants and their ecotoxicological effects on organisms (Kerambrun et al., 2013; Oikari, 2006). The recent emerge of microplastics as the pollutant of the decade has led to considering potential approaches for MPs monitoring. The importance and advantages of caging in pollutant monitoring allowed its consideration for microplastics studies. Biomonitoring for microplastics assessment has been recently tested. The first study used caging for microplastics monitoring was tested in 2017 (Avio et al., 2017) and, since then, it has not been very well investigated. To this day, four studies in total have been realized for microplastics biomonitoring: all of them using bivalves as sentinel species (three of them using blue mussels (marine) and one using Painter’s mussels (freshwater)).

The preference of using bivalves for microplastics biomonitoring is because they are easy to manipulate and maintain, filter-feeders that ingest the particles of their surrounding environment and are prone to take up microplastics. These studies transplanted their cages in areas affected by anthropogenic pressure and near important microplastics sources: wastewater discharge (Domogalla-Urbansky et al., 2019; Railo et al., 2018), wreck removal (Avio et al., 2017) and urban port (Catarino et al., 2018).

As shown in Table 5, each study had different characteristics: cage size, exposition time, deployed species and the digestion method. Three of them were conducted in the marine coastal systems using different species of blue mussels. These differences make the comparisons between studies harder. Ingested microplastics by caged bivalves varied from one individual to another but showed higher concentrations for those transplanted near the wastewater treatment plant discharge. Even though transplantation experiments have just recently started but each conducted study did not fully focus on their caging experiment and did not investigate thoroughly for its validation. Beyer et al. (2017) and Li et al. (2019) suggested on the important role that mussels would play in microplastics monitoring and suggested them as sentinel organisms for MPs pollution. But a lot of analyses are to be done before this consideration: Recent experimental studies showed that the size of fragments as well as the length of fibers and their ratio-aspect play an important role in the uptake, retention and rejection of microplastics by mussels (Fernández and Albentosa, 2019; Ward et al., 2019).

Table 5: Studies done on microplastics caging using bivalves with their different characteristics and results.

Location	Cages characteristics	Encaged species/depuration	Duration	Mussels digestion method	MPs concentration	Identification method	Most common polymer	Reference
Giglio Island (Tyrrhenian Sea)	N/A; 2 deployments: - 1.5 m from the surface - 30 to 40 m at the bottom	<i>Mytilus galloprovincialis</i> / None	4 weeks	Density separation (NaCl) + digestion (H ₂ O ₂)	2 ± 1 /individual (surface) 1 ± 0 /individual (bottom)	N/A	N/A	Avio et al., 2017
Port Edgar (Estuary of the Forth River)	Cylindrical stainless-steel cages (10 x 8 cm, height and diameter respectively)	<i>Mytilus edulis</i> / None but they were acclimated	1 year (seasonal variation)	Enzymatic digestion	3.4 ± 0.48 particles/individual (0.74 ± 0.125 particles/g ww)	FT-IR	PET	Catarino et al., 2018
Southern coast of Hanko (Baltic Sea)	35 × 35 cm, mesh size 0.5 cm/ 2 cages: - 30 m from WWTP discharge - 700 m from the first cage	<i>Mytilus trossulus</i> / None	4 weeks	Enzymatic digestion	0.1 ± 0.2 MP/individual (0.4 ± 1.9 MP/g ww)	FT-IR	Cotton, Linen, Viscose, Polyester	Railo et al., 2018
Northern Bavaria	N/A ; 4 cages: - 2 cages 245 m upstream a WWTP discharge and - 2 cages 1100 m downstream	<i>Unio pictorum</i> / None	28 days and 6 months	Nitric acid (65%)	N/A	Raman	PP, PVC and pigments	Domogalla-Urbansky et al., 2019

5. OBJECTIVES OF THE THESIS

With the limited knowledge on microplastics sources, the **first main objective** of this thesis was to investigate two important microplastics sources and occurrence in Le Havre harbor (the Seine estuary), Sainte-Adresse and the Lebanese coast. This part relied on the following sub-objectives:

- **Assessing the contribution of a major tertiary WWTP** located in Le Havre harbor (France) in MPs discharge into the marine coastal environment and to **follow the released MPs** (dispersion) in the marine environment along a distance gradient from the discharge pipe in three matrices.
- **Evaluate, for the first time, the role of coastal landfills** as a source of MPs entry into the marine coastal environment. Two coastal areas were chosen, each one consisting of different characteristics: Sainte-Adresse (near Le Havre) and the Lebanese coast (Eastern Mediterranean).

Three different matrices were analyzed for the occurrence of microplastics: water, sediments and marine organisms. Then, they were compared in order to find a similarity among them.

Passive biomonitoring is practical when the same marine species is available at all sampling sites. But this is not always the case and species would be absent in some sampling sites of the studied area. Also, when analyzing fish samples, animals that are in constant movement, may not necessarily reflect a site's pollution. These difficulties that are encountered in the field led to the **second main objective** that focused on **active biomonitoring experiments**. This technique was only conducted in The Seine estuary, the Canche and the Liane. Optimally, caged individuals were compared to native ones when possible.

Given the important ecological role of estuaries as nursery grounds for many marine fish species and implications of microplastics (MP) in ecosystems, we tested for the first time the caging method as a tool to quantify and assess MP contamination of estuarine juvenile fish. For this, the occurrence, number, size, and polymer types of MP ingested by wild and caged juvenile European flounder (*Platichthys flesus*) from a same site were compared in different estuaries (Seine, Canche and Liane).

The blue mussels is suggested as suitable sentinels for monitoring of MPs contamination (Beyer et al., 2017; Li et al., 2019). The **effectiveness of blue mussels caging approach** was assessed, for the first time, to quantify and assess MPs pollution in the field. First, we tested farmed mussels' depuration using filtered seawater throughout different periods (from 16 hours up to 72 hours and 7 days). Then, an **assessment of different exposition periods of caged mussels** was investigated at a same site and the variations in weekly ingested microplastics were **examined and compared with the surrounding seawater contamination**. The objective was to test different deployment periods to find an optimal duration.

Finally, we deployed a 6 week caging study along a pollution gradient originating from a WWTP discharge and near an abandoned coastal landfill. The characteristics of ingested MPs by caged depurated mussels were compared with those ingested by native mussels collected at the same site as well as with those found in their surrounding environment (surface water and sediments).

The thesis is organized in five chapters.

The first chapter will be an introduction and a literature review concerning microplastics pollution. The first part will be a general overview on microplastics. The second part will describe the origin of microplastics, whether primary and secondary. In the third part, microplastics potential sources and entry routes will be denoted and describing respectively in details both wastewater treatment and coastal landfills. The fourth part will include the information concerning the occurrence of microplastics to this day in different aquatic systems and matrices. plants and coastal landfills role as microplastics entry routes. The last part in the introduction concerns biomonitoring tools for pollution in general and microplastics in particular. These parts will offer enough elements to define the thesis objectives that will be thoroughly described.

In the second chapter, the different study areas and sampling sites are described: estuaries (the Seine, the Canche and the Liane), coastal environments (Le Havre and the Lebanese coast) and the wastewater treatment plant. Sampling protocols and methodologies as well as laboratory analyses are detailed in this chapter.

In the third chapter, microplastics sources and occurrence in two coastal zones are investigated in two studies. The first study evaluated a wastewater treatment plant and coastal landfill in Le Havre coastal waters as sources of microplastics. The second study evaluated coastal landfills along the Lebanese coast and their contribution to the alarmingly high microplastics pollution in the Levantine Basin. In both studies, surface water, sediments and biota samples were analyzed.

Then, the fourth chapter conducted experimental designs concerning active biomonitoring tools for microplastics assessment. Three experiments were conducted in Le Havre coastal zone and estuaries to test for the first time the caging method as a tool to quantify and assess MPs contamination. The caging experiments were conducted both with juvenile fish and the blue mussels.

Finally, the general discussion and perspectives will be presented in the fifth chapter. A synthesis will be made about the major results obtained and comparison of different study areas as well as methodologies. Then, all the potential perspectives that can be developed from this thesis are described, in addition to the promising subjects that can be tackled after this study.

CHAPTER 2: STUDY SITES, METHODOLOGIES AND ANALYSIS PROTOCOLS

CHAPTER 2: STUDY SITES, METHODOLOGIES AND ANALYSIS PROTOCOLS

1. STUDY SITES

We were interested in this thesis in testing both active and passive biomonitoring for microplastics assessment. As shown in Figure 6, passive biomonitoring was conducted along the Lebanese coast and in the Seine estuary: sampling included fish (European flounder and European anchovies) and bivalves (hybrid blue mussels, spiny oysters and blue mussels) as well as water and sediment samples. Whereas for the active biomonitoring, cages were tested in the Seine estuary area and native individuals were collected when possible.

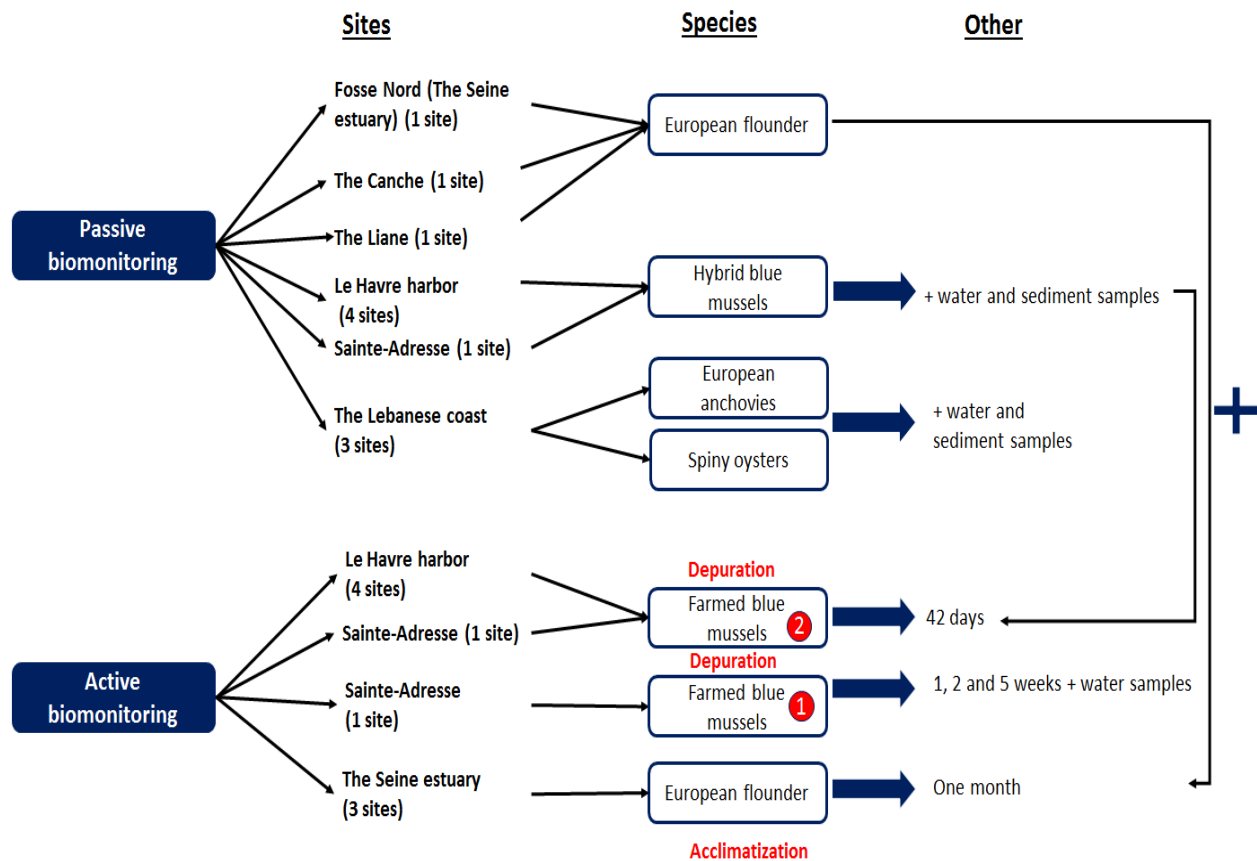


Figure 6: Simplified schematic representation of different sampling sites as well as the different collected matrices done in this thesis.

Different sites studied during this thesis: estuaries, coastal waters and wastewater treatment plant. Microplastics pollution in estuaries was assessed in the Seine, the Canche and the Liane estuaries. In the coastal water regions, Sainte-Adresse (Northwest of France) and the Lebanese coast (Eastern Mediterranean basin). Edelweiss WWTP (Le Havre, France) was chosen to evaluate its microplastics input into the aquatic environment.

1.1 ESTUARIES: THE SEINE, THE CANCHE AND THE LIANE

These three estuaries are located along the Eastern English Channel differing mostly in size and degree of pollution.

A. The Seine estuary

The Seine river has a length of 780 km, a mean discharge of 400 m³/s forming a catchment area of 78 000 km² serving 17.5 million inhabitants and strongly urbanized and industrialized. The Seine estuary forms a basin of important economic value leading to approximately 40% of the economic activity of France. It is divided into 3 parts from Poses to Le Havre and varying in their salinity gradient: the upstream zone (freshwater), the “in-between” (interaction of both fresh and seawater/ brackish water) and the funnel shaped downstream zone (seawater) (Figure 7). This macrotidal estuary exhibits semi-diurnal tides with a tidal range that varies from 3 m (neap tides) and 7.5 m (spring tides) (Avoine et al., 1986) with a sediment cover mostly composed of silt and clay (fine-grained material). The Seine estuary is subjected to both water currents and marine parameters. The river currents effects decrease downstream, whereas tidal forces decrease upstream. These two currents have the highest energy in an internal narrow segment of the estuary (further upstream). The Seine estuary is known for its ability to stock chemical contaminants due to its limited dilution factor. Until the 70's, several sources had played an important role in the pollutants' entry in this basin (Fisson, 2017)

- 1) The inputs coming from the upstream catchment area via the Seine river (Poses)
- 2) Groundwater bodies and affluent inflow
- 3) Direct discharges into the estuary via urban and industrial wastewater treatment plants

4) Runoff or sediments resuspension

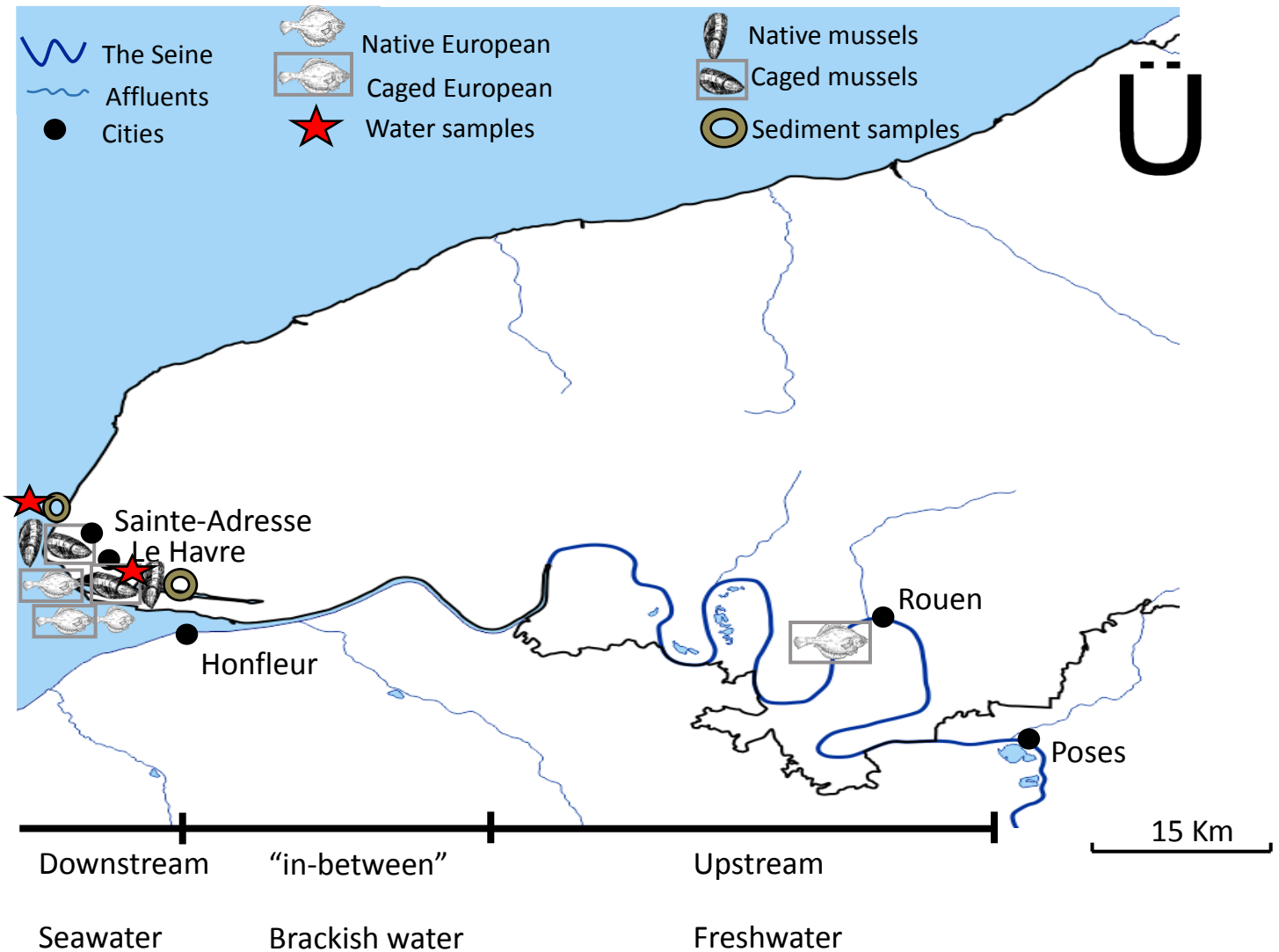


Figure 7: Map of the Seine estuary (Northwestern of France, Normandy) with its three parts: downstream, "in-between" and upstream. Sampling sites along the Seine estuary are indicated as the species' symbol.

Even though in last decades, several efforts have been done in 1980 to decrease several contaminants (trace metals, PCB, pesticides...), it remains to be one of the most chemically polluted estuaries in western northern Europe (Dauvin et al., 2007). Plastics floating the Seine river have been evaluated by Gasperi et al. (2014), and recent estimations indicated the potential entry between 1,100 and 5,900 tons per year of plastic litter flowing from the Seine river into the sea (Tramoy et al., 2019). There is a lack of information concerning microplastics pollution in downstream part of the estuary with only the upstream and the in-between areas assessed for

microplastics abundance (Alligant et al., 2018; Dris et al., 2015). Several sampling sites were chosen in this area as well as wild European flounder (*Platichthys flesus*) were sampled in Fosse Nord in the Seine estuary (Figure 7). At the mouth of the Seine estuary located Le Havre harbor with an area of 102 km² (Figure 8). This harbor is in cooperation with that of Rouen and Paris making it the second biggest French harbor and the sixth in Europe. Its total traffic reached 73

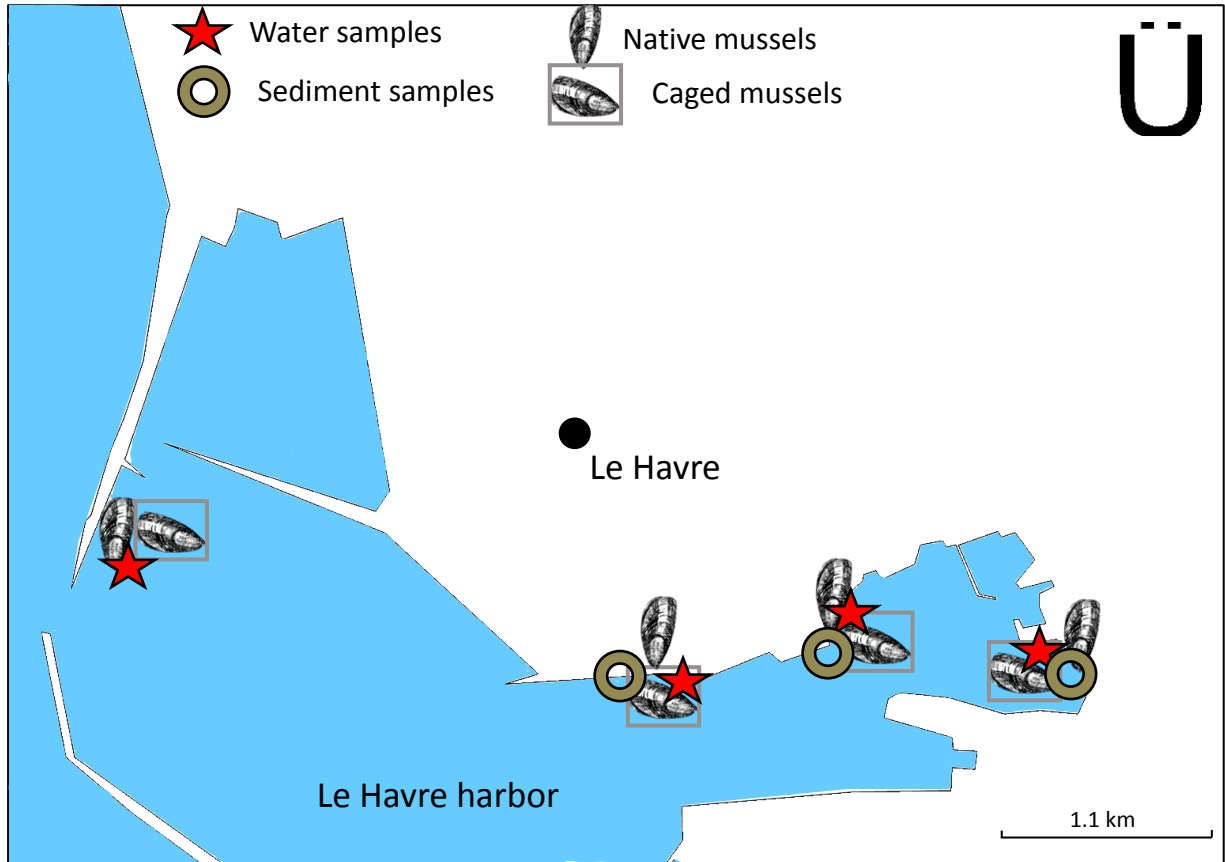


Figure 8: Map of Le Havre harbor with its various sampling sites chosen for this study.

tons). Le Havre harbor is the biggest harbor in France for petrochemical complexes: treating 24.3 tons of crude oil per year. The harbor's area forms an important international industrial and logistic pole with 1200 establishments.

B. The Canche estuary

The Canche river has a length of 85 km, and a mean flow of $15 \text{ m}^3/\text{s}$ forming a catchment area of 1274 km^2 . The Canche estuary is a small estuary of 12 km in length and 1 km in width, and is characterized by a sandy mud substratum. It can be considered as a macrotidal estuary (McLusky and Elliott, 2004) with a tidal range varying from 1 m (neap tides) and 6 m (spring tides). Due to

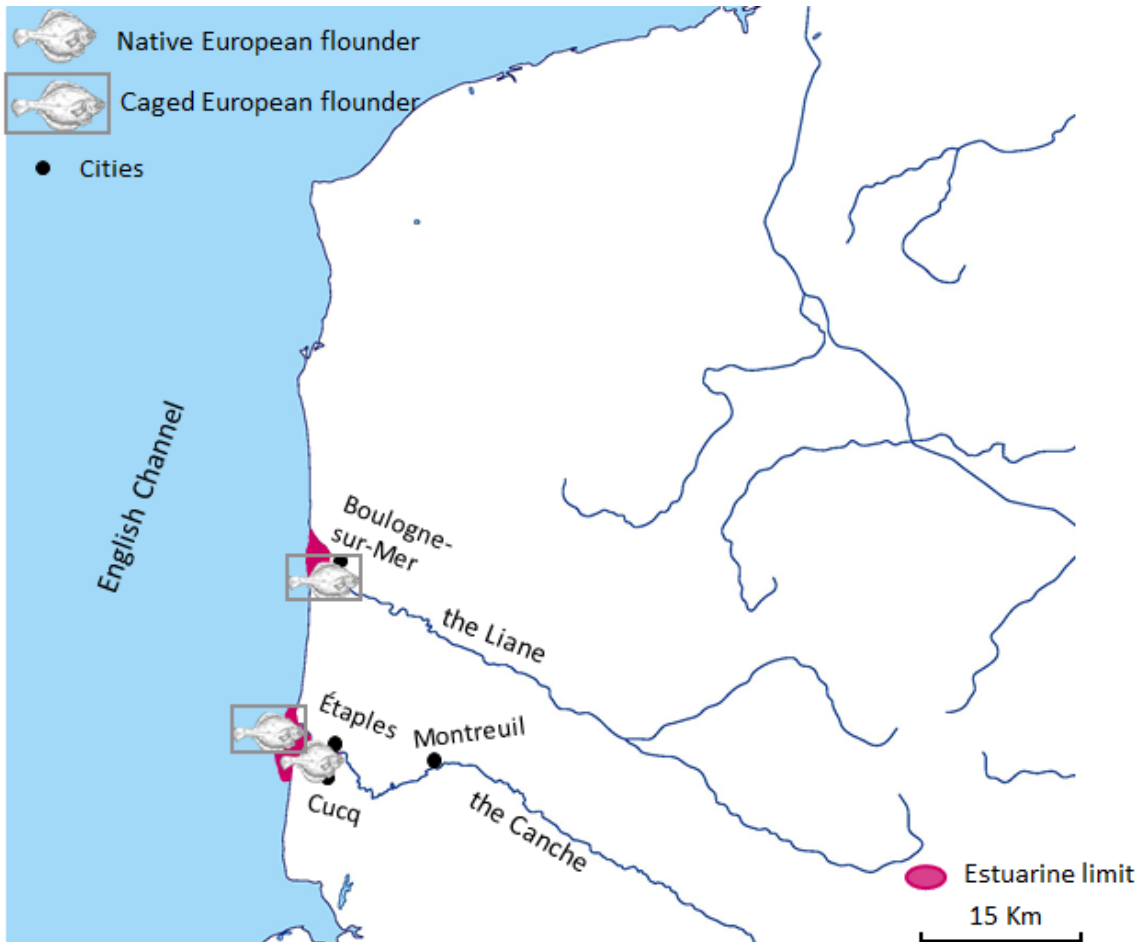


Figure 9: Map of the Canche and the Liane estuaries (Pas-de-Calais).

a low freshwater input ($\sim 13 \text{ m}^3/\text{s}$), its water circulation depends mainly on the tidal forces. The water quality in this estuary might be affected by the effluent water of three small wastewater treatment plants located in Étaples, Montreuil and Cucq (see Figure 9) and treating water for 16 communities; as well as the discharged water by six industries in this area. The Canche estuary is classified under “Natura 2000” and is considered to be a lowly impacted system (Amara et al., 2007). Wild European flounder were caught in the Canche estuary and were used later for MPs analysis and comparison with those caught from the Seine estuary.

C. The Liane estuary

The Liane river has a length of 36 km, a mean flow of 3 m³/s (in winter) and 0.76 m³ (in summer), and forming a catchment area of 244 km². The Liane estuary is the most industrialized among the Picardy estuaries with several industries and economic activities (harbor, commercial and industrial zone, ICPE sites). A municipal wastewater treatment plant (WWTP), treating wastewater for the inhabitants of Boulogne-sur-mer agglomeration, discharges its water into the estuary. A lock is constructed in order to limit the tides' potential influence on the Liane valley (floods for example).

1.2 COASTAL SYSTEMS: SAINTE-ADRESSE AND THE LEBANESE COAST

A. Sainte-Adresse

Sainte-Adresse is a coastal zone located at the bottom of the Dollemard cliffs (see Figure 7). This coast is a part of the "Littoral Cauchois" and is classified under "Natura 2000". Its substratum is composed of sand and pebble and is directly affected by tidal forces (Unknown, 2011). The cliffs of Dollemard are considered as a ZINEFF (Zones Naturelles d'Intérêts Ecologique, Faunistique et Floristique) for its floristic, phytosociological (plant communities), avifaunistics, landscape and geological importance. Yet, along the coast and on the cliffs exist large amount of ancient waste (metals, tires, toxic wastes, plastic and domestic wastes...). These wastes originate from the ancient Dollemard coastal landfill that was closed in the year 2000. This landfill was constructed in 1962 and received large amounts of domestic, industrial and special wastes that were either buried or thrown down from the cliffs. Due to the enormous weight of disposed wastes, the erosion of the cliffs is prematurely increasing (10 to 20 m) and the amount of wastes along the coast is constantly expanding (Figure 10), and is prone to enter coastal waters during high tides. Plastics (such as groceries bags, bottles, containers, caps...) constitute an important amount of the disposed wastes that are distributed on a coastal length of 750 m. High amount of plastics were present in these wastes and had different colors: green, blue and white, and they are agglomerated in 20m² zones (Unknown, 2011). Due to the morphology of the shore, a tidal coefficient of 70 can lead to the abrasions of the lower part of the cliff by the sea and thus introducing wastes into the water (Unknown, 2011). An estimated annual volume of plastics entering by the sea is approximately 30 m³.



Figure 10: Different wastes from the Dollemard discharge along Sainte-Adresse coastal zone

B. The Lebanese Coast

Lebanon is located in the Eastern Mediterranean (Levantine Basin) with a coastal length of 220 km. The Lebanese littoral zone is open to winds and waves (swell), and it is 75% constituted of rocky substrates (Lakkis, 2011). The general circulation along the coast is dominant northwards during most of the year along with the general anticyclonic gyre of the Eastern Mediterranean (Goedicke, 1972). This current is locally modified by the configuration of the coastline and the topography of the narrow continental shelf resulting in a series of clockwise directed eddies and small gyres associated with bays and headlands. Water movements along the coast are also strongly related to surface currents and seasonal meteorological factors (Goedicke, 1972). Anthropogenic pressure on this coast is highly increasing with a 5 million population producing around 2 thousand tons of Municipal Solid Waste (MSW) per year. Among these MSW, 15% are of plastics with no proper disposal. More than 50% of waste are disposed in uncontrolled dumpsites (about 940 dumpsites), 35% are put in sanitary landfills and the remaining 15% undergo material recovery. Three study sites were chosen according to their degree of anthropogenic pressures and are considered as pollution hotspots (Figure 11).

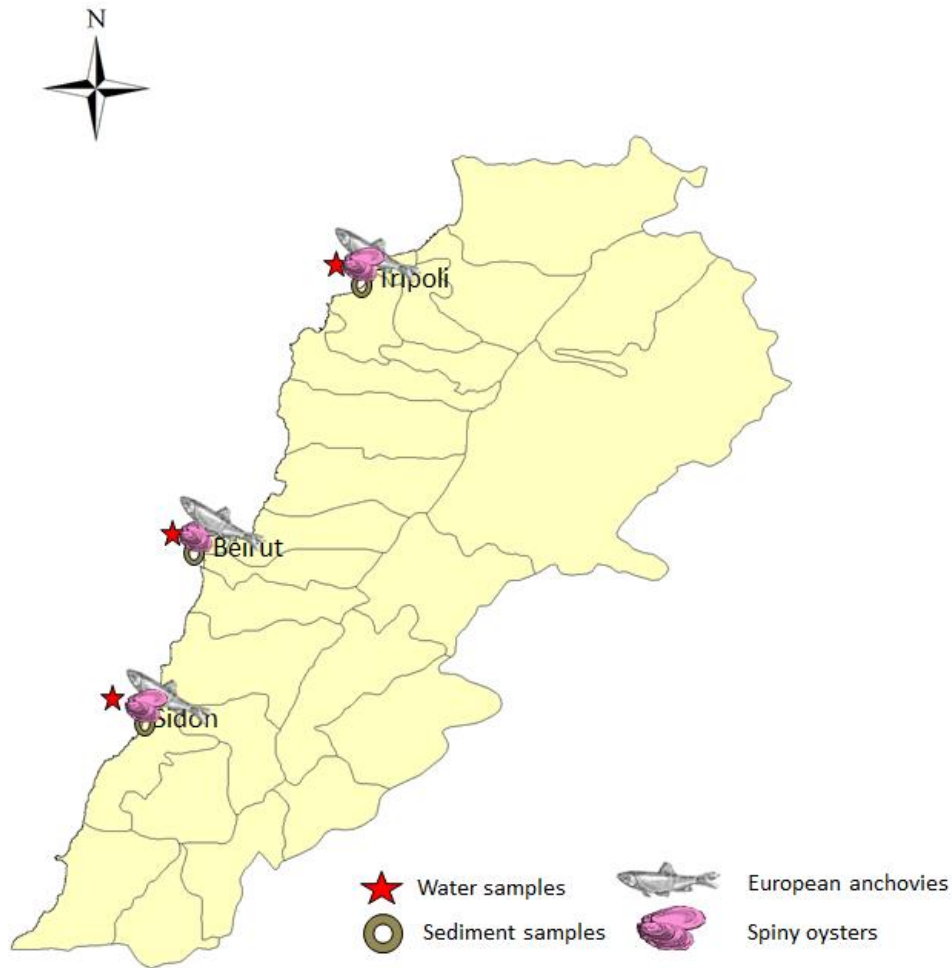


Figure 11: Sampling sites located along the Lebanese coast (Tripoli, Beirut and Sidon).

The first sampling site, Tripoli, is located in the north of the country facing the town's public beach and is characterized by the existence of a coastal landfill. This landfill was constructed in 1980 covering an area of approximately 0.06 km², with an estimated volume of waste of 1.1 million m³ and receiving an average of 400 tons of wastes per day. The landfill that was ought to be closed in 2012, reached today 45 m of height (Figure 12) with no gas nor leachates control (Halwani et al., 2020).



Figure 12: Tripoli's coastal landfill with its protective peripheral wall

Beirut is the country's capital generating 50% of the total MSW. The most important harbor in the country is located in this site along with three coastal landfills: One closed and two actives reaching their maximum capacities of 55 meters of height. These landfills have been active since 1975 even though they were closed and re-opened several times with some additional area (Figure 13).

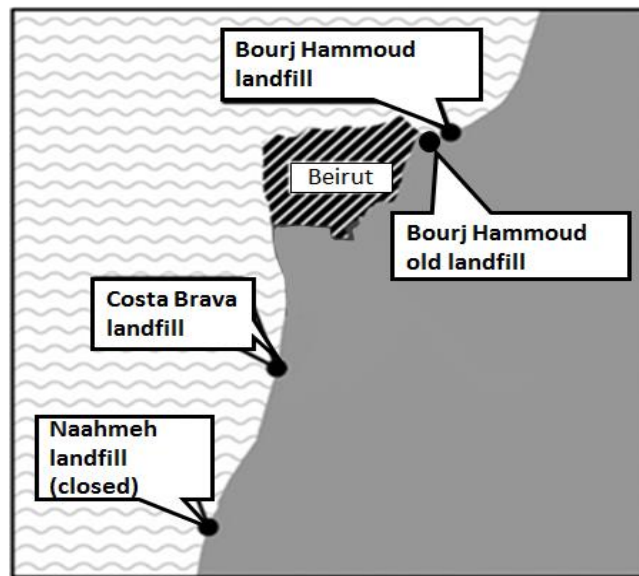


Figure 13: Different landfills surrounding Beirut region

Sidon's site is where the biggest "mountain of garbage" was located in Lebanon. A closed landfill that covered an area of 60 000 m², received an average of 300 tons of wastes/day, reached 58 m of height and an estimated volume of 1 500 000 m³ until its transformation to a public park (Maroun, 2012). This project was interrupted and traces of wastes can still be observed underwater.

None of these landfills have/had any basal lining barrier nor an appropriate leachates treatment, and leachates are directly dumped to the sea.

1.3 WASTEWATER TREATMENT PLANT: EDELWEISS

“Edelweiss” wastewater treatment plant is installed inside Le Havre harbor. This WWTP was commissioned in 2011 receiving effluents from 20 towns (serving capacity of 415 000 population equivalent). It is a tertiary wastewater treatment plant that treats domestic and industrial water in three (during dry time) or five different steps (during wet weather conditions) and discharges a mean volume of 80 000 m³ per day (Figure 14).



Figure 14: Aerial view of Edelweiss WWTP with its various treatment steps (adapted from the report of CODAH, 2013).

Edelweiss WWTP is equipped with three Degremont[®] technologies: Cyclor[™], Densadeg[®] and Thermylis[™]. These techniques have an object of acquiring good water quality, decreasing the ecological footprint, managing all potential hazardous products while respecting the surrounding environment (Figure 15). This establishment is relatively new and is constructed to obtain a good treated water quality. But no treatment or equipment for microplastics removal were taken into

consideration which makes WWTP an important MPs entry source for MPs. As shown in Figure 15, three sampling points were taken into consideration: influent, sludge and effluent. They were sampled on the 4th of April 2018 allowing to give a preliminary assessment of MPs variation inside the WWTP and, eventually, evaluate the amount being discharged.

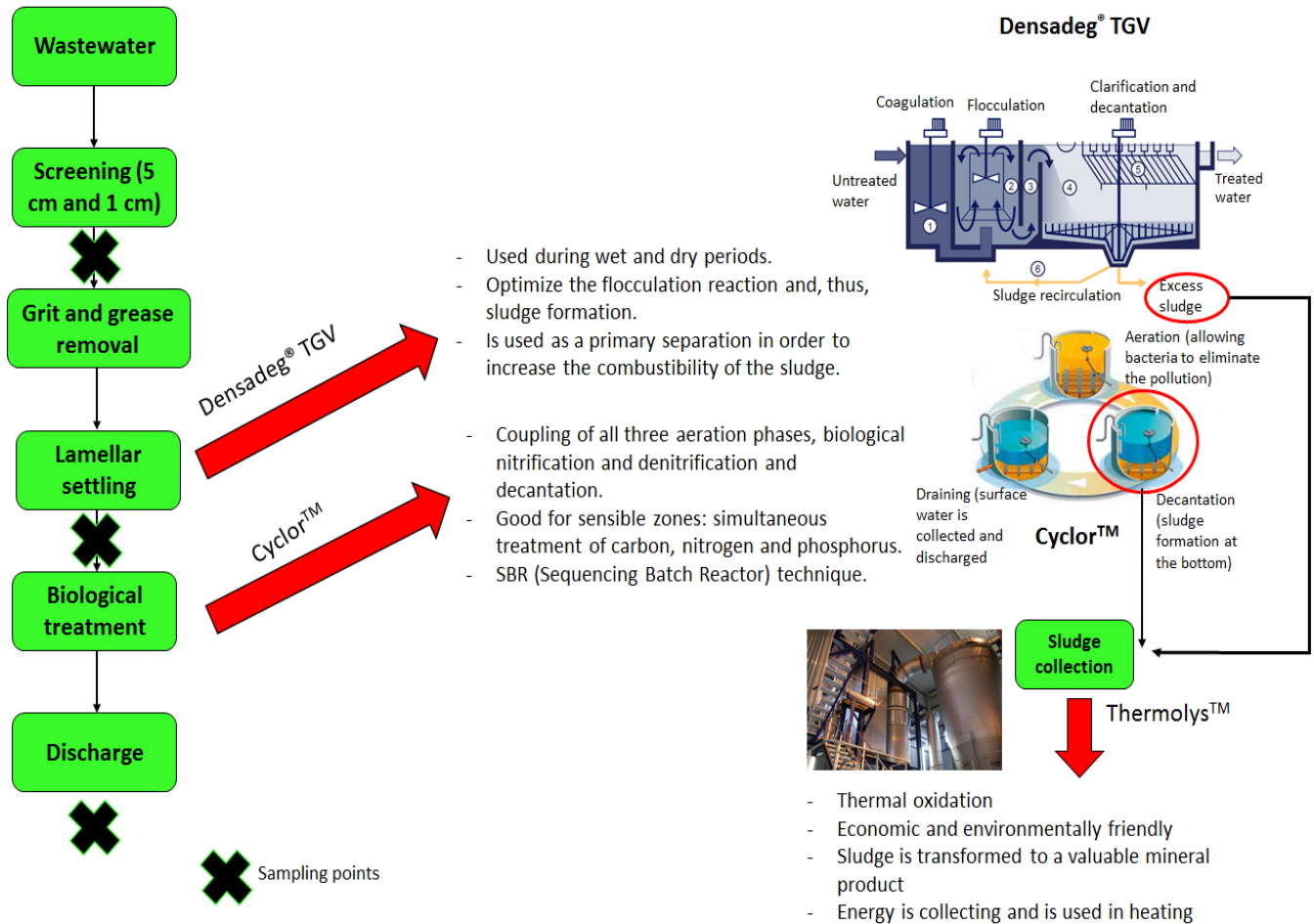


Figure 15: Wastewater treatment steps inside Edelweiss WWTP with the different treatment phases and characteristics the water undergoes. Also, the three sampling points are indicated in the diagram (adapted from Edelweiss, 2016).

2. SAMPLING TECHNIQUES OF ALL THREE MATRICES (WATER, SEDIMENTS AND NATIVE BIOTA)

2.1 WATER SURFACE

A. Le Havre harbor and Sainte-Adresse

As indicated in Figure 7 and Figure 8, water samples were collected in four sites inside Le Havre harbor and one site in Sainte-Adresse. These sites were distributed at a respective distance of 0.05 km, 1.1 km, 1.6 km, 5.3 km and 10.3 km from the Edelweiss WWTP effluent. In both areas, the same sampling technique was used. This technique consisted of a water pump (Bilge pump, SEAFLO[®]) connected to a flowmeter (Turbine Digital Pro K24) in order to follow the exact volume of filtered water (Figure 16). This pump was connected to a pipe and washed with Milli-Q water before sampling. Water filtration was done on five sieves of different mesh sizes: 500 µm, 200 µm, 80 µm and 20 µm. Sampling was conducted during low tide, and the filtered water volume varied from 1 m³ to 2 m³ except for the 20 µm sieve that was clogged after filtering a volume ranging between 20 and 250 L depending on the water turbidity. Then these sieves were covered in aluminum foil and they were rinsed using Milli-Q water inside clean glass bottles for laboratory analysis.

The same methodology was used for the collection of the WWTP effluent. Whereas for the influent, 2 bottles of 500 mL were sampled inside clean glass bottles.

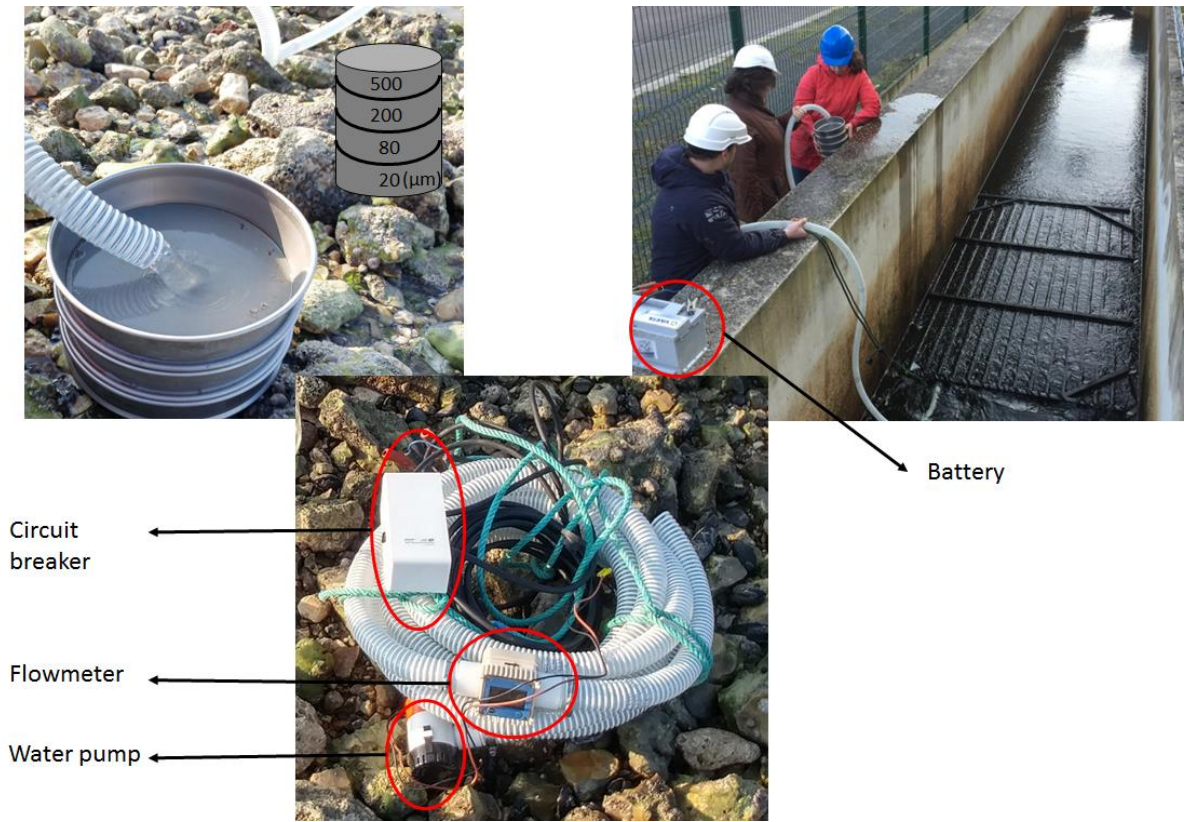


Figure 16: Water filtration method used (water pump, flowmeter) and the sieves of different mesh sizes used.

B. The Lebanese coast

Lebanese sea surface water was collected using a manta trawl net (52 μm mesh size) with an opening of 0.63 m and a length of 0.255 m equipped with a flowmeter (Figure 17). As shown in the previous Figure 11, the sampling was conducted in the three sampling sites: Tripoli, Beirut and Sidon; facing the coastal landfill and public beach of each site.

Sites	Length (m) Width (m)	Distance (m)	Filtered volume (m ³)
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Figure 17: Manta trawl used in collecting the water samples off the Lebanese coast.

The net was trawled for 10 minutes, parallel to the coast at about 500 m from the coast with a small artisanal vessel (3 m of length) at a speed of 2 knots. The filtered volume in each site is indicated in **Table 6**.

After trawling, the net and the collector were rinsed inside clean glass bottles pre-rinsed with filtered distilled water

Table 6: Length and width of the manta net, the distance and the volume of water sampled along the Lebanese coast.

Tripoli	0.63 0.255	680	101.184
Beirut		780	123.318
Sidon		580	91.698

2.2 SEDIMENT SAMPLES

A. Le Havre harbor and Sainte-Adresse

Three sites were sampled inside Le Havre harbor and one site in Sainte-Adresse (Figure 7 and Figure 8). In the former, one site was not sampled due to its rocky substrate. A stainless-steel sediment core sampler pre-rinsed by Milli-Q water was used in collecting 1 kg of sediment samples in each site (Figure 18). Samples were put inside aluminum foil and frozen at -10°C.

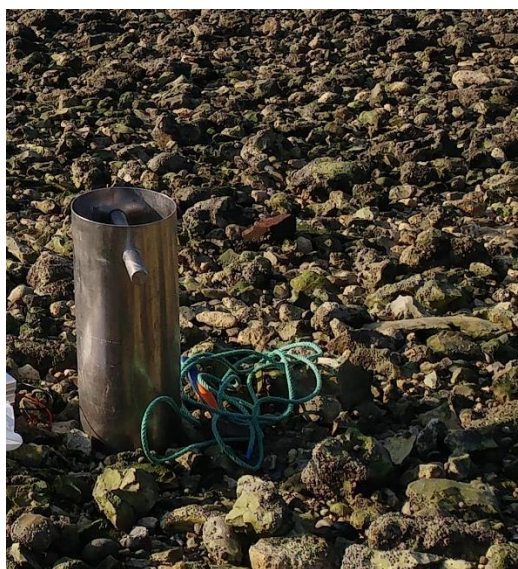


Figure 18: Core sampler used in collecting sediment samples in Le Havre and Sainte-Adresse.

B. The Lebanese coast

The collected sediments were from public beaches in all three sampling areas. The chosen beaches' substrates were made of sand and the sublittoral zone was chosen. A steel ring with two stainless-steel plates, pre-rinsed with filtered distilled water, were used to sample the first two

centimeters (Figure 18). Sediment samples were collected and conserved inside clean glass jars and frozen at -10°C.



Figure 19: Steel ring with two stainless-steel plates used in collecting sediments along the Lebanese coast.

2.3 NATIVE BIOTA

Passive biomonitoring was evaluated using different species depending on the studying area:

- European flounder: demersal fish (Fosse Nord and the Canche)
- Blue mussels spp.: filter-feeding mussels (Le Havre Harbor and Sainte-Adresse)
- European anchovy: filter-feeding pelagic fish (the Lebanese coast)
- Spiny oysters: filter-feeding oysters (the Lebanese coast)

A. European flounder: *Platichthys flesus*

Wild juvenile European flounder (*Platichthys flesus*) were collected in Fosse Nord (the Seine estuary; highly polluted) and The Canche estuary (used as reference) in the first week of September 2017. In European estuarine waters, European flounder is one of the most important components of the juvenile demersal fish assemblage (Selleslagh et al., 2009). Juvenile European flounders feed mainly on crustaceans, polychaetes and small fish, and are mostly distributed in estuaries. Estuaries play a key role in their population renewal and constitute an important nursery ground (Selleslagh et al., 2009). Juveniles individuals have been reported to be highly sensitive with their mortality and condition are affected by the degree of contamination

concerning their surrounding environment (Amara et al., 2009; Kerambrun et al., 2013). They live in contact with the sediments and, thus, are exposed to chemical stress (Minier et al., 2000). Microplastics have been already found in the adult digestive tracts of this species (McGoran et al., 2017). Wild juveniles in this study were caught using a small beam trawl (Figure 20). The collected individuals were put inside aluminum foil and frozen at -20°C.

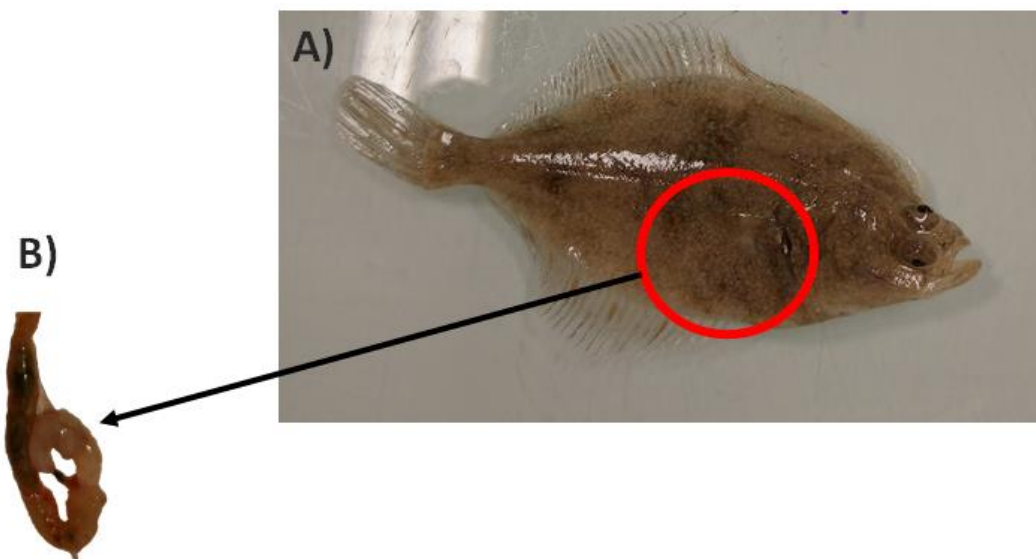


Figure 20: A) Wild juvenile European flounder caught in the Canche estuary and B) its dissected digestive tract that is later digested.

B. Blue mussels: *Mytilus* sp.

More than 70% of the species existing in Le Havre harbor are hybrid blue mussels species (J. Couteau, personal commentary). These native species are filter-feeders, fixed on substrates and are widely distributed in the harbor (Figure 21). They constitute an essential ecological value by provide food and habitat to a variety of species. Due to their large ciliated gills, these organisms feed on phytoplankton by pumping and filtering large volumes of water (Reviewed by Beyer et al., 2017). This behavior allows them to bioaccumulate the contaminants existing in their surrounding environment (*in situ*) (Beyer et al., 2017). These species allow anthropogenic pollutants transfer through the trophic chain (from the abiotic phase and primary production to higher trophic levels) (Farrell and Nelson, 2013; Larsen et al., 2016). Blue mussels are considered as sentinel species in anthropogenic contaminants monitoring (Farrington et al.,

2016) and have been also suggested for microplastics pollution (Bråte et al., 2018). Therefore, in order to evaluate the microplastics content in these native mussels, samples were collected on the 3rd and 4th of April 2018, covered individually with aluminum foil and directly frozen at -4°C (in order to stop mussels from egesting any ingested particles).



Figure 21: Individuals of hybrid mussels collected inside Le Havre harbor. The right photo indicates the wide distribution of these mussels along the shore.

C. European anchovies: *Engraulis encrasicolus*

Anchovies are filter feeders, feeding mainly on planktonic organisms and they have two different feeding behaviors making them opportunistic species: they either filter suspended particles existing in the water column or they visually detect their prey and feed by predation (Bacha and Amara, 2009; Plounevez and Champalbert, 2000). They play an important role in coastal ecosystems since they are among the most important commercial fish species of the Mediterranean and are the main diet for pelagic predators (Fossi et al., 2017). This pelagic-neritic species occurs on the surface water and may reach a depth of 400 m and microplastics were already found in their gut contents in the Mediterranean (Compa et al., 2018; Renzi et al., 2019b; Rios-Fuster et al., 2019). Anchovies are wholly eaten by the Lebanese population (with

no degutting) as *bizree* and are distributed all along the coast (Figure 22 - A). In order to evaluate the occurrence of microplastics in European anchovies caught off the Lebanese coast, ten individuals were collected using purse seine in each sampling site (Figure 11) and frozen at -20°C.

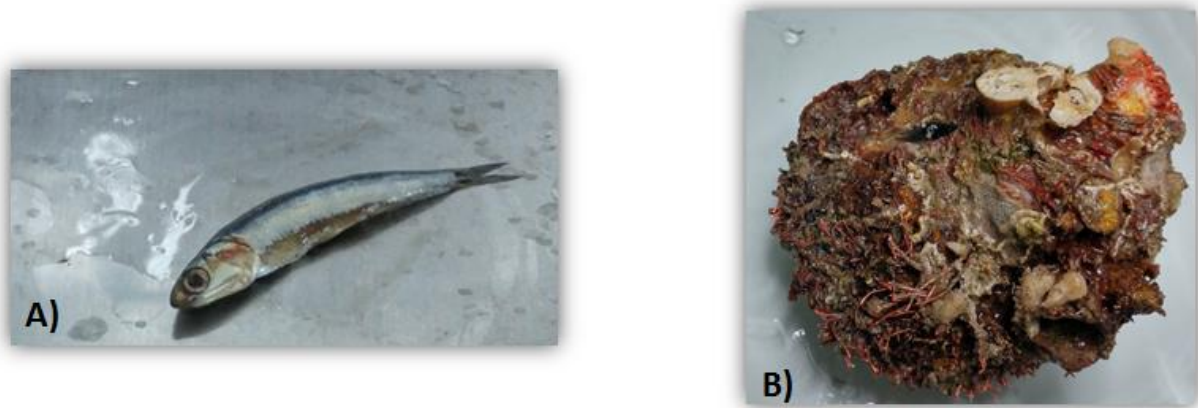


Figure 22: A) European anchovies and B) *Spondylus spinosus* caught in the Lebanese coastal waters.

D. Spiny oysters: *Spondylus spinosus*

The spiny oyster is a filter-feeder benthic bivalve species found firmly attached to subtidal rocks (Figure 22 – B). This Indo-West Pacific native species invaded the Eastern Mediterranean over a decade ago and progressed northward toward the Eastern Mediterranean. (Mienis et al., 1993; Moazzo, 1939; Zenetos et al., 2009). It is observed in many areas at a depth range of 1 - 40 m and can form a density of dozen individuals per 1 m² (Çeviker and Albayrak, 2006; Zenetos et al., 2009). This invasive species replaced the common habitat-forming species (Crocetta et al., 2013) and have been widely found along the Lebanese coast. Spiny oysters are wholly eaten by the Lebanese population which is important to evaluate the number of potential microplastics ingested by this species. Individuals were collected by fisherman along the coast (see Figure 11) and frozen directly. This is the first work that assessed microplastics ingestion by *Spondylus spinosus*.

3. CAGING EXPERIMENT

The Active biomonitoring technique used consisted of caging European flounder and blue mussels in the Seine estuary and Le Havre harbor, respectively. This technique was accompanied by collection of wild individuals in most of the sampling sites allowing a comparability with native individuals. Different caging systems were used in each experiment that would be detailed below.

3.1 JUVENILE FLOUNDER CAGING EXPERIMENT

A. Preparation of European flounder before caging

Juvenile flounders (150 individuals; total length: 7 to 9 cm) were collected from the Canche estuary using a small beam trawl. Then, they were transferred to Mareis Aquarium, Etaples, Pas-de-Calais, France where they were acclimatized for 7 days in a 500 L aquarium. They were supplied with the open seawater circuit of Mareis and were daily fed on frozen Mysidacea and brine shrimps (*Artemia* sp.).

One day before the experiment, flounders were anesthetized using Eugenol 35 mg/L (dissolved in seawater). They were individually weighted (g), measured (cm) and marked using Alpha visual implant tags (1.2 mm x 2.7 mm, Northwest Marine Technology; Figure 23).



Figure 23: Procedure of implanting the Alpha tags on European flounders individuals prior to their caging deployment.

B. Caging deployment

Stainless-steel cages were fabricated without any plastic material in order to prevent plastics contamination. Their length was of 1 m, whereas their width and height were of 0.6 m. Their mesh size was 15 mm allowing water circulation and enough space for fish to feed. The cages

were fixed to the bottom with a screw anchor secured by scuba divers at depths varying between 4 to 8 m (Figure 24).

Five sites were selected: three in the Seine estuary (Rouen, Fosse Nord and Le Havre harbor), one site in the Canche estuary and one in the Liane estuary (see Figure 7 and Figure 9). Cage deployment was conducted on the 12th and the 13th of September 2017. Inside all cages, 15 to 20 individuals were put inside except for the Canche, where 27 specimens were caged. The caging experiment lasted for one month before retrieval. Survival rate was $> 70\%$ in all sites except in the Canche because the cage was partially silted obtaining, therefore, the lowest survival percentage of 37%. Each individual was identified by his Alpha tag and directly frozen.

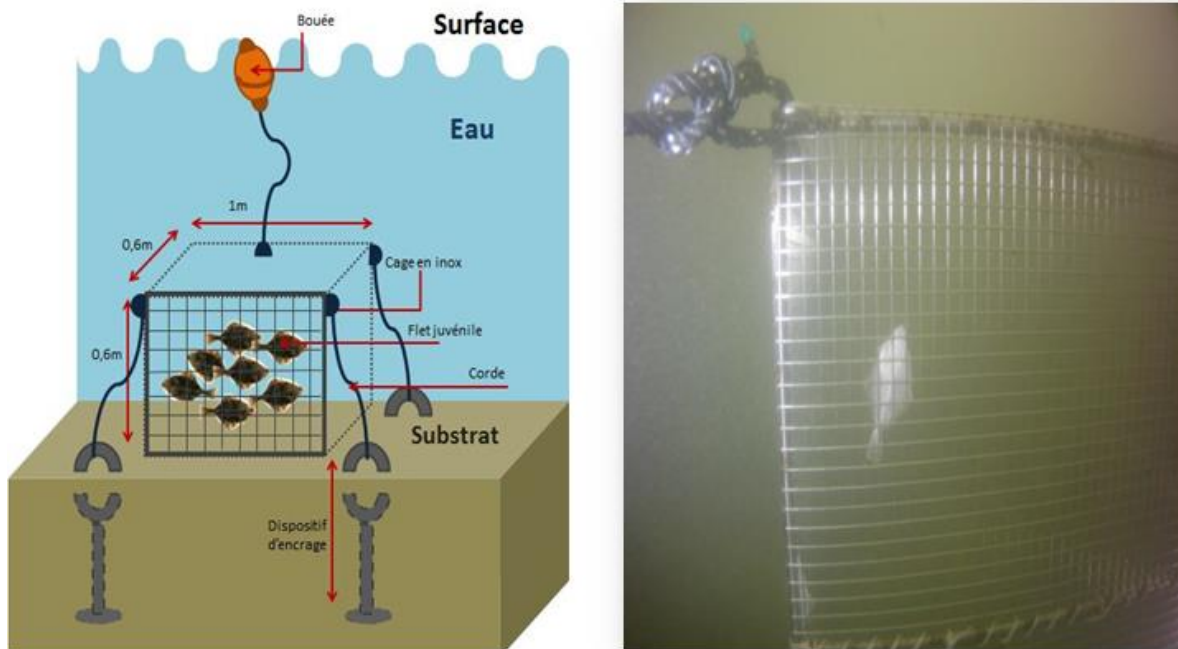


Figure 24: Caging model and real photos of cages constructed for *Platichthys flesus* deployed in three different estuaries along the Eastern English Channel

3.2 FARMED BLUE MUSSELS CAGING EXPERIMENTS

Two active biomonitoring experiments were conducted using farmed blue mussels (Bouchot mussels) collected from the same farm (Hardelot plage, Boulogne-sur-mer, France): the first

caging experiment was conducted inside Le Havre harbor (4 sites) and Sainte-Adresse (1 site) that lasted 42 days. The second caging experiment was conducted only at Sainte-Adresse with weekly follow up (1, 2 and 5 weeks). Table 7 indicates the difference in caging factors used in each campaign.

Table 7: Differences between the two conducted caging experiments using *Mytilus edulis*.

	Caging location	Caging duration	Aquarium tank volume	Room T° (°C)	Filtered seawater	Number of mussels	Fed daily	Follow-up
February-April 2018	Le Havre harbor and Sainte-Adresse	42 days (21 February till 4 th of April 2018)	160 L	8 ± 1	0.1 µm; changed every 24 hours for 7 days	150	Yes (except for Saturday and Sunday of the depuration week)	No
July-August 2018	Sainte-Adresse	1, 2 and 5 weeks	160 L	17 ± 1	0.1 µm changed in the first 16 hours, 24 hours then every 24 hours for 6 days	220	Yes (except for Saturday and Sunday of the depuration week)	Yes, in the first 16, 24, 48 and 72 hours

A. Blue mussels collection and depuration

The depuration process consisted of putting the individuals inside a 160 L closed aquarium pre-rinsed with Milli-Q water (**Figure 25**). The aquarium was placed inside a thermoregulated room and was supplied by seawater filtered on Polycarbonate filters with a pore size of 0.1 µm. Every 24 hours and for 7 days, the water inside the aquarium was drained then the aquarium was rinsed with Milli-Q water and supplied with newly filtered seawater. During depuration, the mussels' were put in a 12 h light-dark illumination regime and their filtration capacity was enhanced by daily feeding with algal cultures (a concentration not exceeding 10⁷ of *Rhodomonas* sp. and *Isochrysis* sp. in order to have a high clearance rate (Widdows et al., 1979)). Thus, mussels would be able to egest all ingested microlitter. To assure that the depuration experiment was valid, samples of non-depurated and 7 days depurated mussels for each experiment were put in aluminum foils and frozen at -20°C. For the second experiment (in July and August), a keener

follow-up was realized: individuals were taken at different time intervals (16, 24, 48 and 72 hours) that reflected the ability of mussels to filter clean water and egest any potential microplastics. The temperature in the thermoregulated room changed from the first and the second experiment due to the meteorological conditions during the caging. Mussels were depurated and acclimated in temperatures that were more or less similar to those found in the natural environment: February and April water temperature ranged between 8 and 10°C; whereas for July and August, the water temperature varied between 17 and 20°C.

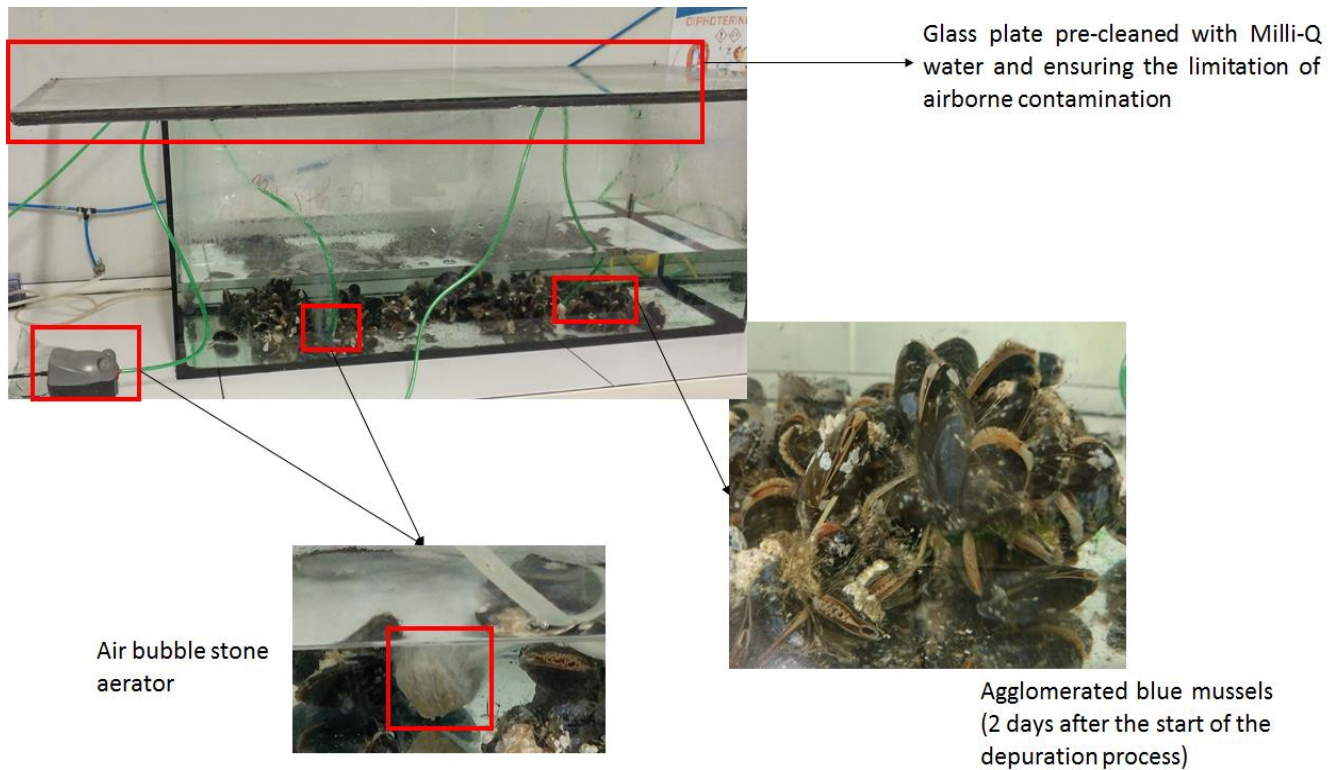


Figure 25: Farmed mussels depurated inside 160 L aquarium equipped with several airstones to ensure constant oxygenation of the individuals.

B. Cages deployment

Stainless-steel cages (50 cm x 50 cm x 50 cm, length x width x height) with no plastic materials to avoid contamination was deployed (Figure 26). The cage's mesh size was 15 mm allowing water circulation and stopping mussels from falling out of the cages. A stainless-steel grid was placed in the mid-height of the cages as a suitable substrate allowing mussels' attachment and avoiding direct contact with sediments. The cages were deployed during low tide in the lower intertidal shore zone and attached to the bottom using four reinforced iron rods.

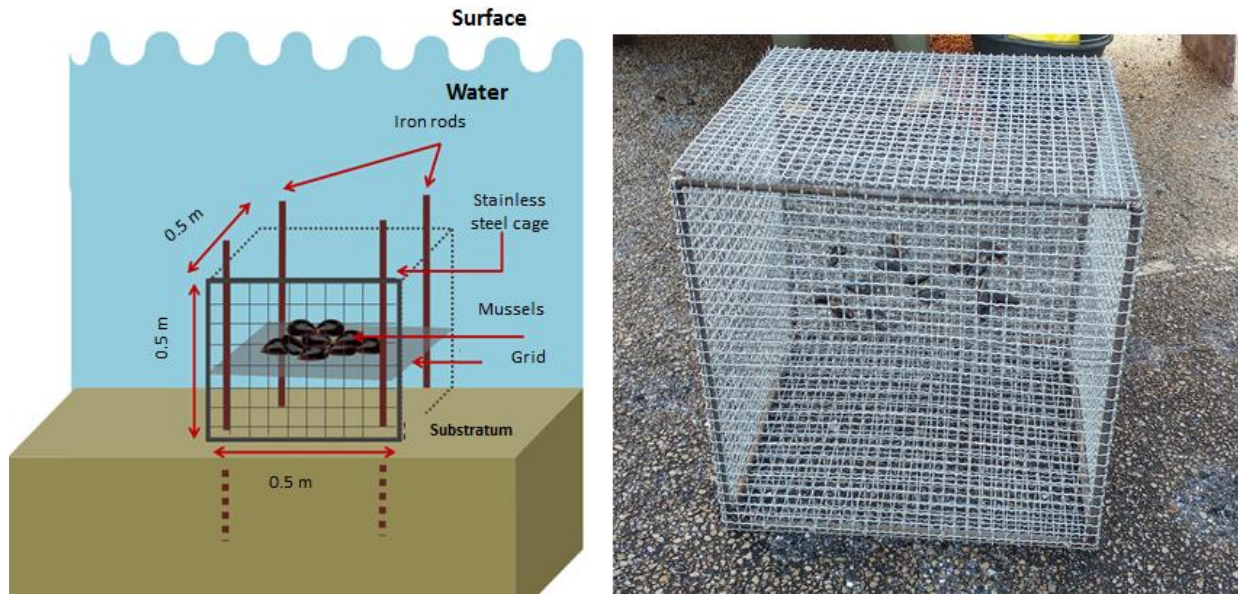


Figure 26: Caging model and real photos of cages constructed for *Mytilus edulis* deployed in Le Havre harbor and Sainte-Adresse.

The first caging experiment, 17 to 23 individuals were put inside each cage. Cages were deployed in four sites inside the harbor and one site in Sainte-Adresse with respective distances from the WWTP influent (see Figure 7 and Figure 8). After 42 days, they had an acceptable survival rates (73% > 70%) even though water temperature were low (winter season). Whereas for the second experiment, 50 individuals were put inside each cage (3 cages) and transplanted in Sainte-Adresse. Each cage had a really high survival rates exceeding 90%.

3.2 CAGING MODEL COMPARAISON

Table 8: Summary of different characteristics for caging models used for fish and mussels.

	Length (cm)	Width (cm)	Mesh size (cm)	Grid	Fixation	Depth	Animal tagging
<i>Platichthys flesus</i>	100	60	1.5	No	Screw anchors (1.2 m)	4 to 8 m	Yes (Alpha tags, Northwest Marine Technology)
<i>Mytilus edulis</i>	50	50	1.5	Yes	Iron rods (1 m)	infralittoral /intertidal	No

4. LABORATORY ANALYSES AND SAMPLES PREPARATION

4.1 CONTAMINATION PREVENTION

This step was conducted thoroughly throughout the whole thesis. It is considered an essential step in any microplastics study. Most of the materials used were made of glass (Petri dishes, Erlenmeyers, Filtration system, bottles...) or from stainless-steel with no plastics used during manipulation. All these equipment were rinsed with filtered ethanol 70% and Milli-Q water or filtered distilled water. During all analyses, several solutions were used: ethanol 70%, potassium hydroxide KOH 10%, zinc chloride $ZnCl_2$, hydrogen peroxide H_2O_2 and distilled water. These solutions were filtered more than 3 times using Buchner filtering system (constituted of a glass flask and funnel) under a laminar flow hood using glass fiber filters GF/A with a porosity of 1.6 μm : The Buchner system was carefully cleaned clean by Milli-Q water and 70% ethanol solution was prepared and filtered. Then, all materials were rinsed with this ethanol 70% followed by Milli-Q water.

Following each analysis step (whether biological tissue digestion, organic matter oxidation, solutions filtering, dissection...), a blank was always put in place. It consisted of a clean Erlenmeyer (filtered ethanol 70% and Milli-Q water) with a respective volume of a solution that is being used for the samples. For example, for mussels digestion, clean Erlenmeyer containing a filtered solution KOH 10% would be put open inside the laminar flow hood during individuals dissection, addition of their respective KOH 10% volume and digestion. This same control would undergo the same digestion conditions (60°C for 24 hours) then it would be re-opened during the filtration of digested mussels solutions and would be eventually filtered. This tracking would be able to give an idea of a potential airborne contamination. This same procedure was done for density separation (using $ZnCl_2$) and organic matter digestion (using H_2O_2). Each batch (constituted of a specific number of Erlenmeyers) has its appropriate control filter.

All observed items of a specific control filter and were also observed in the same batch filters, are directly eliminated as potential microplastics. i.e. The same yellow fiber was observed in one sample filter and in its control, that fiber would be not be taken into consideration and subtracted from the visual counting.

4.2 WATER SAMPLES

A. Le Havre harbor and Sainte-Adresse

All collected water samples (including WWTP influent) underwent density separation (DS) using a filtered solution of zinc chloride (ZnCl_2) with a density of 1.8 g/cm^3 . This solution was used to its high efficiency in retrieving microplastics (Rodrigues et al., 2018) with a retrieval percentage $> 80\%$. In order to determine the exact volume of needed ZnCl_2 for our samples, different volumes were tested (50 mL, 100 mL and 150 mL). These volumes were tested on solutions that were similar to our samples: 600 mL of water with 5 g of sediments. Then, seventeen polymers of different densities (PET, PE, PP, PVC and PS) were added to the solution and they were left 24 hours (shaken twice in the first eight hours, then they were left to rest for 16 hours). Two separate phases were formed with the supernatant consisting of particles with a density $< 1.8 \text{ g/cm}^3$ (Figure 27).

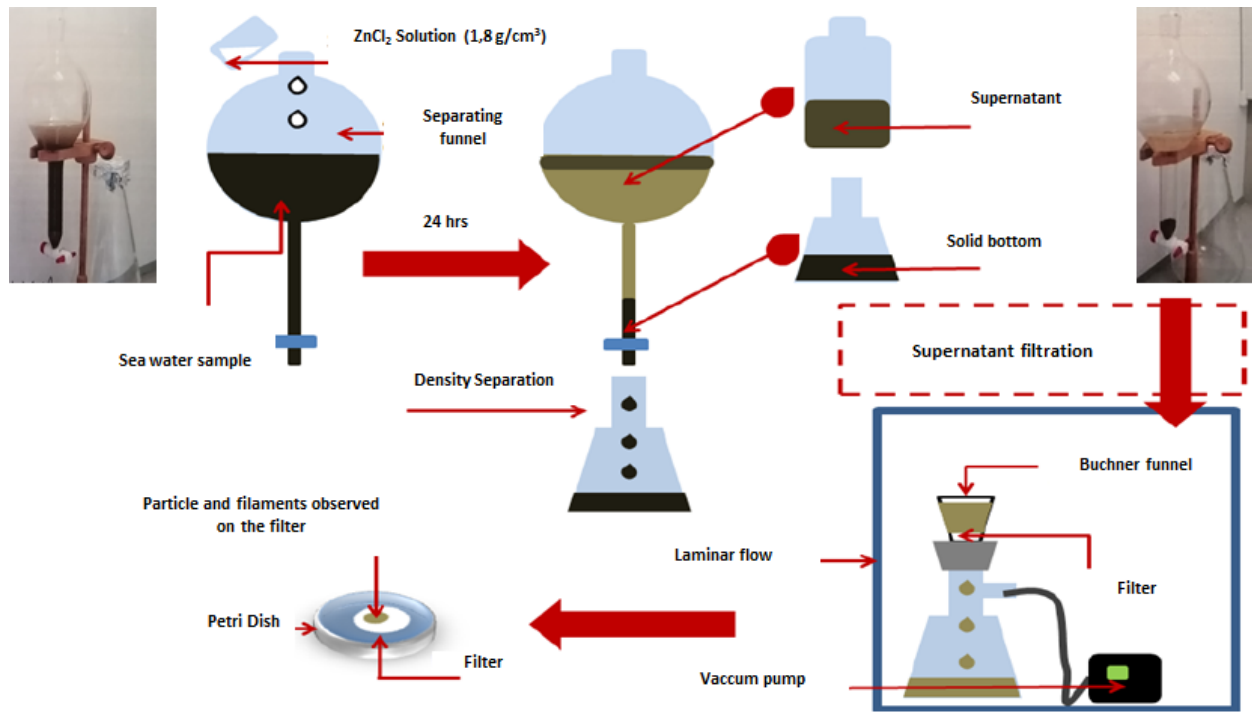


Figure 27: Density separation protocol adapted for the collected water samples

The density separation efficacy was calculated following the formula (Phuong et al., 2018):

$$\%Efficacy = \left(\frac{\text{Number of MPs after DS}}{\text{Number of MPs before DS}} \right) \times 100$$

The percentage obtained was $82.7 \pm 0.5\%$ (above 80 %) and the volume of 50 mL was chosen. Therefore, all water samples were put inside clean separating funnel and a volume of 50 mL of $ZnCl_2$ was added and the density separation was done as described above. After 24 hours, the supernatant is filtered on GF/A filters (90 mm) and were put inside clean glass Petri dishes. Each site had up to 4 filters depending on the sample.

B. The Lebanese coast

Due to difficulty in acquiring materials and solutions in Lebanon, no density separation was done on the water samples. The collected water samples were filtered on a stainless-steel sieve of a mesh size of 850 μm and the recovered water was vacuum filtered on GF/A filters with a diameter of 47 mm. The suspected MPs present on the 850 μm sieve were sorted visually under the stereomicroscope, fixed on a microscopic slide and conserved inside a clean glass Petri dish. Whereas the obtained filtered were put inside clean glass Petri dish and prepared for visual observation and Raman analysis.

4.3 SEDIMENT SAMPLES

A. Le Havre harbor and Sainte-Adresse

Before analysis, wet sediments were thawed at room temperature for 24 hours. For each site, 3 subsamples of 100 g each were taken and dried at $60^\circ C$ for 24 hours. From each subsample, 10 g of dried sediments was weighed (Liebezeit and Dubaish, 2012; Mathalon and Hill, 2014) and placed inside a clean Erlenmeyer. Organic elimination protocol was adapted from other published studies (Figure 28). A volume of 40 mL of hydrogen peroxide 30% (H_2O_2) was added (Nuelle et al., 2014) and the Erlenmeyers were placed on heating stirrer plate for 24 hours at $40^\circ C$ with a rotation speed of 100 rpm. After removing organic debris, the solution was transferred to a separating funnel and 40 mL of $ZnCl_2$ was added (Imhof et al., 2013; Liebezeit and Dubaish, 2012). The mix was agitated for 5 min then let rest for 24 hours. The supernatant was filtered on GF/A filters (90 mm) and put inside clean glass Petri dishes. No more than two filters were obtained for each site.

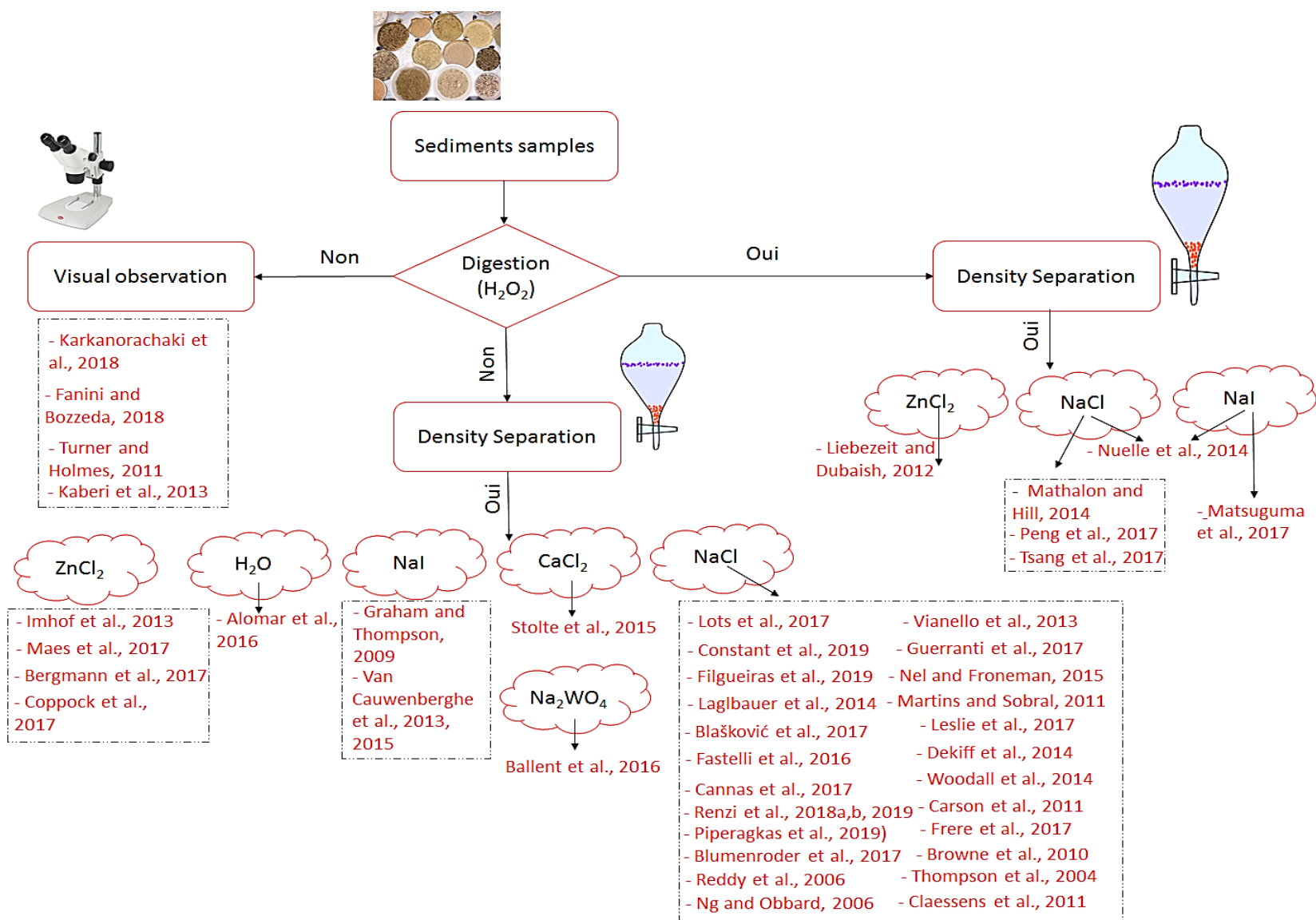


Figure 28: Summary of studies that used different solutions to extract microplastics from sediments. (H_2O_2 : hydrogen peroxide, $ZnCl_2$: zinc chloride, H_2O : water, NaI : sodium iodide, $CaCl_2$: Calcium chloride, Na_2WO_4 : sodium tungstate).

B. The Lebanese coast

Then, 100 g of sediments were analyzed and, firstly, particles ranging from 1 to 5 mm were visually sorted (Figure 29). These sediments constituted of sand were not dried and directly underwent density separation protocol using ZnCl_2 (1.8 g/cm^3) (Imhof et al., 2013; Nuelle et al., 2014). A volume of 50 mL was added to the sample and shaken for 5 minutes (once every four hours) then let rest for 16 hours. The supernatant was recovered after 24 hours and filtered on GF/A filters (90 mm).



Figure 29: Sieved sediment on a 2 mm mesh size and visually sorted on a microscopic slide for Raman analysis.

4.4 BIOTA SAMPLES

All collected biota samples underwent the same digestion protocol using KOH 10%. This solution is considered to be the best compromise between different digestions methods used with no observed alteration in any polymers except for cellulose acetate and biodegradable polymers (Dehaut et al., 2016; Karami et al., 2017; Kühn et al., 2017). Fish species were dissected and their digestive tracts underwent KOH 10% digestion whereas the whole soft tissue of mussels and oysters was digested. Figure 30 shows the general protocol used during biota digestion: Erlenmeyer put at 60°C for 24 hours with an agitating speed of 300 rpm.

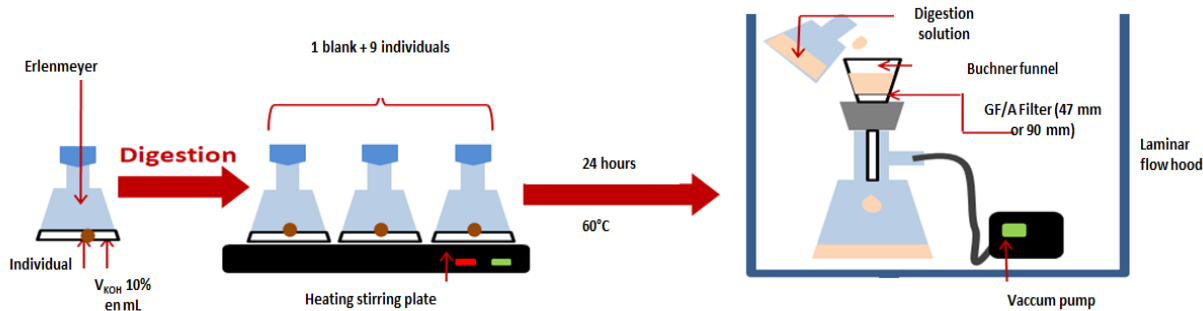


Figure 30: Digestion protocol used for collected marine organisms.

European flounders and anchovies had a digestive tract with a weight < 1 g and 150 mL of KOH 10% was used for each individual. Erlenmeyers were put on a heat stirring magnetic plate at 60°C for 24 hours. After digestion, each each solution was filtered on GF/A filters with a 47 mm diameter (Figure 31) and put inside clean glass Petri dishes for visual observation and Raman identification. Each individual lead to one GF/A filter (47 mm)

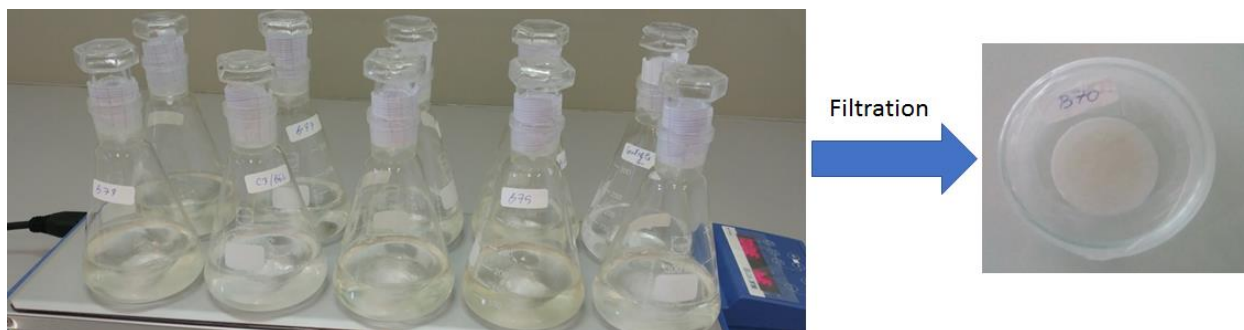


Figure 31: Ten Erlenmeyers containing digestive tracts and KOH 10% solution after 24 hours of digestion (including one blank with filtered KOH 10% only) and the obtained filter after filtration.

Due to the limited number of spiny oysters collected, digestion efficiency was impossible to calculate in order to find out the exact volume of KOH 10% necessary for digestion. A volume of 400 mL was used for each individual and the obtained solution was filtered on GF/A filters of a 90 mm diameter (no more than one filter was obtained for each individual).

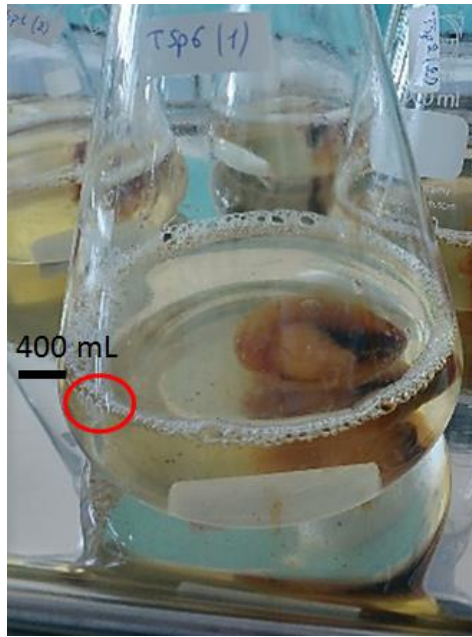


Figure 32: Spiny oysters dissected and put inside clean Erlenmeyer filled with a volume of 400 mL of KOH 10%.

Whereas for the caged and native blue mussels, more than enough individuals were collected and the digestion efficiency was calculated for each species. Hybrid native blue mussels (*Mytilus* sp.) were bigger and had a net weight higher than farmed blue mussels (*Mytilus edulis*) (Figure 33). First tests done on these mussels, led to an uncertainty in the volume of KOH 10% necessary for their soft tissue digestion. So, the digestion efficiency was calculated in order to have the proper volume of KOH 10% needed for each species to have the optimal digestion percentage.



Figure 33: Hybrid blue mussels (*Mytilus* sp.) on the left and blue mussels (*Mytilus edulis*) on the right.

The digestion efficiency (%) was calculated as follows (Dehaut et al., 2016):

$$\%DE = 100 - \left[\left[(DFW - CFW) / TW \right] \times 100 \right]$$

DE: Digestion efficiency

DW: Dry filter weight after digestion (g)

CFW: Clean filter weight (g)

TW: Mussel total weight (g)

Nine individuals of farmed blue mussels, *Mytilus edulis*, (symbol: F1 to F9) and nine individuals of hybrid native mussels, *Mytilus* sp., (symbol: W1 to W9) were used to calculate the digestion efficiency. For each tested volume, three individuals were taken into consideration and their digestion efficiency was calculated. The volume with the highest digestion efficiency was used for the remaining samples collected in each study sites. Individuals total weight and drained net weight of soft tissue was measured to the nearest g. Clean filters weight was calculated: Weight of clean Petri dish with clean filter — Weight of clean Petri dish without filter. After digestion filters were dried at 20°C for 4 hours and their weight was measured as follows: Weight of Petri dish with after digestion dried filter — Weight of clean Petri dish without filter. Digestion efficiency are, therefore, calculated following the equation written above and all obtained results are shown in Table 9.

Table 9: Calculated digestion efficiency (%) for farmed mussels (F1 to F9) and native hybrid mussels (W1 to W9).

KOH 10% volume (mL)	Individual	Total weight (g)	Net Weight (g)	Clean filter weight (g)	After digestion filter weight (g)	Digestion efficiency (%)
50	F1	10,2	4	0,2	0,4	95
50	F2	7,6	3,5	0,4	0,4	100
50	F3	10,5	4	0,3	0,4	97,5
100	F4	7,1	2,3	0,3	0,4	95,65
100	F5	11,3	4,6	0,4	0,4	100
100	F6	11,3	5,1	0,3	0,3	100
150	F7	10,1	3	0,3	0,3	100
150	F8	9,1	3,4	0,4	0,4	100
150	F9	8,7	3,2	0,3	0,4	96,88

150	W1	13,8	5,9	0,3	0,4	98,31
150	W2	53,4	24,6	0,3	0,4	99,59
150	W3	43,3	17,1	0,3	0,4	99,42
200	W4	56,2	31,5	0,3	0,5	99,37
200	W5	63,9	16,5	0,3	0,4	99,39
200	W6	42,5	22,7	0,3	0,4	99,56
250	W7	63,8	19	0,3	0,4	99,47
250	W8	38,8	20,5	0,3	0,3	100
250	W9	37,4	25,3	0,3	0,3	100

A volume of 150 mL and 250 mL was chosen for farmed blue mussels and native hybrid mussels, respectively. Individuals were thawed four hours prior to digestion, then they were dissected under a laminar flow hood, digested using KOH 10% for 24 hours at 60°C and filtered on GF/A filters of 90 mm diameter put inside clean glass Petri dish. No more than one filter was obtained for each individual.

5. MICROPLASTICS ANALYSES

After sample collection and preparation, filters and obtained items were ready to be analyzed for microplastics presence. This analysis was divided into two steps:

- Visual observation: suspected items were counted and noted.
- Raman microscopy: an essential identification step used to confirm that the suspected items are considered as polymers.

5.1 VISUAL OBSERVATION

After filtration, the filters existing inside the glass Petri dishes were observed under 120x magnification using a Leica M165C stereomicroscope and photos of suspected MPs were taken using a Leica M170 HD camera via Leica Application Suite (LAS) software (Figure 34).

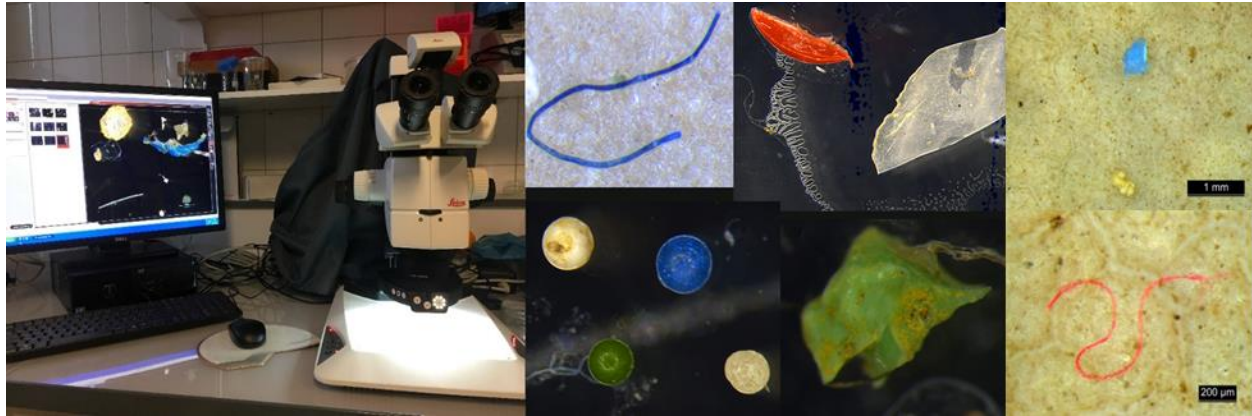


Figure 34: Samples observed using Leica stereomicroscope with several examples of observed microplastics (microbeads, fibers and fragments)

The filters were methodically examined to allow the detection of all items present: from the highest edge to the lowest along the first column, lowest to highest edge along the second column and so on. The observed items were counted and characterized by shape (films, fragments, microbeads or fibers) and color (dark, red, blue, green, white, transparent). Several criteria were followed in order to minimize any confusion with organic matter: (1) no cellular or organic structure, (2) homogenous thickness along the particle, and (3) homogenous colors (Hidalgo-Ruz et al., 2012). Whereas for visually identifying synthetic fibers, these following criteria were taken into consideration (Song et al., 2015): consistent in thickness with no taper toward the ends and are clear and homogeneously colored (red, blue, green and black).

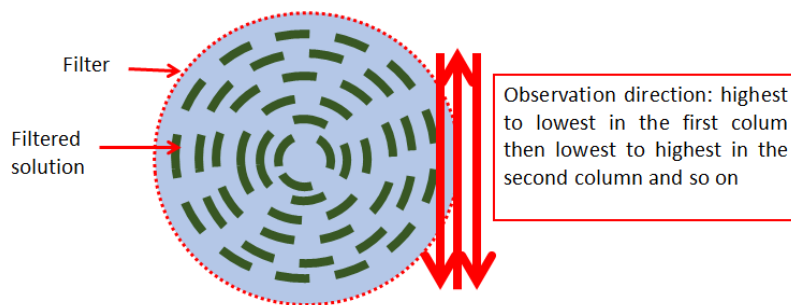


Figure 35: Observation direction used to observe filters under the stereomicroscope.

All photos were processed by “Piximètre” software version 5.9 and suspected items were measured at their longest dimension. Suspected items were divided into several size classes depending on the objective of each conducted study in this thesis.

5.2 MICRO-RAMAN SPECTROSCOPY ANALYSIS AND POLYMER IDENTIFICATION

Visual observation alone is not enough in any microplastics study and, most often, leads to an overestimating in the number of microplastics (Dekiff et al., 2014; Lenz et al., 2015). Raman spectroscopy use a monochromatic light i.e. laser as a source. The emitted radiation interacts with the sample: an energy shift is emitted by the scattered photons which give information concerning molecule vibrations inside the sample (Käppler et al., 2015). In simpler words, the laser beam passes through the particle and puts it in an excited state (high energy virtual state). The molecule relaxes to a vibrational energy level (different that that of its beginning state) producing a photon of different energy. The difference between these two energies lead to the Raman shift. Raman spectroscopy highly depends on a molecular polarity: polar chemical bonds aromatic bonds C-H and double bonds C=C are easily excited.

In this thesis, micro-Raman spectroscopy was used in microplastics identification: Micro-Raman Xplora Plus (HORIBA Scientific®, France). The acquired machine is equipped with an automated plate protected by a closing door (in order to minimize airborne contamination during identification) (Figure 36).

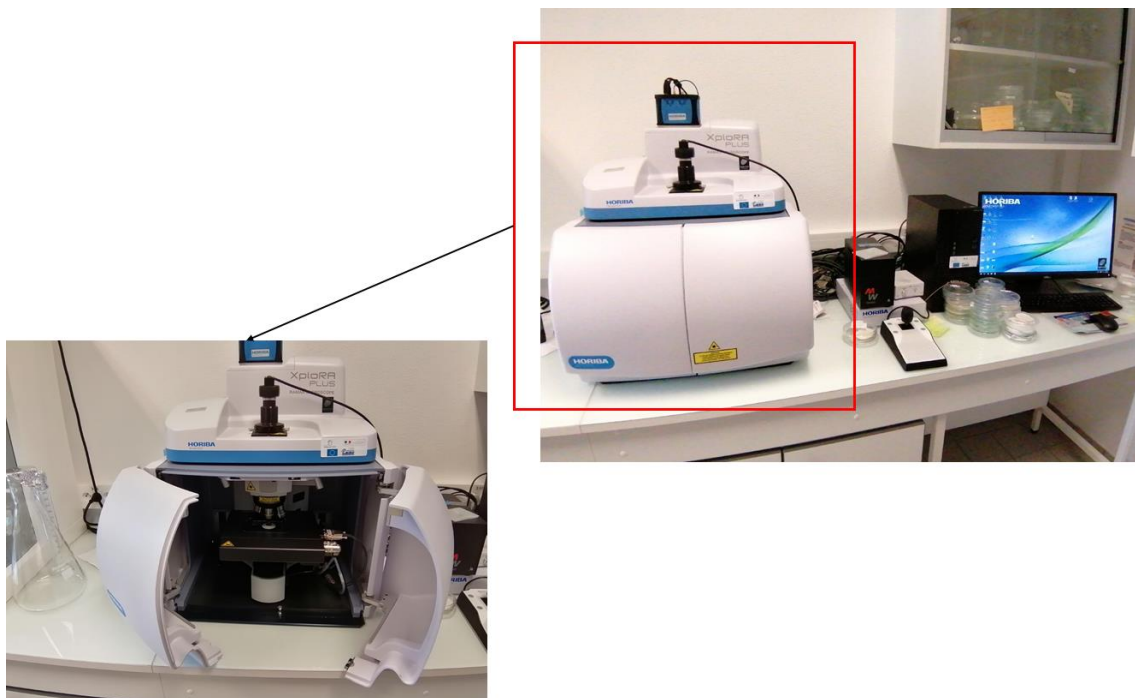


Figure 36: *Micro-Raman Xplora Plus (Horiba Scientific, France) used for microplastics identifying throughout this thesis. The open door (on the left) is closed (on the right) during samples analyses (limiting airborne contamination to filters).*

During identification, two lasers were used with wavelengths of 532 nm and 785 nm with a range of 200-3400 cm^{-1} . Samples were analyzed using two objectives (Olympus): x10 and x100. Due to the high time consumptions, half of the filters were analyzed during each study; whereas for the items, the highest possible amount was identified. The identification was done either manually or using the automated ParticleFinder module for LabSpec 6. ParticleFinder is an automated application that locates particles and performs automated Raman analysis by moving each particle beneath the laser spot. This application is easier to analyze small flat filters (47 mm) rather than bigger filter (90 mm), and remain highly time consuming. Using Raman microscopy has different advantages and limitation (Table 10). One of the most occurred problem in this thesis is that the obtained spectra would be sometimes affected by the underlying substrate and the obtained peaks are considered noisy (fluorescent). In some cases of highly fluorescent spectra, some of the obtained peaks might represent compounds which requires in changing in the acquisition parameters. Whether it is the acquisition time, number of accumulations, the laser, the grating and the laser wavelength, all these parameters often changed from one item to another making identifying high time consuming. Reduced acquisition time and

low number of accumulation lead to poor spectrum quality (which in some cases is hard to identify).

Table 10: Advantages and disadvantages of using micro-Raman spectroscopy during microplastics analysis (adapted from Käppler et al. 2016)

Advantages	Disadvantages
Down to 1 μm particles can be identified	Polyesters are difficult to be identified correctly
C=C compounds, aliphatic and aromatic compounds are detectable	Spectrum is related to sample purity, measurement parameters, dye pigments and is partially fluorescent
Long measurement time but can be reduced	Reduced measurement time lead to loss of spectrum quality
Is not destructive	Topographic imaging is difficult on small transparent particles

Spectra underwent baseline correction in order to have a cleaner spectrum which can more easily be identified (Figure 37). Each obtained spectrum was compared to a polymer database identification software (KnowItAll, BioRad[®]) and a personal library done with standard polymers: HDPE and LDPE (both considered as PE), PP, ABS, PS, ePS, PVC, PET, PUR, PTFE, and PA 6 and PA 12 (both considered as PA), obtained from Goodfellow (France). KnowItAll software gives a resemblance (correlation) between the obtained spectra and the one existing in its database. The identification is considered correct when the HQI (Hit Quality Index) was above 70 (ranging from 0 to 100).

Another problem faced while using this identification method is the dye pigments spectra. In some of the colored particles, the observed spectrum is that of the dye (Copper phthalocyanine, Cobalt phthalocyanine, Hostasol Green, Astra Blue....) instead that of the polymer. On the obtained polymer, some of the polymer characteristic peaks can be observed (Figure 38).

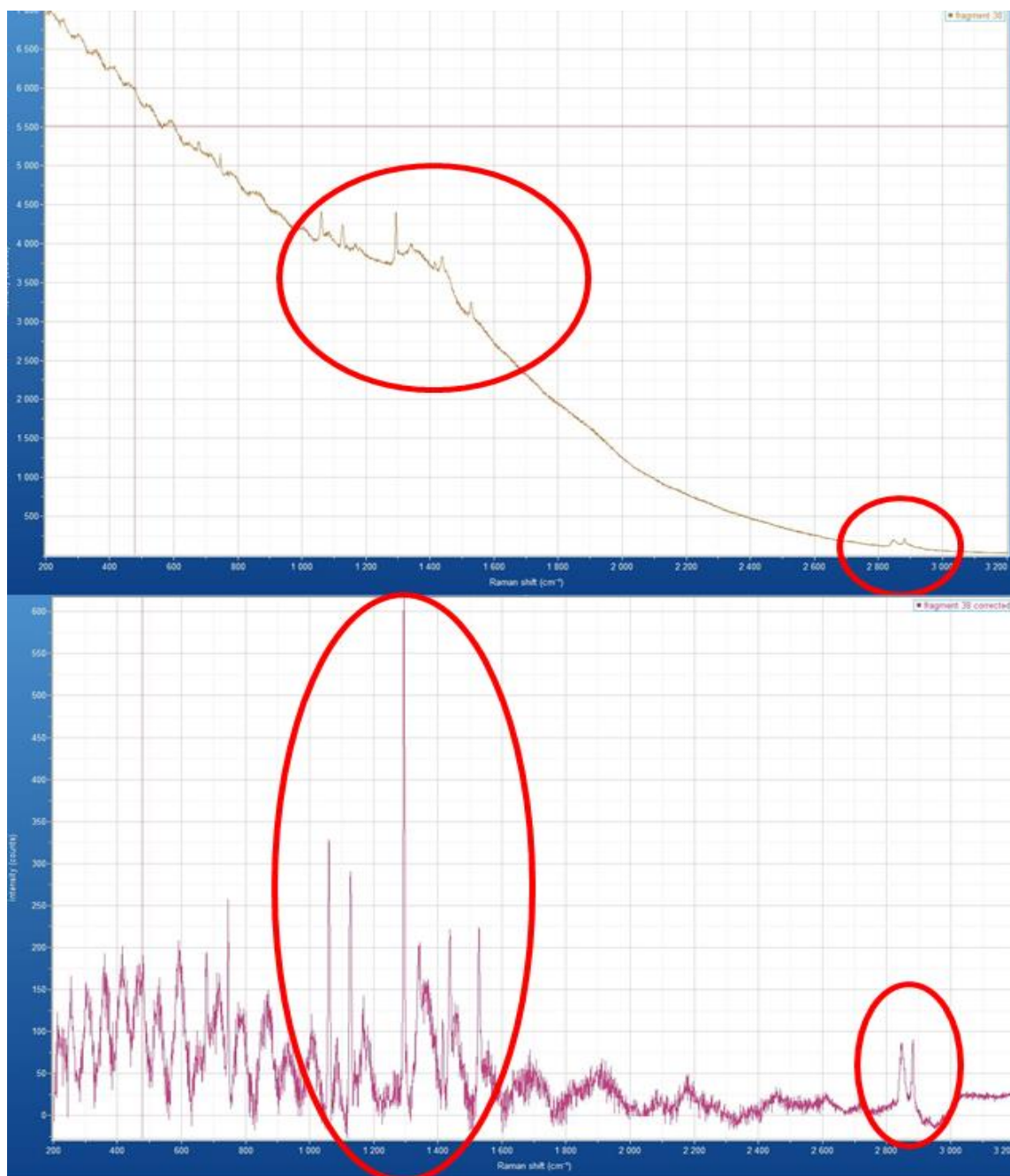
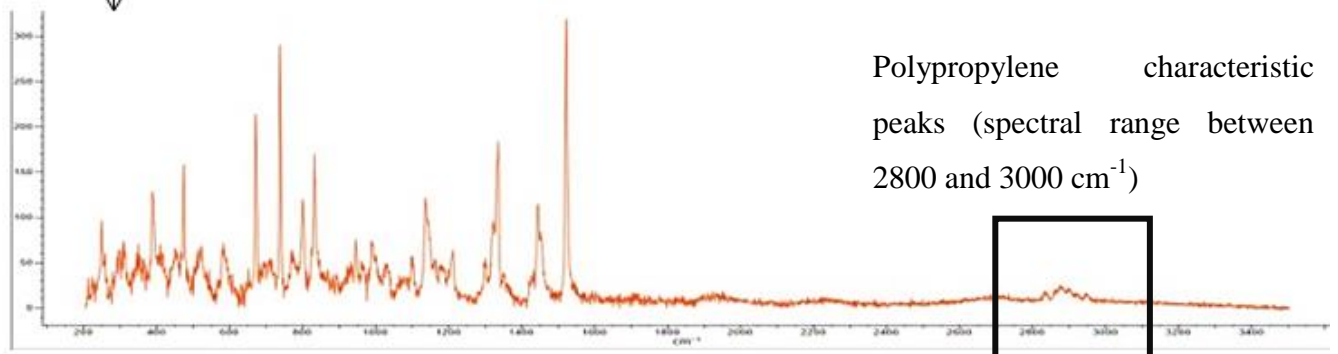
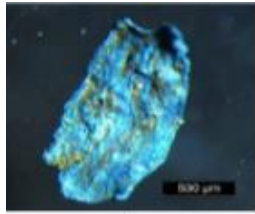


Figure 37: Baseline correction for a polyethylene spectrum.

KnowItAll software license allows the use of “Mixture analysis” which has a role in decomposing a spectrum into two spectra. It consists of comparing the potential polymer spectrum with that of the obtained dye (Figure 39). After each Mixture analysis, a HQI is obtained consisting of both spectra. The identification was considered correct when the highest HQI was obtained between the dye and that of a suspected polymer.



Polypropylene characteristic peaks (spectral range between 2800 and 3000 cm^{-1})

Copper Phthalocyanine (Dyestuff)

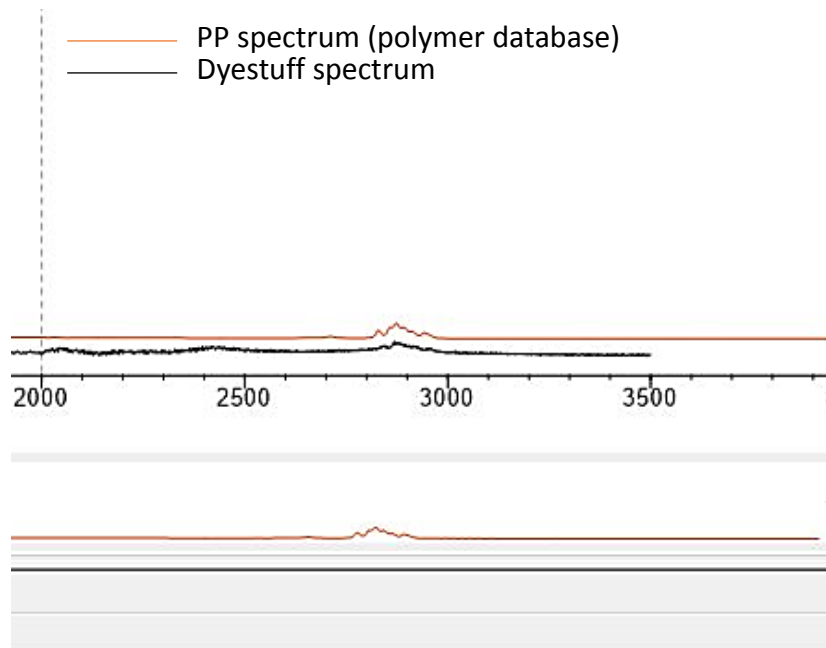


Figure 38: Polypropylene characteristic peak observed in an obtained dye spectrum.

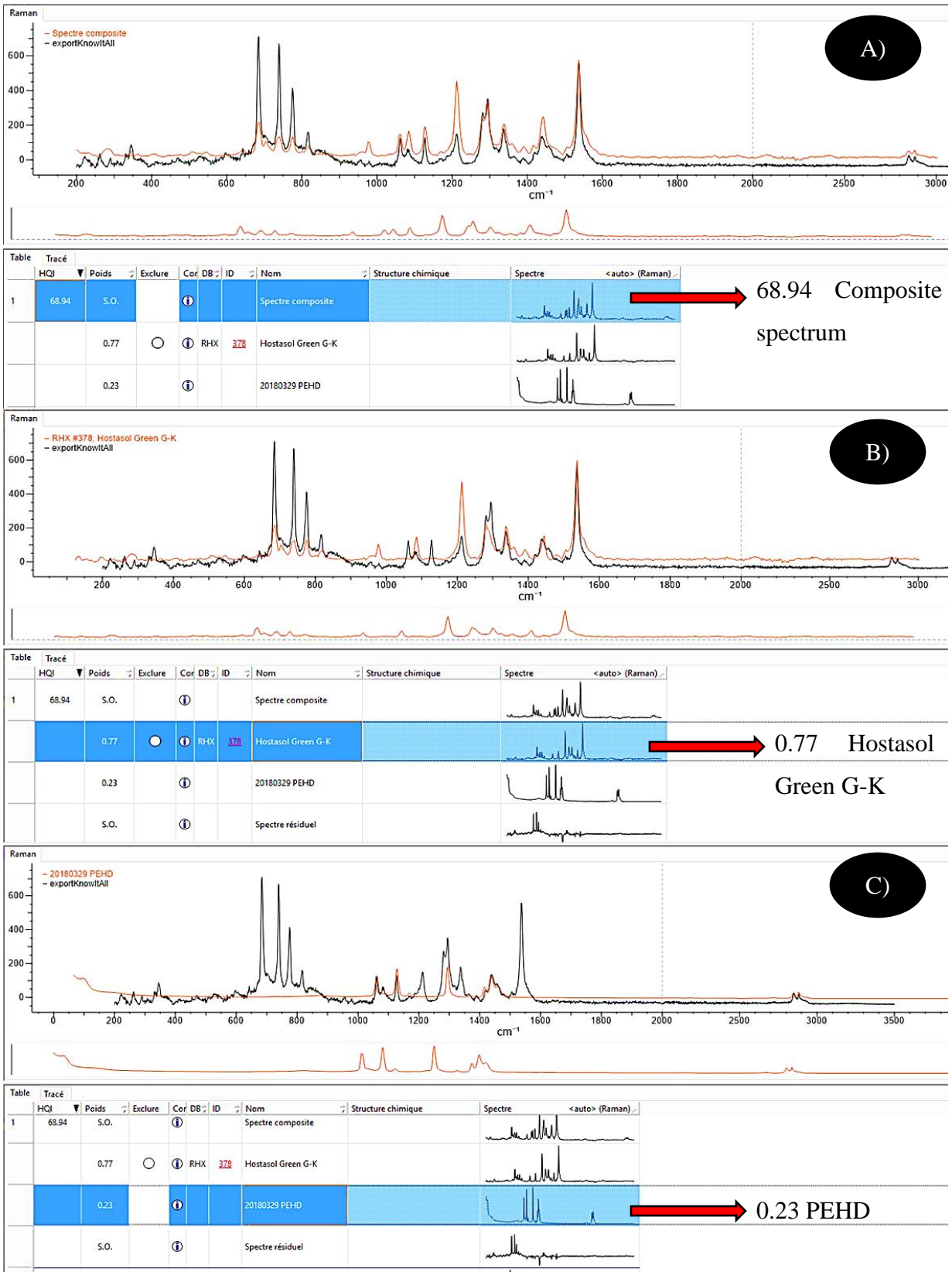


Figure 39: Mixture analysis realized on a dye spectrum (Hostasol Green G-K): A) represent the obtained composite spectrum, B) represent the pigment spectrum and C) represents the polymer (PE) spectrum.

CHAPTER 3: SOURCES OF MICROPLASTICS AND PASSIVE BIOMONITORING

In this Chapter 3, microplastics sources were evaluated by analyzing surface water and sediment samples. In addition, passive biomonitoring was also conducted by collecting wild biota samples in the vicinity of the studied sources. These sources were consisted of Wastewater Treatment Plant and coastal landfills which their role in MPs is not really well-known. **Article 1** will consist of an assessment of a tertiary WWTP and an abandoned coastal landfill in Le Havre, France; whereas **Article 2** will contain the evaluation of three coastal landfills and wild dumps along the Lebanese coast.

A. Synthesis of Article 1:

Edelweiss tertiary wastewater treatment plant located in Le Havre harbor as well as the Dollemard abandoned coastal landfill were chosen in order to assess their role as a MPs entry sources. In the former, the MPs in its influent, sludge and effluent were evaluated and then the MPs dispersion (discharged by the effluent) was assessed on a distance gradient (1.1 km, 1.6 km, 5.3 km and 10.3 km) from the discharge pipe. MPs were analyzed in three matrices: sea surface water, sediments and native blue mussels (*Mytilus* sp.) that were collected at four sites inside the harbor and the fifth at the vicinity of the coastal landfill.

Inside the WWTP, MPs retention percentage was 98.83% with 2.84 items/L observed in the effluent. From the effluent, this concentration decreased 96% to reach its lowest (0.099 items/L) at a 5.3 km distance from the discharge pipe. Instead of reaching even lower values at 10.3 km away, MPs concentration doubled to reach 6 items/L near the abandoned coastal discharge. The analyzed sediments had a different pattern than that of the surface water which is probably due to the water currents, dispersion velocity, resuspension of particles and the different densities of polymers. Blue mussels were highly contaminated in MPs and varied significantly from 0.41 ± 0.33 items/g to 2.75 ± 3.08 items/g. Mussels, being filter-feeders individuals, would be exposed to the MPs existing in their surrounding environment explaining their high contamination by MPs and especially by those of a size $< 300 \mu\text{m}$. The most dominant polymers found were polystyrene (PS), polyurethane (PUR) and acrylonitrile butadiene (ABS). These can be linked to important cargo and ships traffics as well as to the fact that Le Havre harbor is a highly industrialized zone.

The results highlighted on the importance of WWTP and coastal landfills as MPs sources in the coastal environment, and their potential harm on biota. Also, they indicated that blue mussels are a promising sentinel species for small MPs (< 200 μm) in the marine environment.

Chapter 3: Sources of Microplastics and passive biomonitoring

I. ARTICLE 1: SOURCES OF MICROPLASTICS POLLUTION IN THE MARINE ENVIRONMENT: IMPORTANCE OF WASTEWATER TREATMENT PLANT AND COASTAL LANDFILL

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Published in "Marine Pollution Bulletin", 146 (2019), 608-618

ABSTRACT

This study investigated the role of a municipal wastewater treatment plant (WWTP) effluent and an abandoned coastal landfill as pathways for microplastics (MPs) input into the marine environment. MPs were first analyzed in raw sewage influent, sludge and effluent samples, and their fate was studied along a distance gradient from the WWTP in three matrices: surface water, sediments and wild mussels. All suspected MPs were characterized according to their polymer nature using micro-Raman spectroscopy. The investigated WWTP had an estimated daily discharge of 227 million MPs. MPs were found in all matrices with a decreasing abundance from the effluent. Strong MPs abundances (higher than those found near the WWTP effluent) were observed in the vicinity of the coastal landfill suggesting its importance as a MPs entry route into the marine coastal environment. Our study supports the idea that blue mussels are a promising sentinel species for MPs (< 200 μm).

Keywords: Microplastics; Wastewater Treatment Plant; Coastal Landfill; Mussels; Micro-Raman

1. INTRODUCTION

The recent increase of microplastics (MPs) occurrence into the aquatic environment is raising concern about their potential harm on diverse organisms (Gall and Thompson, 2015). These plastics of a size below 5 mm (GESAMP, 2015) can be divided into either primary microplastics (directly produced MPs) or secondary microplastics (resulting from macroplastics fragmentation (GESAMP, 2015; Koelmans et al., 2014). Their entry routes into the marine environment mainly come from land-based origin: accidental loss or mishandling plastics, dumping plastic wastes and mismanaged landfills i.e. an absence of a fence surrounding the landfill with no appropriate synthetic material for covering wastes (reviewed by Duis and Coors, 2016). Wastewater treatment plants (WWTPs) add up to these entry routes and is considered as an important MPs pathway into the aquatic environments (Talvitie et al., 2015). These WWTPs may collect both industrial and domestic water containing MPs used in various products (Eerkes-Medrano et al., 2015; Zbyszewski et al., 2014). Several factors influence the MPs quantities being discharged by the WWTPs such as the volume of treated water, the flow rate, the filtration processes and sewer systems (Mason et al., 2016; Ziajahromi et al., 2017). As indicated in previous studies, most of the MPs can be effectively removed by secondary and tertiary treatment (Lares et al., 2018; Murphy et al., 2016), but it doesn't exclude that a large number of MPs is still being released in the aquatic systems (Browne et al., 2011; Mathalon and Hill, 2014; Murphy et al., 2016) and most specifically into river catchments (Roex et al., 2013). Removal efficiencies of microplastics in WWTPs range generally between 72 and 99.4% (Gatidou et al., 2019; Sun et al., 2019) suggesting that most particles are either accumulated during the sedimentation process or the grease

removal (Magni et al., 2019; Murphy et al., 2016). These rates remain limited by current detection techniques.

Both fibers and fragments are observed in the WWTPs water (Magnusson and Norén, 2014). Their dispersion into the aquatic environment leads to their ingestion by aquatic species like bivalves (Browne et al., 2008; Railo et al., 2018). Mussels, *Mytilus* spp. are considered as suitable animals for monitoring chemical pollutants in aquatic environments (Green et al., 2017). They are sessile animals capable of giving precise spatial information, easily collected where they form mussel's beds in shallow waters and contain enough tissue amounts for laboratory analysis. They are filter-feeders (feed mainly on phytoplankton) which increase their probability to ingest MPs and integrating them in the food chain (Wright et al., 2013). MPs ingestion might lead to several physiological issues for bivalves such as strong inflammatory responses in *Mytilus* sp. (Avio et al., 2015) or adverse consequences in *Mytilus edulis* digestive glands (Von Moos et al., 2012). Mussels are important prey source for many fish and they are highly consumed by humans making it critical to evaluate the amount of MPs ingestion.

Even though several studies evaluated WWTPs discharge into the aquatic systems, most of them focused on treatment plants discharging solely in the freshwater systems (Dris et al., 2015; Gies et al., 2018; Lares et al., 2018; Mintenig et al., 2017; Murphy et al., 2016; Wolff et al., 2019). A few assessed WWTPs discharges in the marine environment (Leslie et al., 2017; Talvitie et al., 2017b, 2015; Ziajahromi et al., 2017). To our knowledge, no study has evaluated microplastics input, dispersion and accumulation in coastal marine environment via wastewater treatment plant outlet in three different matrices. Also, no study has evaluated the role of coastal landfills in MPs entry into the coastal marine environment making its relevance not well-known.

The aims of this research were (1) to assess the contribution of a major tertiary WWTP located in Le Havre (France) in MPs discharge into the marine coastal environment, (2) to follow the released MPs in the marine environment along a distance gradient from the discharge pipe in three matrices (surface water, bottom sediments and mussels), and (3) to evaluate the role of an abandoned coastal landfill as a source of MPs entry into the marine coastal environment.

2. MATERIAL AND METHODS

2.1 STUDY ZONE AND SAMPLING SITES

The sampling area was located in Le Havre, Northern France. The study zone investigated was divided into three areas: The Edelweiss Wastewater Treatment Plant (WWTP), Le Havre harbor and Sainte-Adresse coastal zone. The WWTP is located inside Le Havre harbor and receives the effluent of 20 municipalities (serving a population of 415,000 habitants and a volume of 80,000 m³ per day). This tertiary WWTP was constructed in 2011 and is capable of treating collected water in five different steps before discharging it into the harbor's basin. Le Havre harbor is located at the mouth of the Seine estuary, in the Eastern English Channel. It is the second biggest French harbor in tonnage and receives 59% of containers traffic (Particulier, 2015) The Sainte-Adresse's site is located in the vicinity of the Dollemard coastal landfill. This landfill was constructed in 1960 and received tons of household, industrial and special wastes. This landfill was closed in 2000, yet the huge amount of dumped garbage led to its collapse on the bottom of the cliff resulting a 750 m waste distribution along the coast. During high tide, the waves collect the

wastes sitting on the coast promoting their entry into nearby coastal waters (Dollemard, 2011).

In the WWTP, water samples from raw influent and effluent were collected as well as a sample of sewage sludge. In the marine environment, samples of water, sediments and mussels were collected in four sites (1 to 4) inside the harbor, and one site (site 5) in the vicinity of the coastal landfill (Fig. 1). The sites were located from the WWTP outlet with respective distances of 50 m, 1.1 km, 1.6 km, 5.3 km and 10.3 km. These sampling sites were selected in order to follow MPs dispersion and concentration in the three analyzed matrices.

2.2 SAMPLING METHODS

The sampling was done between the 3rd and the 4th April 2018. Inside the WWTP, two samples of 500 mL raw influent and a sample of 100 g of sewage sludge were taken for analysis on the 3rd of April at 3:00 pm. Since we wanted to sample all sites the same day to have the same environmental conditions, we chose to sample a large volume of water (1 to 2 m³) representative of the analyzed water body rather than making replicates. The first 10 cm of the water column was pumped and filtered on stainless steel sieves of different mesh sizes of 500 µm, 200 µm, 80 µm and 20 µm. The filtering system consisted of a water pump (Bilge pump, SEAFLO©) connected to a flowmeter (Turbine Digital Pro K24) that calculate the exact volume being filtered throughout the sieves. Before collecting the water samples, the pump and the pipe were rinsed with the seawater then with Milli-Q water. The 20 µm sieve was the fastest to get clogged so it was analyzed separately. Each sieve was rinsed with Milli-Q water inside clean glass bottles (previously washed with Milli-Q water) covered in aluminum foil and conserved at 4 °C until analysis.

One kilogram (kg) of sediment samples was

taken with a stainless- steel sediment core sampler from all sites except in site 4 where it was a rocky substrate. They were conserved inside aluminum foil then stored at $-10\text{ }^{\circ}\text{C}$ until analysis.

Wild mussels (*Mytilus* sp.) specimens were collected in all sites (1 to 5). Around 20 individuals were sampled near the water filtration location. Each individual was immediately embedded in aluminum foil then frozen at $-10\text{ }^{\circ}\text{C}$ until analysis.

Water temperature, salinity, dissolved oxygen and turbidity were measured at each site with a multiparameter probe (Mutiparameter HI 982, HANNA instruments).

2.3 MICROPLASTICS ANALYSES

2.3.1 CONTAMINATION PREVENTION

In order to prevent contamination, several precautions were followed. Cotton lab coats were worn throughout the experiments, and all steps (filtration, measurements and digestion) were realized under a laminar flow hood. All laboratory materials used were made of glass: bottles, petri dishes, Erlenmeyers, filtration system; and they were covered immediately after manipulation. All surfaces and equipment were rinsed before usage with filtered ethanol 70% and MilliQ water. Whereas for the solutions: Ethanol 70%, KOH 10%, ZnCl_2 , H_2O_2 30%; they were filtered 3 times on GF/A filters (Whatman, France) to eliminate any unwanted particles or fibers. Controls were made with every step, in order to track the contamination during the work.

2.3.2 WATER SAMPLES

All water samples have undergone density separation (DS) using a solution of zinc chloride (ZnCl_2) of a proper density of 1.8 g/cm^3 . This solution was chosen due to its high efficacy in retrieving microplastics (Rodrigues et al., 2018).

Before adding ZnCl_2 to our samples, a series of density separation trials were tested: different volumes of filtered ZnCl_2 (50 mL, 100 mL, and 150 mL) were added to different separating funnels containing a solution of 600 mL of water and 5 g of sediments with seventeen different polymers of different densities (in order to replicate a solution similar to our samples). Samples were left 24 h; they were shaken twice: once every 4 h then left to rest for 16 h to ensure the formation of two separate phases where the supernatant consisted of particles with a density $< 1.8\text{ g/cm}^3$. The density separation efficacy was calculated using the formula (Phuong et al., 2018a):

$$\%Efficacy = \left(\frac{\text{Number of MPs after DS}}{\text{Number of MPs before DS}} \right) \times 100$$

The percentage obtained was $82.7 \pm 0.5\%$ (above 80%) and the volume of 50 mL was chosen.

2.3.3 SLUDGE SAMPLES

Sludge samples were put in aluminum foil and dried at $60\text{ }^{\circ}\text{C}$. After testing several solutions (hydrogen peroxide (H_2O_2 30%), hydroxide potassium (KOH 10%) and sodium hypochlorite NaClO) and encountering filtration difficulties or a small sample analysis ($< 1\text{ g}$), visual observation was chosen. Three dried subsamples of 3 g were put inside clean Petri dishes and observed under a stereomicroscope (Murphy et al., 2016). Microplastics (including fibers, fragments and particles) were put on a microscopic slide for further counting and analysis (color, length and polymers type).

2.3.4 SEDIMENT SAMPLES

Wet sediments collected were defrosted at room temperature. For each site, 3 subsamples of 100 g were taken and oven dried at $60\text{ }^{\circ}\text{C}$ for 24 h. From each subsample, 10 g of dried sediments (Harrison et al., 2012; Liebezeit and Dubaish, 2012; Mathalon and Hill, 2014) was weighed

and placed inside an Erlenmeyer pre-rinsed with Milli-Q water. Firstly, 40 mL of 30% hydrogen peroxide H₂O₂ (Avio et al., 2015; Nuelle et al., 2014) was added to the Erlenmeyer that was placed on a heating stirrer for 24 hour at 40°C

with a rotation per minute of 100 rpm in order to remove organic debris. Afterwards, a density separation solution was used. Briefly, the solution

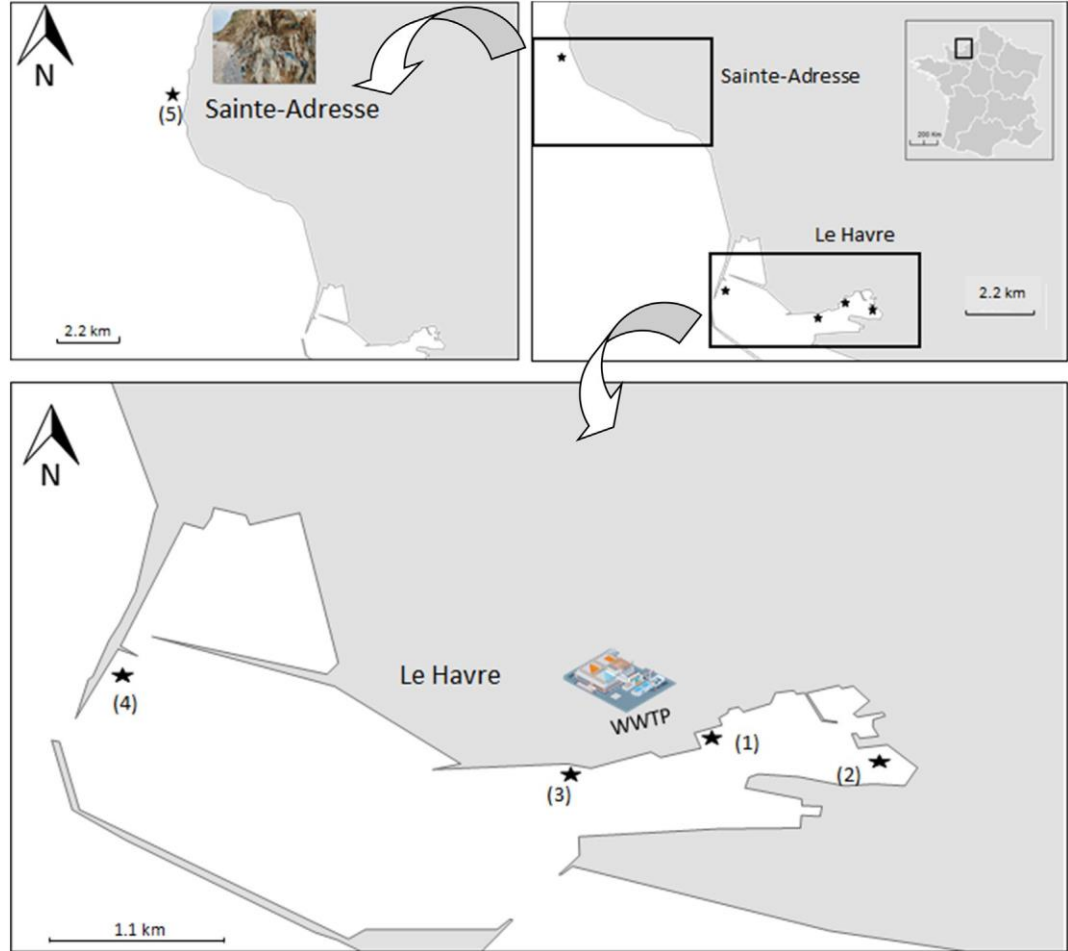


Fig. 1. Localization of the WWTP (Le Havre wastewater treatment plant) and the sampling sites: 1 (near the effluent of the WWTP), (2) upstream and (3) & (4) downstream the effluent, (5) Sainte-Adresse's site (near the coastal landfill)

containing the sediments were put inside a separating funnel and a volume of 40 mL of ZnCl₂ (1.8 g/cm³) was added (Imhof et al., 2013; Liebezeit and Dubaish, 2012; Nuelle et al., 2014); the mix was then agitated for 5 minutes and let sit for 24 hours. The supernatant containing the MPs was filtered on GF/A filters and stored inside clean glass Petri dishes.

2.3.5 MUSSEL SAMPLES

Mussels were thawed 4 hours prior to digestion. Ten mussels were taken from each site: the total

length, width (cm), total and net weight (g) was taken for each individual. Their condition index

$$CI = \left(\frac{\text{Net Weight (g)}}{\text{Total weight (g)}} \right) \times 100$$

(CI) was calculated as follow (AFNOR, 1985):

This index is a good indicator for the physiological condition (tissue growth, energy reserves) of mussels (Orban et al., 2002)

Mussels were digested using a solution of potassium hydroxide (KOH) 10% (m/v,

ChimiePlus, France) was used in order to eliminate any organic material (Dehaut et al., 2016; Kühn et al., 2017). The correct KOH 10% volume was chosen after testing different volumes (150 mL, 200 mL, and 250 mL) added inside Erlenmeyers containing mussels. Then, these Erlenmeyers were transferred to a heating

$$\%DE = 100 - \left[\left[\frac{(DFW - IFW)}{TW} \right] \times 100 \right]$$

magnetic stirrer at 60°C for 24 hours. After digestion, the digestat of each individual was filtered on GF/A filter and the digestion efficiency was calculated following the equation:

DE: Digestion efficiency

DW: Dry filter weight after digestion (g)

IFW: Initial filter weight (g)

TW: Mussel total weight (g)

The volume with the highest digestion efficiency was chosen for all wild mussels individuals. A volume of 250 mL of KOH% was chosen for the samples (%DE= 99.82 ± 0.3%).

2.4 MICROPLASTICS OBSERVATION AND IDENTIFICATION

2.4.1 VISUAL OBSERVATION

After digestion, filters were observed under 120x magnification using Leica M165 C Stereomicroscope and images of suspected MP particles were taken with a Leica M170 HD camera and LAS (Leica application suite) software. To prevent double-counting of MPs, filters were methodically examined from the highest edge to the lowest along the first column, lowest to highest edge along the second column and so on. All suspected MPs particles were counted, categorized by type (films, fragments, microbeads or fibers) and color (dark, red, yellow, blue, white or green). The following criteria were taken into consideration

while counting as mentioned by Hidalgo-Ruz et al. 2012): (1) absence of cellular or organic structures; (2) a homogenous thickness across the particles; and, (3) homogenous colors. Specific criteria were taken for counting synthetic fibers: consistent in thickness with no taper toward the ends and are clear and homogeneously colored (red, blue, green and black) (Song et al., 2015). Measurements were done on the suspected items at their longest dimension using “Piximètre” software version 5.9 and they were divided into five different size classes: <20 µm; 20 – 80 µm; 80 – 200 µm; 200 – 500 µm; >500 µm.

2.4.2 RAMAN SPECTROSCOPY

Micro-Raman Xplora Plus (HORIBA Scientific®, France) was used in order to identify the polymer nature of suspected plastics. The machine is equipped with an automated plate protected by a door that can be shut during analysis so the filter placed inside would not be affected by any airborne contamination.

Due to the time consumption that takes when analyzing filters under micro-Raman, five filters of mussels containing the highest number of potential MPs were chosen and analyzed. All sludge and sediments items, and subsamples of water items were also taken for Raman analysis. For identification, two lasers with a wavelength of 532 nm and 785 nm and a range of 200-3400 cm⁻¹ were used with x10 and x100 objectives (Olympus). Filters were either analyzed manually or using automated ParticleFinder module for LabSpec 6. Each particle spectrum is compared to a polymer database identification software (KnowItAll, BioRad®) and a personal library made with standard polymers obtained from Goodfellow (France). Pigments spectra (Copper phthalocyanine, Hostasol Green, etc...) obtained were identified using an option called “Mixture Analysis” existing in “KnowItAll software” that is capable to decompose a mixed

spectrum into two spectra consisting of the dye and its associated polymer.

2.5 STATISTICAL ANALYSIS

All statistical comparison tests were done using SPSS software (IBM SPSS STATISTICS 20).

Microplastics found in sediments and mussels were reported per gram of sample and in unit volume (per L) for the MPs in water samples. After a Shapiro-Wilk test, mussel's and water data did not follow a normal distribution and non-parametric tests (Kruskal-Wallis and Mann-

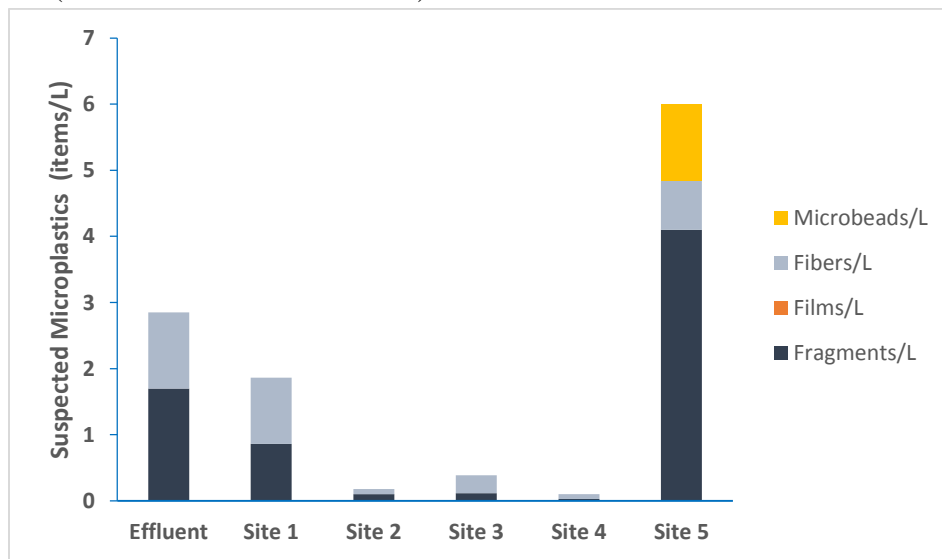


Fig. 2. Suspected microplastics concentration (items/L) by shape categories from the effluent to the different surface water sampling sites (site 1 being the closest to the effluent to site 5 being the farthest but close the landfill; see Fig. 1).

Whitney U tests) were used to test for difference of ingested MPs between all sites (significance level: 0.05). The mussels' condition index and microplastics found in sediments followed a normal distribution and one-way analysis of variance (ANOVA) test with a post-hoc Tukey test was used to determine if there was significant difference between sites ($p < 0.05$ significance level). Spearman's rank correlation was used to test any correlation between the number of ingested MPs and the mussels' condition index.

Non-metric Multidimensional Scaling (n-MDS) was performed on a Bray-Curtis distance matrix created from transformed data (square root). This test was used as an ordination method alongside ANOSIM and SIMPER tests to compare the contribution of each observed polymer in the similarity and dissimilarity between water, sediments and mussels of all

sites. These tests were done using PRIMER v7.0 (Primer-E Ltd., Plymouth Marine Laboratory, UK).

3. RESULTS

3.1 CONTAMINATION CONTROL

Blank samples only showed the presence of fibers with an average of 1.71 ± 1.5 during the dissection and digestion of mussels (representing the average found for a batch of 9 mussels representing a total one hour constant lab work under the laminar flow hood), 0.5 ± 1 for water samples density separation and 0.25 ± 0.43 for sediment samples, H_2O_2 digestion and density separation (also representing an hour of laboratory work). Fibers on background blanks were dominated by black (66.7% of total) and clear (33.7%). The absence of fragments, films, foams and beads indicates that the

contamination was only limited to airborne fibers. The average numbers of fibers found in the control blanks were similar to other studies (Bråte et al., 2018; Horton et al., 2017). These blanks are important in any microplastics study

in order to evaluate the number of procedural contamination every batch of samples is undergoing and, hence, correcting the results.

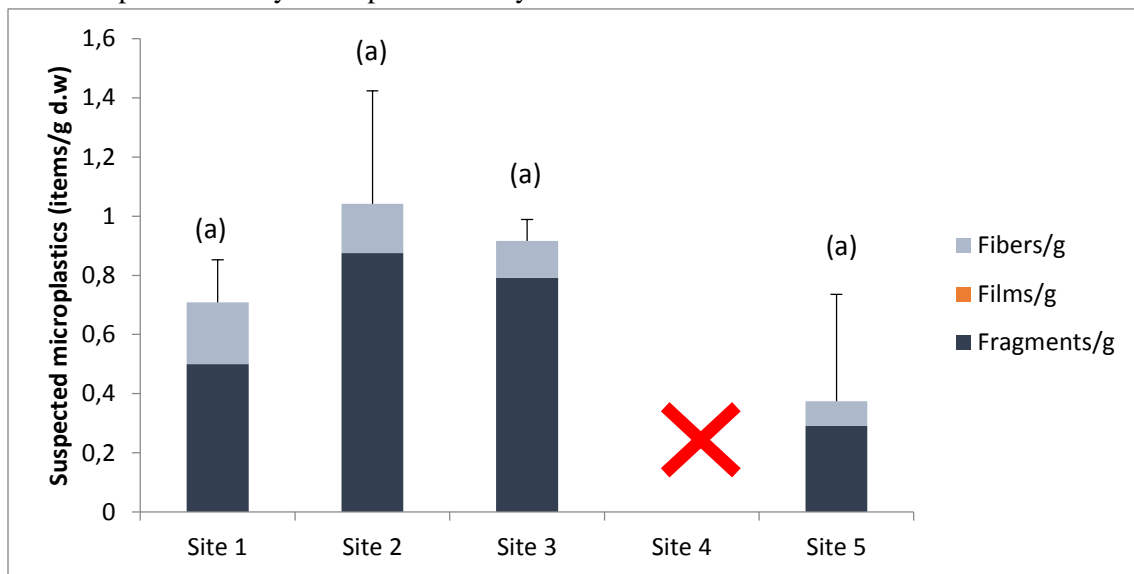


Fig. 3. Average (\pm SD) number of suspected microplastics (items/g of dried sediments) by shape categories in sediments from different sampling sites except for site 4 where sediments could not be sampled (rocky substrate) (Statistics: letters indicate significant differences between sites; $p < 0.05$.)

3.2 WASTEWATER TREATMENT PLANT AND MICROPLASTICS DISPERSION

The amount of suspected MPs items in the raw sewage influent was 244 items/L; dominated by fragments with 183 fragments/L. This number decreased to 2.84 items/L in the effluent leading to an estimated MPs retention of 98.83% by the WWTP. Synthetic fibers constituted 25% and 40% of suspected MPs found in the influent and the effluent water sample, respectively. Blue fragments and black fibers dominated both the influent and the effluent. The collected sewage sludge had an average of 16.13 ± 1.2 suspected MPs/g with a dominance of fibers (12.46 ± 1.08 fibers/g). Red fibers, and blue and green fragments dominated the sludge samples.

Supplementary Table 1 shows the environmental parameters measured during sampling. Temperature ranged between 8°C in site 3 and

9.1°C in site 1. Turbidity ranged between 4.23 N.T.U in site 2 and 17.6 N.T.U in site 1; it reached a higher value in site 5 (121 N.T.U). The number of suspected MPs in the surface water decreased with the increasing distance from the outlet flow of the WWTP (Fig. 2). The number of suspected MPs decreased by 96% at site 4 (5.3 km from effluent) when comparing to the amount of MPs initially emitted from the plant (2.844 items/L to 0.099 items/L). Yet in site 5, near the landfill, the amount of MPs was alarmingly high reaching 6 items/L (double amounts of MPs discharged from the WWTP). Particles (fragments, films or beads) dominated sites 2 and 5 whereas fibers dominated in sites 1, 3 and 4 (1; 0.27 and 0.073 fibers/L, respectively). Site 5 had the presence of secondary microplastics (fragmented shapes) but also primary microplastics (polystyrene raw microbeads represented 22% of identified

particles). Blue fragments (45%) and black fibers (51%) dominated the water samples.

3.3 MICROPLASTICS IN SEDIMENTS

Microplastics analyzed were expressed in number of plastics found in 1 g of dried sediments. Although there was no significant difference in MPs concentration among the analyzed sites ($p > 0.05$), the highest concentration was recorded in site 2 (1.04 ± 0.07 MPs/g) and the lowest in site 5 (0.41 ± 0.36 MPs/g) (Fig. 3). Fragments dominated the type of MPs found in all sampling sites representing 70.5%, 84%, 86.9, and 77.7% at sites 1, 2, 3 and 5, respectively. Site 1 had the highest number of fibers with an average of 0.2 fibers/g. Films were not observed in all sediment samples. Blue color dominated all fragments (69%) whereas blue and green colors constituted 71.4% of observed fibers.

3.4 MICROPLASTICS IN MUSSELS

For all sites, mussels collected had a shell length ranging between 1.4 and 7.72 cm with an average of 5.6 ± 1.6 cm. The net weight ranged between 0.5 and 20.4 g with an average of 6.52 ± 4.2 g. There were significant differences in mussels' wet weight between sites ($p < 0.05$): mussels from site 5 were significantly smaller than those from the others sites. To avoid disparity between different sized mussels, the number of ingested MPs was converted per gram of soft tissue (wet weight) and not per individual (Bråte et al., 2018). 93% of collected mussels had ingested suspected MPs. Mussels from site 5 ingested a significantly higher number of suspected MPs (2.75 ± 3.08 items/g) compared to the others sites ($p < 0.05$) except for site 4 (Fig. 4). Mussels from site 2 had the lowest number of ingested MPs (0.41 ± 0.33 items/g). Fragments dominated mussels in all sites (52% in sites 1, 3 and 4; 72% in site 5) except in site 2 where fragments and fibers were equally distributed (~40%). Among the investigated fragments, blue, red and transparent fragments (~

20% for each color) dominated. Whereas for the fibers, both blue and black colors represented 80% of the observed fibers. There was significant difference between the mussels' condition index (CI) ($p < 0.05$) with site 1 being significantly different from site 5. Yet, there was no significant correlation (Spearman's correlation, $r = -0.084$; $p > 0.05$) between the CI index and the ingested items.

3.5 ITEMS SIZE DISTRIBUTION IN THREE DIFFERENT MATRICES

Suspected MPs were categorized into five different size classes as shown in Fig. 5. Inside the WWTP (Fig. 5 A), two MPs size classes (20 - 80 μm and 80 - 200 μm) dominated in the raw sewage influent samples and were equally distributed (30%) whereas the other size classes: < 20 μm and 200 - 500 μm represented a respective 14% and 17.5% of the sample. The effluent water sample was dominated by small MPs from the size class (20 - 80 μm) representing 42% of the sample. Two size classes: 80 - 200 μm and 200-500 μm were equally distributed in the effluent; each one represented a percentage of 20.8%. Whereas in the sludge, the size class 200-500 μm dominated with 56.25% of the suspected MPs followed by size classes 80-200 μm and > 500 μm (18.75% each). Smaller items were less abundant in the sewage sludge with only 6.25% items in the size class of 20-80 μm with no observed items in the size class <20 μm .

The size class 20 - 80 μm was the dominant size class in all water samples (Fig. 5 A). The size class 200 - 500 μm had a higher dominance in site 2 (37.7%) in comparison to other sites. All sites had the presence of < 20 μm size class with an abundance less than or equal to 5%.

For the sediments, the dominant MPs size class in all sites was the 20 - 80 μm representing between 40% at site 3 and 56% at sites 3 and 5 (Fig. 5 B). Plastics with a size class > 500 μm consisted mainly of fibers with an abundance

ranging between 12 % (sites 2 and 3) and 22% (sites 1 and 5).

Ingested items of a size < 200 µm were the most abundant in mussels representing 78.5%, 84.4%,

72.2%, 63.1%, 61.9% at sites 1, 2, 3, 4 and 5, respectively (Fig. 5 C). Among them, suspected MPs of size class 20 - 80 µm were the most abundant in all sites with an average of 41% of the ingested items.

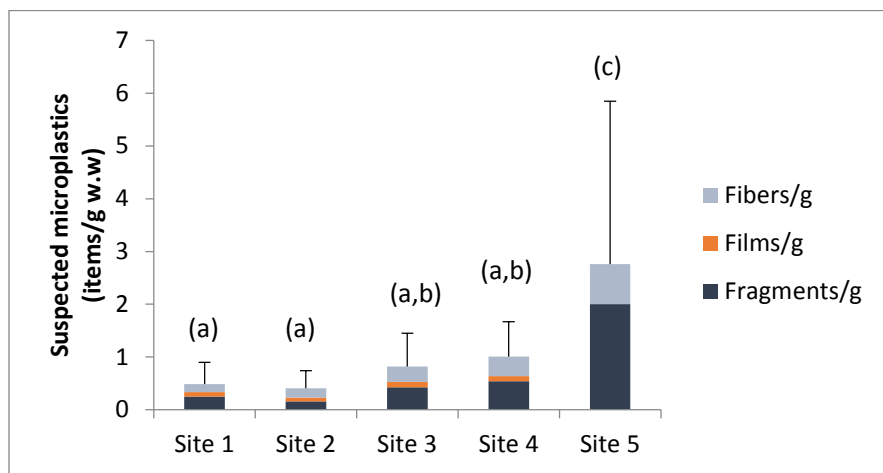


Fig. 4. Average (\pm SD) number of suspected microplastics (items/g of mussels' wet weight) by shape categories in mussels collected in all the investigated sites (statistics: letters indicate significant differences between sites; $p < 0.05$).

3.6 POLYMERS IDENTIFICATION

In our study, approximately 19% of all items were identified with the Raman spectroscopy: half of mussels' filters and all items found in the sludge and sediments samples were analyzed. For the water, an average of 27 MPs was identified per site. Of the 398 items analyzed, 344 (86.4%) were positively identified as a known polymer. Among the non-polymers items, only 2% were identified as cellulose-based fibers. The rest were non identifiable (for example if a sample is too thin, Raman tends to detect the underlying substrate instead of the sample K appler et al., 2015).

In the WWTP, 10 types of polymers were identified with a dominance of polystyrene (PS) and polyethylene (PE) in the influent, sludge and effluent samples (Fig. 6 A). PS was dominant in the influent (37.5%) and the sludge (25%) whereas PE (39.7%) dominated the effluent

sample. The other polymers only represented a limited proportion (~3 - 17%) of total MPs in the wastewater.

In the surface water samples, 11 types of polymers were identified with polystyrene (PS = 40%), polyamide (PA =18%) and polyethylene terephthalate (PET =14%) the most detected polymers. Polypropylene (PP) and PET were mainly detected in site 5 and PA in sites 2, 3 and 5.

Sediments samples had eight different polymer types (Fig. 6 B). A copolymer ethylene-propylene was observed in site 1; it was the first copolymer identified in this study. PS was the most abundant polymer (38%) and observed in all sites.

Nine types of microplastics polymers were ingested by mussels (Fig. 6 C) with PS (33%),

acrylonitrile butadiene styrene (ABS = 19%) and PP (16%) the most abundant polymers.

Polystyrene (PS) and acrylonitrile butadiene styrene (ABS) tend to be found in blue or green colored MPs with a size below 200 μm . Polypropylene (PP) and polyethylene (PE) were found either in transparent or in multicolor shades (green, blue and red colors) with a size class greater than 200 μm . All PET found were of

fibers whereas those in PA were both fibers and films. There was no specific relationship between colors and size for the other polymers.

Comparison of the polymers composition identified in the different matrices (water, sediments and mussels) indicated a high dissimilarity between groups (ANOSIM, $R=0.857$, significance level = 0.1%). Non-metric

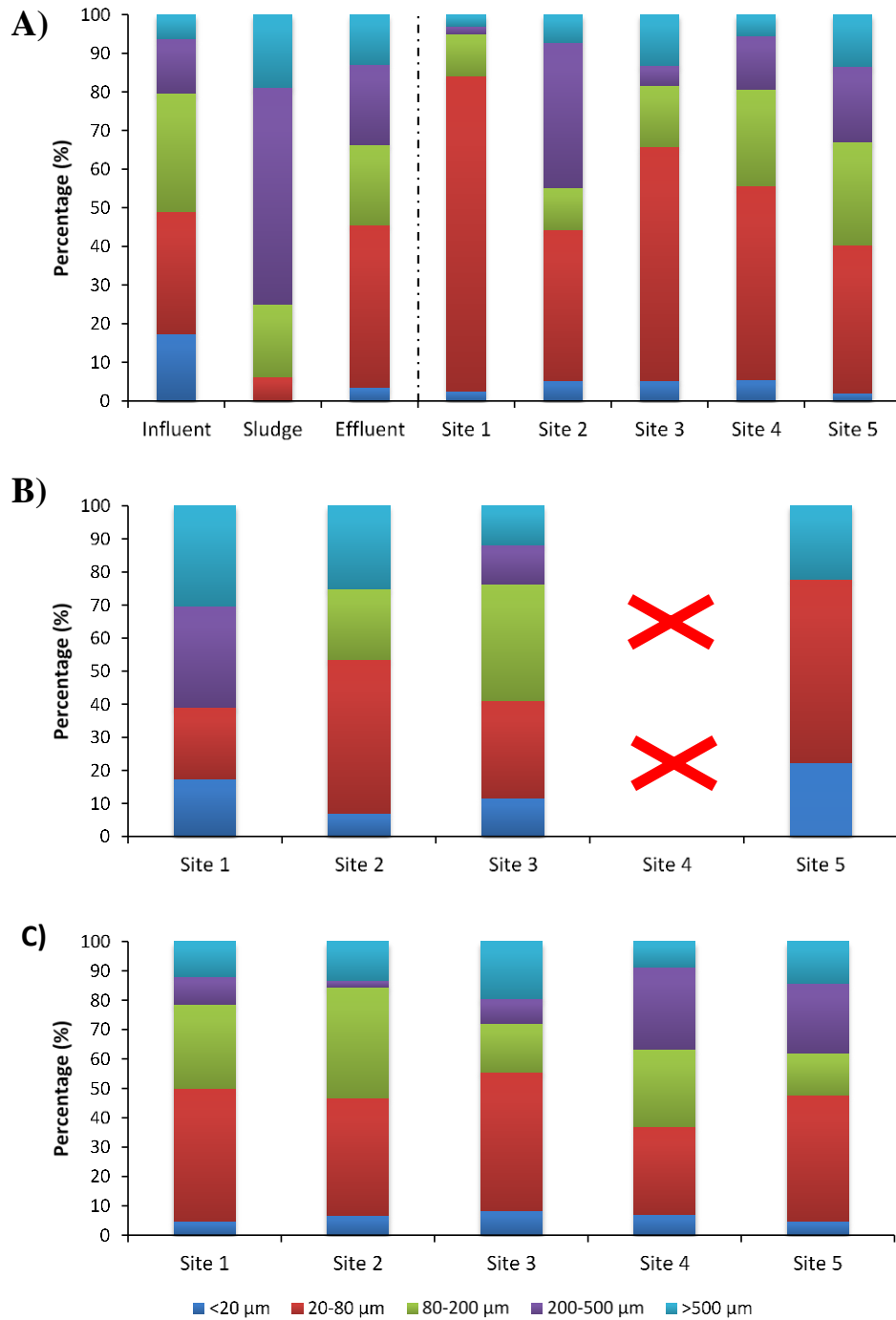


Fig. 5. Proportion of size class composition of suspected microplastics in the three analyzed matrices A) WWTP and the surface water, B) sediments and (C) mussels from the different sampling sites (site 1 being the closest to the effluent to site 5 being the farthest near the landfill; see Fig. 1).

multidimensional scaling (n-MDS) showed the dimensional distance separating these groups (a, b, c, d and e) (Fig. 7). Mussels and sediments of all sites (except sediments from site 5) were

regrouped in the same category, indicating a similarity in polymers composition between mussels and sediments. Water samples (except site 5) formed two more or less similar groups (SIMPER test indicated a similarity of 57%). At

site 5, polymer composition of water and sediment samples were very different from the other sampling sites (65% of dissimilarity). They are characterized by the high abundance of PET and PS.

4. DISCUSSION

4.1 WASTEWATER TREATMENT PLANT AS A MPS SOURCE IN THE MARINE ENVIRONMENT

Many recent studies have highlighted the crucial role of municipal wastewater treatment plants as potential pathways of microplastics entry into the aquatic environment. The detected amounts of suspected MPs items in the raw sewage influent (244 items/L) and treated wastewater effluent (2.84 items/L) were in the range of those reported in other WWTPs in Europe, USA and Australia: between 1–3160 particles/L and 0.0007–125 particles/L for raw and treated wastewater, respectively (reviewed by Sun et al., 2019). According to the results of the present study, Le Havre WWTP had a MPs removal efficiency of 98.83%. Removal efficiencies of microplastics in WWTPs range generally between 72 and 99.4% (Gatidou et al., 2019; Sun et al., 2019). Variations between studies are mainly caused by different types of wastewaters and treatments used in studied WWTPs with different sampling size limitations, preparation and identification methods (Lares et al., 2018; Gatidou et al., 2019). The dominance of fibers and fragments in WWTP effluent is consistent with other studies (Mason et al., 2016; Michielssen et al., 2016; Sutton et al., 2016), yet we found a higher proportion of fragments. Generally, fibers were the most abundant type of microplastics in the wastewater (Lares et al., 2018; Leslie et al., 2017). Le Havre harbor has a strong presence of industrial activities

(petrochemicals, refineries) and is also the leading harbor in containers traffic. Several accredited industries in Le Havre collect both solid wastes (plastics, groceries, metal, glass, ashes for example) and liquid wastes (bilges water, hydrocarbon mud, wastewater, slops) resulting from cargo and ships' self-generated wastes. These collected wastes by Le Havre industries could end up in the treated water of the WWTP and potentially be an important fragment source in the influent. The concentration of suspected MPs items in the sludge samples (16.13 ± 1.25 MPs/g of dried weight) is in the average of what has been observed in other WWTPs such as in Glasgow (19.67 ± 4.51 MPs/g; Murphy et al., 2016). Microplastics concentration in sludge generally varies between 1 and 170 MPs/g (Gatidou et al., 2019). Normally, the detected amounts of MPs found in the sludge are in orders of magnitude higher than those found in treated wastewaters (Talvitie et al., 2017; Gatidou et al., 2019) suggesting the high potential of WWTPs in retaining microplastics from raw wastewater. Even though the number of MPs retained in the sludge might seem high, but when converting the number of sludge found per g to per m³ (the studied WWTP generates about 800 g of sludge per m³ of treated raw influent) a concentration of about 13,000 MPs per m³ of raw wastewater treated are retained in the sludge. Compared to the number of MPs found in influent (244,000 MPs per m³), a large number of MPs seems to have been either removed during the pretreatment processes, during grease and grit removal steps or during the secondary/tertiary treatments. For example, Murphy et al. (2016) observed a high proportion of micro- plastics' removal during grit and grease

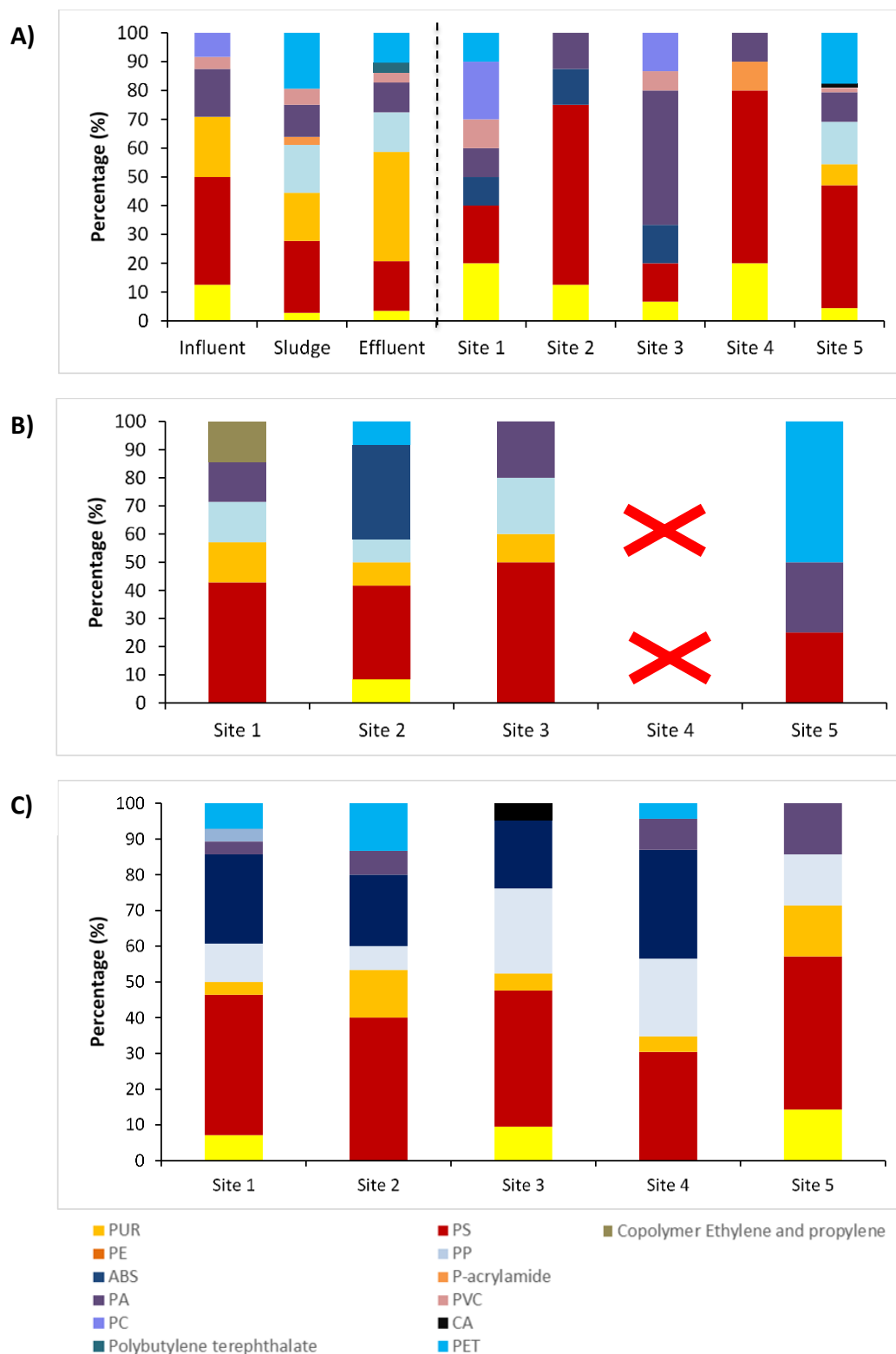


Fig. 6. Proportion of different polymers identified by micro-Raman spectroscopy in the three analyzed matrices: A) WWTP and surface water; B) sediments, C) mussels from the different sampling sites (site 1 being the closest to the effluent to site 5 being the farthest near the landfill; see Fig. 1).

removal (44.6%). The contribution of the different processes on MPs removal is still limited. However, recent experiments using a lab-scale sequencing batch biological WWTP showed that 52% of microbeads were accumulated in the activated sludge (Kalčíková et al., 2017). Inside the Edelweiss WWTP, activated sludge is collected, dehydrated, incinerated and transformed to a non-harmful valuable mineral product. In case of agricultural usage, this obtained product will be free of plastic (MPs will be incinerated and won't cause any potential harm to the soil). Yet, WWTP incinerate their sludge without taking into consideration the plastics existing in their samples; plastics combustion can lead to the formation of two by-products: airborne particulate emission (soot) and solid residue ash (black carbonaceous color) (Reviewed by Verma et al. (2016)). Soot has a high potential of health and environmental risks (particulate bound heavy metals and dioxins (Valavanidis et al., 2008)). Incinerating sludge should be reconsidered due to the plastics presence and its toxicity when burned. We found differences in suspected MPs size classes in the raw influent, effluent and sludge. Large sized MPs ($> 200 \mu\text{m}$) were observed in the sludge indicating their retention during treatment and explaining their low presence in the effluent. This is generally the case where larger and heavier particles are more easily retained (e.g. during sedimentation), whereas smaller particles remain in suspension (Murphy et al., 2016; Magni et al., 2019). The dominance of smaller particles (20–80 μm) in the effluent may also have resulted from particles degradation during treatment. As reviewed by Sun et al., 2019, polystyrene (PS = 27%), polyethylene (PE = 24%), polyamide (PA = 12%), polyethylene terephthalate (PET) and polypropylene (PP). (11% each) were the most detected polymers in WWTPs. Polystyrene (PS) are used in packaging (Marsh and Bugusu, 2007) such as polystyrene foam used for coffee

cups, trays, takeaway food containers, eating utensils (Issam et al., 2009; Thompsett et al., 1995). The PET and PA are all widely used in synthetic clothes; while PE are used in food packaging, films, plastics bottles and beauty care products (Lares et al., 2018; Ziajahromi et al., 2017). Some of the polymers found in our samples can be traced to maritime activities: for example, PUR is mainly used as rigid and flexible foams, coatings, adhesives, and binders (Zhang et al., 2015).

4.2 RELEASE, DISPERSION AND CONCENTRATION OF MICROPLASTICS IN THE MARINE ENVIRONMENT

Low concentrations of MPs (e.g. $< 0.1 \text{ MP/L}$) in the effluent may contribute significantly to MPs pollution in the receiving environment due to the large volumes that are continuously discharged by WWTPs (Carr et al., 2016; Mason et al., 2016; Mathalon and Hill, 2014; Michielssen et al., 2016; Talvitie et al., 2017b, 2015; Ziajahromi et al., 2017). With an average discharge volume of $80,000 \text{ m}^3/\text{day}$, the daily amount of MPs released in the marine environment by Le Havre WWTP would be approximately $227 \times 10^6 \text{ MPs/day}$. Correcting this number with our Raman identification data results in an estimated daily discharge of $210 \times 10^6 \text{ MPs/day}$. The daily discharge of microplastics by WWTPs varies according to the number of serving habitants, the volume of treated raw influent and also depending on the seasons or environmental conditions. For example, Talvitie et al. (2017) estimated that a Finnish WWTP treating a volume of $270,000 \text{ m}^3/\text{day}$ may discharge MPs with values as high as 1.7×10^6 and 1.4×10^8 particles/day in different days of the week.

The results of this study reveal the presence of MPs in all examined matrices (water, sediments and mussels) and in all sites, indicating their extensive dispersion in the coastal marine environment. To our knowledge, this is the first study that assessed MPs dispersion in the coastal marine environment along a gradient from the WWTP effluent in three different matrices. Our

results showed that the number of MPs in the surface water decreased with the increasing distance from the WWTP outflow. The number of MPs decreased by 96% at site 4 (5.3 km from the outflow) when comparing to the amount of MPs in the effluent. Such spatial gradient of decrease in MPs was not observed for sediments and mussels samples. There was no relationship between MPs concentration found in the water

and concentrations in sediments or mussels samples. The reason for this discrepancy between surrounding water and sediments or mussels samples may be related to several factors including weather conditions, water circulation, dispersion velocity and MP polymers density that may affect MPs sink to the water column and seabed. In addition, the concentration of MPs in surface

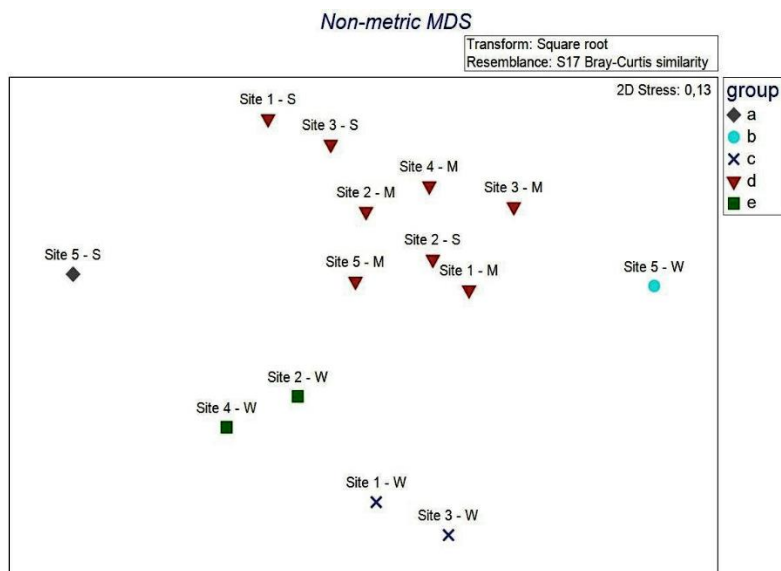


Fig. 7: Non-metric multidimensional scaling (n-MDS) of polymer composition for the three compartments (water, sediments and mussels) analyzed in all sites. The represented data regrouped the sampling site with its appropriate collected sample (W: Water, M: Mussels, S: Sediments)

water is directly affected by the amount of MPs found in the effluent which varies temporally (diurnal or daily variations) (Talvitie et al., 2017). When comparing different matrices or compartments, a more adapted sampling strategy should take into account the time lag necessary for

MPs to sink in the water column, to be ingested by mussels and to be sedimented at the bottom. So, the concentration of MPs measured in the surface water near a source of MPs does not necessarily reflect their bioavailability to organisms living in the water column or in the bottom. Railo et al. (2018) showed no significant difference in MPs concentration between mussels located 30 m or 700 m from a WWTP discharge pipe. Another study demonstrated that bivalves

located downstream and upstream the WWTP accumulated more particles than those located directly near the effluent (Domogalla-Urbansky et al., 2019). The heterogeneity of the MPs distribution and of its concentration levels in different matrices are likely reflecting a complex interplay between the distance from the pollution sources, hydrodynamic features such as currents, gyres, fronts and shape of the coast line (Schmidt et al., 2018). In open aquatic environment, far from direct sources of MPs, a correlation was found between microplastic levels in the water and in the mussels. This is the case in open coastal waters along the China coasts where Qu et al. (2018) found a positive and quantitative correlation of MPs in mussels and in their surrounding waters.

In comparison with the MPs concentrations reported for other water bodies nearby the discharge of WWTP effluents, the concentrations of the present study were considerably higher. For example, MPs concentrations of 0.001–0.002 MPs/L were reported for seawater collected 20–200 m from the WWTP effluent outlet in Sweden (Magnusson and Norén, 2014). In the Seine River (near Paris) MPs concentrations of 0.013–0.1 MPs/L were recorded downstream the Seine-Centre waste-water treatment plant (Dris et al., 2015). In the bay of Brest (France), near an important military harbor, the mean MPs concentration in surface water was 0.00024 ± 0.00035 MPs/L (Frère et al., 2017). In sediments, as for water, the MPs concentrations were also higher than those recorded in the Southern North Sea in the harbor of Nieuwpoort 166.7 ± 92.1 MP/kg DW (Claessens et al., 2011) or in the bay of Brest 0.97 ± 2.08 MPs/kg DW (Frère et al., 2017), but in the range of those recorded in Mediterranean coastal sediments (Alomar et al., 2016) or in the Venetian Lagoon (Vianello et al., 2013). Concerning mussels, the number of MPs ingested ranged between 0.4 and 1 MPs/g w.w and was similar to those found by Li et al. (2018) in the UK coastal water (0.7–2.9 items/g w.w) but higher than those recorded by (Vandermeersch et al., 2015) in Belgian, Dutch and French North Sea coasts (0.2 ± 0.3 items/g w.w) or in the French Atlantic coast by (Phuong et al., 2018c) (0.23 ± 0.20 items/g d.w).

The high number of MPs in surface water, sediments and mussels found in Le Havre harbor can be linked to the continuous discharge of the WWTP in this semi-enclosed basin. Harbor's geometry might contribute to a low flushing rate (Claessens et al., 2011); its morphology (the basin) along with a great amount of daily discharged MPs will result to a high accumulation of MPs and raises their chances of ending at the bottom. Small water

bodies have higher MPs concentration than that in open sea (Luo et al., 2019) explaining the great abundance of MPs found inside the harbor.

Several studies showed that fibers were the most dominant in sediments (Alomar et al., 2016; Blair et al., 2018; Cannas et al., 2017; Lots et al., 2017) or mussels (Brâte et al., 2018; Catarino et al., 2017; De Witte et al., 2014) whereas in our study, fragments were the most dominant as observed in the sediments of the Venetian Lagoon (Vianello et al., 2013). The dominance of fragments over other types of MPs in sediments and mussels samples suggests an additional source of their entry other than the WWTP where textile-derived fibers are dominant (Frère et al., 2017; Gatidou et al., 2019). The main source of MPs fragments in the harbor may be related to anthropogenic activities and to the breakdown of larger plastic debris (Cole et al., 2011; Vianello et al., 2013). The shape and color characteristics of fragments found indicate that they are mainly secondary microplastics resulting from the decomposition of larger plastics. The MPs polymers composition identified in the sediments and mussels' samples were different from those identified from the effluent or surface water samples (Fig. 6) suggesting that there are other sources of MPs inputs into the harbor. Le Havre is a highly industrialized city and a leading chemical industrial platform in Europe. It is the 1st French petrochemical complex, with 24.3 tons of crude oil processed per year. Many refineries and plastic companies (about 200 companies) are located in and around the harbor.

4.3 COASTAL LANDFILL AS IMPORTANT SOURCE AND ROUTE FOR MPS IN THE COASTAL ENVIRONMENT

This study revealed strong MPs abundances in the coastal environment near the Sainte Adresse's abandoned coastal landfill (site 5). To our knowledge, no study to date has evaluated MPs concentrations in coastal areas

near landfills. The concentrations found in the water and mussels samples are 2 to 4 times higher than those found in Le Havre Harbor. However lower MPs concentration in sediments was observed in site 5. This can be explained by the difference in sediments composition between sites. Site 5 consists of sandy sediments whereas for the other sites (inside the harbor), the sediments are sandy-muddy to muddy. Strand et al. (2013) demonstrated that there is a strong relationship between MPs abundance and both organic (%TOC) and fine fraction ($< 63 \mu\text{m}$) content in sediments, supporting the hypothesis that MPs will accumulate in depositional areas. In the Lagoon of Venice, Vianello et al. (2013) detected the lowest microplastic concentrations in the outer Lagoon, where water currents are higher ($> 1 \text{ m/s}$). Consequently, the highest concentrations were encountered in the inner Lagoon which is characterized by lower hydrodynamics and a higher fine particle ($< 63 \mu\text{m}$) fraction in the sediment.

Our results highlight the significant role that coastal landfills play in

MPs entry into the marine coastal environment. Many environmental factors and microbial degradation (He et al., 2019; Zettler et al., 2013) may lead to the fragmentation of plastics to microplastics, and small plastic debris would be carried out by the discharge of leachate. Microplastics were identified in the leachate from both active and closed landfills (He et al., 2019). Microplastics polymer composition found at site 5 near the landfill was different from other sampling sites although this cannot provide conclusive evidence of these microplastics origins. This site had also a dominance of polystyrene (PS) (~25–42%) with a high abundance of PS microbeads (22%) in the water sample. PS is generally more prone to weathering by UV radiation than other plastics (Alimi et al., 2018). The landfill could potentially carry more PS plastic into the environment than the WWTP

discharge. Although fragmentation and release of microplastics is a long-term process, our results confirm that landfill isn't the final sink of plastics, but a potential source of microplastics (He et al., 2019). This can pose serious environmental problems since microplastic is not listed as pollutant in the any country's regulations of landfill.

4.4 MUSSELS AS A SENTINEL SPECIES FOR MPS POLLUTION

Mussels from all sites have mainly ingested small sized MPs ($< 200 \mu\text{m}$). This was similar to other studies that demonstrated that MPs below $300 \mu\text{m}$ were the most found in bivalves (Leslie et al., 2017; Naji et al., 2018; Phuong et al., 2018c, 2018b). Although we found that small sized MPs dominated the water and sediments samples, it is currently unknown whether their abundance in mussels are due to mussels size preferences in regards to prey, or because the majority of MPs in the environment were typically in this size range. In laboratory exposure experiments, Browne et al. (2008) previously demonstrated that mussels are capable of ingesting plastic particles between 3 and $10 \mu\text{m}$ in size that they filter from the water phase. Several studies showed that bivalves are capable to ingest different type of polymers and the most common plastic inside their soft tissues differed from one study to another (Avio et al., 2017; Bråte et al., 2018; Digka et al., 2018; Phuong et al., 2018c, 2018b). Polystyrene (PS) was the most common polymer found in our mussels, followed by acrylonitrile butadiene styrene (ABS) and polypropylene (PP). They are the most common plastics used worldwide whether in packaging or other household materials. The comparison of ingested polymers by mussels indicated a high similarity with sediments (69% of average similarity – SIMPER test). Mussels, such as the blue mussel *Mytilus edulis* is often selected as model species as they inhabit a wide geographic range, are sedentary, and filter large volumes of water. Our study supports the idea that the blue

mussels are a promising sentinel species for small MPs (< 200 μm) in the marine environment (Bråte et al., 2018; Li et al., 2018). However, if they are suggested to be used as a standard bioindicator for MPs contamination in the marine environment (OSPAR, 2015), it is necessary to resolve a standardized method for mussel sampling and MPs extraction and identification in order to ameliorate the comparability between MPs data studies.

5. CONCLUSION

The investigated WWTP had a microplastics retention efficiency of 98.83% yielding to daily discharges of 227 million MPs predominantly composed of fibers and fragments. These MPs were found in all examined matrices (water, sediments and mussels) and in all sites, indicating their extensive dispersion in the coastal marine environment. Polystyrene (PS), polyethylene (PE), polypropylene (PP) and acrylonitrile butadiene styrene (ABS) were the most common polymers identified by Raman spectroscopy. The number, size and type of MPs in the water, sediments and mussels differed but a similarity in polymer composition was found between mussels and sediments samples. Our study supports the idea that the blue mussels are a promising sentinel species for small MPs (< 200 μm) in the marine environment.

To our knowledge, this study is the first that assessed MPs dispersion in the coastal marine environment along a distance gradient from the WWTP outflow in three matrices. Our results showed that the number of MPs in the surface water decreased with the increasing distance from the WWTP outflow. It revealed strong MPs abundances (2 to 4 times higher than those found near the WWTP outlet) in the vicinity of the abandoned coastal landfill suggesting that it contributes more than the WWTP effluent as routes of MPs entry into the marine coastal environment. This study was limited by the number of water samples taken even though high volumes of water were filtered. Yet, it gives an

idea about the MPs being released by the WWTP and coastal landfill. However, more researches should concentrate on microplastic sources and their temporal variations in relation with different factors such as environmental conditions (seasons, precipitation, wind, water current, tide,...) and factors associated with the operation of the WWTP (the volume of treated raw influent, timing of effluent discharge,...).

Such knowledge could help controlling different MPs sources in order to prevent related MPs pollution and, should be, therefore, more comprehensively investigated in the future.

Acknowledgments

The authors would like to thank the administration of Le Havre harbor (especially Miss. Magali HAUTOT who helped obtaining permissions to enter the harbor and was our companion during sampling), the CODAH and the wastewater treatment plant “EDELWEISS” board and crew for allowing us to collect our samples.

Declaration of Competing Interest

None

SUPPLEMENTARY FILES

Supplementary Table S1: Environmental parameters measured in all sites (S: Salinity, T: Temperature, O₂: Dissolved Oxygen and Turbidity).

	S (p.s.u)	T (°C)	[O₂] (mg/L)	Turbidity (N.T.U)
Site 1	23.5	9.1	8.72	17.6
Site 2	27.5	8.3	9.82	4.23
Site 3	27.2	8	9.6	5.47
Site 4	23.5	8.3	9.59	7.8
Site 5	25.4	8.2	9.69	121

B. Synthesis of Article 2

This second part of Chapter 3 evaluated, for the first time, the microplastics pollution along the Lebanese coast (Eastern Mediterranean). Three matrices were also chosen: sea surface water, sediments and biota which were all collected near important coastal landfills. The conducted passive biomonitoring consisted in analyzing the MPs abundance in the digestive tract of *Engraulis encrasicolus* (European anchovy) and, for the first time, in the soft tissue of *Spondylus spinosus* (Spiny oysters).

MPs in the sea surface waters had a mean abundance of 4.3 ± 2.2 items/m³ with the highest abundance of 6.7 MPs/m³ found near a former big coastal landfill that was buried on the coast. The mean value of MPs in sediments was 2433 ± 2000 MPs/Kg d.w with the highest number observed in the north of the country (4.68 MPs/g) probably in relation to the northward direction of the water general circulation and movements. This high abundance of MPs in both of the abiotic matrices would increase the chances of marine organisms to anthropogenic particles ingestion. Both analyzed species had a high ingestion percentage (>80%) and a high number of observed MPs. European anchovies ingested an average of 2.5 ± 0.3 MPs/individual and spiny oysters ingested an average of 7.2 ± 1.4 items/individual and 0.45 ± 0.3 items/g ww. Among the various polymers found: polyethylene (PE) was the most abundance in the sea surface water, polypropylene (PP) in the sediments and polystyrene (PS) in biota.

The form and shape of observed MPs (abrasions, biofilms...) as well as their polymer type allowed to give an idea of their origin and trace them back to the coastal landfill. The observation of pellets and microbeads (both considered as primary microplastics) indicate that other MPs sources are playing also a role in the coastal marine MPs pollution (e.g. plastic industries). The high occurrence of MPs in anchovies that are eaten without degutting as *Bizree* and inside the spiny oyster which are served in the Lebanese population ; raises the concern on the human consumption of these contaminated seafood.

II. ARTICLE 2: MICROPLASTICS POLLUTION ALONG THE LEBANESE COAST (EASTERN MEDITERRANEAN BASIN): OCCURRENCE IN SURFACE WATER, SEDIMENTS AND BIOTA SAMPLES

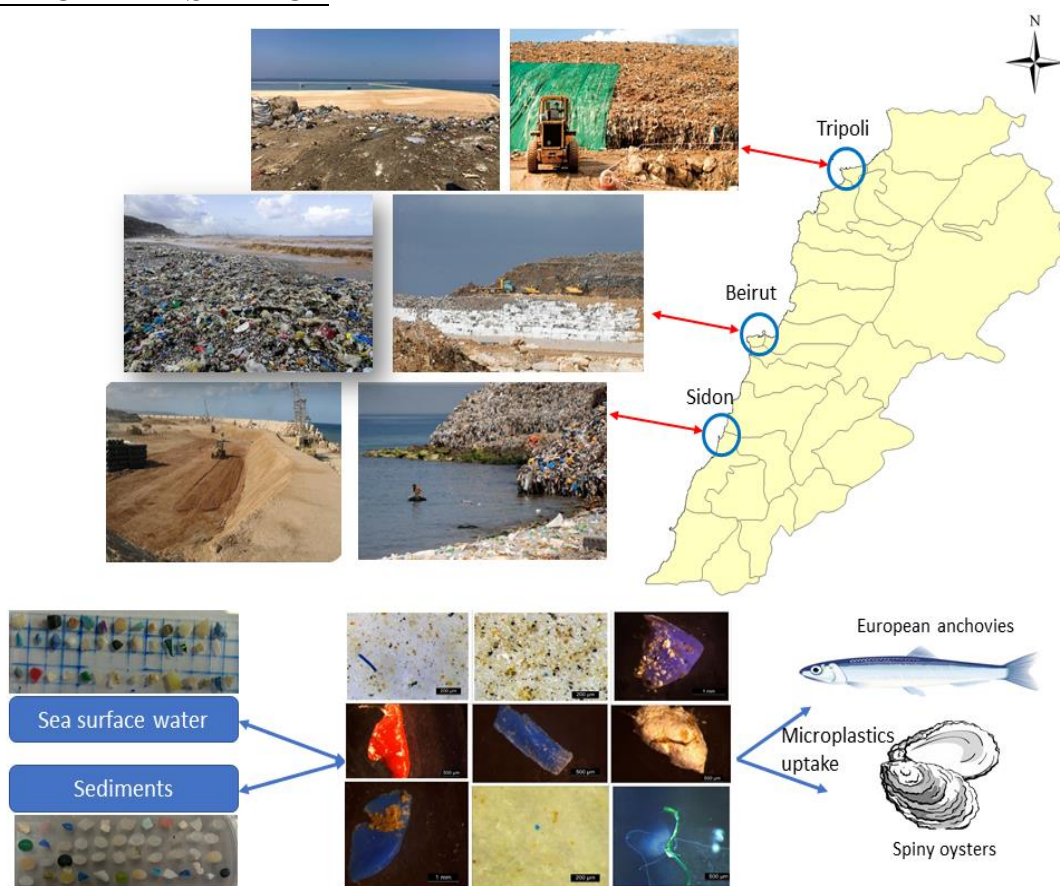
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Published in "Science of the Total Environment", 696 (2019), 133933

GRAPHICAL ABSTRACT



ABSTRACT

The Mediterranean Sea is the largest semi-enclosed sea and one of the worst affected regional seas with sub-basin scale heterogeneity in plastics concentration. Few studies on microplastics (MPs) pollution have been conducted in the Eastern part of the Mediterranean basin. This study aims to evaluate, for the first time, the MPs pollution of the Lebanese coast (Levantine Basin) as well as the most common polymers found, and to assess the potential role of coastal landfills in this pollution. Two important seafood species that are wholly consumed by the Lebanese community: the European anchovy, *Engraulis encrasicolus*, and the spiny oysters, *Spondylus spinosus*, were sampled in three different sites englobing the littoral (Tripoli, Beirut and Sidon). Sea water and sediment samples were also collected from the same sites. Results showed different patterns of MPs concentration in the analyzed matrices: Sidon water sample was highly contaminated in MPs (6.7 MPs/m^3) while Tripoli had the highest MPs in sediments (4.68 MPs/g). The occurrence of MPs in the biota was high (83.4% and 86.3% in anchovies and spiny oysters, respectively). Both anchovies and oysters from Beirut region had the highest ingested MPs/individual (2.9 ± 1.9 and $8.3 \pm 4.4 \text{ MPs/individual}$, respectively). This study is the first that investigated microplastics ingestion by *Spondylus spinosus* while indicating the most common polymers found in the three matrices (water, sediments and biota) in the Eastern Mediterranean. These results highlighted the high MPs pollution found in the Levantine Basin in comparison to other Western Mediterranean regions. In addition, the obtained results indicate a potential contribution of coastal landfills to this pollution.

Keywords: Microplastics; Levantine Basin; Coastal landfill; Filter-feeders; Micro-Raman

1. INTRODUCTION

The world plastic production is continuously increasing, reaching 350 million tons in 2017 (PlasticsEurope, 2018). Among the produced plastics, only 6–26% are recycled. Landfills receive 21–42% of wastes, whereas the rest are wildly released into the environment due to mismanagement through a variety of pathways (Ellen MacArthur Foundation, 2016; O'Connor et al., 2016). Most of the manufactured plastics will eventually end up in the ocean. By the year 2025, about 250 million tons of wastes are expected to enter the ocean water (Jambeck et al., 2015) leading to their accumulation in the marine environment (Thompson et al., 2004; UNEP, 2014). Plastic pollution in the marine environment is becoming a problem of global concern. These plastics are capable of transforming into dangerous small particles known as microplastics (MPs) existing as particles of a size below 5 mm ubiquitously available in the aquatic environments (Thompson et al., 2004; Andrady, 2011). They are either intentionally produced in small sizes such as polystyrene beads for example, or they are the resultant of macroplastics degradation (whether by photochemical reaction, or mechanical actions).

The Mediterranean Sea is prone to several anthropic pressures and has recently been described as one of the most affected marine environments with regards to marine litter and microplastics (Cincinelli et al., 2019; Lebreton et al., 2012). Mainly owing to the semi-enclosed situation and limited outflow of surface waters, a densely populated coastline and intensive fishing, shipping, touristic and industrial activities, substantial amounts of marine litter are accumulating in the Mediterranean Basin. Also, most studies on microplastics have been conducted in the northwestern Mediterranean Sea (e.g. Collignon et al., 2014; Fossi et al., 2016; Lefebvre et al., 2019; Ruiz-Orejón et al., 2016; Schmidt et al., 2018). Results showed that their

distribution and composition are not homogeneous and geographical differences exist between Mediterranean sub-basins (Cincinelli et al., 2019; Suaria et al., 2016). Such heterogeneity in MPs distribution and of its concentration levels are likely reflecting a complex interplay between pollution sources, hydrodynamic features such as currents, up and down-welling, gyres, fronts, and the shape of the coastline (Suaria et al., 2016; Schmidt et al., 2018; Cincinelli et al., 2019). In addition, the absence of a standardized sampling protocol for the identification and quantification levels of MPs could also be responsible for this heterogeneity in results.

Plastics in general, and microplastics specifically, are prone to be ingested by a wide range of organisms (Lusher et al., 2016) and about one hundred species in the Mediterranean have been affected either via ingestion or entanglement (Deudero and Alomar, 2015). Microplastics were already found in several Mediterranean fish species such as *Boops boops*, *Sardina pilchardus*, *Mullus barbatus* and *Merluccius merluccius* (Bellas et al., 2016; Giani et al., 2019; Rios-Fuster et al., 2019) and in wild mussels (*Mytilus galloprovincialis*) caught off the Adriatic Sea and central Tyrrhenian Sea (Gomiero et al., 2019; Renzi et al., 2018c).

Although it is suggested that it is the most polluted region, few studies have been conducted in the Eastern part of the Mediterranean Basin (Gündoğdu and Çevik, 2017; Güven et al., 2017; van der Hal et al., 2017). In the Levantine basin, the Lebanese coast is highly affected by numerous anthropogenic pressures such as harbor activities, fishing, domestic and industrial wastewater discharges (generally without prior treatment). Waste production in Lebanon has increased significantly during the last decade and the country has experienced waste crises. The regulated dumping sites could no longer manage the tremendous quantity of municipal garbage

generated and, therefore, numerous unregulated and open dumping sites were created throughout the country mainly along the coast (Khalil et al., 2018). The average composition of municipal solid waste generated shows an important percentage of plastics (15%) which is two to three times higher than that found in Sweden, France and the United States (El-Hoz, 2019). Though the processes may be slow, landfills are affected by UV radiation and mechanical abrasions leading to the fragmentation of plastics to microplastics, and small plastic debris would find their way into the aquatic environment via leachates discharges (Alimi et al., 2018).

Despite these concerns, there have been no studies specifically examining the occurrence and abundance of microplastics along the Lebanese coastal environment. The aim of this study was to investigate the quantity and types of microplastics found in three matrices (sea water, sediments and marine biota) along the Lebanese coast. All suspected MPs were characterized by size, color and identified according to their polymer nature using micro-Raman spectroscopy.

Fish and bivalves are known to ingest microplastics (Collard et al., 2015; Kazour et al., 2019b; Phuong et al., 2018c), making them a potential vector of toxic chemicals through food chains and into human diets. In the present study we analyzed two marine species that are wholly consumed by the Lebanese community: one pelagic fish species: the European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758) and a bivalve species, the spiny oyster, *Spondylus spinosus* (Schreibers, 1793).

2. MATERIALS AND METHODS

2.1 STUDY AND SAMPLING AREA

Lebanon is located in the Eastern Mediterranean (Levantine Basin) with a coastal length of 220 km (Fig. 1). Three sampling sites were chosen along the coast according to their degree of

anthropogenic pressures making them pollution hotspots (Shaban, 2008). The first site, Tripoli, located in the north, is facing the town's public beaches, characterized by the presence of a fishing harbor, and the existence of a coastal landfill. This latter was constructed in the year 1999 and reached today 36 m height receiving about 400 tons of wastes/day. The second site Beirut, located in the middle, is the country's capital. This site is near the most important harbor in Lebanon and surrounded by three coastal landfills: One closed and two active landfills that reached their maximum capacities (55 m of height) in July 2019. The third site Sidon, located in the south, is facing a river's mouth and is near a closed landfill operated between 1982 and 2013. With a height of 58 m, this landfill was the biggest in the country receiving 300 tons of wastes/day. Actually, its transformation to a public park led to the burying of all wastes underground; traces of these wastes can always be observed underwater. These landfills lack an appropriate leachates treatment or it is not operational, and leachates are directly dumped to the sea. The sampling for all three matrices was done in May 2018 (spring).

2.2 CONTAMINATION CONTROL

Several precautions were followed to minimize air-born contamination during sampling, samples preparation and analysis. Cotton lab coats were worn and different procedures (filtration, measurements and digestion) were done under a laminar flow hood. All surfaces and equipment were rinsed before usage with filtered ethanol 70% and distilled water. Laboratory supplies were made of glass (bottles, Petri dishes, Erlenmeyers, filtration system) and they were covered immediately after manipulation. All solutions (distilled water, Ethanol 70%, potassium hydroxide KOH 10% and zinc chloride ZnCl₂) were filtered 3 times on glass fiber filters (GF/A filters, 1.6 µm porosity, Whatman, France) to eliminate unwanted particles or fibers.

Controls were made to follow up the contamination occurring with every step of preparation and observation.



Fig. 1. Localization of the sampling sites (Tripoli, Beirut and Sidon) along the Lebanese coast.

2.3 WATER AND SEDIMENTS SAMPLING AND ANALYSES

Sea surface water and sediments samples were collected during spring 2018. Sea surface water was collected using a manta trawl net (52 μ m mesh size) with an opening of 0.63 m and a length of 0.255 m equipped with a flowmeter.

The net was trawled for 10 min parallel to the coast at about 500 m from the coast with a small artisanal vessel (3 m of length) at a speed of 2 knots. On average, 100 m³ of sea water was sampled at each site (Supplementary Table 1). The net was washed with filtered distilled water and all collected residues were rinsed inside clean glass bottles. The collected water was filtered on a stainless-steel sieve of a mesh size of 850 μ m and the recovered water was vacuum filtered on

GF/A filters. The suspected MPs present on the sieve were sorted visually under the stereomicroscope, fixed on a microscopic slide and conserved inside a clean glass Petri dish.

The first two centimeters of sublittoral sediments were sampled using a steel ring with two stainless-steel plates, rinsed with filtered distilled water before usage. The samples were put inside clean glass jars and stored at -4°C . Samples were thawed at room temperature 12 h prior to analysis. Then particles with a size range of 1–5 mm were visually sorted and the remaining sediments underwent a density separation using filtered ZnCl_2 ($d = 1.8 \text{ g/cm}^3$) (Imhof et al., 2013; Nuelle et al., 2014). Briefly, 50 mL of filtered ZnCl_2 was added to the sample; the solution was then shaken for 5 min twice (once every 4 h) and let rest for 16 h. The supernatant was then recovered and filtered on GF/A filters. Microplastics found were reported per gram of sediments sample and per m^3 in sea water samples.

2.4 BIOTA SAMPLES AND ANALYSES

Anchovies and spiny oysters were also collected during spring 2018. Anchovies were caught using purse seine nets. Ten individuals of approximately the same size ($9 \text{ cm} \pm 0.9$) were chosen from each sampling site and were immediately individually conserved in aluminum foil and conserved at -4°C . In the laboratory, their morphometric characteristics (total length and weight, digestive tracts weight) were measured and their digestive tracts were dissected, conserved in aluminum foil and frozen at -4°C . For the spiny oysters, ten individuals were collected by fishermen from each sampling site. In the laboratory, the whole individual was removed from its shell and was conserved in aluminum foil. The net weight of the animal ranged between 4.8 g to 38.2 g-with an average of $20.2 \text{ g} \pm 9.4$.

All samples were defrosted 12 h prior to digestion. Fish digestive tracts and oyster soft tissues were digested using potassium hydroxide KOH 10% (Dehaut et al., 2016). In short, the organic material of each individual was put inside separate Erlenmeyer containing KOH 10% solution and then they were incubated at 60°C for 24 h. Then, the digestate was recovered and vacuum filtered on GF/A filters and put inside clean glass Petri dishes.

2.5 STEREOMICROSCOPE AND MICRO-RAMAN SPECTROSCOPY

All samples were first observed under a Leica M165 C Stereomicroscope with a $120\times$ magnification. Photos of items were captured using a Leica M170 HD camera connected to LAS (Leica Application Suite) software. They were listed, measured, and their morphology, color and shape were noted. Several criteria were taken into consideration while counting the suspected items: (1) absence of cellular or organic structures; (2) a homogenous thickness across the particles; and, (3) homogenous colors (Hidalgo-Ruz et al. 2012). Suspected microplastics were divided into 6 size classes: $<200 \mu\text{m}$, $200\text{--}400 \mu\text{m}$, $400\text{--}600 \mu\text{m}$, $600\text{--}800 \mu\text{m}$, $800\text{--}1000 \mu\text{m}$, and $>1000 \mu\text{m}$.

Suspected microplastics were analyzed using Micro-Raman Xplora Plus (HORIBA Scientific® France). This machine is equipped with a closing chamber that isolates the filter from its surrounding, hence minimizing any airborne contamination.

Due to the high amount of time it takes when analyzing filters under micro-Raman, five filters of anchovies and five filters of spiny oysters from each site containing the highest number of potential MPs were identified. Subsamples of sediments and water were analyzed in a way that 25% of particles were analyzed. For MPs polymers identification, two lasers were used with a wavelength of 532 nm and 785 nm with a

range of 200–3400 cm⁻¹. Two objectives (Olympus, Rungis, France) were used: ×10 and ×100. Filters were either analyzed manually or using ParticleFinder module for LabSpec 6. Each particle spectrum is compared to a database polymer identification software (KnowItAll, BioRad®) and a personal library made with specific polymers obtained from Goodfellow (France).

2.6 DATA AND STATISTICAL ANALYSIS

Anchovy Fulton's K condition index was calculated as an indicator of the fish general well-being: $K = (\text{Body weight} / \text{total length}^3) * 100$. The feeding intensity for the fish was calculated using the Gastro Somatic Index (GaSI) (Desai 1970): $\text{GasI} = \text{stomach weight} / (\text{body weight} - \text{stomach weight})$. The mean (\pm SD) abundances of microplastics were calculated for anchovies (MPs/ind) and spiny oysters (MPs/g). After a Shapiro-Wilk test, data distribution of ingested MPs were non normal and a non-parametric tests (Kruskal-Wallis and Mann-Whitney U tests) were used to determine differences in microplastic numbers among species and sites. GaSI values followed a normal distribution and a one way analysis of variance (ANOVA) test with a post-hoc Tukey test was used. Spearman's rank correlation was used to test any correlation between the number of ingested MPs and the fish condition index or GasI. Non-metric Multidimensional Scaling (n-MDS) was performed on a Bray-Curtis similarity matrix created from transformed data (square root). This test was used as an ordination method alongside ANOSIM and SIMPER tests to compare the contribution of each observed polymers in sea water, sediments and biota to the similarity and dissimilarity between sites. Significance level was set at $P < 0.05$. All statistical tests were done using SPSS software (IBM SPSS STATISTICS 20) and PRIMER v7.0 (Primer-E Ltd., Plymouth Marine Laboratory, UK).

3. RESULTS AND DISCUSSION

3.1 MICROPLASTICS CONCENTRATIONS IN SEA SURFACE WATERS AND SEDIMENTS

Microplastics found along the Lebanese coastal waters consisted mainly of fragments (77.5%), fibers (15.25%) and microbeads (7.25%) (Fig. 2-A). For all sites, blue and red colors were the most abundant representing 70% of all MPs. Concentration in the surface waters (4.3 MPs/m³) were high compared to other Mediterranean regions and particularly the western part where MPs concentrations vary between 0.17 and 0.62 MPs/m³ (Table 1). In the Eastern Mediterranean, the MPs concentration fluctuates importantly across regions, ranging from 0.7 items/m³ in Northeast Levantine coast of Turkey (Güven et al., 2017) to 7.68 ± 2.38 MP/m³ in the Israeli surface water (van der Hal et al., 2017). The heterogeneity of the MPs distribution and of its concentration levels could be related to several factors. The mesh size of the net used could play an important role on the number of microplastics caught during sampling. The high concentration of MPs found in the present study maybe due to the low mesh size manta trawl net used (52 μ m) compared to other studies (generally between 200 and 500 μ m). Also, the different methodological analyses and the lack of a standard protocol could also be the cause of that difference. In fact, the water samples in this study were filtered on GF/A filters and all suspected items were observed; whereas other studies used organic material digestion (Gündoğdu and Çevik, 2017; Güven et al., 2017) or just visual observation (Fossi et al., 2016; Lefebvre et al., 2019) which can lead to an underestimation and a bias in microplastics counting. In addition, the distance of the sampling location from the coast may also influence the concentration of MPs

found. Our samples were collected in the coastal waters (about 500 m from the shore). MPs concentration might have differed if the collected samples came from the open sea (van der Hal et al., 2017; Pedrotti et al., 2016). On the other hand, the influence of hydrodynamic features such as currents, up and down-welling, gyres and fronts could also be responsible for this

heterogeneity in MPs concentrations. The Eastern Mediterranean has been reported for its high contamination of floating plastic debris with a dominance of small fragments <2.5 cm in comparison to the Western part (Cózar et al., 2015).

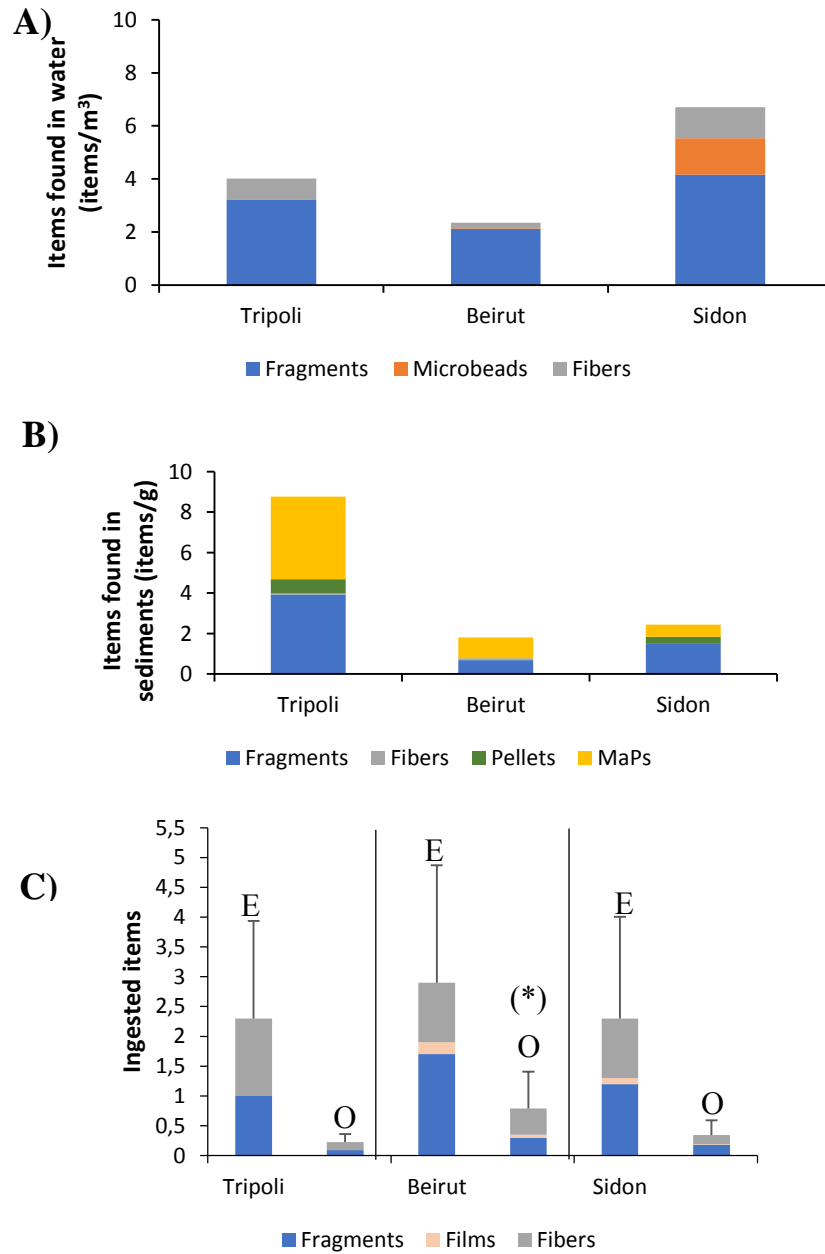


Fig. 2. A) Concentration of suspected microplastics (MPs/m³) according to their shapes found in the surface water; B) suspected microplastics (MPs) and macroplastics (MaPs) concentration (items/g) found in one gram of sublittoral sediments; C) average number (\pm SD) of ingested suspected microplastics by shape categories in the digestive tract of (E) European anchovies (MP/ind) and in the soft tissues of (O) spiny oysters (MP/g) at the three sampling sites (* indicate statistically significant difference).

High fluxes of plastic debris (10–30 kg/km·day) were observed along the Levantine Basin coast from Egypt, Lebanon, Syria and Turkey (Constantino et al., 2019). Differences between the two parts of the Mediterranean Sea can be explained by the different circulation patterns: water currents in the Western part promote circulation; whereas currents in the Eastern part are somewhat attractors leading to the accumulation of floating debris (Mansui et al., 2015). In addition, the complexity of circulation with the existence of mesoscale and sub-basin scale eddies in the Levantine basin (most specifically Shikmona anti-cyclonic eddy) may also explain the high number of MPs found on the Lebanese coast (Menna et al., 2012). High concentrations of MPs in sediments were also found along the Lebanese coast (2433 ± 2000 MPs/Kg d.w.). These concentrations were higher than those found in others Mediterranean regions (Supplementary Table 2). This important difference can be either caused by the different density separation solution used ($ZnCl_2$ used in our study is of higher density than NaCl used in other studies and more microplastics can be identified) or by the mismanaged waste along the Lebanese coast where high plastic items were visually observed during sampling. Both micro and macroplastics (MaPs) were present in all collected sediments samples. Lebanon is a potential landfall of plastics along with other Eastern Mediterranean regions (Duncan et al., 2018) explaining the high abundance of macroplastics found in our samples (consisting of half of the observed anthropogenic materials found). All kind of shapes were found: square, rectangular, spherical and cylindrical (Fig. 3). White was the dominant color in Sidon and Tripoli (46.3% and 36.8%, respectively); blue color was the most abundant in Beirut (23.7%). Pellets were highly abundant in two of our sampling sites (Tripoli and Sidon) which explain the high abundance of white color in these two locations. Several studies investigating MPs abundance in the Mediterranean sediments

also reported the high presence of plastic pellets among their samples (Fanini and Bozzeda, 2018; Kaberi et al., 2013; Karkanorachaki et al., 2018; Turner and Holmes, 2011). The first signaled pellets on the Lebanese coast were due to direct waste discharge of plastic factories on the beaches or in the rivers (Shiber, 1979). To this day, and with the lack of an appropriate treatment, these industries are still releasing their wastes into the aquatic system and are susceptible to unintentional losses leading to this high accumulation of plastic pellets. All of which indicate their persistence whether in the Eastern or Western Mediterranean and they (pellets and fragments) could consist a potential harm to the benthic organisms inhabiting sandy beaches (Carson et al., 2011; Nelms et al., 2016).

Along the Lebanese coast, inter-site differences in MPs concentrations were observed. Surface water samples showed that Sidon was the most contaminated site with 6.7 MPs/ m^3 (Fig. 2-A). This site is characterized by a high presence of microbeads with 1.16 microbeads/ m^3 . Beirut had the lowest concentration of MPs with 2.35 MPs/ m^3 consisting mainly (90%) of fragments. The high number of MPs and pellets observed in the south of Lebanon can be explained by site's localization near the biggest landfill. Although this landfill is closed since 2013, results of our study provide preliminary evidence that landfill isn't the final sink of plastics, but a potential source of microplastics (He et al., 2019). Fragmentation and release of microplastics is a long-term process. Microplastics inside leachates from active and closed landfills have been identified (He et al., 2019). After the stop of post-closure care, microplastics in leachate would be discharged directly into coastal waters. This can pose serious environmental problems since microplastic is not listed as a pollutant in any country's regulations of landfill. This area is also characterized by the presence of the mouth of a major river, Al-Awali River. During sampling, we observed fragments

and other wastes being brought into the coastal waters via the river runoff. This river extends for 48 km of length and collects all the urban water from nearby villages due to the non-existence of

Table 1. Literature data on microplastics abundance in Mediterranean surface waters and instrumental analysis methods used for MPs detection. To compare with our results, only studies expressing concentrations in m^3 are presented.

Location	Sampling nets	Mesh size (μm)	Identification method	MPs abundances	Reference
NW Mediterranean (the Rhône River and the Têt River)	Manta trawl net	333	Stereomicroscope/FTIR	0.18- 0.19 items/ m^3	(Constant et al., 2018)
NW Mediterranean Sea (Ligurian Sea and Sardinian Sea)	WP2 standard net	200	Stereomicroscope	0.62 ± 2 items/ m^3	(Fossi et al., 2012)
NW Mediterranean Sea (Gulf of Lion)	WP2 plankton net	200	Stereomicroscope/FTIR	0.23 ± 0.20 items/ m^3	(Lefebvre et al., 2019)
NW Mediterranean Sea (Gulf of Asinara and the Sardinian Sea)	Neuston net	200	Stereomicroscope	0.31 ± 1.17 items/ m^3	(Fossi et al., 2016)
NW Mediterranean Sea (North Western of Sardinia; Gulf of Asinara)	WP2 standard ring net	200	Stereomicroscope	0.17 ± 0.32 items/ m^3	(Panti et al., 2015)
NW Mediterranean Sea (Tuscany coastal water)	Manta trawl net	330	Stereomicroscope/FTIR	0.26/ m^3	(Baini et al., 2018)
Central-Western Mediterranean Sea (Sardinian Sea)	Manta trawl net	500	Binocular stereomicroscope	0.15 items/ m^3	(de Lucia et al., 2014)
Eastern Mediterranean (Turkish Mediterranean Coast)	Manta trawl net	333	Microscopic observation/FTIR on 24 items	0.7 items/ m^3	(Güven et al., 2017)
Eastern Mediterranean (Iskenderun Bay)	Manta trawl net	333	Microscopic observation	7.26 items/ m^3	(Gündoğdu, 2017)

Eastern Mediterranean Sea (Iskenderun Bay and Mersin Bay)	Manta Trawl net	333	Microscopic observation	2.73 items /m ³	(Gündoğdu and Çevik, 2017)
South Eastern Mediterranean Sea (Israeli surface waters)	Manta Trawl net	333	Stereomicroscope	7.68 ± 2.38 items/m ³	(van der Hal et al., 2017)
Lebanese coast	Manta Trawl net	52	Stereomicroscope/Raman	4.3 ± 2.2 items/m ³	Present study

sewage treatment system. These microplastics are prone to be washed from surface waters onto beaches and, therefore, integrate within the sediments (Barnes et al., 2009; Poeta et al., 2014). The general circulation along the coast of Lebanon is dominant northwards and the water movements are strongly related to surface currents and seasonal metrological factors (Goedicke, 1972). This northwards movement might explain why MPs and MaPs were the most abundant in sediments samples of Tripoli (4.68 MPs/g and 4.08 MaPs/g, respectively) (Fig. 2-B). The high levels of MPs found in the surface water in Sidon and Beirut might have been carried by surface circulation ending up washed on Tripoli beaches.

3.2 MICROPLASTICS IN ANCHOVIES AND SPINY OYSTERS

Microplastics can be ingested by a large variety of marine species and more specifically by filter-feeders (Van Cauwenberghe and Janssen, 2014; Catarino et al., 2018; Collard et al., 2015) raising the alarm for commercial species that are highly consumed by humans. In our samples, 83.3% of European anchovies analyzed had ingested MPs. Such high level of ingestion is in agreement with what was described for anchovies collected from the Adriatic Sea (90%) (Renzi et al., 2019b). However, in the Western Mediterranean Sea, lower occurrences of MPs were described in anchovy digestive tracts (11 to 40%) (Collard et al., 2015; Compa et al., 2018; Lefebvre et al., 2019) (Table 2). In the present study, anchovies ingested an average of 2.5 ± 0.3 MPs/individual which was higher than that found in anchovies collected on the Northwestern Mediterranean coasts such as Spanish coasts (0.18 ± 0.20 items/individual) (Compa et al., 2018) and Gulf of Lions (0.11 to 0.85 items/individual) (Collard et al., 2015; Lefebvre et al., 2019) but lower than those collected in the Adriatic Sea (6.62 ± 3.67 items/individual) (Renzi et al., 2019a). Anchovies caught in the Western

Mediterranean Sea had lower ingested MPs than those caught in the Eastern and Central Mediterranean confirming the higher pollution of these regions (Table 2). For instance, Japanese anchovies, *Engraulis japonicus* from the heavy polluted area of Tokyo bay displayed the same MPs ingestion (2.30 ± 2.50 MP/ind Tanaka et al., 2013) as in our study.

Three shapes of plastics were found: fragments, films and fibers (Figs. 2–C, Fig. 3). There was a slight dominance of fragments (1.3 ± 0.36 fragments/fish) over of fibers (1.1 ± 0.17 fragment/fish), whereas films had the lowest occurrence (0.1 ± 0.1 films/fish). The items had different colors: black, blue, red, white, transparent, and green. For the three sites, blue fragments were the most abundant representing >50% of the sample; whereas for the fibers, black was the dominant color. This predominance of blue plastic items has been previously reported in fish and bivalves (Compa et al., 2018; Digka et al., 2018; Güven et al., 2017; Romeo et al., 2015).

Along the Lebanese coast, anchovies from Beirut had ingested the highest number of MPs items (2.9 ± 1.9 MPs/individual) and those from Tripoli the lowest number (2.3 ± 1.6 items/individual) (Supplementary Table 3). However, there were no significant differences between the numbers of ingested items at the three analyzed sites ($P > 0.05$). No relationship was highlighted between the concentration of MPs in anchovy digestive tracts and in the surface water as it has been described in the western Mediterranean (Compa et al., 2018; Lefebvre et al., 2019). As a pelagic species, anchovies are a highly moving fish so they potentially do not ingest MPs at the site they were collected, making correlations harder to underline. Gastro-Somatic Index (GaSI) and Fulton condition index were higher in Beirut yet not significantly different (Supplementary Table 3). There was no correlation between GaSI and the number of ingested items ($r = 0.067$;

P > 0.05). Fulton condition index and the number of ingested items showed a weak but not



Fig. 3. Microplastics of different shapes and colors collected along the Lebanese coast in three different matrices: e.g. A) blue microbead and B) green fiber found in the collected surface water, C) Macro and microplastics collected on the 2 mm sieve and D) white and black pellets collected in Tripoli sediments sample, E) blue fiber and F) blue fragment observed on biota filter.

significant correlation ($r=0.316$; $P=0.089>0.05$). In the western Mediterranean Sea, recent studies also revealed that anchovies' MPs ingestion was not related to the body condition (Compa et al., 2018; Lefebvre et al., 2019).

Spiny oyster is a Lessepsian species of high commercial value in Lebanon. The collected individuals had an average of 7.2 ± 1.4 items/individual and 0.45 ± 0.3 items/g ww. To date, this is the first study done on MPs ingestion in *S. spinosus* in the Mediterranean and worldwide. Therefore, our results can only be compared to other oysters or bivalves species. Oysters from the present study had the same average of items found in *Crassostrea gigas* collected from the Atlantic Ocean with an average of 0.47 ± 0.16 particles/g ww (Van Cauwenberghe and Janssen, 2014), yet lower than that found in wild mussels (*Mytilus galloprovincialis*) collected from the central Tyrrhenian Sea (Mediterranean) with an average of 7.2 items/g ww (Renzi et al., 2018c) and from the Adriatic Sea with an average of 1.06–1.3 items/g ww (Gomiero et al., 2019). Our samples were less contaminated than those collected from the Mediterranean which is probably due to the difference in the analyzed species and not the geographical MPs concentration in the environment. Oysters tend to have more flesh than mussels explaining the difference in MPs ingestion per gram of ww. Oysters from all sites had the presence of fragments, films and fibers with 86.3% of analyzed individuals having suspected MPs in their soft tissue (Fig. 2-C). Due to a difference in individual total body soft tissue weight, the number of items was also reported to the number of items per gram. Oysters from Beirut had the highest items concentration with 8.3 ± 4.4 items/individual (or 0.78 ± 0.62 items/g)

out of which fibers constituted >50%. Sidon had the highest number of fragments/individual reaching 4.3 ± 4.4 fragments/individual. Tripoli had the lowest number of items/g with 0.22 ± 0.13 items/g. Beirut was significantly different when comparing to the other sites ($P<0.05$). The observed items had six different colors similar to those found in the anchovies; with blue color being dominant in the oysters' samples.

3.3 SIZE CLASS DISTRIBUTION AND POLYMERS CHARACTERIZATION

In the water samples, the size class <200 μm dominated in Tripoli (48.78%) and Sidon (51.8%); whereas for Beirut, the size class >1000 μm dominated (31.7%) (Fig. 4 - A). Suspected items <1000 μm represented a percentage of 88%, 69.3% and 87% in Tripoli, Beirut and Sidon, respectively. All sediment samples had a high abundance of the size class >1000 μm representing >70% of the samples (Supplementary Fig. 1). For the biota, the smallest size class <200 μm was the most abundant in all sites (Fig. 4 - B and C). For anchovies, the ingested size class 200–400 μm was the second most dominant in Tripoli and Beirut with a percentage of 34%. The size class 600–800 μm was only present in Sidon's anchovies, whereas the size class >1000 μm was absent in Tripoli. These results agree with the feeding ecology of the European anchovy which is a filter-feeder that feeds on planktonic organisms generally <2 mm (Bacha and Amara, 2009). No relationship between the size of the anchovies and the size of the ingested items was observed. Although Beirut's anchovies were significantly ($P<0.05$) bigger than those from the other two sites, they ingested items of a similar size than those ingested in Tripoli and Sidon. This indicates that the size of ingested items may be related to the available particles during feeding rather than the morphometric characteristics of organisms (i.e. mouth opening size). Plastics of

all size classes were present inside the oyster's body soft tissue. The smallest size class (0–200 μm) was the most abundant for all sites.

Table 2. Average (\pm SD) of ingested items found in the digestive track of European anchovies (*Engraulis encrasicolus*) collected in different areas of the Mediterranean Sea.

Sites	Digestion method	Concentration (items/ind)	Occurrence	Polymers Identification method	Main polymer	Reference
Spanish Western Mediterranean coasts	None. Visual observation	0.07 (\pm 0.35) to 0.33 (\pm 0.26)	15%	FTIR	PET	(Compa et al., 2018)
Western Mediterranean Sea (Iberian Peninsula coast and the Balearic Islands)	None Visual observation	0.07 \pm 0.26	2.56%	None	-	(Rios-Fuster et al., 2019)
North Western of the Mediterranean Sea (Gulf of Lions)	None. Visual observation	0.11 \pm 0.31	11%	ATR-FTIR	PET	(Lefebvre et al., 2019)
Northwestern Mediterranean Sea	NaClO	0.85	40%	Raman spectroscopy	PE, PP, and PET	(Collard et al., 2015)
Northern Mediterranean (Adriatic Sea)	H ₂ O ₂	1.25	91%	μ -FT-IR	PVC	(Renzi et al., 2019b)
Eastern Mediterranean Sea (Lebanese coast)	KOH 10%	2.5 \pm 0.3	83.4%	Raman Spectroscopy	PS	Present study

The two size classes: 200-400 μm and $>1000 \mu\text{m}$ were the second most abundant between the ingested MPs. Even though there was a significant difference in spiny oyster's net weight ($P < 0.05$) between sites, all MPs items size classes were present in all collected individuals. This suggests that the size of ingested items is not related to the individual weight, but to the available particles existing in the surrounding water. Among the suspected MPs analyzed, 94% were identified as microplastics. Nine types of polymers were identified in our samples (Fig. 5). Six types were common to all three matrices (water, sediments and biota): polypropylene (PP), polyethylene (PE), polystyrene (PS), polyamide (PA), polyethylene terephthalate (PET) and polyurethane (PUR). In addition to these polymers, polyvinyl chloride (PVC) was present in water and sediments; polylactic acid (PLA) was only present in the water whereas acrylonitrile butadiene styrene (ABS) was only present in biota. Each of the three matrices had a specific polymer that dominated over the others: PE was the most abundant in water samples (70.83%, 50% and 47.37% at Tripoli, Beirut and Sidon, respectively). Polyethylene is the most common plastic on a global scale and consequently the most dominant plastic debris in the Mediterranean Sea and worldwide, deriving mainly from plastic bags and bottles (Suaria et al., 2016). It is well documented that plastics such as PE float in the water surface, as its density is lower than this of the water. Polymers with a density higher than that of water (PVC, for example) were observed in the surface water. The existence of polymers denser than seawater have been already reported (Enders et al., 2015; Suaria et al., 2016) with no clear mechanism leading to their existence. During sampling, there was strong waves and the water was agitated which might explain their presence due to the high velocity and turbulent mixing which increases the chances of denser polymers to persist on the sea

surface (Moritomi et al., 1982; Suaria et al., 2016). Polypropylene was the most abundant in sediments (48.11%, 55.88% and 43.33% at Tripoli, Beirut and Sidon, respectively), and polystyrene dominated in the biota specimens (having a percentage equal to or above 25% for anchovies and spiny oysters). Polypropylene was observed in all anchovies and spiny oysters of all sites except in the anchovies of Beirut where PE was observed instead. Polymers distribution didn't have a pattern with shapes or colors. For example, pellets found were mostly white (several were colored) yet their polymer type was of PE, PA, and PP. The same for fragments, different colors were related to different polymers with no similar pattern. Comparison of the polymers composition identified in water, sediments and oysters indicated a dissimilarity between groups (ANOSIM, $R = 0.796$, significance level = 0.2%). Cluster dendrogram led to the existence of two distinct groups (a and b) regrouping on the one hand the water and sediment samples and on the other hand the biota (anchovies and oysters) (Supplementary Fig. 2). Non-metric multidimensional scaling (n-MDS) showed the dimensional distance separating these groups (a and b) based on their polymer composition. Water and sediments formed 'group a' and were characterized by the high presence of PP and PE. Whereas 'group b' composed by biota samples, and were characterized by the high abundance of PS. Similarity of Percentages (SIMPER) indicated that PE, PP, PA and PUR contributed to 72.12% of dissimilarity between these two groups.

3.4 MICROPLASTICS POTENTIAL ENTRY INTO THE MEDITERRANEAN BASIN VIA COASTAL LANDFILLS, AND THEIR FATE AND DISTRIBUTION IN THE COASTAL ENVIRONMENT

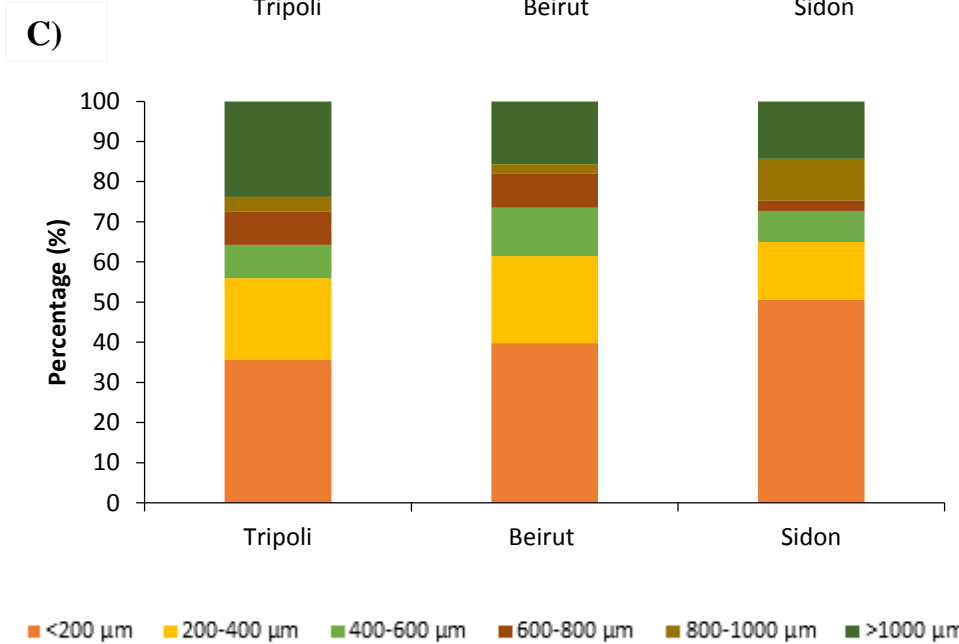
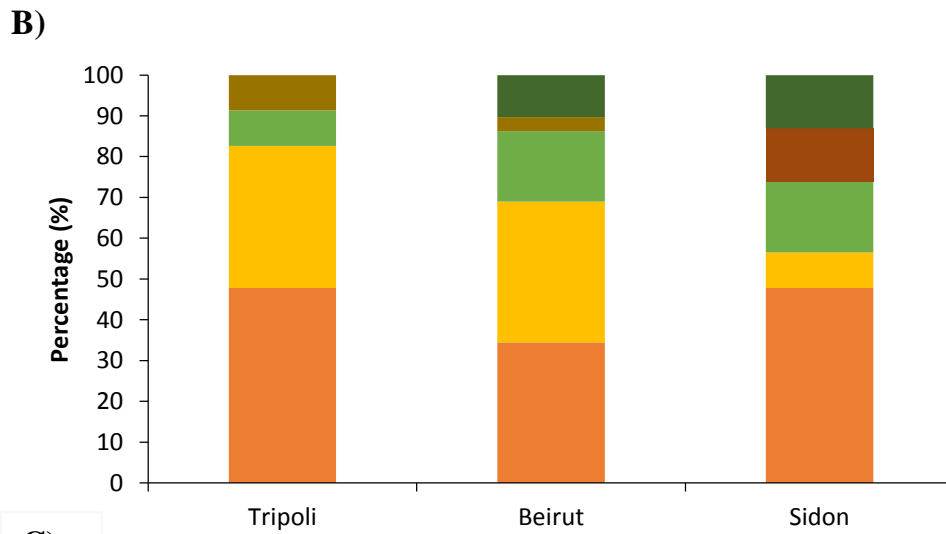
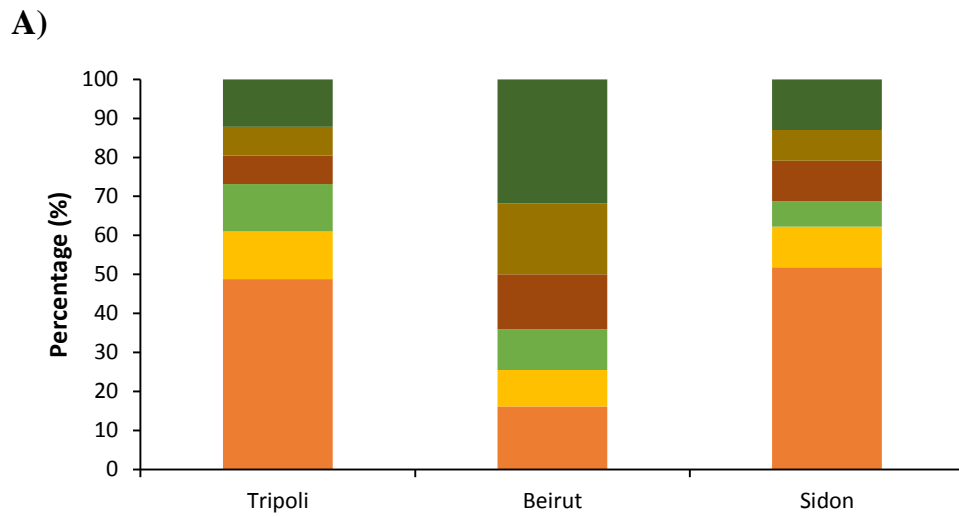


Fig. 4. Proportion of size class composition of suspected microplastics in the A) Water surface, B) anchovies and C) spiny oysters from the different sampling sites

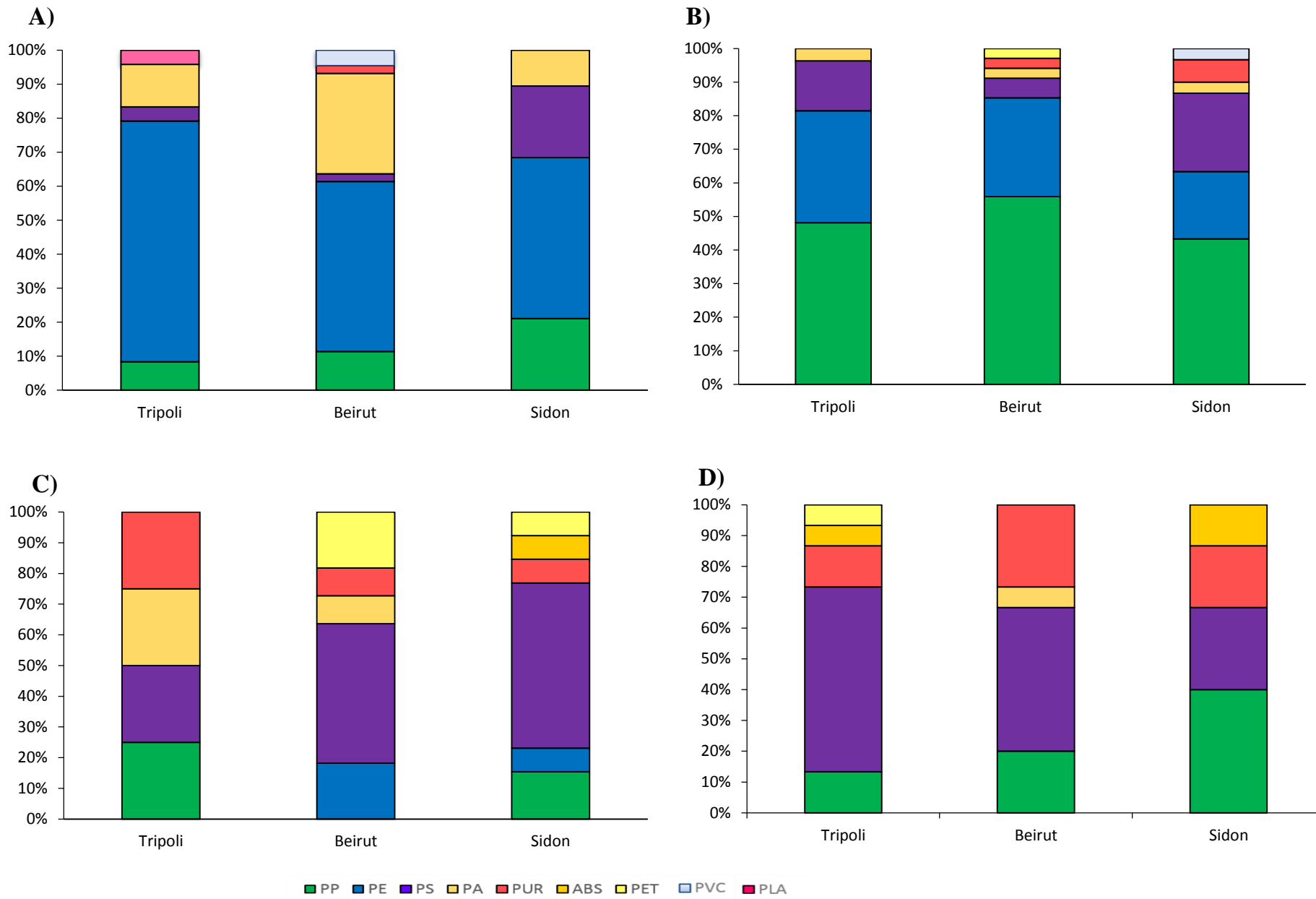


Fig.5. Proportion of different polymers identified by micro-Raman spectroscopy in the three analyzed compartments: A) water; B) Sediments, C) anchovies and (D) spiny oysters.

This study showed a high microplastics pollution in areas located in the vicinity of coastal landfills. Even though some of them are no longer active, but their waste continues to find its way into the sea due to mismanagement and lack of proper treatments. Some of the polymers found in our samples such as polyethylene, polypropylene and polystyrene were observed in transparent, white and different colors. They are widely used in packaging, consumer products, pipes and fitting, and are the common polymers found in landfills and their leachates (He et al., 2019; Kazour et al., 2019b; Praagh et al., 2018). The high abundance of these types of polymers in sea surface water and sediment samples can be traced back to mismanaged landfills along with accidental garbage disposal. Even though Lebanon is considered as a potential sink of plastics in the Eastern Mediterranean (Duncan et al., 2018), it should also be considered as an important microplastics source in the Mediterranean basin. In most studies, whether in the surface water, sediments or biota, fibers were generally the dominant microplastic shape found (Compa et al., 2018; Lefebvre et al., 2019; Renzi et al., 2019a). In the present study, fragments constituted >77%, 90% and 50% of items found in surface water, sediments and biota samples, respectively. The same pattern was found in a study done near an abandoned coastal landfill, where fragments constituted the majority of observed items (Kazour et al., 2019b). Such dominance of fragments in the analyzed samples highlights on the potential role of Lebanese coastal landfills in fragments input into the Mediterranean Basin. Other factors can also lead to this severe microplastics contamination and, most precisely, on fragments abundance on the Lebanese beaches. Munari et al. (2017) indicated a high number of fragments (approximately 90% of the samples) was observed near beaches affected by riverine run-off. All of the sampled Lebanese beaches are affected by riverine runoff as well as

a significant anthropogenic pressure leading to a major microplastics pollution along the beaches (100 times higher than other reported studies, see Supplementary Table 2). These microplastics are prone to re-enter the surface water with waves hitting the shoreline (directed by wind speed and direction) increasing their chance to end up in nearby regions via the northward general water circulation pattern. The above stated factors increase microplastics availability to marine organisms leading to potential human health risks. In Lebanon, an average of 19.7 g of fish and seafood per person is consumed (Nasreddine et al., 2006) making it around 7 kg per caput per annum. Converting the average of ingested MPs/g in oysters to the number of seafood ingested by a Lebanese person, we would have approximately 31,500 MPs ingested per person (4500 MPs/kg of oysters). This is similar to that obtained by Catarino et al. (2018) (13,731 and 68,415 particles/y/person) yet lower than that obtained by Van Cauwenberghe and Janssen (2014) (11, 000 MPs/y/person). European anchovies are eaten wholly by the Lebanese population as *Bizree* (juvenile pelagic fish caught by purse seiners) as well as spiny oysters that are served in various Lebanese restaurants raising the concern on the human consumption of these contaminated seafood.

4. CONCLUSION

These results should be considered as a preliminary analysis on MPs levels recorded off the Lebanese coast in three different matrices. This is the first contribution to characterize the area and to understand the degree of its MPs pollution. More studies are necessary to evaluate the ecological impacts of these MPs on the marine biodiversity in the Levantine Basin. The levels of MPs found were alarmingly high in comparison to other Mediterranean regions. The high number of ingested items by both anchovies and oysters could suggest a potential health concern since these two species are wholly

consumed (without being degutted). This study highlighted the role of landfills in MPs entry into the coastal marine environment. Coastal landfills and mismanaged wastes are important issues existing in 80% of the Mediterranean Basin explaining the elevated number of MPs found in this semi-enclosed basin. The whole Mediterranean should be regarded as an important case study for microplastics pollution where more initiative should be taken to insure an appropriate waste management and, hence, decreasing plastics pollution.

Acknowledgments

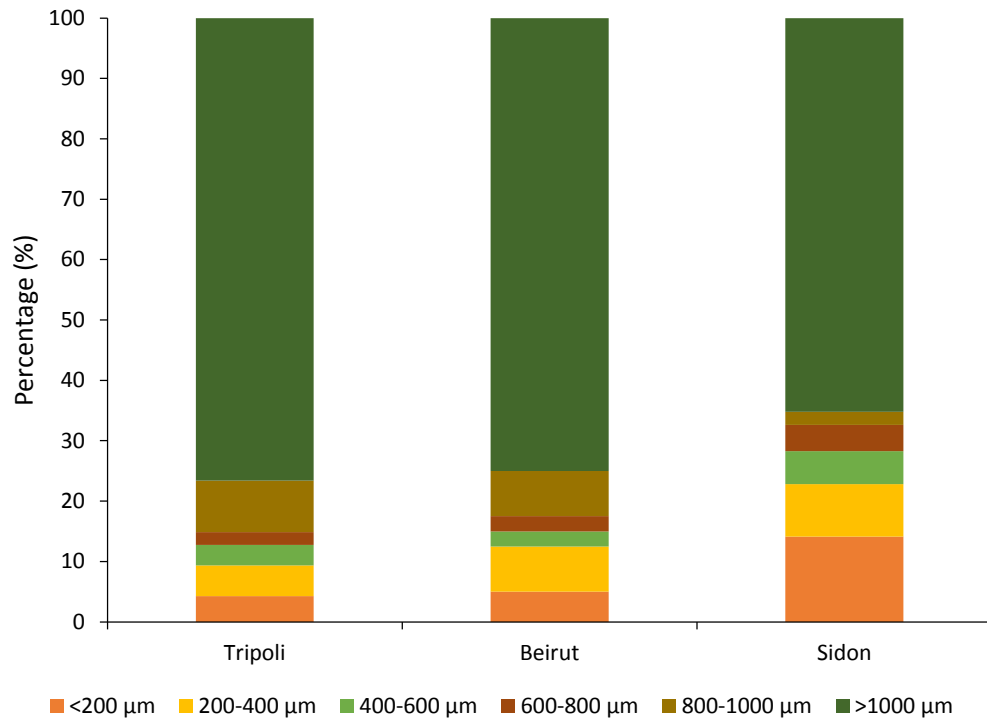
The authors would like to thank G. Duflos, A. Dehaut and L. Hermabessiere from the Agence Nationale de Sécurité Sanitaire de L'alimentation, de L'environnement et du Travail (ANSES) – Boulogne-sur-mer for their help and advices during the sampling preparation. The authors would also like to thank Elie Tarek from the National Center of Marine Sciences - Batroun for helping us acquiring our field samples.

Declaration of competing interest

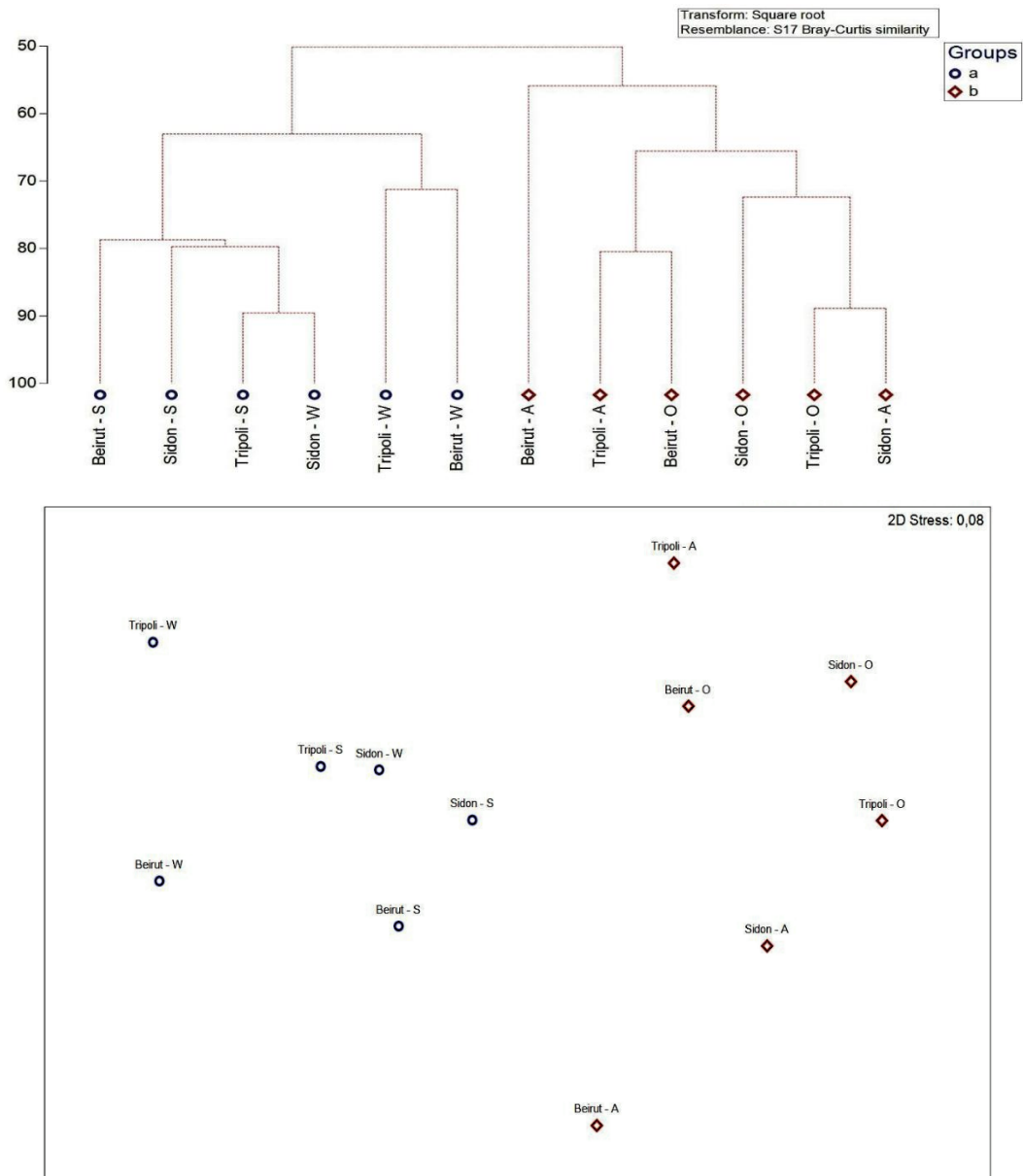
None

SUPPLEMENTARY FILES

Supplementary Figure S1: Proportion of size class composition of suspected microplastics in the sediments from the different sampling sites



Supplementary Figure S2: Cluster dendrogram and Non-metric multidimensional scaling (n-MDS) based on polymer composition of the three compartments (water, sediments and biota) analyzed in all sites. The represented data regrouped the sampling site with its appropriate compartment (W: Water, A: Anchovies, O: Oysters, S: Sediments).



Supplementary Table S1: Length, width, distance and the filtered volume of water sampled in all three sites.

Sites	Length (m)	Width (m)	Distance (m)	Filtered volume (m³)
Tripoli	0.63	0.255	680	101.18
Beirut	0.63	0.255	780	123.31
Sidon	0.63	0.255	580	91.69

Supplementary table 2: Concentration of microplastics found in sediments from different Mediterranean beaches according to the different methodologies and treatments used.

Location	Sample treatment	Concentration	Reference
Mediterranean beaches	Dried at 60°C, sieved (5 mm) Density separation using NaCl	147 ± 14 items/kg d.w (Western Mediterranean) 387 ± 100 items/kg d.w (Eastern Mediterranean)	(Lots et al., 2017)
Gulf of Lion (Northwestern Mediterranean Sea)	Dried at 50°C, sieved Density separation using NaCl	58 - 166 items/kg d.w	(Constant et al., 2019)
Spanish Mediterranean continental shelf (Western Mediterranean)	3 g dried at 70°C Density separation using NaCl	45.9 ± 23.9 items/kg d.w	(Filgueiras et al., 2019)
Gulf of Trieste (North central Mediterranean)	Dried at 60°C, and underwent two-step decantation (NaCl and 250 µm sieve)	Infralittoral: 155.6 items/kg d.w Shoreline: 133.3 items/kg d.w	(Laglbauer et al., 2014)
Coastline of Northern Crete (Central Mediterranean)	Visual separation	4.4 - 1195 pellets/m ² 2.5 - 1197.5 fragments/m ²	(Karkanorachaki et al., 2018)
Eastern Adriatic Sea (Central Mediterranean)	Dried at 60°C, sieved (63 µm) Density separation using NaCl	32.3 - 377.8 items/kg d.w	(Blašković et al., 2017)
Aeolian Archipelago (central Mediterranean Sea, south Tyrrhenian Sea)	Dried at 60°C, sieved (63 µm) Density separation using NaCl	151 - 678.7 items/kg d.w	(Fastelli et al., 2016)
The coasts of south Tuscany (central Mediterranean, Tyrrhenian Sea)	Dried at 40°C sieved (4-2-1 mm and 63 µm) Density separation using NaCl	62 - 466 items/kg d.w	(Cannas et al., 2017)
Northern Adriatic Sea from Caorle (Italy) to the Slovenian coasts (Central Mediterranean)	Density separation using NaCl	137 – 703 items/kg d.w	(Renzi et al., 2018a)
Salina Island (central Mediterranean)	Dried at 40°C sieved (4-2-1 mm and 63 µm) Density separation	99 - 431 items/kg d.w.	(Renzi et al., 2018b)

Sea, south Tyrrhenian Sea)	using NaCl		
Silba and Grebena Islands (Central Adriatic Sea)	Dried at 40°C sieved (4-2-1 mm and 63 µm) Density separation using NaCl	180 –526.7 items/kg d.w. (Silba Island) 273.3–360.0 items/kg d.w. (Grebena Island)	(Renzi et al., 2019a)
Island of Malta (Central Mediterranean)	Visual observation, pellets were only considered	<1 to 167 pellets/m ²	(Turner and Holmes, 2011)
Kokkini Chani sandy beach (Northeastern of Crete; Central Mediterranean)	Visual observation	7112 pellets	(Fanini and Bozzeda, 2018)
Northern Crete (Central Mediterranean)	Fluidization and flotation stages (Nuelle et al., 2014)	5 ± 5 items/kg d.w to 85 ± 141 items/kg d.w	(Piperagkas et al., 2019)
Tunisian Mediterranean coast (Central Mediterranean)	Samples were dried and underwent a density separation using NaCl	141.20 ± 25.98 and 380 items/kg d.w (in two sites along the Tunisian Mediterranean coast)	(Abidli et al., 2018)
The shoreline of Kea Island (Aegean Sea, Eastern Mediterranean)	Visual separation	275.75 ± 311 items/m ² (between 4 and 2 mm) 330 ± 403 items/m ² (between 2 and 1 mm)	(Kaberi et al., 2013)
Eastern Mediterranean	Dried at 60°C, sieved (2 mm) and underwent a density separation using ZnCl ₂	2433 ± 2000 items/Kg d.w	Present study

Supplementary Table S3: Average (\pm SD) Fulton’s condition index (K), Gastro Somatic Index (GaSI), and the numbers of ingested items for the European anchovies (*Engraulis encrasicolus*) in all sites.

	Site	Condition index		GaSI (%)		items/individual	
		Mean	SD	Mean	SD	Mean	SD
<i>Engraulis encrasicolus</i>	Tripoli	0,56	0,03	5,73	2,11	2,30	1,64
	Beirut	0,68	0,03	6,32	2,66	2,90	1,97
	Sidon	0,61	0,04	6,06	2,02	2,30	1,70
	Mean	0,61	0,06	6,03	0,29	2,50	0,35

CHAPTER 4: ACTIVE BIOMONITORING AS A TOOL FOR MICROPLASTICS ASSESSMENT

This chapter focuses on active biomonitoring experiments for MPs monitoring which will be described in **Article 3, Article 4 and Article 5**. This technique was tested in estuaries and coastal water. Since fish are organisms of constant movement and their MPs analysis would not truly reflect that of the sampling site, caging experiments were conducted on juvenile estuarine fish and the results are described in **Article 3**. Whereas both **Article 4 and Article 5** will be an assessment of the blue mussels (*Mytilus edulis*) caging approach and its future use as a uniform active biomonitoring tool for MPs contamination. When possible, native individuals were sampled as a passive biomonitoring but also to have a comparison with the native organisms living in the sampled area.

A. Synthesis of Article 3

Estuaries are among the aquatic environments that are affected by various anthropogenic pressures and are the most modified. Juvenile fish such as European flounder are one of the most important species occupying European estuarine water and has been already suggested as a biomonitoring species chemical contamination. Thus, in this article, the species was selected and prepared for a one-month transplantation experiment. The objective of this approach is to test this method, for the first time, as a tool to quantify and assess MPs contamination in juvenile fish.

Three sites were chosen in the Seine estuary, one site in the Canche and one in the Liane (making a total of five sites). Wild fish were only found in the Canche and Fosse Nord (The Seine estuary). Caged fish had a lower MPs average (1.67 ± 1.43 items/individual) but a higher ingestion percentage (75%) than wild fish (2.04 ± 1.93 items/individual and 58%). There was no correlation between the number of ingested items and the fish condition index nor with the mortality percentage. Fibers (69%) dominated fragments and polyamide (PA), polyethylene terephthalate (PET) and polyurethane (PUR) were the most abundant inside the fish digestive tracts.

The high ingestion of MPs along with an acceptable survival rate of 70%, caged fish are suitable to assess microplastic contamination in aquatic environment. Their important occurrence in digestion tracts raises also the concern on the MPs potential negative effects for fish recruitment success and population renewal.

III. ARTICLE 3: JUVENILE FISH CAGING AS A TOOL FOR ASSESSING MICROPLASTICS CONTAMINATION IN ESTUARINE FISH NURSERY GROUNDS

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Published in "Environmental Science and Pollution Research", (2018), 1-12

ABSTRACT

Estuaries serve as nursery grounds for many marine fish species. However, increasing human activities within estuaries and surrounding areas lead to significant habitat quality degradation for the juveniles. In recent years, plastic pollution has become a global environmental issue as plastic debris are found in all aquatic environments with potential adverse impacts on marine biota. Given the important ecological role of estuaries and implications of microplastics (MP) in ecosystems, here we assess the occurrence, number, size and polymer types of MP ingested by wild and caged juveniles European flounder (*Platichthys flesus*). We deployed caged fish for one month at five sites in three estuaries in the Eastern English Channel. The Seine estuary, heavily impacted by manmade modifications and one of the most contaminated estuaries in Europe, was compared to two smaller estuaries (Canche and Liane) less impacted by industrial activities. We found that juvenile flounders (7- 9 cm) were vulnerable to plastic ingestion. 75% of caged fish and 58% of wild caught fish had the presence of MP items in their digestive tract. Fibers (69%) dominated in the fish's digestive tract at all sites. An average of 2.04 ± 1.93 MP items were ingested by feral juveniles flounder and 1.67 ± 1.43 by caged juveniles flounder. For the caged fish, the three sites impacted by wastewater treatment plant (Liane, Le Havre Harbor and Rouen) were those with the highest percentage of individuals that have ingested MP items. Most of the isolated items were fibers and blue in color. Polymers identified by micro Raman spectroscopy were Polycaprolactam, Polyethylene Terephthalate and Polyurethane. Although other environmental factors may have affected caged fish condition and mortality, we found no significant correlation with the number of ingested MP. However, the high occurrence of MP ingested by juvenile fish on nursery grounds raises concerns on their potential negative effects for fish recruitment success and population renewal. Finally, this study describes, for the first time, the feasibility of using caged juvenile fish as an assessing tool of MP contamination in estuarine nursery grounds.

Keywords: Microplastics; Caging; Juvenile Flounder; Estuaries; Raman Spectroscopy.

1. INTRODUCTION

The occurrence of microplastics (defined as particles <5 mm in their longest size) in aquatic ecosystems (marine and freshwater) is well documented (for review: (Cole et al., 2014; Van Cauwenberghe et al., 2015; Wright et al., 2013). Due to their different densities ranging from 0.9 g/cm³ (Polystyrene and Polypropylene) to 1.39 g/cm³ (Polyethylene terephthalate and Polyvinyl chloride), they are either found at the water surface layer (Ivar Do Sul and Costa, 2014) or sunk to the bottom (Woodall et al., 2014). Therefore, both pelagic (Collard et al. 2015) and benthic species (McGoran et al., 2017) may be affected by these plastic pieces. Many aquatic species have now been reported to ingest plastics from the environment. Microplastics (MP) can enter the food web of aquatic environments via direct or indirect pathways, including inhalation, entanglement, ingestion from incidental capture, being mistaken for food, or by the ingestion of a prey species already containing microplastics (Au et al., 2017; Setälä et al., 2018). Because of their ubiquitous presence, their small size, and the chemical pollutants existing in plastics (such as additives or adsorbed contaminants from the surrounding environment), MP could threaten the health of various organisms (Auta et al., 2017). Indeed, the ingestion of MP may cause both direct physical and toxicological effects. Physical effects include internal abrasions and gut blockages, which may lead to starvation (Wright et al. 2013; Gall and Thompson 2015). Among other potential effects, the ingestion of MP instead of food may lead to a delay in growth (e.g. due to starvation), a decrease in the individual fitness, and even causing death, with likely negative effects on population dynamics (Critchell and Hoogenboom, 2018; Luís et al., 2015; Rochman et al., 2013). In recent years, an increasing number of studies have been carried out to assess the occurrence and effects of MP in marine fish species (e.g., Lusher et al., 2017,

2013; Nadal et al., 2016; Neves et al., 2015). However, few studies have concerned estuarine fish (but see Bessa et al., 2018; Ferreira et al., 2018; McGoran et al., 2017; Vendel et al., 2017). These studies focused mainly on tropical estuaries and on wild-caught adult fish. Estuaries are known as essential fish habitats because they act as nursery grounds for juveniles of various marine fish species, providing refuge, food, and habitat (Beck et al., 2001; Selleslagh and Amara, 2008). Despite their ecological importance, estuaries are amongst the most modified and threatened aquatic environments (Halpern et al., 2008). These areas are exposed to a growing anthropogenic pressure, particularly through acute and chronic pollutions such as industrial and wastewater effluents discharge. Estuarine ecosystems have been identified as microplastics hotspots (Browne et al. 2011, Wright et al. 2013).

In order to compare different sites or estuaries, it is necessary to investigate the same species of the same age range at each site. However, it is almost impossible to find a species that is present in all sites of interest. To cope with this problem, transplant experiments can be conducted (Kerambrun et al., 2011; Oikari, 2006). Caging experiments present many advantages (Oikari 2006) including the selection of well-characterized homogenous organisms (number, age, size, weight, and sex) and the control of their exposure (location, time, and season). In addition, this technique offers advantages over simple field collection of organisms since it is possible to study an impacted zone surrounding a relatively precise outlet discharging pollutants. Such approach was successfully used to monitor microplastics contamination in mussels (Catarino et al. 2018; Railo et al. 2018). To the best of our knowledge, juvenile fish caging, as a tool for assessing estuarine microplastics contamination, has not been investigated before.

The aims of this research were to estimate the occurrence, number, size and polymer types of MP ingested by wild and caged juveniles European flounder (*Platichthys flesus*) and to test the caging method as a tool to quantify and assess MP contamination of juvenile fish. We also tested the hypothesis that ingested plastic adversely affects the condition and survival of caged fish. The European flounder, was selected for the study because it is one of the most important components of the juvenile demersal fish assemblage in European estuarine waters (Selleslagh et al., 2009). This benthic species is commonly used for environmental monitoring studies in northern European waters (e.g. Amara et al., 2009; Marchand et al., 2003).

2. MATERIAL AND METHODS

2.1 STUDY SITES

The study area was located along the French coast of the Eastern English Channel. Three estuaries were investigated: the Liane, Canche and Seine estuaries (Fig 1). Liane and Canche are small estuaries with small freshwater input: 3 and 7 m³.s⁻¹, respectively. The Liane estuary is mainly affected by a municipal wastewater treatment plant (WWTP) that treats the wastewater of ca. 200,000 inhabitants. The Canche estuary is not impacted by any important human activity and is considered as clean estuary (Amara et al. 2009). The Seine estuary, the largest one in the English Channel (150 km² at high tide), displays a strongly urbanized and industrialized basin since it concentrates approximately 40% of the economic activity of France. In spite of significant efforts to restore environmental quality during the past few decades, it remains one of the most chemically polluted estuaries in Western Northern Europe (Dauvin et al., 2007).

2.2 SAMPLING AND CAGING EXPERIMENT

In September 2017, 150 0-group juveniles' flounder (7- 9 cm total length, TL) were collected in the Canche estuary using a small beam trawl. After capture and before deployment in cages, the fish were acclimatized for one week in a 500 liter aquarium supplied with an open seawater circuit and were daily fed on frozen Mysidacea and brine shrimps (*Artemia* sp.).

One day before the caging experiment, flounders were anaesthetized (Eugenol 35 mg/L), weighed (to the nearest 1 mg), measured for total length (within 0.1 mm), and individually marked (Visual Implant Tag, 1.2 mm×2.7 mm, Northwest Marine Technology).

Cage placement was carried out the 12th and 13th of September 2017 at five sites. Three sites were chosen in the Seine estuary: Rouen (49°22.995' N; 01°00.676' E), Le Havre harbor (49° 28. 853' N; 00° 05.590' E) which are both affected by a wastewater treatment plant (Emeraude and Edelweisse, respectively), and Fosse Nord (49°27.328' N; 00°07. 493' E) in the main channel of the estuary. Two other cages were put in the Canche (50°30.982' N; 01°37.852' E) and the Liane estuaries (50°42.08' N; 01°36.59' E). The number of fish placed inside the cages was between 15 and 20 fish per cage. The cages were made of Stainless steel without any plastic material to avoid contamination. Their length was of 1 m, whereas their width and height were of 0.6 m. Their mesh size was 15 mm allowing water circulation and enough space for fish to feed. The cages were fixed to the bottom with a screw anchor secured by scuba-divers at depths varying between 4 to 8 m. Following the one month caging exposure, all fish were rapidly transferred to the laboratory, identified (tag), weighed, and measured. In order to evaluate the potential effect of microplastics contamination on juvenile fish, we calculated the Fulton's K condition index as an indicator of the fish general well-being.

$K = 100 W/L^3$; where (W) is the body mass (mg) and (L) is the total length (mm).

Along with the caging experiment, feral juvenile flounders of the same age (G0) and size (7- 9 cm TL) were collected in September 2017 at two

sites near the caging sites: the Canche and the Seine (Fosse Nord) estuaries in order to compare microplastics contamination between feral and

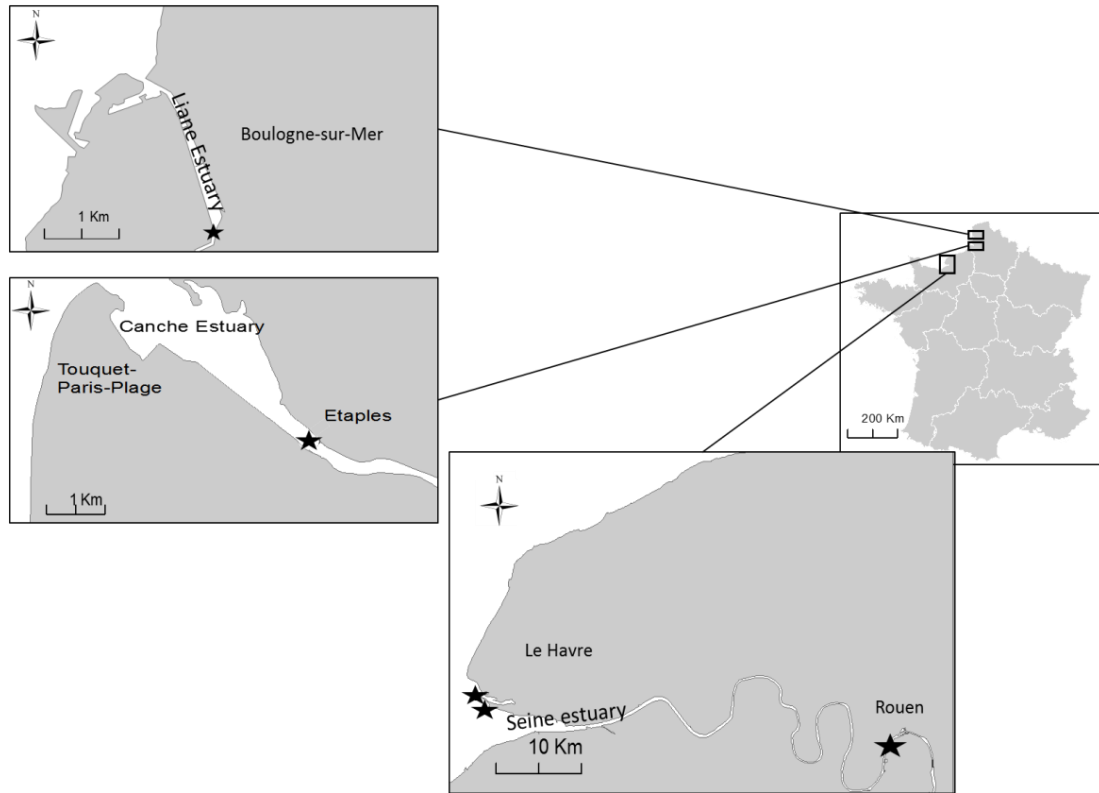


Fig. 1 Sampling and caging sites of juveniles flounder in (1) the Liane, (2) the Canche, and the Seine estuary, (3) Le Havre Harbor, (4) Fosse Nord, and (5) Rouen

caged fish. Although we sampled in all the sites used for the caging experiment, we did not capture flounder at the other three sites.

2.3 MICROPLASTICS ANALYSIS

Flounders were dissected under a laminar flow hood and their digestive tract (stomach and gut) were weighted, preserved in aluminum foil, and conserved at -20°C until analysis. Cotton laboratory coats were worn at all times during samples analysis, dissecting instruments were cleaned with MilliQ water after every dissection, and the usage of plastic material was avoided.

Prior to digestion, digestive tracts were taken out of the freezer and left to thaw. All the following

procedures were done under a laminar flow hood. Solutions used (besides MilliQ water) were filtered three times using glass fiber filters GF/A with a pore size of $1.6\ \mu\text{m}$ (Whatman, France). All materials were cleaned with MilliQ water, filtered ethanol 70%, and MilliQ water, respectively. The digestive tract of each individual was taken and emplaced in an Erlenmeyer with a volume of 100 mL of filtered KOH 10% (m/v, ChimiePlus, France) (Dehaut et al. 2016; Hermsen et al. 2017). With every digested lot constituted of 9 erlenmeyers each one containing one digestive tube; one control was made containing only 100 mL of KOH and has undergone the same digestion conditions as the samples. These Erlenmeyers were put on a

heating magnetic stirrer for 24 hours at 60°C. Then, each solution was vacuum filtered on a 47 mm GF/A filter (Whatman, France). Each filter was put inside a clean glass Petri dish and surrounded with parafilm. Filters remained covered until Raman analysis to avoid prolonged exposure to atmospheric contamination from dust.

2.4 STEREOMICROSCOPE OBSERVATION AND MICRO-RAMAN SPECTROSCOPY ANALYSIS

After digestion, filters were observed under 120x magnification using Leica M165 C Stereomicroscope and images of plastic items recovered were taken with a Leica M170 HD camera and LAS (Leica application suite) software. The filters were methodically examined from left to right along the first row, right to left along the second row and so on, to prevent double-counting of MP. Microplastics were classified according to their physical characteristics into fibers, fragments, and films. They were counted, measured at their longest dimension, and their color was noted. During stereomicroscope inspection, samples remained closed inside the Petri dish. Whereas for Raman analysis, filters were placed inside and the machine was directly closed to avoid airborne contamination.

Five filters containing potential MPs, were randomly selected per site and analyzed using a Micro-Raman Xplora Plus (HORIBA Scientific® France). Each filter corresponds to the digestive tract of an individual fish. Two lasers were used with a wavelength of 532 nm and 785 nm with a range of 200-3500 cm^{-1} . Two objectives (Olympus, Rungis, France) were used: x10 and x100. Filters were either analyzed manually or using ParticleFinder module for LabSpec. This latter is an automated application that locates particles and performs Raman analysis on these particles by moving each particle beneath the

laser spot. Each particle spectrum is compared to Database polymer identification software (KnowItAll, BioRad®) and a personal library made with specific polymers obtained from Goodfellow (France). The identification is considered correct when the HQI (Hit Quality Index) was above 80 (ranging from 0 to 100).

2.5 STATISTICAL ANALYSIS

Data were analysed using XLSTAT software. The conditions for applying parametric tests, i.e. homogeneity of variance and normality, were verified using Fisher and Shapiro-Wilk tests respectively. As result of these tests, non-parametric tests (Kruskal-Wallis (KW) and Mann-Whitney U-test) were used in order to highlight significant differences of MP contamination in flounder caged at different locations and with feral individuals collected at the same site. Differences between groups were considered as significant when $p < 0.05$. The KW test was followed by a post hoc test Multiple Comparisons of p-value (MCP) when it was significant at $p < 0.05$. Data are expressed in mean \pm standard deviation (SD).

3. RESULTS

3.1 CAGING EXPERIMENT

After one-month of exposure, all the cages were recovered. The number of fish in each cage was counted and survival percentage was calculated. The mean percentage of survival was 70.59% and all sites had a high survival rate ($> 70\%$) except for the Canche estuary where the cage was partially silted and, therefore, having the lowest survival percentage of 37% (Fig. 2). The Fulton's K condition factor of each individual flounder analyzed varied between 0.55 and 1.39 $\text{mg}\cdot\text{mm}^{-3}$ (mean value $0.79 \pm 0.11 \text{ mg}\cdot\text{mm}^{-3}$). Individuals from Le Havre Harbor and Fosse Nord had a significantly lower K compared to the Canche and Rouen (Fig. 2).

3.2 MICROPLASTICS OCCURRENCE IN FISH

A total of 86 fish (feral and caged) were analyzed. In all the examined fish, 149 items were identified on the filters using the stereomicroscope as potential MP consisting of 103 fibers, 43 fragments, and 3 films (Fig. 3). Fibers (69%) dominated in fish's digestive tract

whereas films were only observed in feral fish in Fosse Nord and caged fish in Le Havre Harbor with an average number of 0.2 ± 0.42 and 0.083 ± 0.28 , respectively. An average of 75% of caged fish had at least one MP items (fragments, fibers, and films) in their digestive tract (64 caged fish analyzed) with Le Havre harbor having the highest

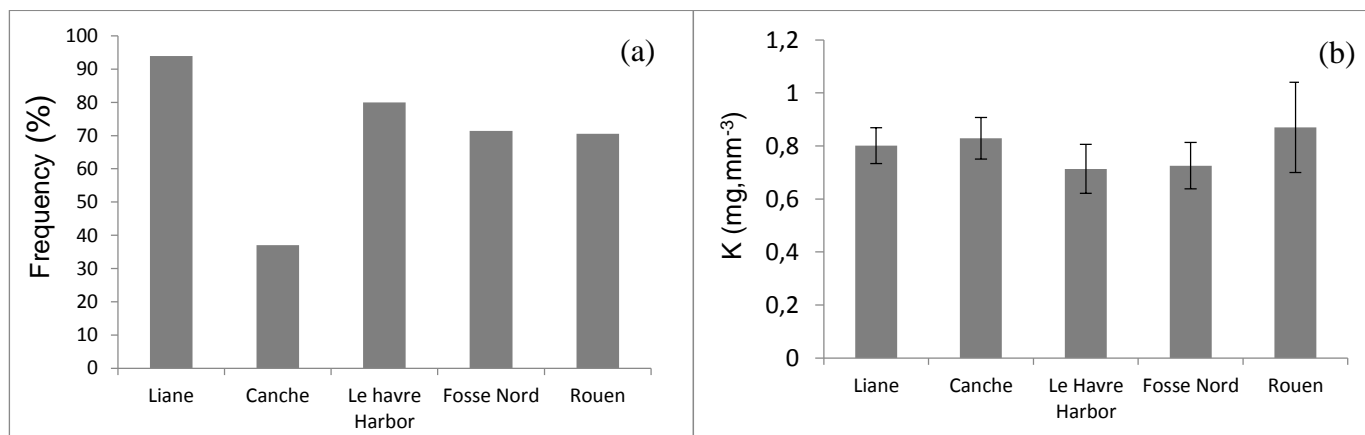


Fig. 2 a Percentage of juvenile flounder following 1-month caging experiment at the different sites and b Fulton K condition factor (mean \pm SD).

percentage of 91.7% (Fig. 4). For the feral fish, Fosse Nord had a higher percentage of contaminated fish (80%) than that of the Canche estuary (36.4%) (Fig. 4). An average of 2.04 ± 1.93 items were ingested by feral juvenile flounder and 1.67 ± 1.43 in caged juvenile flounder (Fig. 5). Although not significantly different (Mann-Whitney U-test, $p=0.097$), the number of MP items in feral fish was higher in the Seine estuary (Fosse Nord) compared to the Canche estuary. For the same site, where both feral and caged fish were analyzed, the number of MP items ingested by feral fish was higher but only significant for Fosse Nord (Mann-Whitney U-test: Fosse Nord $p=0.011$; Canche $p=0.970$) than in caged fish (Fig. 5). For caged fish, the number of ingested items was highest in the Liane (2.47 ± 1.51) and lowest in the Canche (0.90 ± 0.99) and Fosse Nord (0.93 ± 0.70). A significant difference was only observed between the Liane and Fosse Nord (KW test, $p=0.004$). There was no significant correlation between

juvenile fish condition and the number of MP ingested at each site ($p=0.336$). In addition, the mortality rate observed in caged fish at each site is not correlated to the mean number of MP ingested ($p=0.09$). On the contrary, the sites with the lowest mortality (Liane, Le Havre Harbor and Rouen) corresponded to those with the highest number of MP ingested.

3.3 CHARACTERIZATION OF MICROPLASTICS

Color distribution of ingested items was mostly uniform across all analyzed sites, blue MP being the most common (54%), followed by red (21%) and black (13%), while other colors such as pink, white and green were less frequent (Fig. 6a). The size of fibers ranged from $70 \mu\text{m}$ to $4510 \mu\text{m}$ and for the fragments between $5 \mu\text{m}$ and $66 \mu\text{m}$. Fragments, films, and fibers were divided into several size classes: $0-200 \mu\text{m}$, $200-400 \mu\text{m}$, $400-600 \mu\text{m}$, $600-800 \mu\text{m}$, $800-1000 \mu\text{m}$, and $> 1 \text{mm}$. Most of the isolated MP belonged to the smallest ($< 200 \mu\text{m}$) and largest ($> 1 \text{mm}$) size class with

respectively 35.6% and 24.2% while the other size classes had a similar distribution (Fig. 6b). There was no inter-sites difference in ingested item size except for the Canche (for feral and caged fish) where the largest size class dominates.

Five filters were randomly selected from each site and analyzed using μ -Raman to confirm if the particles extracted were plastics by identifying their chemical composition. In the Raman spectrum of fibers, only fluorescence could be observed, although an optimization including the reduction of laser power and bleaching was attempted. In addition, for the colored items, the spectrum was hidden by the additives (dyes) existing on particles. Only 37 fragments were

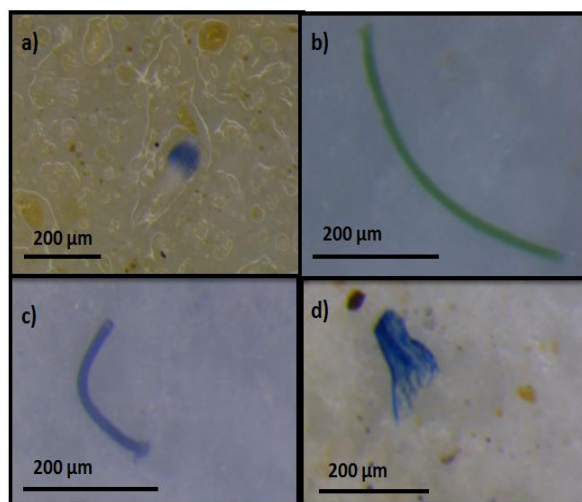


Fig. 3 Examples of microplastics found in the digestive tract of juveniles flounder: a) represents a fragment; b) and c) filaments; and d) films

successfully analyzed with the Raman. Among these fragments, eleven were identified as polymers: Polycaprolactam (PA-6), Polyethylene Terephthalate (PET) and Polyurethane (PUR). For colored particles (blue and green), the observed spectrum was that of the dye. Two corresponding spectra were observed: Copper Phthalocyanine (specific of blue items and the most frequent obtained spectrum) and Hostasol Green G.K (which is characteristic of green items). Fibers were not identified with the Raman due to its delicate procedure when identifying thin and

small fibers; suggesting that microplastics ingestion might have happened in lower proportion than mentioned above.

The spectral range of PA-6, PET, PUR and Copper phthalocyanine are presented in the Supplementary Material: The PA-6 having its characteristic peaks between 900 cm^{-1} and 1500 cm^{-1} , and 2500 cm^{-1} and 3000 cm^{-1} . Whereas for PET, characteristics peaks were between 600 cm^{-1} and 1700 cm^{-1} and 3000 cm^{-1} and 3400 cm^{-1} (decreased trend).

When excluding the colored items, we observed that in wild caught fish from the Canche and Fosse Nord, the MP items were made of Copper Phthalocyanine and PA, respectively. In caged fish, the MP items were made of PA in the Liane and in the Canche. Whereas for the three Seine estuary sites, PET was the predominant polymer (61%) followed by PA and PUR (Fig. 7).

4. DISCUSSION

This research identified and quantified, for the first time, the presence of ingested microplastics in feral and caged juvenile fish ($\leq 9\text{ cm TL}$) from the Eastern English Channel estuaries highlighting their potential negative effects. In this region, estuaries provide nursery areas for a wide variety of marine fish species including commercially important fish such as seabass, sole, plaice, and flounder (Selleslagh et al. 2009). Estuaries are also used by adults as reproduction, migration, and feeding grounds (McLusky and Elliott, 2004). These ecosystems play an important role in maintaining biodiversity and constitute an essential fish habitat supporting future recruitment to adult fish stocks (Beck et al. 2001). However, increasing human activities within estuaries and surrounding areas, lead to a significant habitat loss for the juveniles and a decrease in the quality of the remaining habitats as was reported for the Seine estuary (Courrat et al., 2009; Gilliers et al., 2006).

Several studies have identified the presence of microplastics in the digestive tracts of wild-

caught fish. However, the level of fish contamination in transitional systems such as estuaries is less known. Most of the studies were conducted in tropical estuaries (Bessa et al., 2018; Dantas et al., 2012; Ferreira et al., 2018; Possatto et al., 2011; Ramos et al., 2012; Vendel et al., 2017). Only two studies have been conducted in temperate estuaries: McGoran et al. (2017) in River Thames, UK and Bessa et al.

(2018) in the Mondego estuary (Portugal). We found that estuarine juvenile flounders are vulnerable to plastic ingestion: 75% of caged fish and 58% of wild caught fish had the presence of MP items in their digestive tract. In a recent study, McGoran et al. (2017) found that over 70% of River Thames adults European flounder had ingested plastics. These results are high compared to previously published estimates

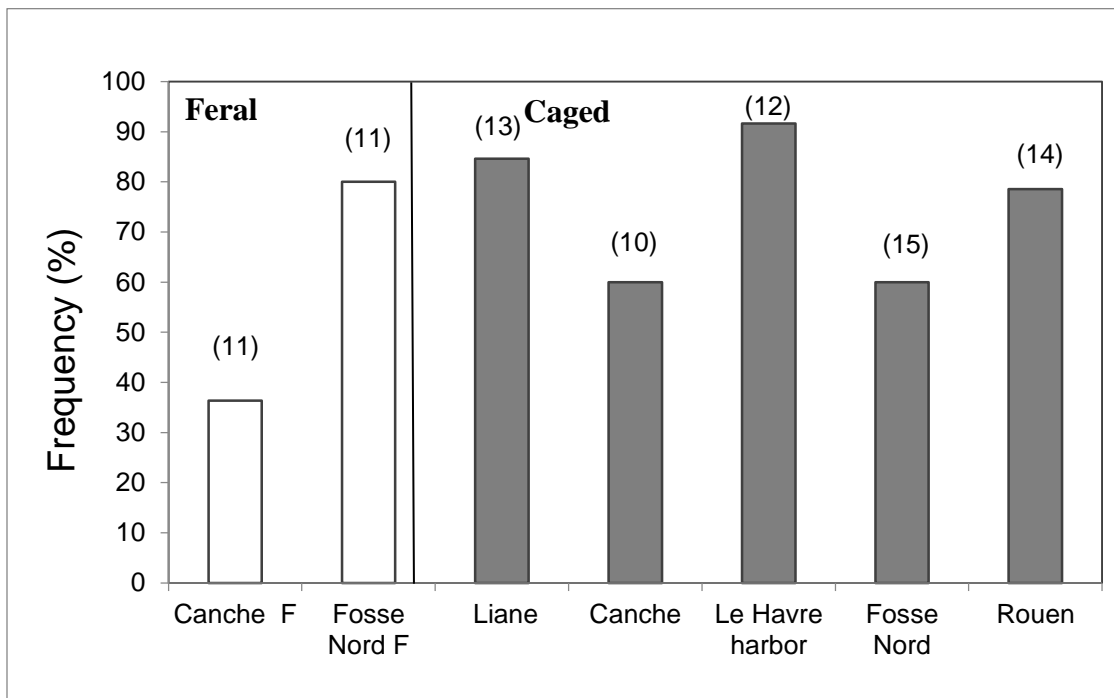


Fig. 4 Percentage of juvenile flounder that have ingested items. White bars: feral fish and grey bars: caged fish. Between brackets are presented the total number of analyzed individuals

of plastic ingestion by marine fish (both pelagic and demersal species) which ranged from 2.6 % in the North Sea (Foekema et al., 2013), 18% in the Central Mediterranean (Romeo et al. 2015), 28% in the Adriatic Sea (Avio et al., 2015), and 41% in the Eastern Mediterranean (Güven et al., 2017). In comparison with our study area, Lusher et al. (2013) reported that 37% of fish in the English Channel had ingested MP, whereas this ingestion was only 5.4% in the southern North Sea (Foekema et al. 2013). The high occurrence of MP in estuarine fish suggests that MP are more common within estuarine water column and sediments than in the marine environment (Anderson et al., 2018). These transitory waters

are important transport routes of MP into the marine environment since about 80% of marine plastics are derived from land-based anthropogenic sources (Andrady, 2011; Schmidt et al., 2017). Mean concentration in rivers is roughly 40–50 times higher than the maximum concentration observed in the open ocean (Schmidt et al. 2017). Estuaries are also dominated by fine sediments in the subtidal and intertidal mudflats which can act as important short-term and longer-term sinks for MP (Browne et al., 2010; Horton et al., 2017; Leslie et al., 2017) as often occurs with other contaminants such as metals, hydrocarbons, and pesticides. For example, in two South Carolina Estuaries,

intertidal sediments contained a greater amount of microplastics than the sea surface microlayer (Gray et al., 2018). Estuaries are considered as hotspots of MP contamination (Browne et al. 2011; Wright et al. 2013). This means that estuarine fish are exposed to a higher concentration of MP and, thus, have a higher probability of MP ingestion than marine species.

Several studies showed higher frequencies of fibers compared with other forms of microplastic

in a variety of marine environments (see (Cole et al., 2013; Wright et al., 2013). In most studies, fibers were the dominant type of microplastics ingested by estuarine fish (> 90%) (Ferreira et al. 2018; Bessa et al. 2018). In our study, fibers (69%) constituted the majority of items found in the digestive tract of the juvenile flounders. This percentage was similar to the one observed in flounders (70%) from the River Thames

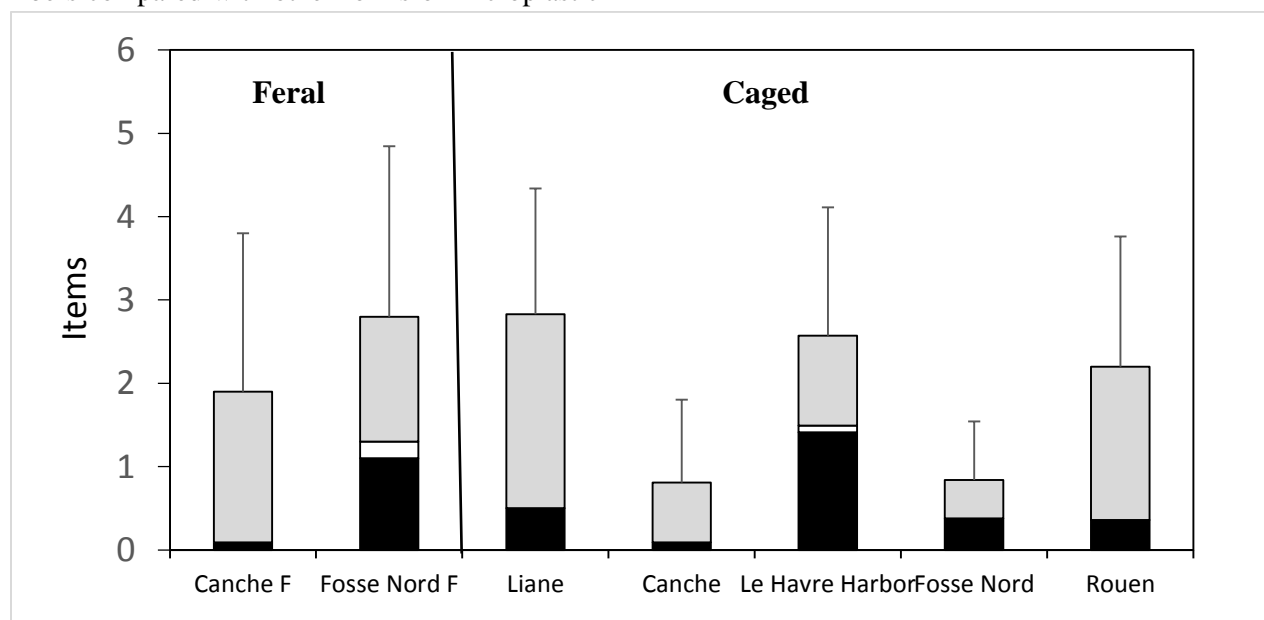


Fig. 5 Average (+ SD) number of items (fragments, fibers, and films) ingested by feral and caged juveniles flounder at the different estuarine sites. Grey: fibers; black: fragments; white: films

(McGoran et al. 2017). The dominance of fibers seems to be a typical pattern for many other demersal fish in other locations (e.g. Lusher et al. 2017; Bessa et al. 2018). As suggested by Ferreira et al (2018), filaments may resemble as natural food items for juvenile flounders (such as nematodes, amphipods, and polychaetes) resulting in mistaking them as preys. The high contamination of fibers in estuarine organisms supports Jabeen et al. (2017) suggestion that freshwater systems and estuaries (transitional systems) are more likely to be contaminated by fibers. For example, in the Solent estuary (UK) more than 80% of particles collected in the water column were fibers (Gallagher et al., 2016). In the Seine River water, Dris et al. (2015) found

that fibers were dominant with an average of 45 fibers/m³ and 0.54 fragments/m³ in the water column. Even though the main sources of fibers in these systems are not fully determined, they could be related with Wastewater Treatment Plants (WWTPs) (Browne et al., 2011; Klein et al., 2015). While they are able to retain a high proportion, e.g., from 83% to 95%, WWTPs effluents still constitute an important source of fibers (Dris et al. 2015; Leslie et al. 2017). Fibers of all colors were found in the gut of juvenile flounders, but blue fibers were predominant. This is also a typical observation, reported worldwide, for estuarine fish species (Possatto et al. 2011; Vendel et al. 2017, Bessa et al. 2018; Ferreira et al. 2018) and also for marine and freshwater

species (Lusher et al. 2017). A recent study investigating the removal of microplastics by WWTPs determined that blue microplastic fibers were most often released from WWTPs (Conley et al., 2019). During the caging experiment, the three sites (Liane, Le Havre Harbor, and Rouen) that are affected by wastewater treatment plant effluent presented the highest ingested number of fibers per fish, suggesting the role of WWTPs as an important source of fibers in estuaries. However, abandoned ropes, fishing gears

(Browne et al. 2011) and atmospheric fallout of fibers (Dris et al., 2017) could be as potential sources of fiber contamination in the aquatic systems.

The characterization of the extracted particles involved an identification of the plastic component using micro-Raman spectroscopy. In the Raman spectrum of fibers, only fluorescence could be observed, although an optimization including the reduction of laser power and bleaching was

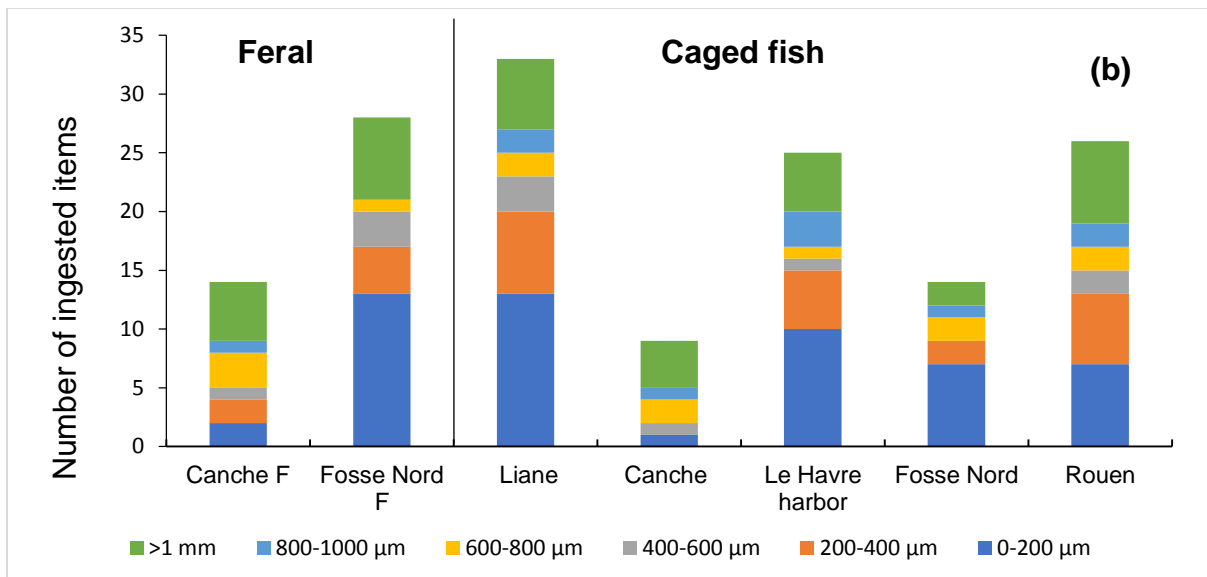
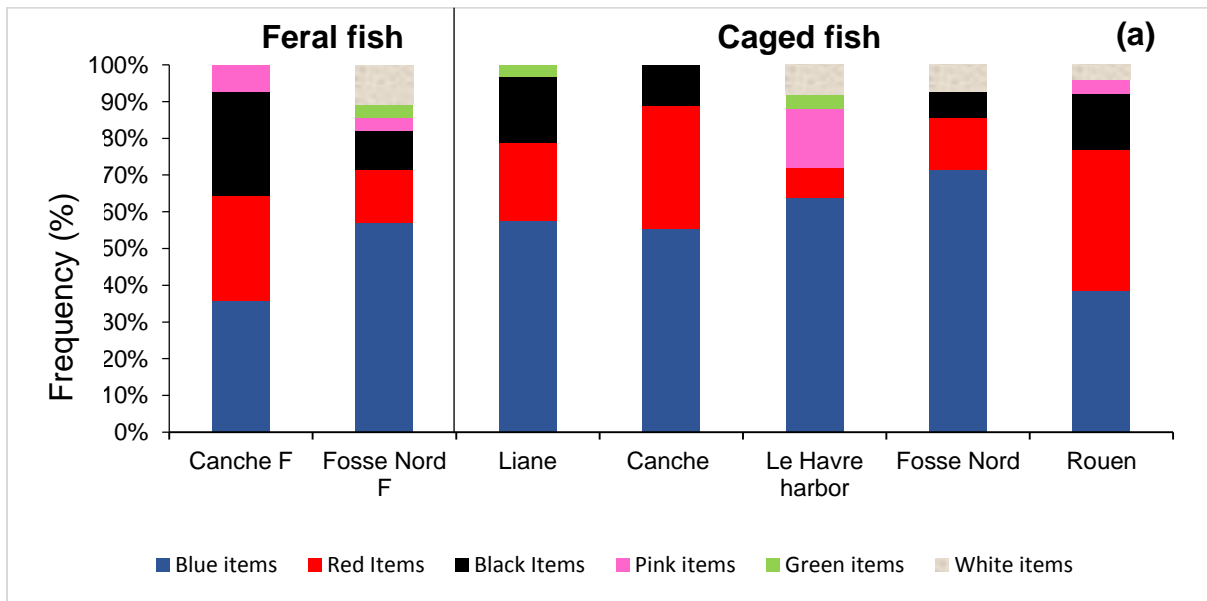


Fig. 6 Percentage of items ingested by feral and caged juvenile flounders at the different estuarine sites categorized by color (a) and the number of items ingested sorted by size class (b)

attempted (see K  ppler et al. (2016)). Yet, when the sample is thin, Raman tends to detect the underlying substrate instead of the sample (K  ppler et al., 2015) which explains the problem we had when identifying fibers' nature. Raman is able to achieve a better spatial resolution (down to 1   m) than FT-IR (10   m) (Lenz et al., 2015) but the identification of fibers relies mainly on FT-IR as Raman analyses did not prove to be efficient so far for this type of microplastics (K  ppler et al., 2016). For the colored items, the spectrum was hidden by the additives (dyes)

existing on particles. Even if these spectra were subtracted, polymers could not be identified due to the intense additives' spectra (Van Cauwenberghe et al., 2015; Van Cauwenberghe and Janssen, 2014). This problem was discussed by many authors (see (Collard et al., 2015; Fr  re et al., 2016; Lenz et al., 2015) and, therefore, preventing polymer identification. As the analysis of fibers was not conclusive with the Raman, most of the analyzed MP items were fragments. As previously

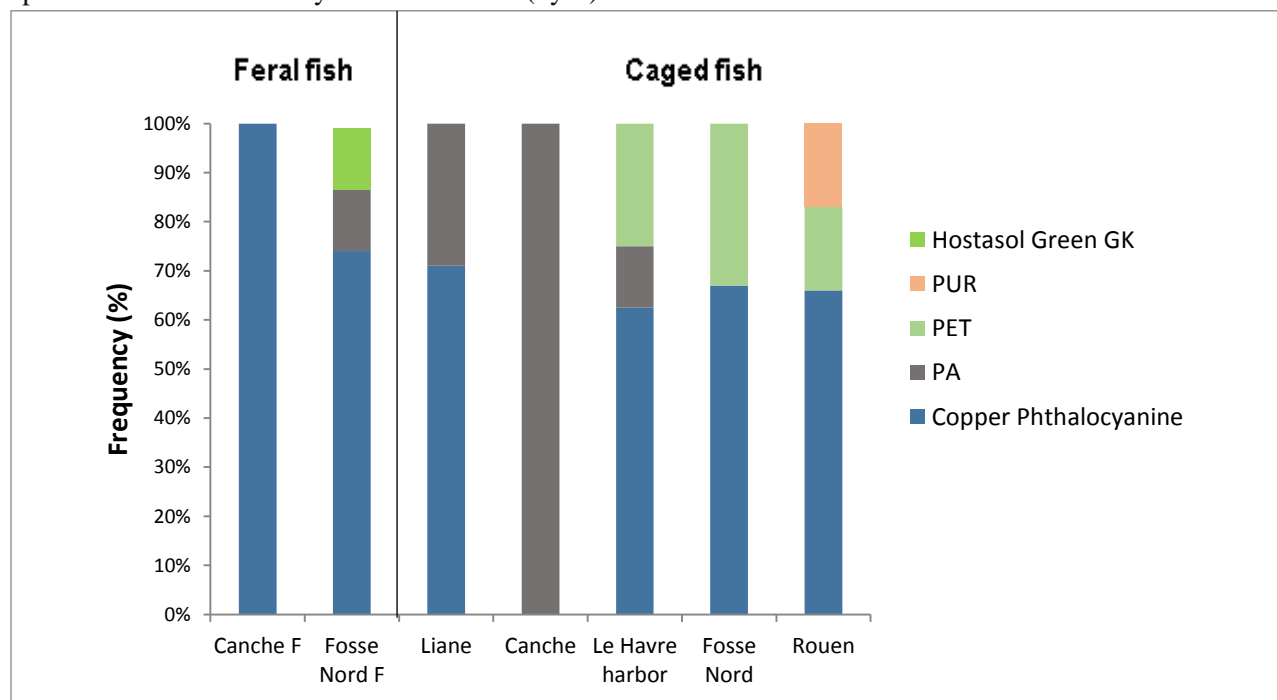


Fig. 7 Different polymers ingested by juvenile flounders (feral and caged) identified using micro-Raman spectroscopy

mentioned, the majority of MP items identified were fibers so we only have a partial representation of the type of polymers ingested by flounders. A combination of identification techniques is necessary for a complete and reliable characterization of the chemical composition of plastics (Hermabessiere et al., 2018; K  ppler et al., 2016). The types of polymers identified were Polycaprolactam (PA), Polyethylene Terephthalate (PET), and

Polyurethane (PUR). Less dense MP such as polyethylene (PE) and PUR can be found on the surface or in the water column while denser plastics like PA and PET sink and reside primarily in sediments (Chubarenko et al., 2016). The presence of Polyurethane in fish caged in Rouen may be explained by the presence of numerous petrochemical industries in and near this site. Another explanation could be that this low dense polymer (PUR) may have sunk to the

bottom since the site of Rouen is characterized by a low water density (salinity = 0.4) compared to the other sites which are characterized by higher water salinity ranging from 17.5 at Fosse Nord and 30.5 at Le Havre Harbor. However, the buoyancy of microplastics can also be affected by chemical contaminants and biofouling.

To the best of our knowledge, all the studies that have investigated the ingestion of microplastics by fish have been conducted from wild caught species or in laboratory experiments. However, the migration of many fish species for feeding and breeding creates uncertainty about how well the analysis made on an individual truly reflects the environmental contamination by MP in or around the site of capture (Oikari 2006). In this study, we tested for the first time the feasibility of using caged juveniles to quantify and assess MP contamination of in estuarine nursery grounds. Such approach was successfully used to monitor microplastics contamination in mussels (Catarino et al., 2018; Railo et al., 2018). Our results demonstrated that the fish caging approach is suitable to assess MP contamination in estuaries and to a lesser extent their effects on fish condition. An average of 2.04 ± 1.93 MP items was ingested by feral juveniles flounder and 1.67 ± 1.43 by caged juveniles flounder. Similar levels (1.9 ± 0.1 items/individual) were previously reported for different adult fish species by Lusher et al. (2013) in the English Channel or in others estuaries: 1.67 items/individual (Bessa et al. 2018), 3.03 (Ferreira et al. 2018) and 1.06 (Vendel et al. 2017).

The higher number of fragments and fibers in wild fish when compared with the caged ones suggest that the latter are probably limited in their feeding zone and, therefore, will have a lower number of ingested items. During the caging experiment, most of the fish have lost weight and it is likely that food availability in the cages was rather low due to the limited cage dimension. The more frequent occurrence of MP in benthic

species compared to pelagic fish (e.g. Neves et al. 2015; McGoran et al. 2017; Jabeen et al. 2017) suggests that plastic occurrence may be high near the sea floor and/or in sediments, or that benthic fish are less selective feeders. In the Thames estuary, McGoran et al. (2017) found that 70% of sampled European flounder had plastic fibers in their gut compared with only 20% of European smelt, *Osmerus eperlanus* (a pelagic species). The generalist feeding behavior of juvenile flounders which feed on benthic preys and ingest large quantities of sediment (Selleslagh and Amara, 2015) suggest that everything is a potential prey to feed on, including microplastics being mistaken as food source.

In this study, we compared exposed juvenile fish from 5 sites in 3 different estuaries. Except the Liane and Fosse Nord, we did not observe significant differences in the number of MP ingested by caged fish. However, the three sites impacted by WWTP (Liane, Le Havre Harbor and Rouen) are those with the highest percentage of individuals that have ingested fibers. This suggest the possible contribution of WWTPs as a source of MP in estuaries.

While microplastic ingestion by fish has been confirmed in laboratory and wild caught specimens, we know little about the impact of MP consumption by fish. However, the quantities observed in fish guts are generally very low, usually less 1 to 2 particles per individual (Lusher et al. 2017). Although other environmental factors may have affected caged fish condition and mortality, we found no significant correlation between the condition factor and the mortality rate with the MP number ingested by fish. Other studies also found that the condition factor of wild captured fish was similar for those with or without MP ingestion (Ramos et al. 2012, Foekema et al. 2013). However, these results did not exclude the possibility of physiological and toxicological consequences. Risks associated with the ingested MP come from the material itself and from the chemical pollutants included

in plastic such as additives or contaminants adsorbed from the surrounding water. Hazards associated with the complex mixture of plastic and accumulated pollutants are largely unknown (Browne et al. 2013; Lusher et al. 2017). Metabolic and physiological negative responses have been only observed under laboratory conditions, where in most cases; very high levels of microplastics were tested under exposure scenarios that were not representative of natural environmental conditions (e.g. Critchell and Hoogenboom, 2018; Pedà et al., 2016; Rochman et al., 2013 and review in Lusher et al. 2017). Recently microplastics were isolated in the gills, liver, and digestive tract of the Zebra danio (*Danio rerio*); which caused inflammation, oxidative stress, and disrupted energy metabolism (Lu et al., 2016). Rochman et al (2013) showed that Japanese medaka (*Oryzias latipes*), exposed to a mixture of polyethylene with chemical pollutants sorbed from the marine environment, can bioaccumulate these chemical pollutants leading to liver toxicity and pathology. Fish behavior may also be affected by microplastic exposure: the common goby (*Pomatoschistus microps*) displayed reduced predatory performance, abnormal swimming behavior, and lethargy (de Sá et al., 2015).

5. CONCLUSION

Regarding the present study, we can conclude that caged fish are suitable to assess microplastic contamination in aquatic environment. Both caged and wild caught European flounder from three estuaries of the Eastern English Channel ingested MP, mainly fibers, in an amount higher to that generally observed in other marine fish species. This would confirm previous studies that have indicated that MP are more common within estuaries than in the marine environment (Schmidt et al. 2017; Horton et al. 2017). European flounder is an opportunistic species that tolerates a wide range of salinity (0 to 35) and can be an ideal indicator to study MP contamination along a salinity gradient. Since

microplastic contamination may vary in space and time, particularly in estuarine systems affected by tide and river flow, the caging approach may be useful for assessing the spatial and temporal variability in MP and the many factors that influence this.

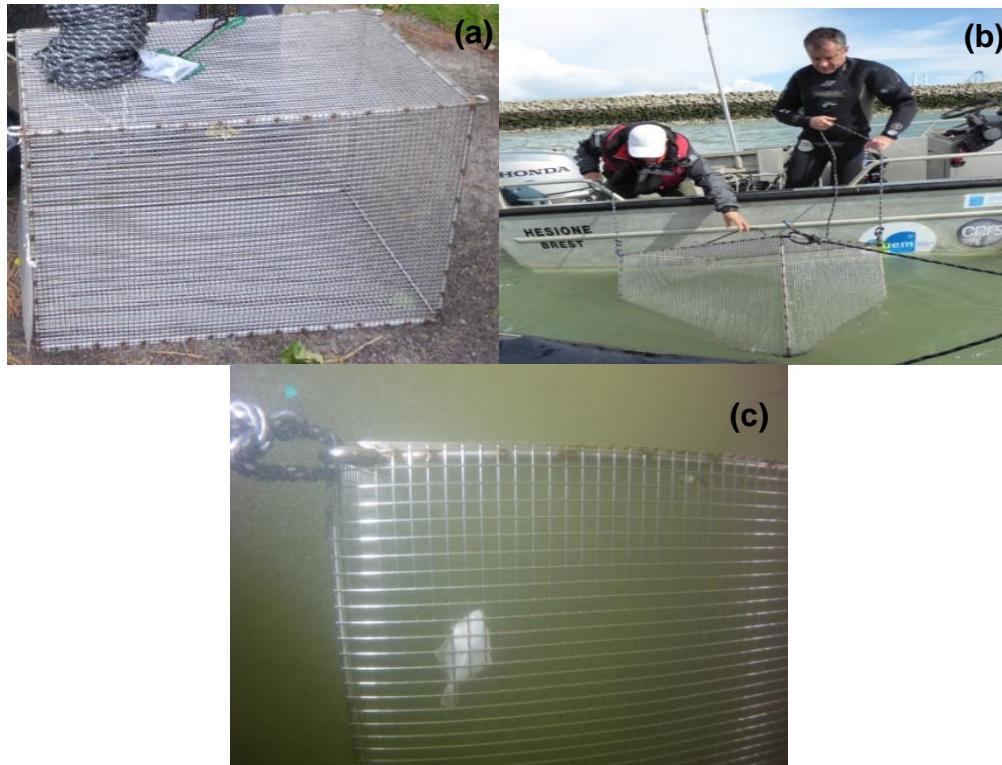
The high occurrence of MP ingested by juvenile fish in nursery grounds raises concerns on their potential negative effects for fish recruitment success and population renewal. No negative effects on juvenile fish condition was observed. However, further researches are required to fully understand the ecological impact of MP within these essential fish habitats. The caging approach may be useful to study the potential effect of MP ingestion on physiological and toxicological responses fish by measuring different biomarkers.

Acknowledgments

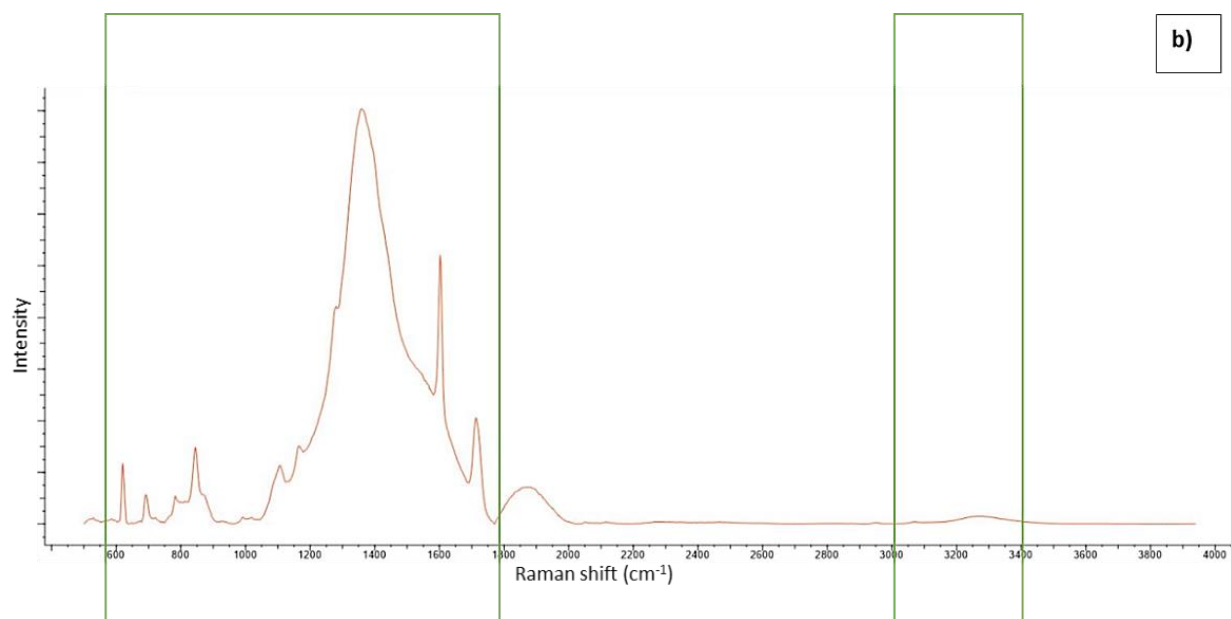
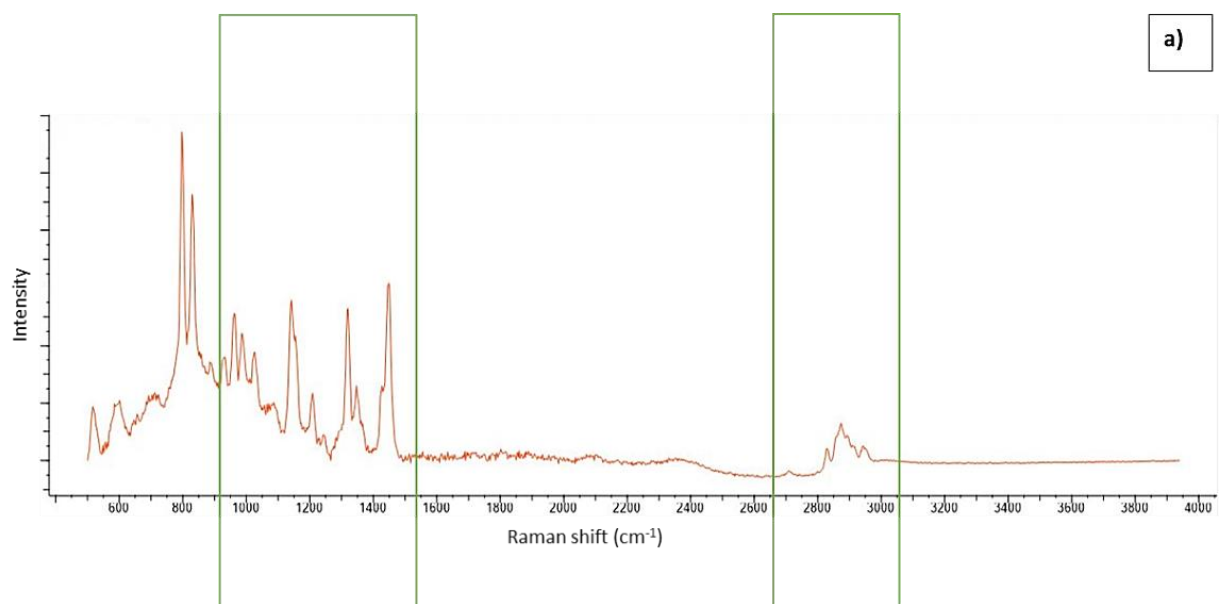
The authors would like to thank Rouen and Le Havre harbors' administration for giving us the permission to install the cages in the sampling sites and the divers' contribution (F. Gevaert, G. Duong; I. Richard and J.L. Lenne) for the cages' deployment.

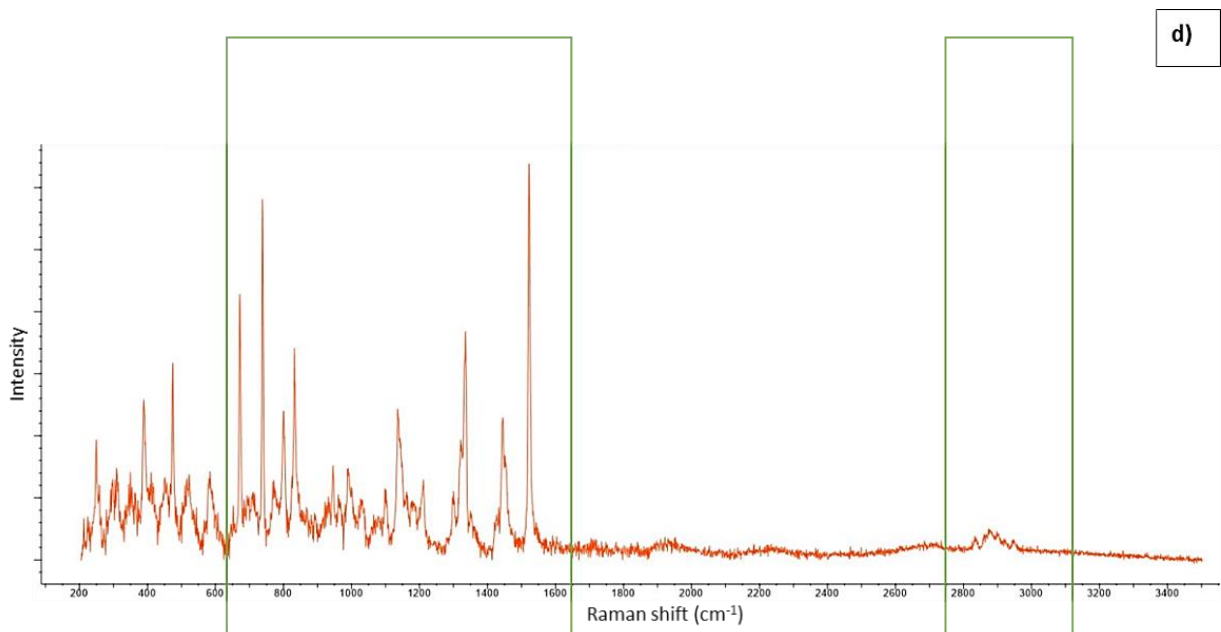
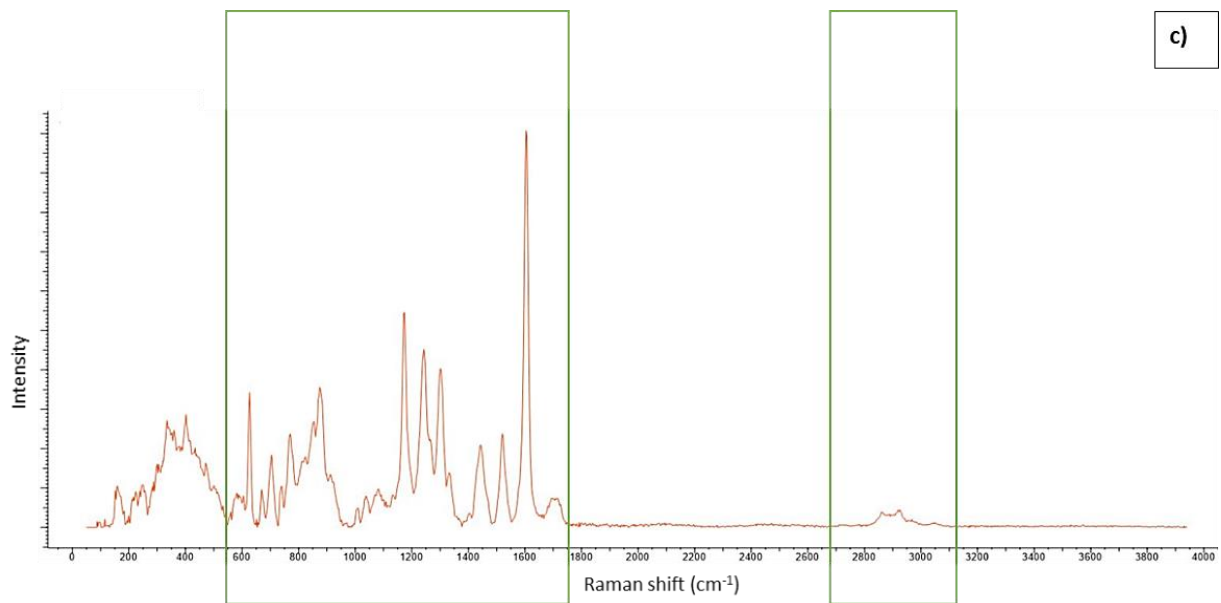
SUPPLEMENTARY FILES

Supplementary Figure S1: The caging setup with (a) representing the stainless steel cage of 1 m length and a 0.6 m height and width. (b) representing the cage deployment and (c) representing *Platichthys flesus* swimming inside the cage after its attachment to the bottom.



Supplementary Figure S2: Spectrum of Polycaprolactam (a), Polyethylene Terephthalate (b), Polyurethane (c), and Copper Phthalocyanine (d) obtained by micro-Raman spectroscopy.





B. Synthesis of Article 4:

Blue mussels are individuals that are easy to handle and manipulate in laboratory experiments. *Mytilus edulis* was chosen to test the effectiveness of caging for MPs monitoring. This assessment started by a depuration experiment process where farmed mussels underwent a 7 days depuration in strictly controlled conditions (e.g. temperature, limiting contamination, feeding, water filtration and renewal...). A follow-up to this procedure was conducted from 0 h, 16 h, 24 h, 48 h, 72 h and 7 days after depuration. Then, these depurated clean mussels equally divided into three different cages that were deployed near a coastal discharge. Different exposure periods were tested: 1, 2 and 5 weeks in order to assess the necessary exposure period for caged mussels to reach the steady-state in microplastics bioaccumulation. In addition to the biota, samples of water were filtered during each period.

A success in the depuration laboratory experiment was observed after 7 days of depuration with a depuration percentage of 98.78%. A significant difference in items was observed 16 hours after depuration and a total elimination of fragments was observed 48 hours after depuration. The obtained results from depuration indicate that the transplanted mussels in the field were clean, and will therefore reflect the contamination of the sampling site.

Both water samples and mussel samples were highly in MPs. But, unlike the mussel samples, the water samples fluctuated with each different sampling date. This is probably caused by the variation of the tidal coefficient: the it is, the bigger the wave would be and microplastics originating from the coastal landfill will enter the sea water. Mussels' MPs concentration did not fluctuate; it continued to increase: 1 week after deployment, a significant increase was observed and continued progressively to reach 1.42 ± 0.76 MPs/g ww. Mussels showed an abundance of fragments with a size strictly $< 300 \mu\text{m}$ whereas fibers were longer and even reaching sizes $> 1000 \mu\text{m}$.

This suggests that a period of deployment of 5 weeks seems to be the minimum exposure period for mussels to reach a bioaccumulation steady-state. And that they are a promising tool for MPs biomonitoring in marine coastal environments.

IV. **ARTICLE 4: EFFECT OF EXPOSURE PERIOD ON CAGED BLUE
MUSSELS (*MYTILUS EDULIS*) MICROPLASTICS
BIOACCUMULATION**

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ABSTRACT

An assessment of periodical mussel caging for active microplastics (MPs) biomonitoring was investigated for the first time. Depurated mussels were transplanted in the marine coastal waters near an abandoned coastal landfill. Different deployment exposure periods were tested: 1, 2 and 5 weeks, and the abundance and characteristics of ingested MPs were compared. All exposed mussels had the presence of fragments and/or fibers. After 1 week of deployment, the number of ingested MPs increased significantly to reach an average of 0.93 ± 0.6 items/g. During the second and the fifth week of caging, the number of ingested MPs increased progressively, yet not significantly, and reached a threshold of about 1.4 items/g. In contrast, the amounts of MPs in water samples collected near the coastal landfill showed temporal variations depending on the tidal coefficient. In both matrices, MPs with a size $< 200 \mu\text{m}$ were the most abundant. Although some polymers were under-represented or totally absent in the caged mussels compared to the surrounding seawater, there was a good similarity in polymer types proportion being found between caged mussels and the surrounding water. Our results indicate that the amount of MPs measured in seawater may be affected by environmental factor such as tide and that mussels are more integrative of MPs pollution. We found that a 5 weeks period was the minimum exposure period for mussels to reach a bioaccumulation steady-state. The present study highlights the relevance of the mussel caging as a tool for the microplastics biomonitoring

Keywords: Microplastics; Caging; Bioaccumulation; Blue mussels; Coastal landfill

1. INTRODUCTION

The pollution of the aquatic systems is constantly rising. Among these pollutants, microplastics (MPs) constitute one of the most important contaminants of the last decade. These small microscopic particles (< 5 mm) are ubiquitously bioavailable to a wide range of organisms. Various sources play an important role in their entry into the aquatic environment. Wastewater treatment plants and coastal landfills are considered as two important entry routes of microplastics (Gatidou et al., 2019; Kazour et al., 2019b; Talvitie et al., 2015). The analysis of different matrices (water, sediment, biota) near these sources indicated the omnipresence of MPs (Kazour et al., 2019a, 2019b; Leslie et al., 2017). Among the analyzed biota, various species have been investigated for microplastics ingestion including invertebrates, bivalves, fish and mammals. Monitoring tools for evaluating microplastics abundance have been recently considered (Avio et al., 2017; Kazour et al., 2018; Li et al., 2019) out of which suggested the use of specific species as bioindicators of MPs (Beyer et al., 2017; Li et al., 2019). Turtles and seabirds are considered as bioindicator of microplastics of a size > 1 mm (Fossi et al., 2018; Van Franeker et al., 2011). For MPs sizes < 1 mm, mussels have been suggested to be used as MPs bioindicator because they fulfill different criteria defining them good bioindicators (Li et al., 2019). For example, mussels have been used as sentinel organisms for monitoring different pollutant contaminants (Beyer et al., 2017; Turja et al., 2014). These filter-feeding bivalves, *Mytilus* sp, have ecological and physiological characteristics that allow them to filter large volume of their surrounding water accumulating various types of contaminants (Beyer et al., 2017; Farrington et al., 2016). They have a high tolerance to difficult environmental parameters as well as a wide spatial distribution. All of which allowed their consideration as a good candidate species in marine biomonitoring programs (Beyer et al., 2017; Scott et al., 2019).

Active biomonitoring by using caged transplanted organisms has been recently adapted for monitoring microplastics. Bivalves and fish caging have been already tested for monitoring microplastics (Avio et al., 2017; Catarino et al., 2018; Domogalla-Urbansky et al., 2019; Kazour et al., 2018; Railo et al., 2018; Kazour et al., in preparation). In these studies, different exposition periods were tested: 28 days (Domogalla-Urbansky et al., 2019; Railo et al., 2018), one month (Avio et al., 2017, Kazour et al., 2018), 42 days (Kazour et al., in preparation), six months (Domogalla-Urbansky et al., 2019) and one year (Catarino et al., 2018). Blue mussels are

organisms that are capable of reaching a steady-state/equilibrium in contaminants bioaccumulation (Beyer et al., 2017). Since the organisms has been proven to ingest and egest microplastics in laboratory experiments (Rist et al., 2019; Ward and Kach, 2009), it is, therefore, important to test several exposure periods in the field in order to reach a “steady-state” of microplastics bioaccumulation. To our knowledge, no evaluation has been done on the exact period needed for caged mussels to ingest microplastics. For chemical pollutants, the optimal deployment time depends largely on the type of contaminants: in caged mussels, trace metals, for example, reach a steady concentration more rapidly than hydrophobic organic contaminants (Beyer et al., 2017). It is necessary to consider both the time and the contaminant specific deployment period necessary for MPs monitoring. Thus, the main objective of this study was to assess the necessary exposure period for caged mussels to reach the steady-state in microplastics bioaccumulation. The amounts and characteristics of MPs (shape, size, color and type of polymers) ingested by caged mussels were compared with those found in their seawater surrounding environment. In this study, we also analyzed experimentally the mussel depuration kinetic after 16 h, 24 h, 48 h, 72 h and 7 days to determine the depuration period necessary to obtain reference mussels (without MPs) that can be used for transplant caging experiments.

2. MATERIAL AND METHODS

2.1 SAMPLING AND CAGE DEPLOYMENT

2.1.1 MUSSELS DEPURATION PROCESS

Blue mussels (*Mytilus edulis*) were collected from farmed Bouchot mussels from Camiers near Boulogne-sur-mer, (French Eastern English Channel coast). Around 220 mussels of approximately the same size (4.9 ± 0.37 cm) were sorted: ten individuals were immediately frozen at -20°C and serve as reference samples and the rest was placed in a 160 L glass aquarium pre-cleaned with Milli-Q water and closed with a glass lid. The aquarium was supplied by filtered seawater ($0.1 \mu\text{m}$) constantly oxygenated, changed every 24 hours for 7 days, and it was placed in a thermoregulated room at $17 \pm 1^{\circ}\text{C}$ with a 12 h light-dark illumination regime. To assure their filtration capacity, mussels were fed on regular basis with live microalgae, *Rhodomonas* sp. and *Isochrysis* sp.. The depuration kinetic was tracked down by taking subsamples of mussels (10 individuals) after 16 h, 24 h, 48 h, 72 h and 7 days. They were immediately frozen at -20°C .

2.1.2 SAMPLING AREA

The study site was located at Sainte-Adresse coastal zone near the bottom of the Dollemard cliffs (Northwestern France). The coast is a part of the “Littoral Cauchois” and is classified under “Natura 2000” with a sand and pebble substratum directly affected by tidal forces (macrotidal regime). The chosen study area is located in the vicinity of the Dollemard coastal landfill. This landfill was constructed in 1960 and received large amount of ancient waste (metals, tires, toxic wastes, plastic and domestic wastes...). Even though the landfill was closed in 2000, it has collapsed on the bottom of the cliffs resulting to 750 m of wastes distributed. The observed wastes could be affected by tidal waves promoting their entry into the nearby coastal waters.

2.1.3 CAGING TRANSPLANTATION PROCEDURE

Depurated mussels were transported to caging sites in a small aquarium supplied by oxygenated filtered water. 150 individuals were distributed equally in three stainless-steel cages (50 cm x 50 cm x 50 cm, length x width x height) of a 15 mm mesh size and equipped by a stainless-steel grid placed mid-height assuring a substrate for mussels' attachment (see Kazour et al., in press for a detailed description of the cages). The cages were deployed during low tide in the lower intertidal shore zone on the 10 of July 2018. One week afterward, one cage was retrieved and the same process was repeated for the 2nd and 5th week. After retrieval, each mussel was put separately inside an aluminum foil and frozen at -20°C until analysis. Environmental parameters (water temperature, salinity and dissolved oxygen) and turbidity were measured during cages transplantation and retrieval using a multiparameter probe (Mutiparameter HI 982, HANNA instruments).

2.1.4 WATER SAMPLING

Water samples were collected during cages' deployment and on each retrieval days. The samples were collected as described by Kazour et al., 2019b. In short, 0.5 to 1 m³ of the surface water was pumped on sieves of different mesh sizes (500 µm, 200 µm, 80 µm and 20 µm) that were rinsed inside clean bottles. Due to low mesh size of 20 µm sieve, it was the fastest to get clogged and only 20 L was filtered.

2.2 MICROPLASTICS ANALYSES:

2.2.1 CONTAMINATION PREVENTION

Several precautions were followed in order to control microplastics contamination. Cotton lab coats were worn throughout the experiments, and all steps (filtration, measurements and digestion) were realized under a laminar flow hood. All surfaces and equipment were cleaned using filtered ethanol 70% and MilliQ water. All used solutions were filtered three times on glass fibers filters (GF/A) Whatman, (France) in order to remove any unwanted particles and fibers. Only glass laboratory materials (bottles, petri dishes, Erlenmeyers, filtration system) were used. Contamination was tracked down by making a control with every procedure that underwent the same conditions as the samples. Observed fibers in the blanks were then eliminated from the samples.

2.2.2 WATER AND MUSSELS SAMPLES

As described by Kazour et al., 2019, collected water samples underwent density separation using zinc chloride (ZnCl_2 , 1.8 g/cm^3) and filtered on glass microfiber filters (GF/A, Whatman, France).

Ten mussels (depurated and caged) were thawed 4 hours prior to analysis. Their total length, width (cm), total and net weight (g) were measured. The condition index (CI) was calculated (AFNOR, 1985): $\text{CI} = ((\text{Net Weight (g)})/(\text{Total weight (g)})) \times 100$. Each mussel was put separately inside Erlenmeyers containing 150 mL of potassium hydroxide 10% (KOH 10%). Then, they were placed on a heating magnetic stirrer for 24 hours at 60°C . The obtained solution was filtered on GF/A filters and visually observed.

2.2.3 VISUAL OBSERVATION AND POLYMER IDENTIFICATION

All mussels and water filters were observed under 120x magnification using Leica M165 C Stereomicroscope and images of suspected MP particles were taken with a Leica M170 HD camera and LAS (Leica application suite) software. All suspected MPs particles were counted, categorized by type (fragments, fibers, microbeads or films) and color. While observing the

items, the following criteria were taken into consideration: (1) absence of cellular or organic structures; (2) a homogenous thickness across the particles; and, (3) homogenous colors (Hidalgo-Ruz et al., 2012). Measurements were done on the suspected items at their longest dimension and they were divided into different size classes of 200 μm . Whereas for the fragments found inside depurated and caged mussels, more precise size classes of 20 μm were considered.

Identification of suspected microplastics was done using Micro-Raman Xplora Plus (HORIBA Scientific®, France). Due to the time consumption analyzing filters under micro-Raman takes, five filters of caged mussels from each cage retrieval and containing the highest number of potential MPs from each sampling site were chosen and analyzed. Subsamples of water items were also taken for Raman analysis. For identification, two lasers with a wavelength of 532 nm and 785 nm and a range of 200-3400 cm^{-1} were used with x10 and x100 objectives (Olympus). Each particle spectrum is compared to a polymer database identification software (KnowItAll, BioRad®) and a personal library made with standard polymers obtained from Goodfellow (France).

2.2.4 STATISTICAL ANALYSIS

Suspected microplastics found in water and mussels were reported in unit volume (per L) and per gram of sample, respectively. Mussels length and condition index followed the assumption of normality and a One-Way Analysis of Variance (ANOVA) with post hoc were used. Number of ingested items did not comply with the parametric assumption of normality (Shapiro–Wilk tests) and homogeneity of variance (Levene tests), the non-parametric Kruskal–Wallis test and Mann–Whitney U were used. Spearman’s rank correlation was tested for the mussels’ condition index and the number of ingested items. All results are expressed as mean \pm SD, and p-value < 0.05 was considered as statistically significant. All tests were performed with SPSS software (IBM SPSS STATISTICS 20).

3. RESULTS

3.1 DEPURATION EXPERIMENT AND CAGE DEPLOYMENT

After seven days of depuration, only 3 individuals were found dead (98.5% survival rate) and there was no significant difference in mussel's condition index ($p = 0.989 > 0.05$). All non-depurated mussels had MPs with an average of 1.82 ± 1.34 items/g (out of which 82% were constituted of fragments) that significantly decreased to 0.28 ± 0.27 items/g after the 16 h of depuration ($p = 0.001 < 0.05$). After 72 hours, only 10% of mussels had MPs and the number of observed items significantly decreased by 98.78% reaching 0.022 ± 0.07 items/g and consisting only of fibers (Fig. 1). After seven days of depuration, fibers were the only shape found among the observed items. Red and blue were the dominant colors for both observed shapes, and several black fibers were also found. During the experiment, the size of observed fragments ranged from $18.65 \mu\text{m}$ to $389.12 \mu\text{m}$ with a total mean size of $63.92 \pm 49.77 \mu\text{m}$. Fibers' size classes ranged from $89.05 \mu\text{m}$ to $1938 \mu\text{m}$ with a mean size of $512.2 \pm 477 \mu\text{m}$. The highest and the lowest sizes of fragments and fibers were observed in non-depurated mussels. As shown in Supplementary Fig. 1, three size classes ($< 20 \mu\text{m}$, $100 - 120 \mu\text{m}$ and $> \mu\text{m}$) were rapidly egested after 16 hours. Whereas the size classes ($60-80 \mu\text{m}$ and $120-140 \mu\text{m}$) were egested after 72 hours.

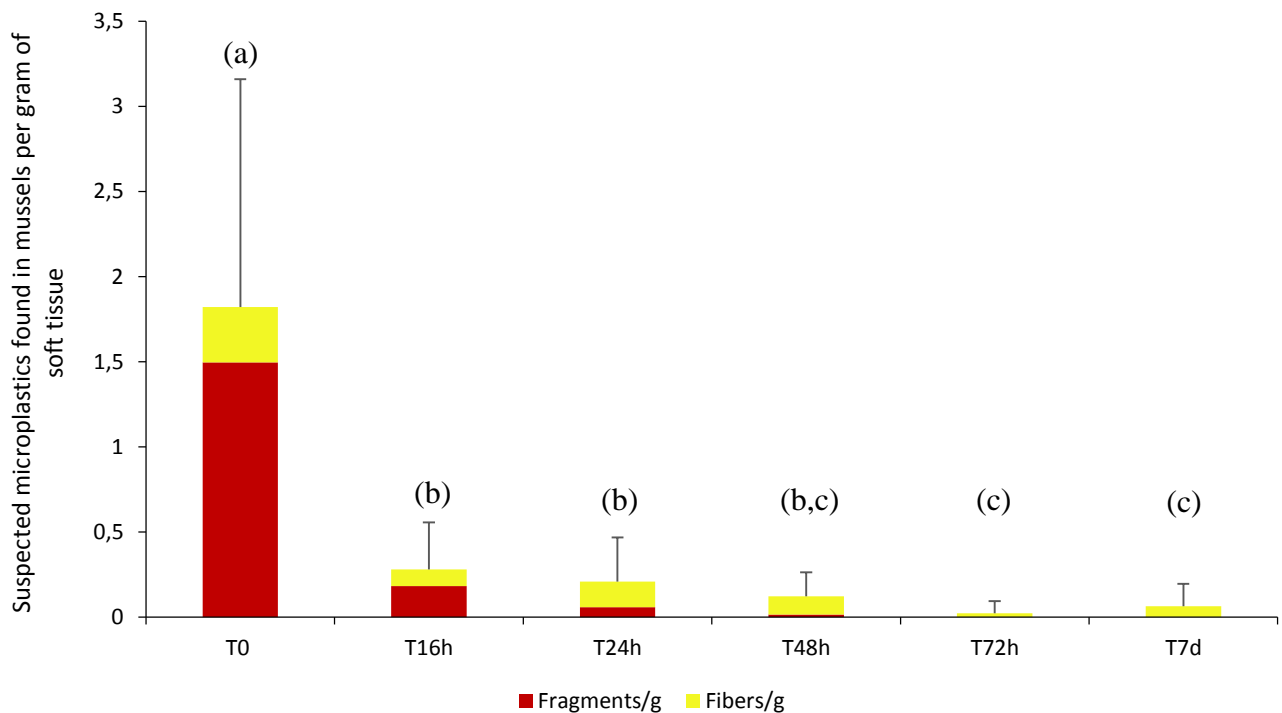


Fig. 1: Average (+SD) number of suspected microplastics (items/g of mussels' wet weight) by shape categories found in the non-depurated mussels (T0: farmed mussels) and the 16, 24, 48- and 72-hours depurated mussels (T16h, T24h, T48, T72, respectively) and 7 days depurated mussels (T7d).

During the caging period, the water temperature ranged between 19.5 and 21.7 °C. The survival rate was 95.6% for the first week and decreased to 94% and 90% for weeks 2 and 5, respectively. No significant difference in mussels' total length was observed during the whole caging experiment ($p = 0.707 > 0.05$). Before cage deployment, mussels had a condition index of 49.43 ± 4.94 %. This CI decreased significantly during the first week reaching 45.05 ± 2.57 % ($p = 0.02 < 0.05$) and continued to decline significantly after 5 weeks of caging reaching 40.02 ± 5.26 %.

3.2 MICROPLASTICS ANALYSIS

3.2.1 WATER SAMPLES

The concentration of microplastic found in the seawater varied throughout the different sampling dates (Fig. 2 – A). The highest number of suspected microplastics per m^3 was observed in W1 (17/07/2018) with 1826 items/ m^3 , whereas the lowest was observed in W2 (24/07/2018) with 374 items/ m^3 . As shown in Fig. 2, lower tidal coefficient led to lower items abundance and vice versa. An average of 1 suspected item was observed in the 20 μm sieve. Fibers and fragments were more or less equally abundant except in W2 where fragments were highly dominant (71%). Microbeads (0.1%) were only observed in W3 (24/07/2018). A wide range of colors was observed for all shapes: blue, red, black, green, white and transparent. Biofilms and abrasions were observed on the items' surfaces, especially those collected from the 500 μm sieve (Fig. 3).

3.2.2 MUSSEL SAMPLES

Suspected microplastics ingestion increased significantly by 87% after one week of caging, reaching an average of 0.93 ± 0.6 items/g ($p < 0.05$) (Fig. 2 – B). All collected mussels had the presence of fragments or/and fibers. The number of ingested items continued to increase in Tw2 and Tw3 with no significant difference with Tw1 reaching 1.28 ± 1.2 items/g and 1.42 ± 0.76 items/g, respectively ($p > 0.05$). The number of ingested items had a moderate negative correlation with the mussels' condition index ($R = -0.411$, $P = 0.007$). Two MPs shapes were found: fibers and fragments with the latter constituting more than 73% among the ingested items. Fragments and fibers were observed in various colors: blue, red, green and transparent; in addition, black fibers were also denoted.

3.2.3 SIZE CLASS AND POLYMER TYPE

Suspected microplastics found in all samples were categorized as shown in Fig. 4. All size classes were observed in water and mussels. In the water samples, the size class $< 200 \mu m$ was the most abundant in most of the sampling dates except in W5 where the class $> 1000 \mu m$ was

slightly more abundant (31.64%) (Fig. 4 – A). The second most abundant size class differed from one sampling date to another: > 1000 μm in W0 (33.9%), 600 – 800 μm in W1 (21.8%) and 200 – 400 μm in W2 (26%). The size classes 400 – 600 μm and 800 – 1000 μm were the least abundant observed sizes in all of the sampling campaigns.

In the subsamples of mussels taken before caging, only two fibers were present, each one belonging to a different size class (200 – 400 μm and 600 – 800 μm). During the caging, mussels were able to ingest items of various sizes (Fig. 4 – B). In the analyzed mussels of all sampling dates, the size class < 200 μm was the most abundant (> 65%) and the bigger size classes constituted only of fibers (the largest fragment was 296.42 μm). The second most abundant size class was 200 – 400 μm for Tw2 and Tw5, and 400 – 600 μm for Tw1. Items of a size 800 – 1000 μm were missing from Tw1 sampled mussels. Items of a size > 600 μm constituted no more than 15% of all the observed items. Fragments with size classes between 20 - 40 μm and 120 – 140 μm were the most abundant in the caged mussels collected through the different sampling dates (Supplementary Figure 2).

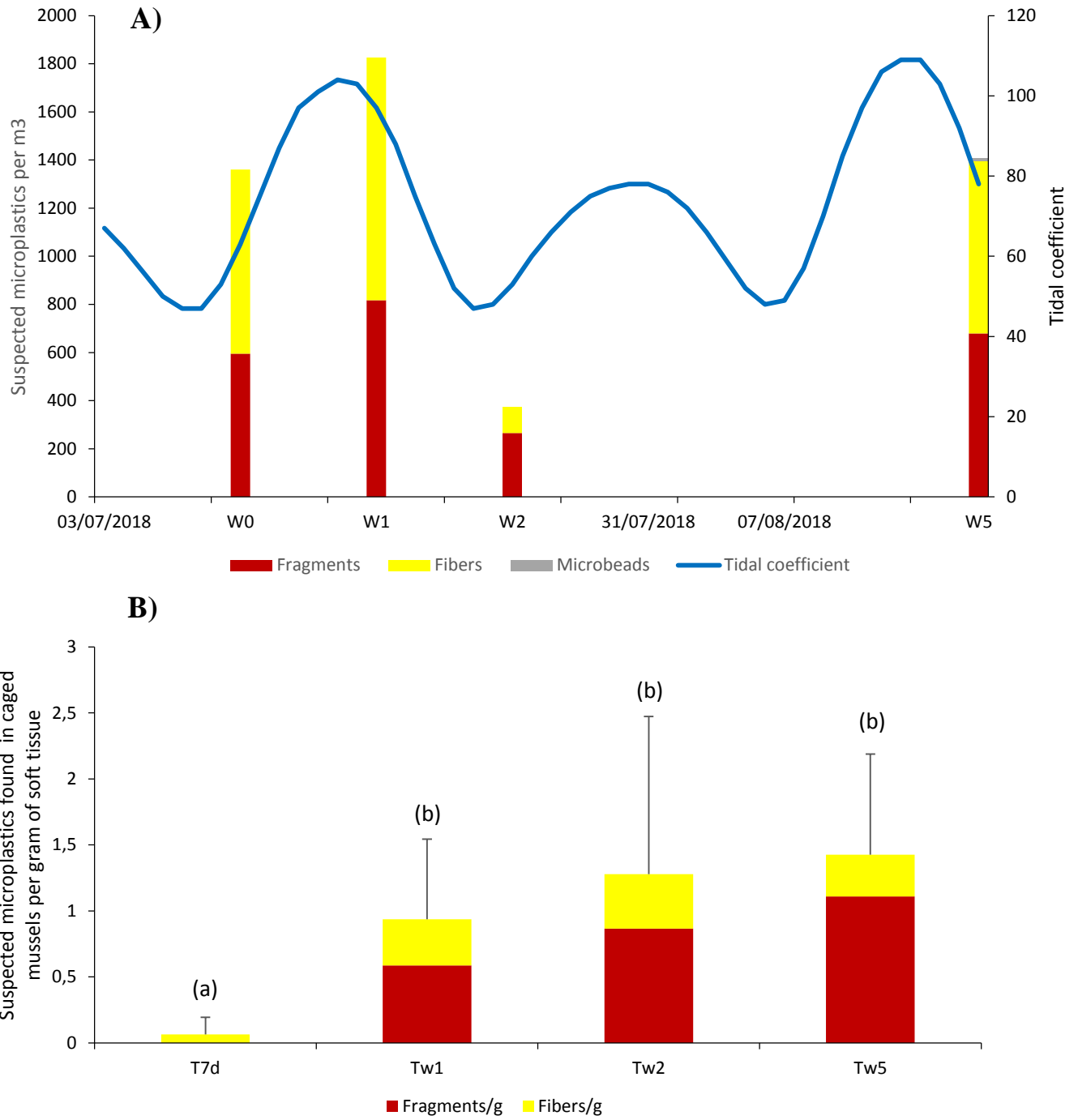


Fig. 2: A) Suspected microplastics by shape categories found in the surface water (items/m³) collected on different sampling dates and the variation of tidal coefficient during the sampling period and B) average (+SD) number of suspected microplastics (items/g of mussels' wet weight) by shape categories found in the caged mussels collected in the first (Tw1), second (Tw2) and fifth week (Tw5).

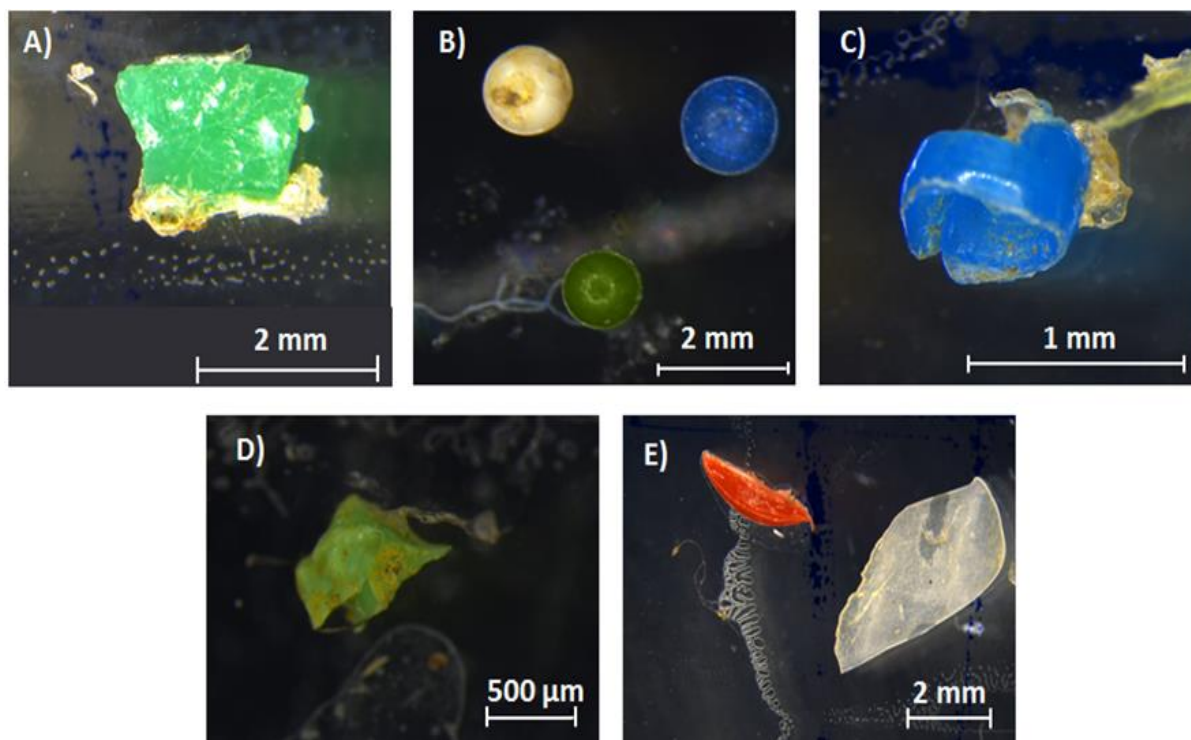


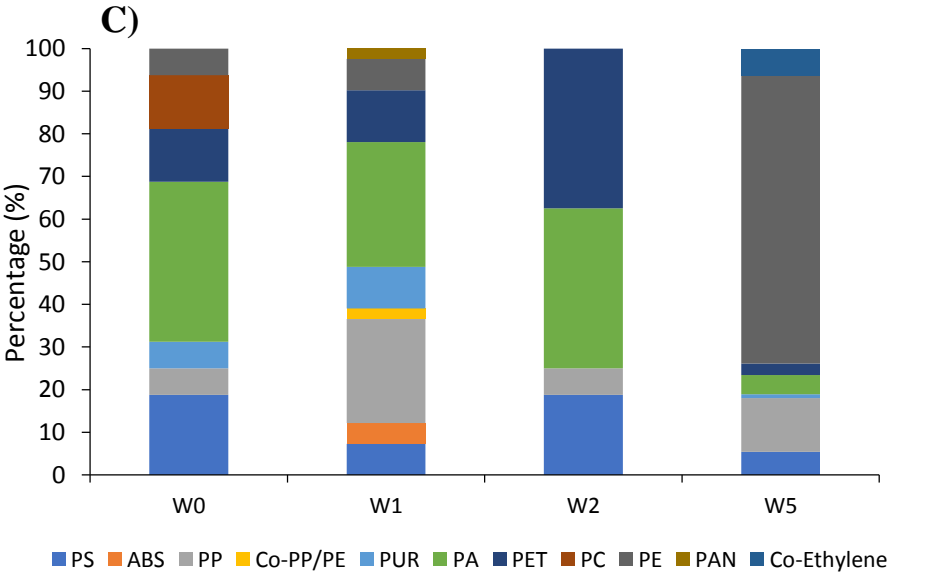
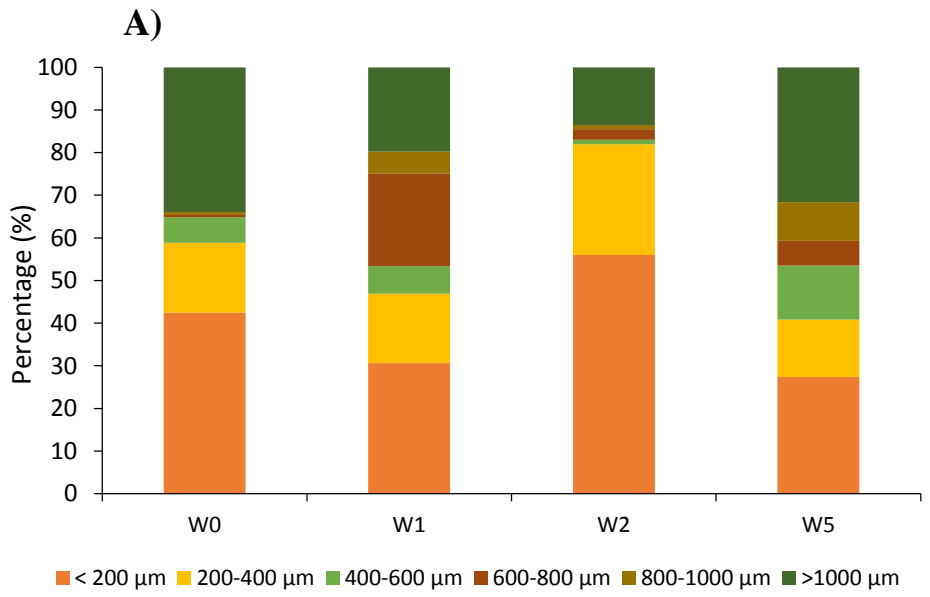
Fig. 3: Example of microplastics of different shapes and colors collected in seawaters surface samples A) green fragment with a biofilm formation on its surface, B) white, blue and green microbeads C) and D) blue and green fragments with biofilms and abrasions observed on their surface and E) red and transparent fragments with abrasions.

Out of 305 items analyzed, 84.26% were identified as polymers. The rest were either identified as cellulose or minerals (3.6%) or they were non-identifiable (12.14%; Raman tends to detect the underlying substrate instead of the sample if it was too thin (Käppler et al., 2015)). Several types of polymers were found in the water and mussels samples (Fig. 4 – C & D). For the water samples, 11 types of polymers were identified out of which 8 of them were common in mussels. The common polymers were: polystyrene (PS), acrylonitrile butadiene styrene (ABS), polypropylene (PP), copolymer PP-PE, polyurethane (PUR), polyamide (PA), polyethylene terephthalate (PET) and polycarbonate (PC). The polymers found in mussels had their color and sizes similar to that found in the water (Supplementary Table 1). In addition, the four polymers that were only found in water (polyethylene (PE), copolymer ethylene and polyacrylonitrile (PAN)) were mostly fragments of a size $> 200 \mu\text{m}$ which explains why they were not ingested by the caged mussels.

In the water samples, four types of polymers were common in all sampling dates: PS, PP, PA and PET (Fig. 4 – C). Three types of polymers (ABS, Co-PP/PE and PAN) were only observed in W1. PC was identified in W0 and Co-Ethylene in W5. In the analyzed samples of W2, neither PUR nor PE were detected. PA was the most abundant in W0 and W2; this polymer along with PP was dominant in W1. PE was highly detected in W5.

In mussels, PS, ABS and PA were common in all analyzed samples (Fig. 4 – D). PP and PET were not observed in mussels analyzed from Tw2. Tw5 samples were the only one that had the presence of PC and no Co-PP/PE were detected. In Tw1 samples, no PUR was observed among the analyzed particles. PS and ABS were the most dominant in mussels collected from Tw1 and Tw2 whereas PA and PS were the most abundant in Tw5.

Seawater



Mussels

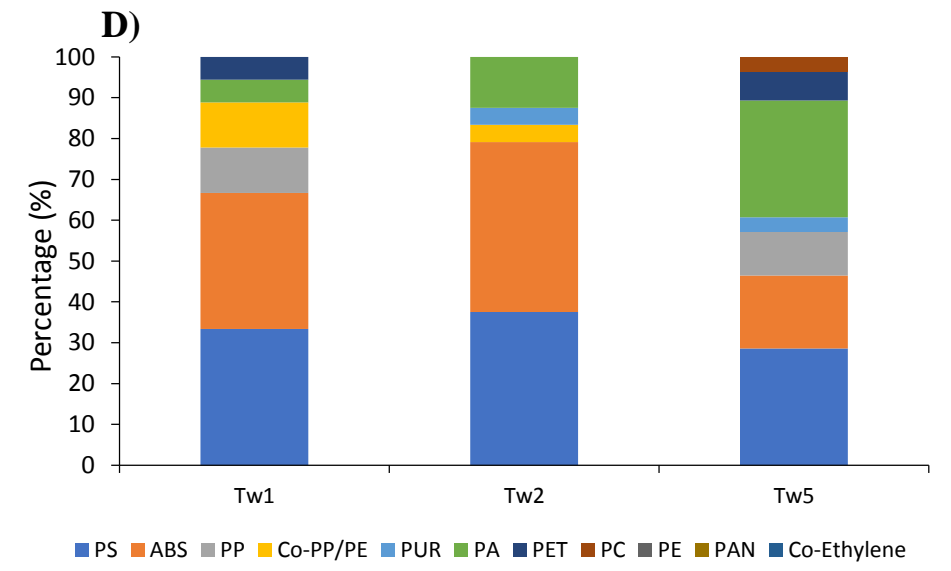
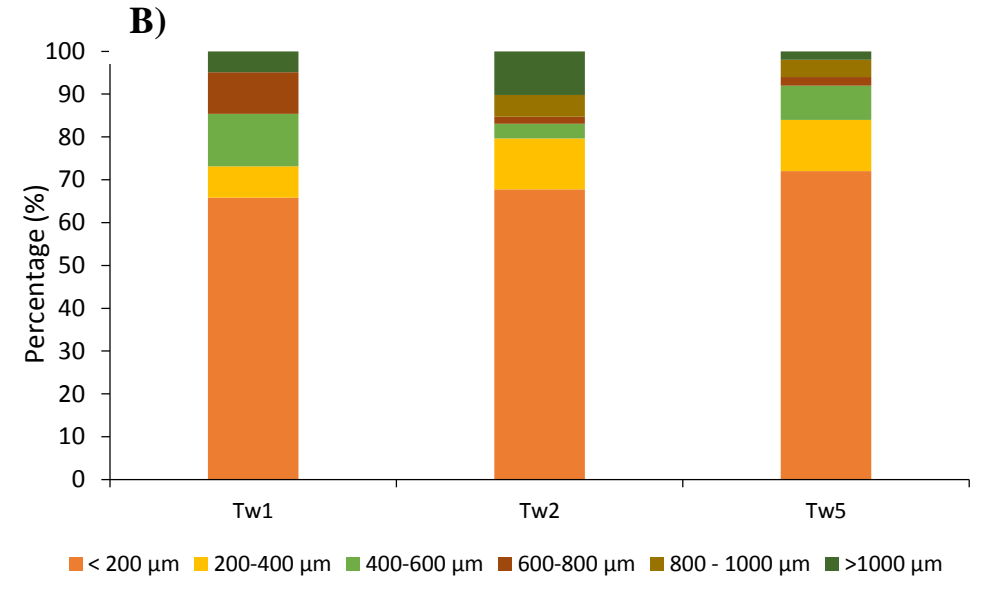


Figure 4: Proportion of size class composition of suspected microplastics in the A) sea surface water samples and B) caged mussels, and the proportion of polymer types found in C) sea surface water samples and D) caged mussels.

4. DISCUSSION

Previous experiences on microplastics depuration rates by mussels have been already tested. The absence of a standardized protocol lead to a non-reliable comparison between studies. The obtained depuration rate in this study (98.78%) indicated that the characteristics used during the experiment (feeding, filtered water change for example) led to an optimum egestion rate by mussels. Rist et al. (2019) indicated that 2 hours was not enough for blue mussels to egest their items and that the depuration period should be extended. A significant decrease in items was observed directly after 16 hours with a total absence of fragments after 48 hours. Fibers have more residence time in the mussels gut (Ward et al., 2019) which might explain their presence 72 hours after depuration. This depuration phase prior to cage deployment is an important asset in MPs caging experiments. It assures the use of clean reference individuals to be deployed in the caged transplantation for a realistic biomonitoring of the site's pollution.

Coastal landfills have been already described as important microplastics sources in coastal water whether by their leachates or their poor management or abandonment (He et al., 2019; Kazour et al., 2019b, 2019a; Praagh et al., 2018). In our collected water samples, microplastics abundances varied from one date to another with values higher than 1380 items/m³ except in 24/07/2018 (lowest abundance: 374 items/m³). Though these values are considered high, they were lower than those observed at the same site in April 2018 (6000 items/m³; Kazour et al., 2019b). The quantity of microplastics entering the marine coastal environment from the coastal landfill may be affected by many environmental factors such as wind, rain, storm, tide, wave... During the study period (July and August 2018), there was no storm nor important winds or rainy events in the studied area. The only factor that might have contributed to the variation of the seawater MPs abundance is the tidal coefficient. As shown in Fig. 2, the number of detected items follows the pattern of the tidal coefficient: high tidal coefficient during one or two days before sampling led to higher amount of MPs in the seawaters. During high tidal coefficients, the sea rises higher on the intertidal zone and comes into contact with the landfill and thus carries MPs, that have been degraded and deposited at the bottom of the landfill, towards the sea.

The high number of items with a size > 1000 µm, as well as their abrade surface and form, indicates that they might originate from degraded macroplastics (secondary microplastics) and might be traced back to the landfill. The high concentrations of MPs in the seawater may also be the result of higher bottom sediments re-suspension during high tidal coefficient. Indeed, we

found that water turbidity was very high during high tidal coefficient (see supplementary Table 2).

Our caged mussels were deployed in highly MPs polluted waters which probably explains the high ingested amount found. Similar studies indicated that high MPs ingestion was found near important anthropogenic sources (industrial sources, sewage effluent discharges and highly frequented harbors) rather than in references sites (Kazour et al., 2019b; Li et al., 2018; Scott et al., 2019). Mussels were already suggested as bioindicators for microplastics pollution (Li et al., 2019) and as sentinel groups for MPs < 500 μm (Bråte et al., 2018; Kazour et al., 2019b). Previous mussels caging experience used a minimum of 28 days for microplastics monitoring (Railo et al., 2018). An essential key in caging experiments is to identify the necessary time needed for mussels to accumulate the pollutant. Short deployment time such as 3 weeks were used to monitor chemical pollutants (Marigomez et al., 2013; Zorita et al., 2006). This work tested short time deployment (1 and 2 weeks) and longer periods (5 weeks). The significant increase in ingested microplastics from Tw0 to Tw1 indicated that mussels were able to adapt to their environment, filter its water and ingest microplastics. After 2 and 5 weeks, a progressive increase, yet not significant, of ingested microplastics was observed. From week 1 to week 2, an increase of 0.34 suspected MPs/g was observed, this increase was reduced to 0.15 between week 2 and week 5 indicating that the mussels were close on reaching their steady state (balance between defecation/egestion). Therefore, 5 weeks seems to be the minimum exposure period for mussels to reach a bioaccumulation steady-state. A longer deployment period is necessary to be tested to confirm such results.

Among the ingested items, the colors and the types of polymer varied from one sampling date to another but the items' size range remained approximately the same. Caged mussels ingested fragments of a size < 300 μm and fibers of a larger sizes (reaching > 1000 μm) which is an accordance to what experimental studies have found. Ward et al. 2019 demonstrated that mussels ingested microspheres of a size < 510 μm and showed high residence rate as well as for longer fibers (587 μm and 1075 μm) in the animal's gut. Therefore, mussels would be selective to surface and size of the item.

There are several factors that should be taken into account when considering mussels as MPs monitoring organisms. Our results indicated that mussels ingested smaller sized fragments and long aspect-ratio fibers which was in accordance conducted experimental exposures (Ward et al.

2019). But in the natural surrounding environment, microplastics are affected by several environmental factors and their abundance in surface water are periodically variable. The size, the shape, the density and other characteristics play a role on the bioavailability of MPs to aquatic organisms (Carbery et al., 2018). For example, the shape of an item influences its accumulation or elimination by organisms (Browne et al., 2013; Ward et al., 2019) which leads to an under-representation of certain items sizes and polymer types (Scott et al., 2019).

5. CONCLUSION

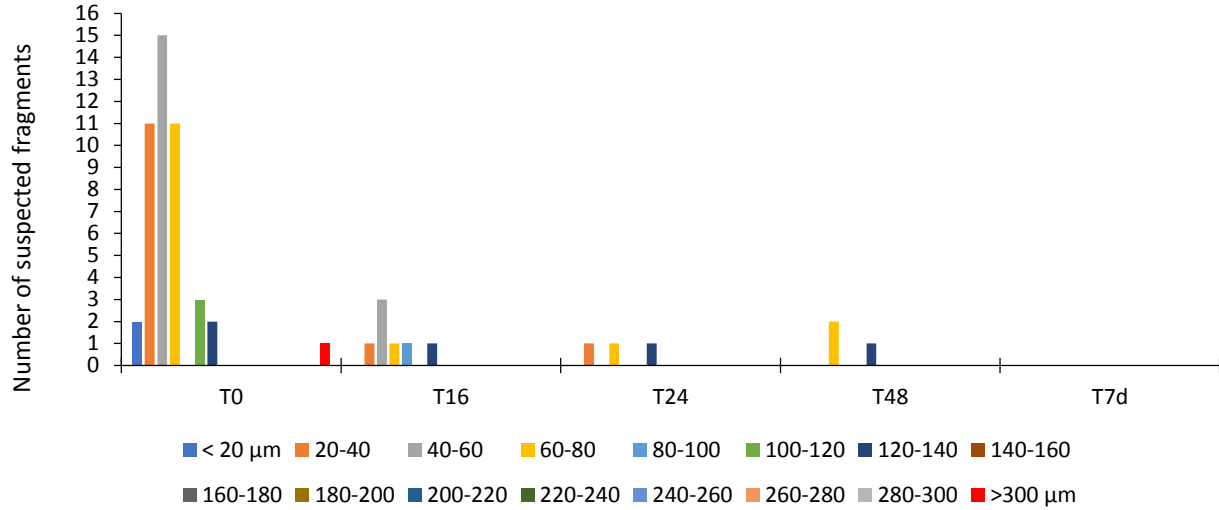
The present study investigated, for the first time, the necessary exposure period for caged mussels to reach the steady-state in microplastics bioaccumulation. After one week of exposure, clean depurated mussels significantly ingested microplastics from the surrounding environment. During the second and fifth weeks, mussels ingested higher number of microplastics yet not significant suggesting that 5 weeks of deployment seems to be the minimum exposure period for mussels to reach a bioaccumulation steady-state. The ability to attain a standardized MPs biomonitoring protocol is still at its primary phases and need further studies. The blue mussels caging may be a promising tool for MPs biomonitoring in marine coastal environments.

Acknowledgments

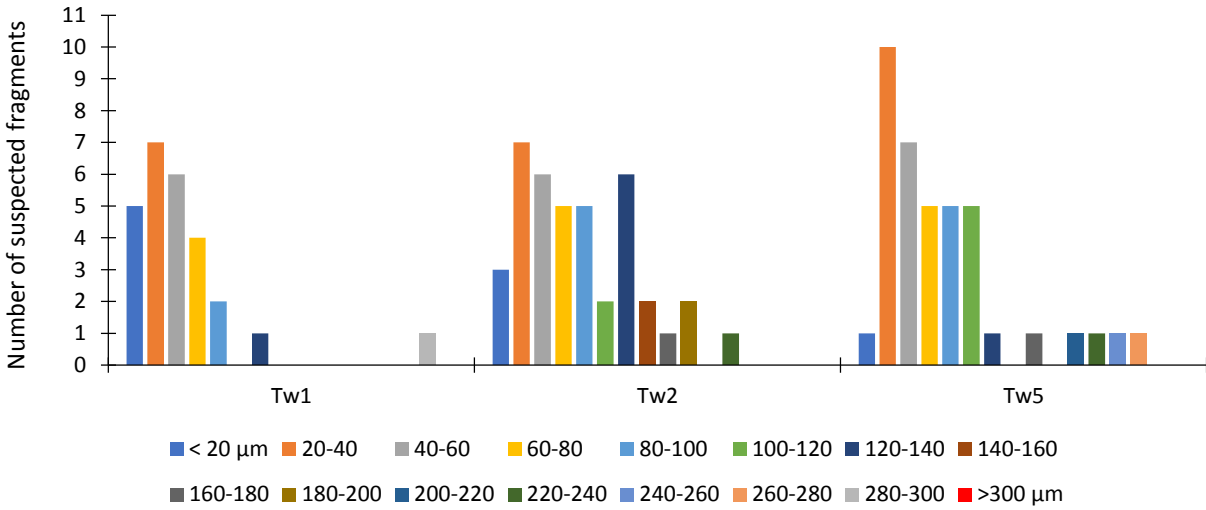
The authors would like to thank Sami Souissi and Capucine Bialais for providing us the live microalgae culture; Khalef Rabhi, Sebastien Monchy and Mamadou Diop for field assistance.

SUPPLEMENTARY FILES

Supplementary Figure S1: Number of suspected fragments with their different size classes found in mussels during the depuration experiment.



Supplementary Figure S2: Number of suspected microplastics fragments of different size classes found in mussels during the five weeks caging experiment.



Supplementary Table S1: Different characteristics (form, size and color) of polymers found in both seawater and mussels samples.

	All observed sizes and forms	Water	Mussels
PS	Fragments and microbeads < 200	Fragments: blue, yellow, and transparent Microbeads: white	blue and red
ABS	Fragments < 200	Blue, red	blue and red
PP	Fragments < 200 and > 200	Blue and green < 200 ; transparent, white and red > 200	blue and green
Co-PP/PE	Fragments < 200	Blue	blue
PUR	Fragments < 200	Blue, red, yellow	red
PA	Fragments < 200 and fibers > 200	Blue, black, red, green, transparent	blue, black, green
PET	Fibers	Blue, black, red	black and red
PC	Fragments < 200	Blue	Blue
PE	Fragments > 200; Microbeads > 200	Blue, red, green, yellow, transparent; microbeads: blue and green	
PAN	Fiber	Blue	
Co-Ethylene	Fragments > 200	Transparent	

Supplementary Table S2: Environmental parameters measured during each sampling campaigns.

	Temperature (°C)	Salinity (P.S.U)	Oxygen (mg/L)	Turbidité (N.T.U)
10/07/2018	19.5	34.7	8,87 mg/L	35.5
17/07/2018	19.7	33.5	8.01 mg/L	112
24/07/2018	21.7	32.1	9,38 mg/L	9.01
17/08/2018	19.7	34.2	8,14 mg/L	65

C. Synthesis of Article 5

A six weeks period of caged mussels caging was tested and MPs was compared with those found in water, sediments and native mussels. Farmed blue mussels underwent a depuration experiment as described above (IV.2.1.1IV). Then, they were transplanted in a high contaminated area by microplastics. The objective was to test the effectiveness of mussel caging approach for MPs biomonitoring on a six weeks deployment time.

Farmed mussels had a high depurated percentage of 97.6% assuring that the transplanted mussels were clean. Caged mussels showed a number of ingested microplastics similar or higher than those found in the field but it was not significantly different. Microplastics in the analyzed matrices had a different spatial pattern but Morisita's similarities overlap was high for MPs sizes was high between caged mussels and native (0.98), and also a high similarity for the types of polymer between caged mussels and sediments (0.93). Fragments dominated our samples and MPs of a size $< 200 \mu\text{m}$ were highly abundant.

A dissimilarity in the distribution of MPs along the matrices is can be avoided if a more adapted sampling strategy was taken into account: several factors are to be considered such as the sampling time according to the tide, the time lag necessary for MPs to float in the water column, to be ingested by mussels and to be sedimented at the bottom. If we rely on the assumption that mussels reach a "steady-state" for MPs concentrations, it shows that a six-week exposition period appear to bioaccumulate MPs and, therefore, reflect the number of MPs ingested by native mussels. This approach will allow a more reliable assessment in the marine coastal environments and to be used as an active biomonitoring for MPs contamination.

V. ARTICLE 5: IS BLUE MUSSEL CAGING AN EFFICIENT METHOD FOR MONITORING ENVIRONMENTAL MICROPLASTICS POLLUTION?

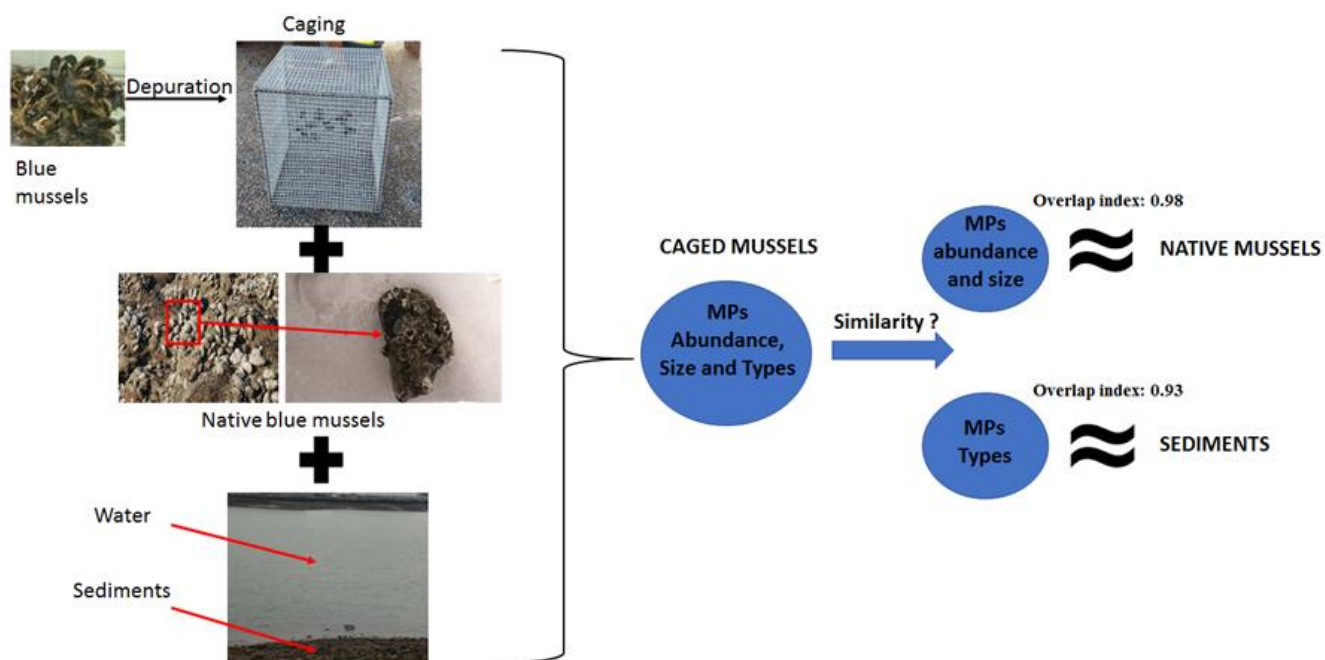
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Published in "Science of the Total environment", Article Number: 135649, DOI: 10.1016/j.scitotenv.2019.135649

GRAPHICAL ABSTRACT



ABSTRACT

The effectiveness of mussel caging for active microplastics (MPs) biomonitoring was investigated for the first time by comparing abundance and characteristics (shape, size, color and type of polymers) of MPs ingested by caged depurated blue mussels with those ingested by native mussels collected at the same sites and with those found in their surrounding environment (surface water and sediments). Mussels were exposed along a pollution gradient originating from a wastewater treatment plant discharge and near an abandoned coastal landfill. After 6 weeks of deployment, the majority (93%) of clean transplanted mussels had ingested MPs with a mean number of items ranging from 0.61 to 1.67 items/g. The occurrence, abundance and properties of MPs ingested by caged mussels were similar to those found in native mussels. Among the debris items detected in caged and native mussels, fragments were the most predominant type, consistent with the MPs found in their surrounding environment. MPs sizes were very similar whether in the water, sediments and both caged and native mussels, with a dominance of items < 150 μm . Although some polymers were under-represented or totally absent in the caged mussels compared to overlying seawater or surrounding sediment, there was a good overlap in polymer types proportion being found between caged mussels and sediments (Morisita's index of similarity = 0.93) or seawater (0.86). Polystyrene dominated all samples in all the different matrices. Our study suggests that blue mussels caging may be a promising tool for MPs biomonitoring making monitoring more reliable with an accurate assessment of the biological effects of MPs over a predetermined exposure period. However, further methodological improvements should be considered to define a uniform protocol for blue mussels caging to allow spatial and temporal microplastics active biomonitoring.

Keywords: Microplastics; Biomonitoring; Caging; Blue mussels; micro-Raman

1. INTRODUCTION

Filter-feeding bivalves such as mussels of the genus *Mytilus* have been widely used as sentinel organisms to monitor coastal water pollution (Beyer et al., 2017; Marigómez et al., 2013; Turja et al., 2014). The popularity of mussels as bioindicators, stems from their biological and ecological characteristics which make them virtually ideal for pollution monitoring. These sedentary organisms filter large amounts of water efficiently and use waterborne particles as food and, thus, are able to bioaccumulate high amounts of different contaminants (Beyer et al., 2017; Farrington et al., 2016). Their broad geographical distribution, abundance and ability to tolerate a wide range of environmental parameters make them species of choice in large-scale and long-term marine monitoring programs (Andral et al., 2004; Scott et al., 2019; Turja et al., 2014). Most often mussel biomonitoring studies involve collection of samples from natural populations, but the adoption of an active biomonitoring alternative by using transplanted mussel (caging) has gained considerable popularity in ecotoxicology research and monitoring (Andral et al., 2004; Brooks et al., 2018; Galgani et al., 2011; Lacroix et al., 2015; Marigómez et al., 2013; Tsangaris et al., 2011). Mussel caging is particularly useful when indigenous mussels are scarce or absent at the planned study sites (Li et al., 2019). In addition, using caged mussels from a single population minimizes confounding factors such as the genetic variability and the physiological, age or reproductive status of the organisms that influence contaminant bioaccumulation (Schøyen et al., 2017; Viarengo et al., 2007). However, several studies have previously reported differences in response between caged and native organisms exposed to environmental pollution. Observed differences were attributed to several factors: absence of cumulative long-term effects of pollutants in caged organisms (Nigro et al., 2006), the existence of adaptive traits or compensatory mechanisms in native mussels chronically exposed to pollution, leading for example to lower bioaccumulation levels than in short-term caged organisms (Greenfield et al., 2014; Lacroix et al., 2015; Marigómez et al., 2013; Schøyen et al., 2017). Others studies found that native mussels accumulated higher concentrations of chemicals (Bolognesi et al., 2004; Piccardo et al., 2001). In some studies, the two monitoring approaches showed similar temporal and spatial changes in chemical contaminant concentrations (Hunt and Slone, 2010; Piccardo et al., 2001). The caging influence on mussels contaminants accumulation is still poorly understood (Schøyen et al., 2017). Many studies have suggested that an integrated use of monitoring data from both native and transplanted mussels may provide a more accurate

assessment of environmental pollution (Bebianno et al., 2007; Brooks et al., 2012; Hunt and Slone, 2010; Marigómez et al., 2013; Serafim et al., 2011).

Microscopic particles and fragments of plastics smaller than 5 mm (commonly called microplastics, MPs) represent a matter of growing concern for the marine environment. Their ingestion has been documented in several marine species worldwide (Cole et al., 2013; Watts et al., 2015), particularly in coastal marine environments acting as recipients for plastics and MPs input sources (Leslie et al., 2017; Kazour et al., 2019). The sources and the occurrence of MPs in the marine environment, and their potential biological impact are subjects to major scientific and public concern. To monitor spatial and temporal trends of MPs pollution and to understand the risk they pose to the health of marine species, it is important to obtain accurate measurements of MPs abundance in environmental compartments (such as water, sediments and biota). However, in highly variable environments like the marine coastal areas, the assessment of MPs abundance in water and sediments is often imprecise and tend to be affected by a variety of environmental factors such as winds, tides, currents and bioturbation (Scott et al., 2019). A key challenge for marine MPs biomonitoring is to develop an appropriate and standardized method with a sentinel organism that can be applied over large spatial and temporal scales to allow cross calibration between studies worldwide. Recently, many studies have focused on the uptake of MPs by mussels, both in laboratory experiments (Browne et al., 2008; Van Cauwenberghe et al., 2015) and in their natural habitats (De Witte et al., 2014; Li et al., 2018; Phuong et al., 2018c; Qu et al., 2018; Santana et al., 2016; Zhao et al., 2018). This species faces a strong exposure to microplastics because of its high filtering activity (Riisgård et al., 2014). Microplastic abundance in field collected mussels is closely related to human activity (Kazour et al., 2019b; Li et al., 2016), and there is evidence for a positive and quantitative correlation between MPs in mussels and surrounding waters (Qu et al., 2018). Their capacity to integrate and amplify the MPs contamination signal (Karlsson et al., 2017) led to their potential consideration as bioindicator for coastal MPs pollution (Beyer et al., 2017; Li et al., 2019; Wesch et al., 2016). The International Council for the Exploration of the Sea (ICES) has suggested blue mussels as suitable sentinels for monitoring of MPs contamination (OSPAR, 2015; Vandermeersch et al., 2015). Although *Mytilus* spp. controlled deployment has been largely used to study diffuse chemical contaminants and biomarkers, this approach was only recently used to study MPs pollution. To our knowledge, only three studies used caged blue mussels in specific areas to

investigate the MPs pollution related to specific anthropogenic activity such as wastewater discharge (Railo et al., 2018), wreck removal (Avio et al., 2017), or to assess seasonal changes in plastic pollution (Catarino et al., 2018). This latter study suggested that the use of cage deployed mussels can be an effective method to quantify and assess MPs pollution in the field; whereas the study of Avio et al. (2017) highlighted the limited capability of transplanted mussels to discriminate MPs pollution around the wreck area.

Mussels are hardy creatures tolerant to handling that are easy to keep in culture, making them suitable for translocation and caging exposure. In addition, the mussels can be depurated in the laboratory before caging and, thus, allow the use of reference individuals to be deployed in different areas. The objective of this research was to investigate the MPs ingestion in caged blue mussels exposed along a pollution gradient originating from a WWTP discharge and near an abandoned coastal landfill (Kazour et al., 2019b). The effectiveness of the mussel caging approach to quantify and assess MPs pollution in the field was assessed for the first time by comparing abundance and characteristics (shape, size, color and type of polymers) of MPs ingested by caged mussels with those ingested by native mussels collected at the same site and MPs found in their surrounding environment (surface water and sediments).

2. MATERIAL AND METHODS

2.1 MUSSELS FIELD DEPLOYMENT AND SAMPLING

2.1.1 MUSSELS DEPURATION

One week prior to field deployment, blue mussels (*Mytilus edulis*) were collected from farmed Bouchot mussels from Camiers near Boulogne-sur-mer, (50°35'43.1"N, 1°34'41.3"E; French Eastern English Channel coast). Mussels were sorted in the laboratory to get 157 individuals of the same size range (5.77 ± 0.5 cm shell length, mean \pm standard deviation). Seven of these mussels were immediately frozen at -20°C (reference samples collected prior to the start of the depuration experiment) and the remaining 150 individuals were pre-cleaned to remove any adhering organisms and placed in a 100 L glass aquarium (1 m x 0.45 m x 0.3 m) meticulously pre-rinsed with Milli-Q water. The aquarium was maintained in a thermoregulated room with a constant temperature of $8 \pm 1^\circ\text{C}$ and a 12 h light-dark illumination regime. The aquarium was

equipped with an air pump and was supplied by filtered sea water (filtered on Polycarbonate filters; 0.1 μm) and the water was changed every 24 hours for 7 consecutive days.

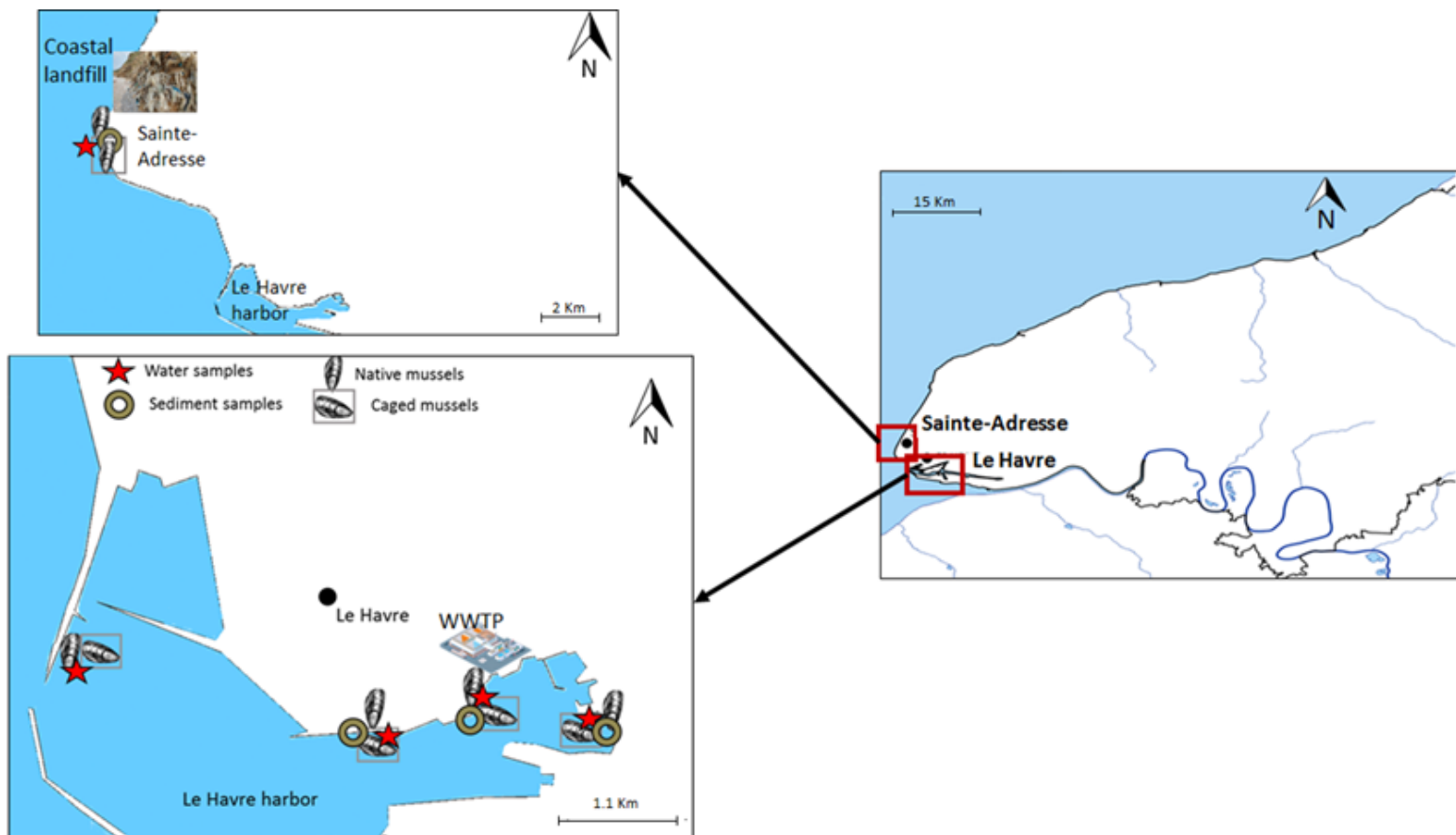


Fig. 1: Mussels caging site location with respect to the wastewater treatment plant (WWTP, Le Havre harbor) and the coastal landfill (Sainte-Adresse).

During the experiment, the mussels were daily fed (except on Saturday and Sunday) with live microalgae, *Rhodomonas* sp. and *Isochrysis* sp. in order to enhance the mussels' filtration capacity. After 7 days of depuration, seven mussels were frozen at -20°C in order to compare the MPs concentration in both non-depurated and depurated mussels. The rest of the mussels were used for caging experiment.

2.1.2 TRANSPLANTATION AND CAGING EXPERIMENT

After the depuration period, mussels were transported to caging sites in a small aquarium (60 L, 0.6 m x 0.35 m x 0.3 m) supplied by oxygenated filtered sea water. The translocation experiment was carried out between the 3rd and the 4th of February 2018 and lasted 42 days (till until the 4th and 5th of April 2018). Five marine coastal sites were chosen for the caging experiment: four sites in Le Havre harbor along a pollution gradient originating from a WWTP discharge and one site near an abandoned coastal landfill (Fig. 1; see Kazour et al., 2019 for more detailed sampling locations). At each site, a stainless-steel cage (50 cm x 50 cm x 50 cm, length x width x height) with no plastic materials to avoid contamination was deployed. The cage's mesh size was 15 mm allowing water circulation and stopping mussels from falling out of the cages. A stainless-steel grid was placed in the mid-height of the cages as a suitable substrate allowing mussels' attachment and avoiding direct contact with sediments. 17 to 23 mussels were put in each cage. The cages were deployed during low tide in the lower intertidal shore zone and attached to the bottom using four reinforced iron rods. The study area is characterized by semi-diurnal tide with an average tidal range of about 1 m at neap tides and 6 m at spring tides.

After retrieval, each mussel was put separately inside an aluminum foil and frozen at -20°C until analysis. Water temperature, salinity and dissolved oxygen were measured using a multiparameter probe (Mutiparameter HI 982, HANNA instruments) during cages transplantation and retrieval.

2.2 WATER, SEDIMENT AND NATIVE MUSSELS SAMPLING

Water, sediments and native mussels were taken on the 3rd and 4th of April as described in Kazour et al. (2019). In short, at each site, 1 to 2 m³ of the first 10 cm of the water column was pumped and filtered on stainless steel sieves of different mesh sizes (500 µm, 200 µm, 80 µm and 20 µm). Infralittoral sediments were also collected at each site except for site 4 (due to its

rocky substrate). Sea water and sediments analyses were performed according to Kazour et al. (2019).

Mussels from the French Eastern English Channel coast are dominated by *Mytilus edulis*, but some areas are also populated by hybrids between *M. galloprovincialis* and *M. edulis*. This is the case of Le Havre Harbor where 70% of the mussels are hybrids (J. Couteau, personal communication). Therefore, for native collected mussels the term *Mytilus* spp. is used in this study. Native mussels (*Mytilus* spp.) were hand collected from the lower intertidal shore zone from the same sites near the deployed cages. At each site, 10 mussels of similar size were collected and immediately conserved inside an aluminum foil and frozen at -20°C until analysis. In the Harbor (sites 1 to 4), mussels were bigger 6.44 ± 0.73 cm (mean shell length \pm standard deviation) than at site 5 (3.2 ± 0.4 cm).

2.3 MICROPLASTICS ANALYSES

2.3.1 CONTAMINATION CONTROL

Microplastics contamination control is an important step during microplastics analysis. Several precautions were followed. Cotton lab coats were worn throughout the experiments, and all steps (filtration, measurements and digestion) were realized under a laminar flow hood. Only laboratory materials (bottles, petri dishes, Erlenmeyers, filtration system) made of glass were used. All used solutions were filtered three times on glass fibers filters (GF/A) Whatman, (France) in order to remove any unwanted particles and fibers. All surfaces and equipment were cleaned using filtered ethanol 70% and MilliQ water. With every manipulation, a control was made following the same conditions as the samples in order to track the contamination.

2.3.2 SAMPLES PREPARATION

Water and sediment samples were prepared as described by Kazour et al. (2019). In short, water samples were rinsed inside a separation funnel and underwent a density separation step using zinc chloride (ZnCl_2 ; 1.8 g/cm^3) and the supernatant was filtered on GF/A filters. Sediment samples were digested using hydrogen peroxide 30% (H_2O_2 30%) then underwent density separation using ZnCl_2 .

Mussels were defrosted 4 hours prior to digestion and their total length, width (cm), total and net weight (g) were measured. The condition index (CI) was calculated as follow (AFNOR, 1985):

$$CI = \left(\frac{\text{Net Weight (g)}}{\text{Total weight (g)}} \right) \times 100$$

This index is a good indicator for the physiological condition (tissue growth, energy reserves) of mussels (Orban et al., 2002).

Mussels were digested using a solution of potassium hydroxide (KOH) 10% (m/v, ChimiePlus, France) (Dehaut et al., 2016). Caged mussels were thawed, dissected and put inside graduated Erlenmeyers equipped with glass stopper containing a volume of 150 mL of KOH 10% (the volume 150 mL was used because it was the necessary volume that led to an optimal digestion efficiency percentage). These Erlenmeyers were maintained on a heating magnetic stirrer at 60 °C for 24 h. After digestion, the digestat of each individual was filtered on a GF/A filter (90 mm diameter). The same procedure was done with native mussels but a volume of 250 mL was added instead (see Kazour et al. 2019).

2.3.3 VISUAL AND RAMAN SPECTROSCOPY ANALYSIS

All filters were observed under 120x magnification using Leica M165 C Stereomicroscope and images of suspected MPs particles were taken with a Leica M170 HD camera and LAS (Leica application suite) software. All suspected MPs particles were counted, categorized by type (fragments, fibers, microbeads or films) and color. The following criteria were taken into consideration while observing the items: (1) absence of cellular or organic structures; (2) a homogenous thickness across the particles; and, (3) homogenous colors (Hidalgo-Ruz et al., 2012). Measurements were done on the suspected items at their longest dimension and they were divided into different size classes of 50 µm.

Suspected microplastics were identified using Micro-Raman Xplora Plus (HORIBA Scientific[®], France). The machine is protected by a door so that the filter placed inside would not be affected by any airborne contamination. Due to the time consumption it takes to analyze filters under micro-Raman, five filters of caged and native mussels containing the highest number of potential MPs from each sampling site were chosen and analyzed. All sediments items, and subsamples of

water items were also taken for Raman analysis. For identification, two lasers with a wavelength of 532 nm and 785 nm and a range of 200-3400 cm^{-1} were used with x10 and x100 objectives (Olympus). Each particle spectrum is compared to a polymer database identification software (KnowItAll, BioRad[®]) and a personal library made with standard polymers obtained from Goodfellow (France). KnowItAll software gives a resemblance (correlation) between the obtained spectra and the one existing in the database. If the obtained spectrum showed a high fluorescence, several acquisition parameters are changed from one sample to another: whether in the acquisition time (0.2s, 1s, 2s, or 5s), the number of accumulations (2, 5, 10, or 15), the laser wavelength and intensity (0.1%, 1%, 10%, 50% or 100%), the slit and the hole, to obtain a better identifiable spectrum. The identification is considered correct when the HQI (Hit Quality Index) was above 70 (ranging from 0 to 100). Baseline correction was realized on spectra in order to have a cleaner identifiable spectrum. Pigments spectra (Copper phthalocyanine, Hostasol Green, etc...) obtained were identified using an option called “Mixture Analysis” existing in “KnowItAll software” that is capable to decompose a mixed spectrum into two spectra consisting of the dye and its associated polymer (see Supplementary Figure 1 for an example).

2.4 STATISTICAL AND DATA ANALYSES

Microplastics found in water, sediments and mussels were reported in unit volume (per L) and per gram of sample, respectively. Statistics were performed with SPSS software (IBM SPSS STATISTICS 20). The *Wilcoxon* signed-rank *test* was used to compare the ingested MPs between depurated and non-depurated mussels and a Student t-test was used to compare the condition index. For inter-site comparisons, if data did not comply with the parametric assumption of normality (Shapiro–Wilk tests) and homogeneity of variance (Levene tests), the non-parametric Kruskal–Wallis test and Mann–Whitney U test for post hoc pairwise comparisons were used. All results are expressed as mean \pm SD, and p-value $<$ 0.05 was considered as statistically significant. Morisita’s index of similarity (or Morisita overlap index) is a measure of how similar or different two sets of data are. This index was calculated to compare the size and the polymer types similarity found in caged mussels with those found in native mussels, water and sediments samples.

$$\hat{C} = \frac{2 \sum_i^n \hat{p}_{ij} \hat{p}_{ik}}{\sum_i^n \hat{p}_{ij} \left[\frac{(n_{ij} - 1)}{(N_j - 1)} \right] + \sum_i^n \hat{p}_{ik} \left[\frac{(n_{ik} - 1)}{(N_k - 1)} \right]}$$

\hat{C} : Morisita's overlap index between j and k

j : Caged mussels

k : Native mussels or water or sediments

\hat{p}_{ij} : Proportion of a size class or a polymer i used by j

\hat{p}_{ik} : Proportion of a size class or a polymer i used by k

n_{ij} : Number of size class or polymer j that use i

n_{ik} : Number of size class or polymer k that use i

$N_j ; N_k$: Total number of size class or polymer in sample

This index ranges from 0 (when no common size or polymer types are shared between the two sample types) and 1 (when all sizes or polymer types are common between the two sample types).

3. RESULTS

3.1 MUSSELS CAGING EXPERIMENT

Before translocation, mussels were depurated. There was no significant difference in mussel's condition index after seven days of depuration ($p = 0.264 > 0.05$). However, a significant difference in the amount of MPs was observed between non-depurated and depurated mussels ($p = 0.018 < 0.05$). Non-depurated mussels had an average of 2.22 ± 1.35 items/g with fragments constituting 82.3% of the observed items (Fig. 1). The number of items significantly decreased by 97.6% after 7 days of depuration reaching 0.05 ± 0.15 items/g consisting only of fibers. The size of observed fibers before depuration ranged from 41.54 μm to 622.04 μm with a mean size of 284 ± 156.8 μm ; these fibers were of different colors: black, green, blue and red. Only two blue fibers were observed after depuration with a respective length of 304.06 and 415.73 μm .

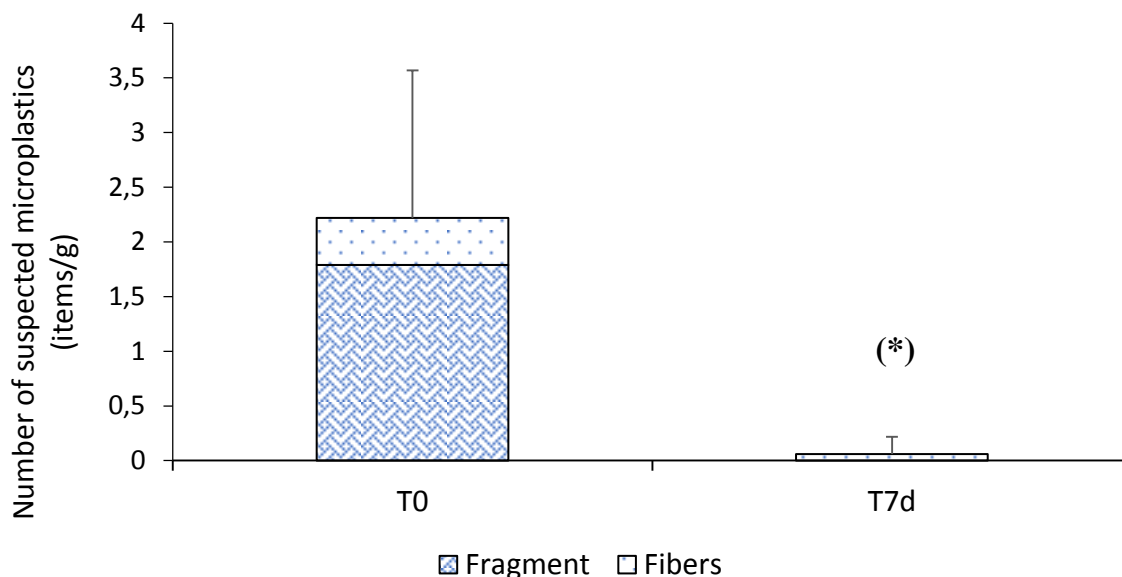


Fig. 2: Average (+SD) number of suspected microplastics (items/g of mussels' wet weight) by shape categories found in the non-depurated mussels (T0: farmed mussels) and the 7 days depurated mussels (T7d).

Even though the cages were transplanted during the winter period when the temperature had been at its lowest (around 6-7°C), the mean mussels survival rate was 73%. After 42 days of caging experiment, mussels shell length (5.7 ± 0.47 cm) and condition index ($54.7 \pm 3.8\%$) were not significantly different from the beginning of the experiment ($p = 0.621 > 0.05$). Also, there was no significant inter-sites difference in the caged mussels condition index ($p = 0.521$).

3.2 MICROPLASTICS ABUNDANCE, TYPE AND COLOR

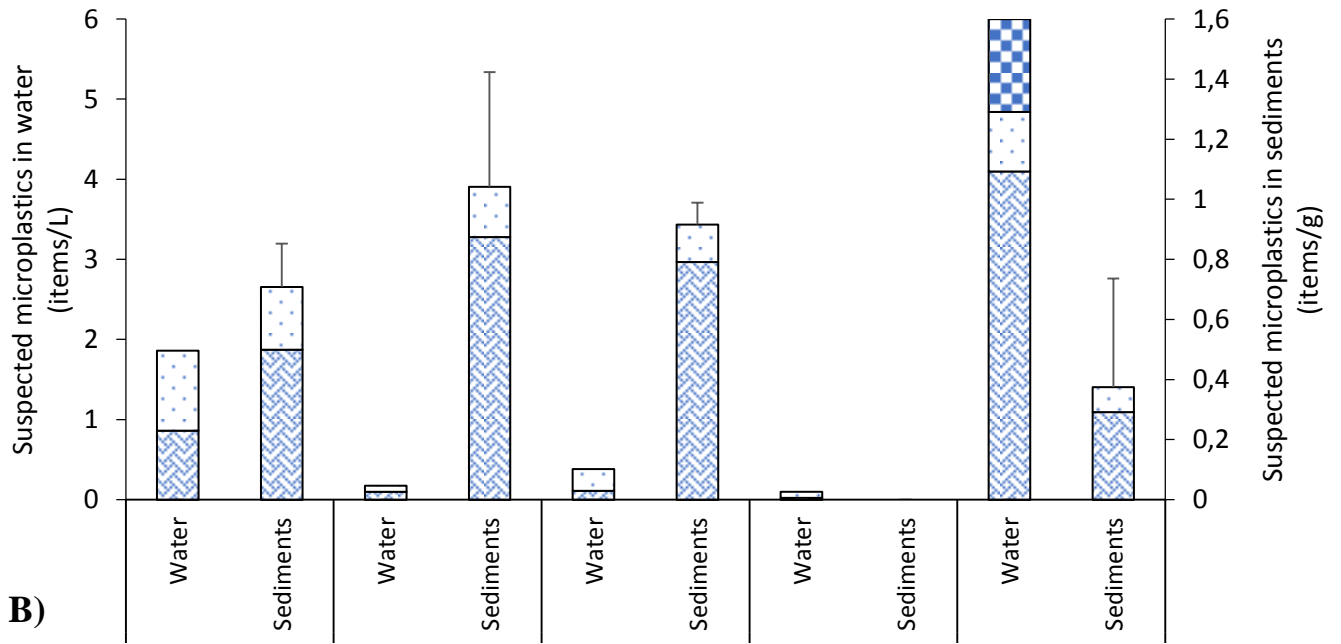
The absence of fragments, microbeads and films in our procedural background blanks indicates that contamination was only limited to airborne fibers. The average fiber contamination during caged mussels dissection and digestion, had an average of 0.72 ± 0.78 fibers (representing the average found for a batch of 9 mussels representing a total one hour constant laboratory work under the laminar flow hood). Similar values were observed for the other three matrices (see Kazour et al. (2019)). The importance of following procedural blanks is that any potential contamination is then eliminated from the analyzed samples.

Microplastic contamination of seawater, sediments, and mussels was evident at all of our sampling sites. The number of suspected MPs in the water decreased with the increasing distance from the effluent of the WWTP and the highest amount was found near the landfill (site 5). Such pattern was not observed for MPs found in the sediments (Fig. 3 - A). Suspected MPs were

found in mussels of all sites; 93% of native mussels and 94.7% of caged mussels had ingested suspected MPs with a mean number of items ranging from 0.41 to 2.76 items/g. The spatial pattern of MP abundances in mussels was different from that observed for water or sediment samples (Fig. 3). Caged mussels ingested the same or higher amount of MPs than native mussels at all sites except for site 5; however for the same site, the amount of ingested MPs by caged and native mussels were not significantly different ($p > 0.05$) (Fig. 3).

Both fragments and fibers were found in the water, sediment and in both caged and native mussels. In all the analyzed matrices, fragments dominated the type of MPs except at sites 1, 3 and 4 for water samples (Fig. 3). Primary microplastics (polystyrene raw microbeads) were found only at site 5 in the water but were not detected in mussels nor in sediment samples. Films were observed only in native mussels. Blue fragments dominated the water (45%) and sediment (69%) samples whereas red and blue fragments dominated in both caged (76%) and native mussels (42% each). For the fibers, black (51%) and blue and green color (71.4%) dominated the water and sediment samples respectively whereas blue and black fibers dominated in caged (85%) and native (80%) mussels. At each site, the proportion of MPs items type or color was similar among caged and native mussels. For fragments, the order of dominant colors was blue, red, green, white and transparent; whereas for fibers, it was blue, black, green and red.

A)



B)

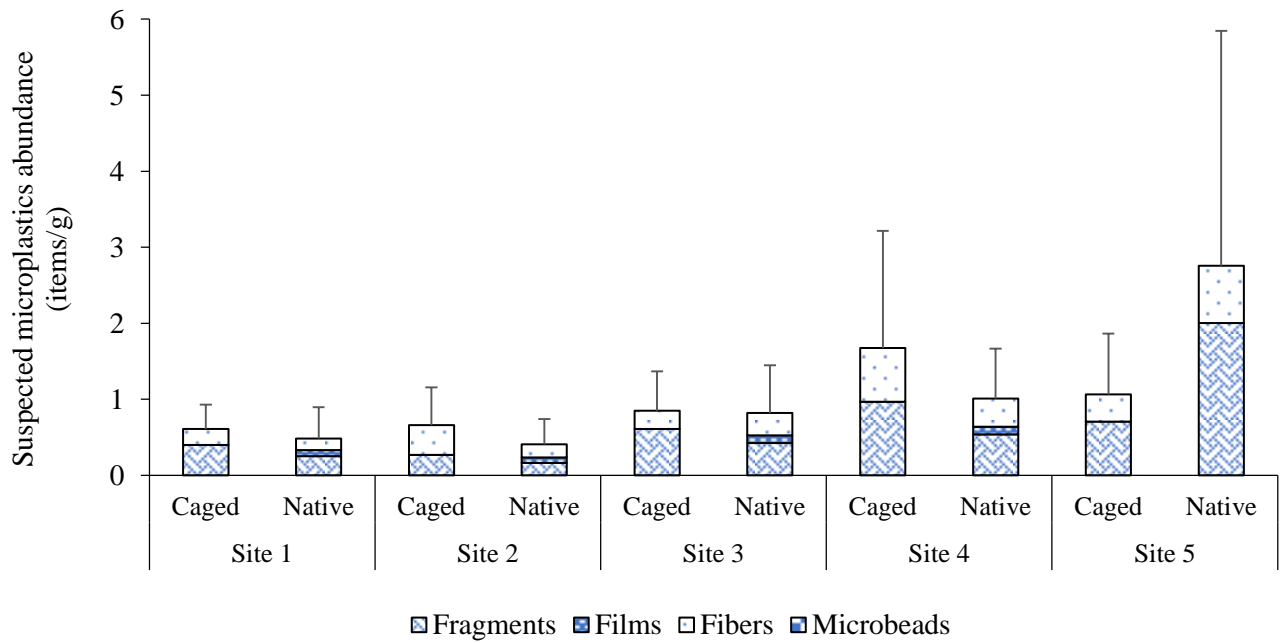


Fig. 3: Average (+SD) number of suspected microplastics by shape categories found in (A) the surface water (items/L) and sediments (items/g), and in (B) the caged and native mussels (items/g of mussels' wet weight).

3.3 MICROPLASTICS SIZE AND POLYMER TYPES

Suspected MPs sizes were very similar whether in the water, sediments and both caged and native mussels, with a dominance of items < 150 μm (Fig. 4). This was confirmed by the very high overlaps between caged mussels vs native mussels (0.98), vs sediments (0.9) and vs water samples (0.84). Smaller size classes (< 200 μm) were more abundant in native mussels compared to caged mussels (Fig. 4 - A). Caged mussels showed greater number of items with a size class > 200 μm compared to native mussels. Sediments and water samples showed higher proportion of items of size class < 150 μm compared to caged mussels (Fig. 4 - B-C).

Of the 503 items analyzed with the micro Raman, 422 (83.9%) were positively identified as a known polymer (examples of various parameters used to identify samples are indicated in Supplementary Table 2). Among the non-polymers items, only 2% were identified as cellulose-based fibers. The rest of the spectra were highly fluorescent and non-identifiable (for example if a sample is too thin, Raman tends to detect the underlying substrate instead of the sample (Käppler et al., 2015)).

Five polymers were identified in caged mussels, by order of dominance: polystyrene (PS), acrylonitrile butadiene styrene (ABS), polyamide (PA), polyurethane (PUR) and polyethylene (PE). A higher number of polymers were identified in native mussels (9), water (11) and sediments (8) (Fig. 4). PS dominated all samples in all the different matrices with ABS and PA the second most abundant polymers. The highest overlap in polymer types was found between caged mussels and sediments (0.93) followed with native mussels (0.88) and water samples (0.86).

4. DISCUSSION

The key advantage of using blue mussels for microplastics biomonitoring, is that they can be depurated in the laboratory, reducing initial contamination bias, and thus, clean reference individuals will reflect the MPs contamination of their specific deployment sites. In mussels, MPs may be retained for extended periods of time, for example, complete clearance of MPs was not achieved after a seven-days depuration period under laboratory conditions with microbeads (2,6 μm) being retained within the digestive tracts (Paul-Pont et al., 2016). Our results showed that after 7 days of depuration, 97.6% of the MPs were eliminated by the mussels. Similar

depuration rate (85%) was observed in the Mediterranean mussel, *M. galloprovincialis*, after seven days depuration period (Fernández and Albentosa, 2019).

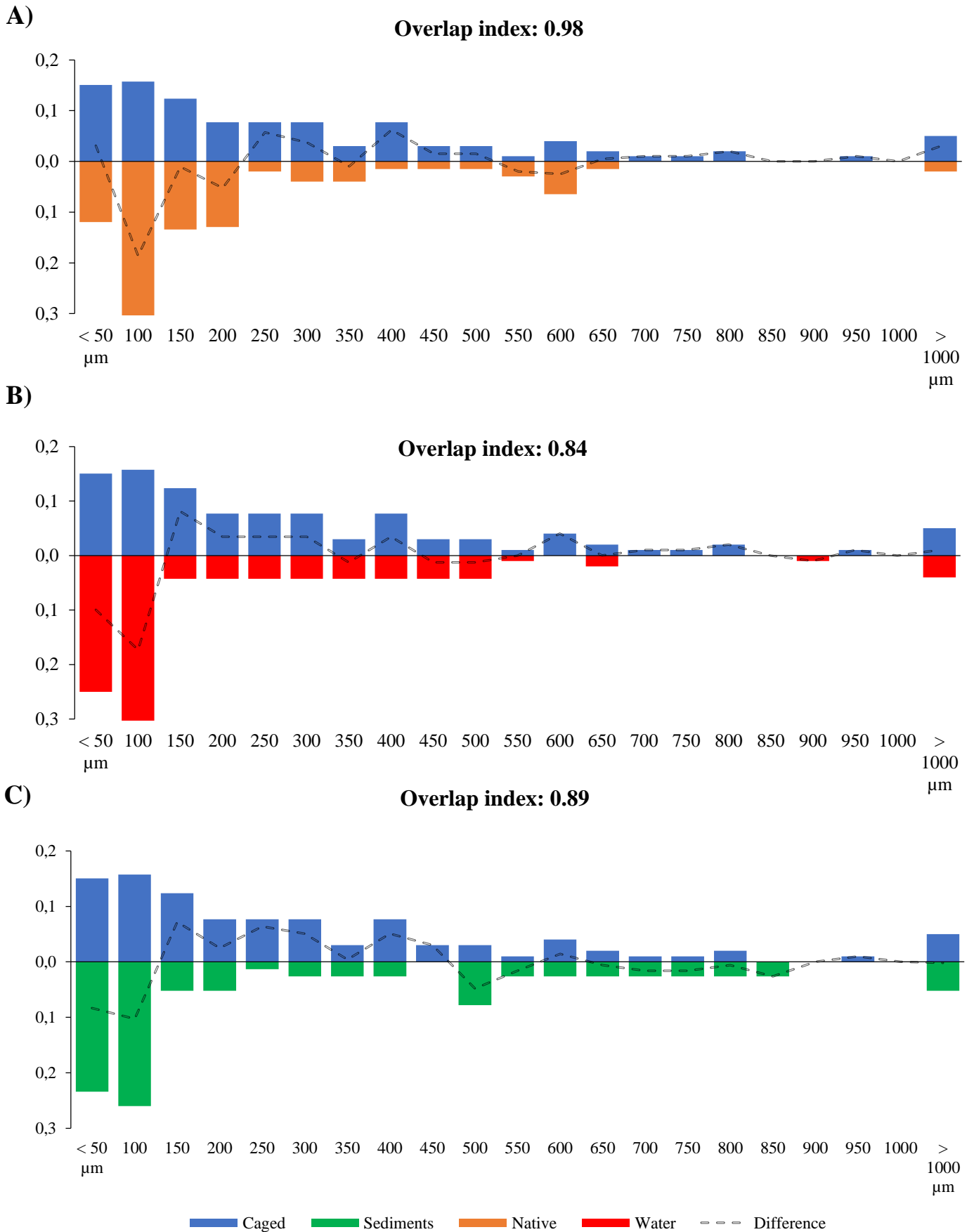


Figure 4: Back to back histograms of size classes of suspected microplastic items found either in the caged mussels (in blue), in the native mussels (orange), in the surface water (in red) and in sediments (in green). The difference in size classes frequency between samples is shown with the dotted black lines, while the overlap index (Morisita's index) is indicated within the graph.

Lower depuration period seems however to be insufficient for total depuration; in a three days depuration experiment of farmed *M. edulis*, Van Cauwenberghe and Janssen (2014) found a reduction of ca. 33% in the MPs quantity. Since depuration significantly decreased the quantity of MP in blue mussels, the eliminated particles were probably located in the digestive system as suggested for the brown mussels (*Perna perna*) by Birnstiel et al. (2019).

The main objective of the study was to test effectiveness of the mussel caging for active microplastics biomonitoring, and this was investigated by comparing MPs ingested by caged blue mussels with those ingested by native mussels collected at the same site and MPs found in their surrounding environment (surface water and sediments). Two aspects can be compared: the quantitative aspect (MPs concentrations in the different matrices) and the qualitative aspect (shape, size, color and type of polymers) of MPs.

4.1 QUANTITATIVE MPS COMPARISON

A key question for microplastics caging studies is how long the blue mussels should stay deployed to be equalized with native mussels and to reflect MPs concentrations of their surrounding environment. For chemical pollution, the reported caging periods lasted generally between 3 to 6 weeks but may last 18 - 24 weeks or up to 2 years (see review in Beyer et al., 2017). Short term mussel deployments, such as one or two months, has been shown to be suitable for chemical pollutant monitoring in coastal waters, e.g. metals (Greenfield et al., 2014; Lacroix et al., 2015; Piccardo et al., 2001). To date, only three studies used caged blue mussels to investigate the MPs pollution (Avio et al., 2017; Catarino et al., 2018; Railo et al., 2018). These studies showed that mussels transplanted for 4 weeks period have accumulated MPs in their tissues. However, they have not compared the ingested MPs with those found in native mussels or in their surrounding environment. In the present study, even though mussels were not transplanted during their optimal condition (the seawater temperature has been at its lowest: around 6-9°C), mussels' condition and growth were maintained after 6 weeks, and their survival rate was high (73%). More importantly, the majority (93%) of transplanted mussels had ingested MPs. This percentage was similar to that of native mussels (94,7%) collected in the same areas. In addition, the spatial pattern in the amount of ingested MPs by native and caged was quite the same except in site 5 where the native mussels ingested higher number of MPs. This difference

of ingested MPs can be due to the significantly lower body weight of native mussels from the site 5 compared to that of transplanted mussels (1.07 ± 0.35 g and 5.15 ± 1.11 g, respectively).

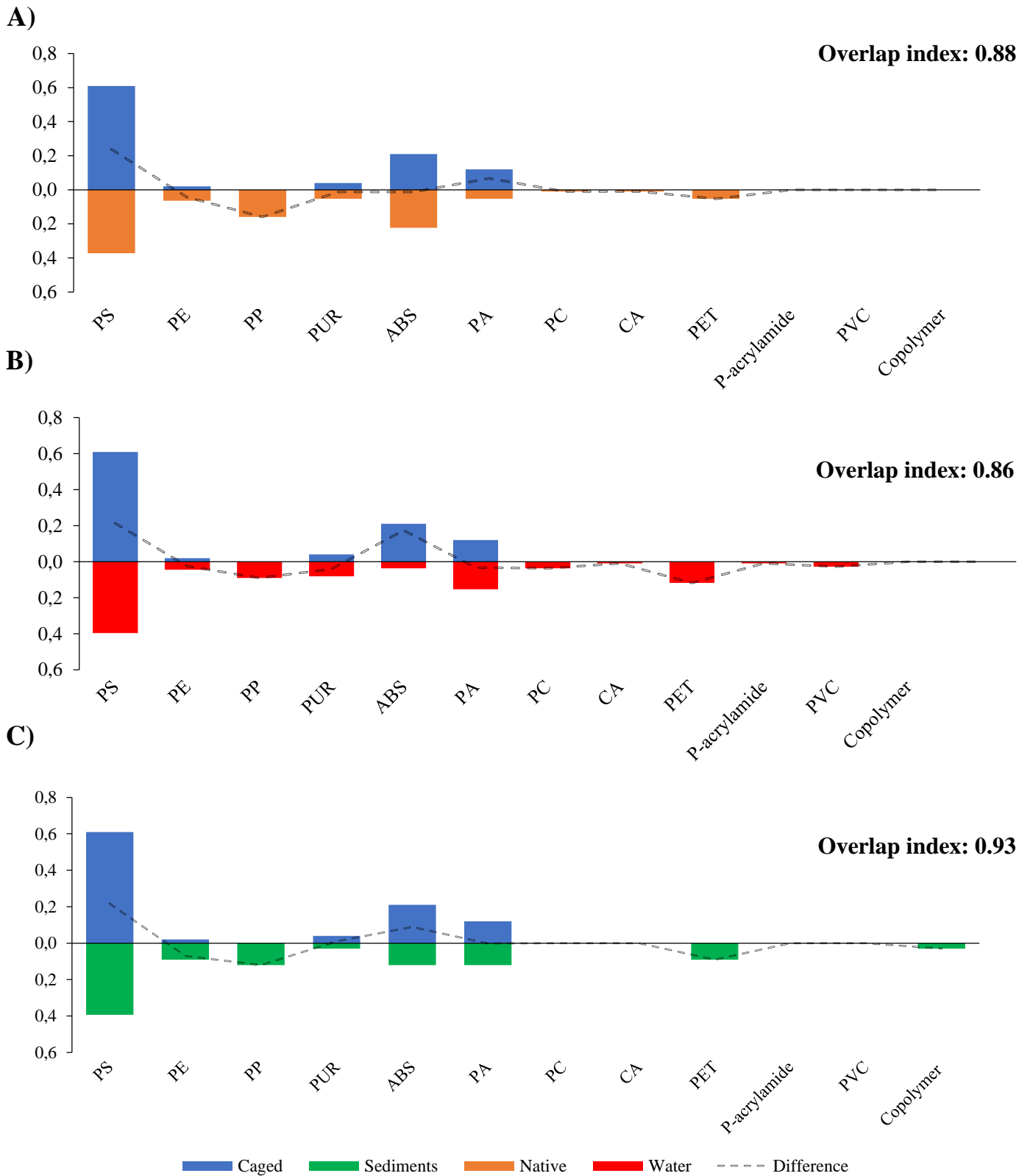


Figure 4: Back to back histograms of polymer types found either in the caged mussels (in blue), in the native mussels (orange), in the surface water (in red) and in sediments (in green). The difference in polymer frequency between samples is shown with the dotted black lines, while the overlap index (Morisita's index) is indicated within the graph.

The increase on the retention time of plastics particles with decreasing size in mussel has been reported (Fernández and Albentosa, 2019; Van Cauwenberghe and Janssen, 2014). Using caged mussels from a single population avoids bias related to the age, the size or the reproductive status of the organisms that can influence ingestion, retention or clearance rates.

Among the detected debris items in native and caged mussels, fragments were the most predominant type observed, consistent with the MPs found in mussels and cockles (Hermabessiere et al., 2019) and juvenile fish (Kazour et al., 2018) collected along the Eastern English Channel coast. This high occurrence and abundance of fragments in mussels reflect those found in their surrounding environment (seawater and sediments). A recent study showed a positive and quantitative correlation of MPs in mussels (*M. edulis* and *Perna viridis*) and their surrounding waters (Qu et al., 2018). The authors suggested that the amount of MPs in mussels can reflect the real abundance of MPs in the coastal water but that mussels were more likely to ingest smaller MPs. In our study, the spatial pattern of MPs concentrations in mussels (both caged and native) was different from that observed for water or sediment samples. Similar results were observed along the coastal waters of the U.K., with no quantitative correlation between MPs in mussels and their ambient seawaters (Li et al., 2018; Scott et al., 2019). Such absence of correlation may be due to the limited sampling sites (only five in our study and seven in Li et al. (2018) study) which are insufficient for robust correlation analyses. In addition, it is likely that a range of factors influence mussel encounter rates with particles within their immediate environment, including hydrodynamics processes in the study area, particle behavior in the water column and the mussels or seawater sampling location in the water column (Avio et al., 2017; Scott et al., 2019). In the study of Avio et al. (2017) mussels were caged for 4 weeks at 2 different depths, approximately at 1.5 m from the surface and from the bottom (30-45 m). They found some differences in the occurrence of ingested MPs as a function of caging depth, with higher ingested particles (32%) in surface deployed mussels compared to those caged in proximity to the bottom (12%). In addition, some variations were observed in terms of typology and size of particles between surface and bottom-caged mussels highlighting the influence of a different distribution of MPs along the water column. The density of the plastic particles will determine their position in the water column, and, therefore, the likelihood of encounter by an organism (Desforges et al., 2014). In our study, we sampled surface water whereas mussels were near the bottom. When comparing different matrices or compartments, a more adapted sampling

strategy should take into account numerous parameters such as the sampling time according to the tide, the time lag necessary for MPs to sink in the water column, to be ingested by mussels and to be sedimented at the bottom (Schmidt et al., 2018). As these conditions have not been implemented in the present study, it is therefore difficult to compare the MP concentrations found in mussels, water and sediments. Thus, the present data should be considered as semi-quantitative until the influence of environmental factors on the measured MP concentrations found in the seawater and sediments and those ingested by blue mussels have been further investigated. In addition, an understanding of MPs bioaccumulation is a prerequisite for the use of mussel as biomonitoring organisms in the aquatic environment.

4.2 QUALITATIVE (SIZE, COLOR AND POLYMERS) MPS COMPARISON

Comparison between native and caged mussels showed that, at each sampling site, the proportion of MPs items shape, size, color and to some extent their composition (polymer types) were quite the same with a high overlap index. Both native and caged mussels from all sites have mainly ingested small sized MPs (< 200 μm). This was similar to other studies that demonstrated that MPs below 300 μm were the most found in mussels (Leslie et al., 2017; Naji et al., 2018; Phuong et al., 2018c). Blue fragments were the dominant items found in all the analyzed compartments. Although small sized MPs dominated the water and sediments samples, it is currently unknown whether their abundance in mussels are due to mussels size preferences in regards to prey, or because the majority of MPs in the environment were typically in this size range (Bråte et al., 2018; Scott et al., 2019).

Concerning polymer types, a good overlap was found between caged mussel and the others analyzed compartments. The highest overlap in polymer types being found between caged mussels and sediments (0.93). Polystyrene (PS) dominated all samples in all the different matrices. This polymer was among the most common plastics used worldwide whether in packaging or other household materials. We found that some polymers were under-represented or totally absent in the caged mussels compared to overlying seawater or surrounding sediment. More polymer types (N=13) were identified in the surface water compared to sediments (N=9) or caged mussels (N=6). In the South West of England, Scott et al. (2019) found significant

differences in the relative abundance of polymer types and particle sizes between seawater, sediment, and mussels. However, they found that particles within mussels reflected more closely those found in the intertidal surface sediment compared to those found in the surface seawater. This is also the case in our study where particles found within caged mussels presented the highest similarity with those found in the sediments. The bioavailability of MPs to aquatic organisms depends on their characteristics, such as polymer type, chemical composition, size, shape, density, etc. (Carbery et al., 2018). The shape of MPs may also influence their accumulation (persistence inside mussels) or elimination (Browne et al., 2011; Thompson et al., 2004). As suggested by Scott et al. (2019), it is likely that both environmental and biological partitioning of microplastic particles and the selective feeding ecology of this species is responsible for the under-representation of certain polymer types and particle sizes within the mussels. This should be taken into consideration when using mussels for MPs pollution biomonitoring.

4.3 MUSSELS BIOMONITORING AS A STANDARDIZED TOOL FOR MICROPLASTICS ASSESSMENT

In this work, we were able to demonstrate the potential use of mussels as a MPs biomonitoring tool. But a lot of factors should be considered to optimize and standardize this tool. The first issue is that mussels represent different filtration rates depending on the region and genotype, for example (Lüskow and Riisgård, 2018; Riisgård et al., 2013). The difference in the uptake rate for analogous species needs to be further studied to appropriately adjust interspecific difference. Algal abundance, temperature and salinity of water, as well as the depth of the transplanted cages could alter mussel's filtration and clearance rates (Avio et al., 2017; Comeau et al., 2008; Riisgård et al., 2014, 2013) and, therefore, their MPs uptake. For example, differences in MPs ingestion have been observed between caged mussel's transplanted at 1.5 m and 30–45 m depth (Avio et al., 2017). In addition, seasonal variation for MPs intake has been observed in caged mussels but it has been more linked to an important water flow from a river rather than the different seasons (Catarino et al., 2018). More progression in this area is highly needed to obtain a standardized protocol. Such tool would constitute plenty of advantages for MPs evaluation such as allowing a microplastics assessment while using similar population (genetic and physiological) in areas lacking mussel beds, controlling exposure period, and to would be

potentially used to study the toxicological impact of microplastics on mussels exposed in the natural environment.

5. CONCLUSION

In summary, the present study demonstrated for the first time the effectiveness of the mussel caging approach. The abundance and properties of MPs found in caged and native mussels were quite similar. If we rely on the assumption that MPs concentrations in native mussels have reached steady-state (balance between intake and defecation/egestion), the results of this study indicate that short term mussel deployments (i.e. 6 weeks) appear to be suitable to reflect native mussels MPs ingestion. Although microplastics monitoring is a complex task in its initial phases (Carbery et al., 2018), caging presents many advantages including the selection of well-characterized homogenous organisms (number, age, size, weight, sex) and the control of exposure (location, time, season). To the best of our knowledge, mussels caging as tool to study microplastics pollution has not been investigated before. Though the abundance and properties of microplastics in mussels does not exactly match those in their environment (seawater and sediments), our study suggests that blue mussels caging may be a promising tool for MPs biomonitoring in marine coastal environments. The caging approach makes monitoring more reliable and may allow accurate assessment of the biological effects of MPs over a predetermined exposure period. However, further methodological improvements should be considered, such as the choice of the deployment time and the caging location (with respect to the shore and the depth of their implantation in the water column), to define a uniform protocol for blue mussels caging allowing an accurate spatial and temporal microplastics biomonitoring.

Authors Contributions

R.A. conceived of the presented idea. R.A. and M.K. planned and carried out the experiment. M.K. performed the samples analyses. All authors discussed the results and contributed to the final manuscript.

Acknowledgments

The authors would like to thank the administration of Le Havre harbor (especially Miss. Magali HAUTOT who helped obtaining permissions to enter the harbor and was our companion during sampling), Khalef Rabhi and Sarah Terki for field assistance. The author would like also to thank Sami Souissi and Capucin Bialais for providing live microalgae cultures.

Declaration of interests

None

SUPPLEMENTARY FILES

Supplementary Table S1: Environmental parameters measured during cages transplantation (C0) and retrieval (C1) in all sites (S: Salinity, T: Temperature, O2: Dissolved Oxygen and Turbidity)

Site	Salinity (p.s.u)		Water Temperature (°C)		Oxygen (mg/L)		Turbidity (NTU)	
	C0	C1	C0	C1	C0	C1	C0	C1
Site 1	27	23.5	6.9	9.1	8.42	8.72	13.7	17.6
Site 2	30	27.2	7.7	8	8.9	9.6	2.21	5.47
Site 3	29.2	27.5	7.6	8.3	8.92	9.82	1.44	4.05
Site 4	30.06	23.5	7.8	8.3	9.06	9.59	44,7	7.8
Site 5	32.5	25.4	5.9	8.2	10.24	9.69	41	130

Supplementary Table 2: Examples of different parameters used for each sample to obtain an optimal spectrum that can be identified.

Laser (nm)	Filter (%)	Spectral range (cm ⁻¹)	Acquisition time (s)	Accumulation	Objectives	Grating	Slit	Hole	Results
532	10	200-3400	3	10	x100	1200	100	300	93.28% PE
532	10	200-3400	1	15	x100	1200	100	300	86.08% PA
785	50	200-3400	1	10	x100	1200	100	300	95.76% PET
785	25	200-3400	2	10	x10	1200	100	300	97.27% PET
785	10	200-3400	3	10	x10	1200	100	300	91.38% PP
532	10	200-3400	1	2	x10	1200	100	300	Dye + 84% PS
785	25	200-3400	1	10	x100	1200	100	300	77% CA
785	10	200-3400	2	10	x100	1200	100	500	77% PA
785	10	200-3400	2	10	x100	1200	100	500	86,85% PS
785	10	200-3400	1	5	x100	1200	100	500	83,19% PUR
785	10	200-3400	1	5	x10	1200	200	500	79,88% PS
785	50	200-3400	2	10	x100	1200	200	500	85,98% PUR
785	10	200-3400	3	10	x100	1200	200	500	91,42% PA
785	25	200-3400	2	10	x100	1200	100	300	80% PELD
785	50	200-3400	5	1	x100	1200	100	500	84,36% PVC
785	50	200-3400	1	10	x100	1200	100	500	88,3% PUR
532	0.1	200-3400	1.5	30	x100	1200	100	500	72% PA

CHAPTER 5: GENERAL DISCUSSION AND PERSPECTIVES

CHAPTER 5: DISCUSSION, CONCLUSIONS AND PERSPECTIVES

The main objectives of this thesis were to evaluate microplastics sources and to test the effectiveness of active biomonitoring in microplastics assessment. In order to answer these objectives, passive monitoring was used, at first, to assess microplastics sources in two different areas (Le Havre and the Lebanese coast) (**Chapter 3**). Then, secondly, active biomonitoring caging experiments using two different species (fish and mussels) were investigated to validate this technique (**Chapter 4**).

1. IS ACHIEVING A STANDARD SAMPLING AND PREPARATION PROTOCOL FAR FROM REALITY?

The increase of published studies related on microplastics research indicates how much knowledge is still needed to develop and reach standardized sampling and preparation methods. Even though recommendations have been already suggested by different authors (see Frias et al., 2018; Gago et al., 2018; González et al., 2016; Hanke et al., 2013); but achieving a standardized sampling protocol for different matrices is still missing. This lack of such homogenous protocols leads to a bias comparison between the results of different studies.

Results obtained during water sampling may differ depending on several factors. First, the variation between sampling methods (see Table 4) as well as the net or the sieve's mesh size may explain the difference in results. In a study done by Dris et al. (2015), a comparison of two sampling nets with different mesh sizes (plankton 80 μ m mesh and manta net 330 μ m mesh) indicated a difference in observed microplastics: only fibers were observed in the plankton net whereas fragments and fibers were found in the manta trawl. Another comparative study between bongo net 333 μ m mesh and a water pump with steel sieves (40 μ m) mesh, suggested that trawl methods are not suitable for water collection due the loss of MPs that can occurs (Cai et al., 2018). Setälä et al. (2016) considered that water pump shows more advantages than manta nets: it is more suitable when analyzing particle < 330 μ m, can be used for sampling different depth and is more precise in the volume of the water filtered. In this thesis, two water sampling methods were used: manta trawl net of a 52 μ m mesh size and a water pump with steel sieves (20 μ m mesh). Both methods were able to collect all shapes of MPs (microbeads, fibers, fragments)

but as the two sampling methods were not used simultaneously, we have not compared their efficiency. Table 11 shows the difference between both water collecting methods; not to mention the necessity of thoroughly cleaning the sieve of the net to avoid MPs underestimation (Gago et al., 2018; Hidalgo-Ruz et al., 2012).

Table 11: Advantages and disadvantages of using water pump or manta trawl during water collection.

Water pump	Manta trawl
Higher number of sampled MPs	Lower number of sampled MPs
Small filtered volume	High filtered volume
Filtering a specific point	Sampling done on a more spatial sector
All shapes can be collected	

It has been shown that the size of the used mesh play an important role in the number of MPs found: a smaller mesh size will lead to higher number of MPs (Cai et al., 2018; Dris et al., 2015; Setälä et al., 2016) but it is important to take into consideration several factors when comparing different studies. For example, the sampling depth is not most often indicated for the water compartment. The lack of such detail is important: a microplastics distribution between water column and surface may vary depending, for example, on the water turbulence (Schwarz et al., 2019). Another important factor is the location of the sampled area. A highly affected area by riverine input or harbor activities, for example, would have higher MPs abundance than nearby sites (Lorenz et al., 2019; Nel et al., 2017). Although water pump has been signaled as a more suitable sampling method, but the choice of a water collecting method would depend on the objectives of the study while taking into account the environmental conditions, ecosystem structure and the sampled depth (Gago et al., 2018; Setälä et al., 2016)

Sediment samplings also vary between studies in terms of sampling volume, area, size, depth and location (tideline, intertidal, shoreline) (Frias et al., 2018; Stock et al., 2019). Sampling methods varied greatly where studies used trowels, sieve bags, spoons, shovels or spatula for samples collected on beaches (Fanini and Bozzeda, 2018; Filgueiras et al., 2019; Klein et al., 2015; Löder and Gerdts, 2015; Nor and Obbard, 2014) while others used Van Veen or Ekman grabber or box corer for samples collected in the sublittoral zones (Löder and Gerdts, 2015; Vianello et al.,

2013). A difference in sampled depth ranged from the first 5 cm surface layer to 32 cm (reviewed by Stock et al. (2019)). Although guidance a document has been released by the MSFD Technical Subgroup of Marine Litter in order to homogenize sediment sample collection (Frias et al., 2018), but it is not yet standardized because sampling method highly relies on the study's objectives reviewed by Stock et al. (2019)).

Even if efforts have started in order to pull a uniform water and sampling methods, but achieving a uniform sample preparation protocol is far from being possible. Methodological differences exist for all matrices including the biota (Annex 2). Most of the used preparations steps include organic material oxidation or digestion and density separation. Various solutions are used for eliminating organic material H_2O_2 30%, KOH 10%, NaClO, HCl, HNO_3 69%, $HClO_4$ (De Witte et al., 2014; Dehaut et al., 2016; Li et al., 2018; Mathalon and Hill, 2014; Phuong et al., 2018c). The compromise when deciding to digest organic tissues before heading to a density separation step is the disadvantages these solutions have on different polymers. Acidic solutions have been shown to dissolve polymers: using HNO_3 leads to a dissolution of PS and PE whereas HCl does not contribute to an efficient organic matter destruction (Avio et al., 2015; Cole et al., 2011). Oxidizing digestion using H_2O_2 solutions lead to a transparency and a thinning of polymers (Nuelle et al., 2014). Alkaline solutions also have negative effects on MPs: NaOH degrade PC, CA, PET and PVC (Dehaut et al., 2016; Hurley et al., 2018). The usage of KOH 10% has been signaled as the best settlement solution for digesting organic material because it has been shown to only degrade CA polymers (Dehaut et al., 2016; Foekema et al., 2013; Karami et al., 2017; Kühn et al., 2017). Enzymatic methods have been recently used and showed no direct dissolution or degradation on polymers (Hurley et al., 2018) but they remain time-consuming, expensive (especially for larger organisms) and different samples need different enzymes (Stock et al., 2019). Then, if samples are to undergo density separation, different solutions are used (Figure 28) which can vary from expensive (sodium polytungstate and sodium iodide) to hazardous (zinc chloride) to a loss in detected polymer (sodium chloride).

A standard protocol for microplastics studies is a must for having more reliable comparisons between studies. But a lot of factors, as stated above, are to be studied and evaluated. With the development and the increase of method improvements, achieving a standard protocol is not yet possible. More thorough investigations are needed depending on the sampling area, the sampling

species, the cost and the most suitable organic digestion solution in order to attain uniform methodologies.

2. MICROPLASTICS SOURCES IN THE AQUATIC ENVIRONMENT

Plastics properties (cheapness, durability and flexibility) resulted to its common use in all everyday life activities (Boucher and Friot, 2017). Due to their characteristics, their usage have been increasing in many sectors such as transportation, construction and packaging (GESAMP, 2017). As indicated in Table 2, there is a wide variety of sources out of which leads to primary or secondary MPs. From these sources, we can identify: tyre wear, synthetic closing, marine coating, personal care products, air dust, wastewater treatment plant, mismanaged wastes, etc (Boucher and Friot, 2017; Dris et al., 2015; Talvitie et al., 2015) (Figure 40)... Identifying the nature and location of a source in a specific study site is important to better understand MPs distribution (GESAMP, 2015). Such knowledge would lead in identifying local hotspots of MPs leading to a better source management and MPs reduction.

Mismanaged wastes have been considered as one of the most important land-based sources in secondary MPs entry leading to about 4.8 to 12.7 million tons per year (Jambeck et al., 2015). These mismanaged wastes derive from uncontrolled and badly managed landfills, wild dump, stormwater, etc where plastics eventually find their way in the recipient waters (Advisors and WWF Mediterranean Marine Initiative, 2019; Jambeck et al., 2015). In the Mediterranean, mismanaged wastes are considered as the main source of plastics leakage into the sea with the Eastern Mediterranean generating the 2/3 of this leakage (Advisors and WWF Mediterranean Marine Initiative, 2019). This thesis evaluated an abandoned coastal landfill and poorly managed dumps and high MPs values were found. The number of MPs found near the Dollermard coastal landfill and their fluctuation (0.374 to 6 items/L) indicate its alarmingly high importance as MPs sources. This variation was linked to the high tidal coefficients when the sea rises higher on the intertidal zone and comes into contact with the landfill and, thus, carries MPs, that have been degraded and deposited at the bottom of the landfill, towards the sea. Whilst the number of MPs found along the Lebanese coast (Levatine Basin) are lower than the latter (an average of 4.3 MPs/m³).

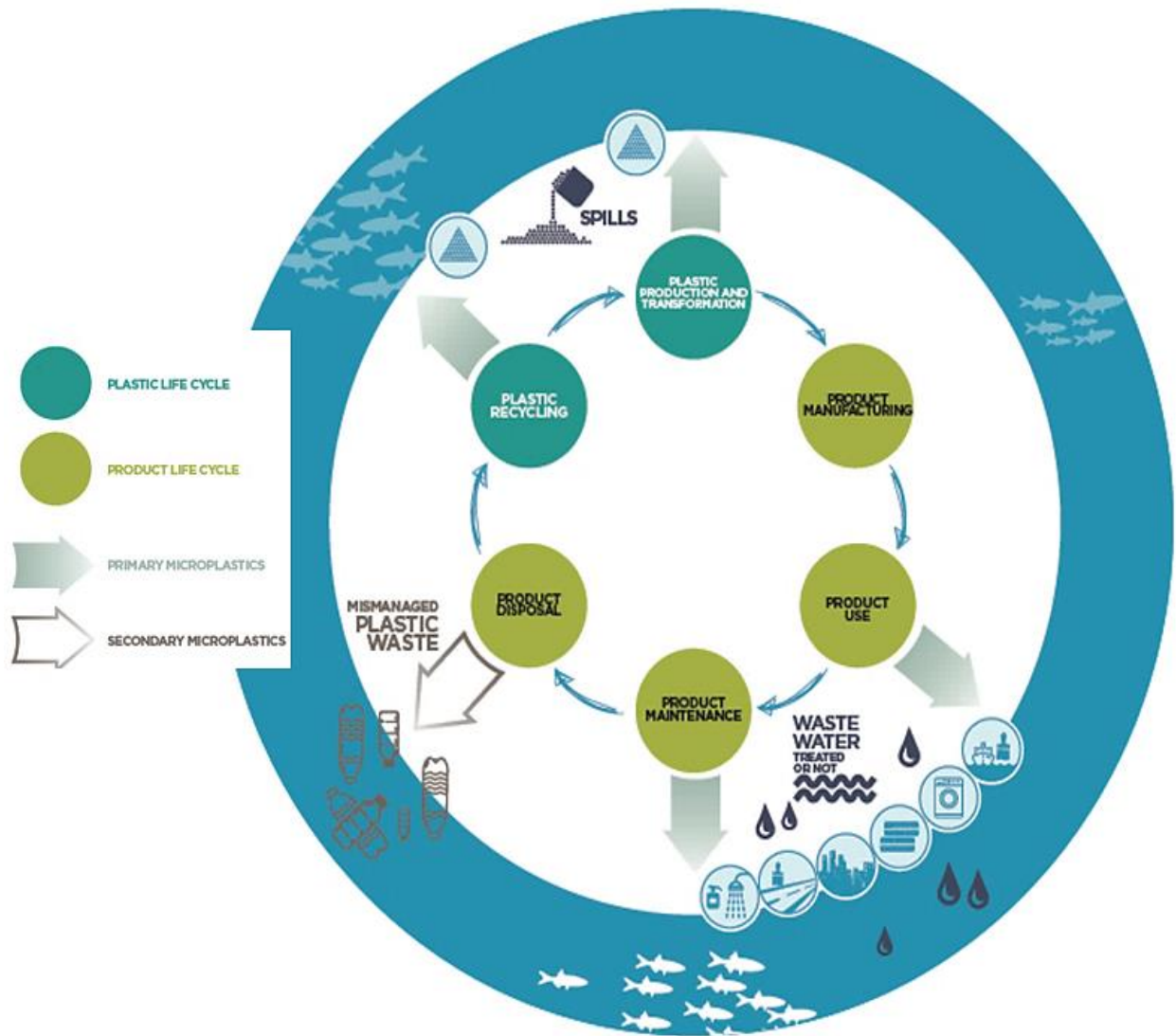


Figure 40: Lifecycle of plastic and plastics products (source Boucher and Friot, 2017)

The Eastern Mediterranean is not affected by tidal waves and the number of MPs released from the landfill to the seawater would be due to factors such as wind, rain, untreated leachates, landfill wall collapse (Halwani et al., 2020; He et al., 2019; Praagh et al., 2018). But the number found remains higher than those found in the Western Mediterranean (Table 4). Other than the water sampling, sediments and biota collected near these sources showed high MPs contamination. The MPs found near these landfills (whether found in the water or in the sediments) showed abrasions, discoloration, biofilm formation and degraded (Auta et al., 2017) and three abundant polymers (PP, PS, PE) which can be retraced back to the coastal landfill as their source. But primary microplastics were also found either as pellets or as microbeads

suggesting that other sources are playing a role in the number of MPs entering near landfills. These virgin microplastics are either lost during transport, accidental or direct waste discharge of plastic factories on the beaches or in the rivers (Shiber, 1979; UNEP, 2005). Also, another source affected our sampling: during Sidon, Lebanon sampling, direct input of microplastics and macroplastics were observed coming from Al-Awali river (Chapter xx, Article xx). Rivers input have been signaled as one of the important source of microplastics in the marine environment which can carry both primary and secondary MPs (Boucher and Friot, 2017; Lorenz et al., 2019; Stock et al., 2019).

The other important MPs source investigated was the WWTP, where high number of MPs (227×10^6 MPs/day) is discharged per day. WWTPs receives industrial, urban and rainwater, treats it and discharge it into the recipient which make does not only make them a source of MPs but also an entry route (Talvitie et al., 2015). Low number of MPs found in the effluent would lead to a high daily released number of MPs ranging from 4.19×10^4 to 4.32×10^{10} MPs/items (Leslie et al., 2017; Mintenig et al., 2017). Even though they are not constructed to treat microplastics, but retention percentages vary from 40 to 99.4% (Gatidou et al., 2019; Sun et al., 2019) (including the 98.83% obtained in this thesis). The removal rate is dependent on the concentration existing in the influent and effluent, and varies with the type of treatment existing in the WWTP (Figure 41): up to 50 - 98% than MPs are removed during the primary treatment (Sun et al., 2019).

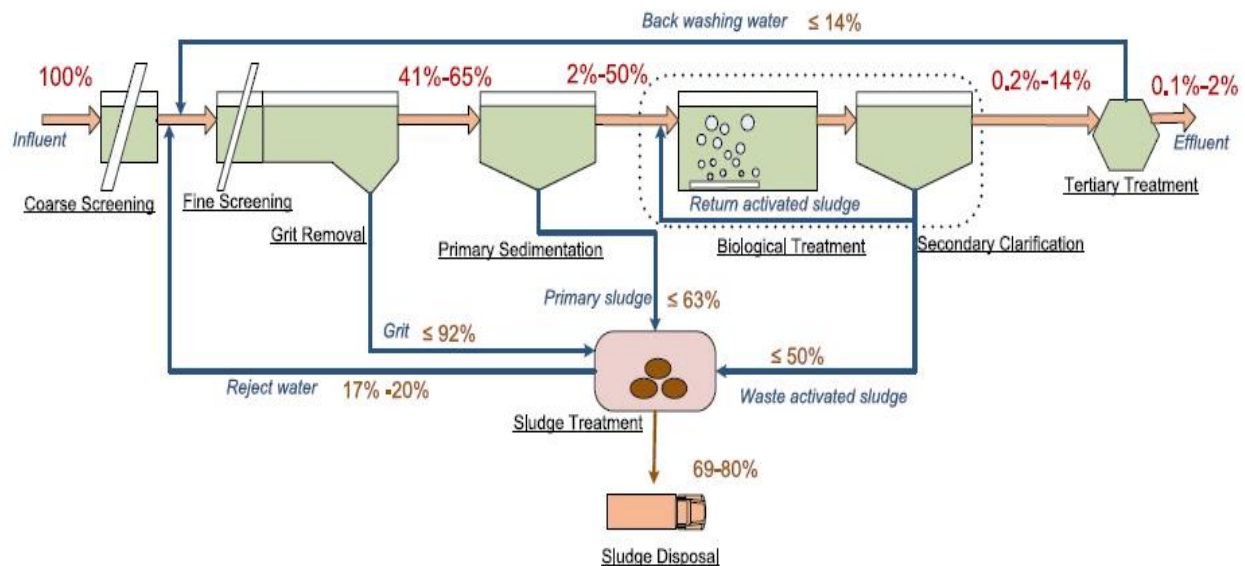


Figure 41: Estimated microplastics particle flow in wastewater treatment plant with primary, secondary and tertiary treatment process (source: Sun et al., 2019)

But the WWTPs can also play another role in indirect MPs entry. Some WWTPs do not incinerate their produced sludge and are, afterwards, used in soil for agricultural usage susceptible to be ingested by living organisms (Rillig, 2012; Sun et al., 2019). Another issue can also be caused by the incineration of the sludge: WWTPs incinerate their sludge without taking into account the plastics that it contains (chapter xx, Article WWTP). Such combustion would lead to the formation of two by-products: airborne particulate emission (soot) and solid residue ash (black carbonaceous color) (Reviewed by Verma et al. (2016)). Soot has a high potential of health and environmental risks (particulate bound heavy metals and dioxins (Valavanidis et al., 2008) indicating its toxicity when incinerated. Several aspects are still needed to be researched when it come to WWTP as a source of MPs:

- A need of a homogenous sampling method: most of the conducted study used water pump but differed in the mesh size. In WWTPs effluent, particles are speculated to be of a small size $< 20 \mu\text{m}$ so a need of a standard $20 \mu\text{m}$ mesh size or smaller is also important.
- A need of a uniform sampling procedure and volume
- Development of treatments or machines targeted for MPs removal with no toxic impact: such control would lead to a decrease and a prevention for MPs entering the recipient environment via WWTP.

In the collected matrices (surface water, sediments and biota) nearby the WWTP, a high MPs contamination was observed. The dominance of fibers and fragments can be traced to the fact that Le Havre harbor is one of the biggest harbor in tonnage in France and is highly industrialized. Some of the abundant polymers found can be linked to other MPs sources rather than WWTP. The abundance of PUR, for example, can be traced back to a high marine traffic (Lorenz et al., 2019). Our sampling area could be considered as “microplastics hotspots”; such areas have been identified near WWTP, sites with important industrial activities, high urbanization and human activities (Abidli et al., 2018; Claessens et al., 2011; Graca et al., 2017; Nor and Obbard, 2014).

Identifying MPs hotspots areas and linking observed polymers to potential sources is the first step and should be followed by reaching the suitable solutions needed to reduce such a high MPs pollution. For example, France was able to ban the distribution of plastic bags in 2016, the introduction of new exfoliating products in 2018 and it is expected, in 2020, to ban cotton swab, cups, glasses and plates, straws, disposable drink lids, etc. (Advisors and WWF Mediterranean Marine Initiative, 2019)... Such issue should be handled by individual countries into taking initiatives, increasing recycling, increase censorship on industries (e.g. avoiding accidental spills), developing waste treatment facilities to stop plastics leakage in order to reduce their potential entry in the aquatic environment.

3. MICROPLASTICS OCCURRENCE IN BIOTA AND THE POTENTIAL USE OF BIOMONITORING AS A TOOL FOR MPS MONITORING

The high occurrence of MPs in the aquatic environment lead to their uptake by biota. The small size and characteristics of MPs leads to being mistaken as food for various aquatic species and be, therefore, integrated within the trophic chain (Cole et al., 2011; Thompson et al., 2009). Evidence of microplastics ingestion has been observed in several marine species: crustaceans (Desforges et al., 2014), bivalves (Li et al., 2018; Phuong et al., 2018b), pelagic fish (Lefebvre et al., 2019), demersal fish (Neves et al., 2015), sea turtles (Duncan et al., 2018; Tomás et al., 2002), seabirds (Rodríguez et al., 2012; Tanaka et al., 2013) and mammals (De Stephanis et al., 2013). In this thesis, the obtained results indicated wild species (benthic and pelagic fish species, mussels and oysters) had the presence of microplastics in their digestive tracts or tissue. Both of our collected fish species had similar number of ingested items (2.04 ± 1.93 items/individual for

European flounder and 2.5 ± 0.3 MPs/individual for European anchovies) and they also had high MPs ingestion rate $> 75\%$. The fish ingestion rate varied greatly between studies from 2 to 100% in sampled fish (Arias et al., 2019; Foekema et al., 2013; Jabeen et al., 2017). The high MPs ingestion rate found in this thesis is probably related to the fact that were also both collected near urban and industrialized areas. Previous studies indicated that fish collected near polluted coastal areas showed a higher MPs contamination (Chan et al., 2019; Lusher et al., 2013). The ingestion rate between demersal and pelagic fish might be significantly different (Rummel et al., 2016) or it may not be (Lusher et al., 2013; Neves et al., 2015; Phillips and Bonner, 2015). Our sampled fish being temporally and spatially, an adequate comparison cannot be done but the number of ingested items indicate both of them are located in a highly MPs polluted environment. The same goes for the blue mussels and oysters. These bivalve species showed high MPs contamination: ranging from 0.41 ± 0.33 items/g to 2.75 ± 3.08 items/g for blue mussels, and 0.22 ± 0.13 items/g to 0.78 ± 0.62 items/g for spiny oysters. Both of them being filter-feeders, their MPs ingestion will reflect that of their surroundings. The continuous ingestion of MPs by these species is more likely to increase their toxic effects on organisms. The additives (e.g. phthalates) existing in plastics are chemical substances that are able to adsorb surrounding organic contaminants and, therefore, presenting potential risk on organisms (Fossi et al., 2017; Wagner et al., 2014). Oxidative stress and decreased in fitness (such as a reduced fecundity and survival rate) are the potential toxic effects caused by microplastics (reviewed by Anbumani and Kakkar, 2018; Strungaru et al., 2019). Accumulating these MPs in organisms has been demonstrated in laboratory experiments to be both size influenced and species specific: $5 \mu\text{m}$ MPs were accumulated in zebrafish (Lu et al., 2016) and $10 \mu\text{m}$ were transported into the mussel's circulatory system (Browne et al., 2008). Following the effects of microplastics during controlled exposition experiments does give information on the potential effects of microplastics; but do the same effects can be seen in the field?

In order to assess the MPs abundance and potential effects on organisms, a biomonitoring approach should be considered. By definition, "biomonitoring is the act of observing and assessing the state and ongoing changes in ecosystems, components of biodiversity and landscape, including the types of natural habitats, populations and species" which relies on using bioindicators to accurately determine the natural environment (Bondaruk et al., 2015). Two types of biomonitoring can be used in the aquatic environment: passive and active biomonitoring

(Lacroix et al., 2015). The passive biomonitoring done in this thesis showed that wild individuals are ingesting high number of MPs. Even though this passive biomonitoring approach is considered as simple, but it possesses many drawbacks (Besse et al., 2012):

- It is highly dependent on the presence of the native studied organisms: they could not be present in all investigated sites, they can be missing during some seasons
- Variety in size, sex, and age of collected native individuals: these differences lead to a bias in data comparison
- Different physiological state
- Different physiological state

Due to these differences, more standardized approach is needed: an active biomonitoring tool for MPs monitoring. Its use would permit the reduction of both physiological and genetical variability by using a single population, a control on the exposure duration, and will allow a more reliable evaluation of toxicological and physiological effects of a specific contaminants on the organism (Andral et al., 2004; Besse et al., 2012; Beyer et al., 2017). From this, caging deployments were tested, in this thesis, as a suitable approach for environmental MPs analysis. The used species were sentinel species used for previous chemical pollutant biomonitoring (Amara et al., 2009; Beyer et al., 2017; Kerambrun et al., 2013; Oikari, 2006): fish (*Platichthys flesus*) and bivalve (*Mytilus edulis*). Starting with the fish, we described in this thesis the first conducted fish caging experiments for microplastics evaluation in estuarine environments. In sampled wild fish, the numbers of ingested MPs do not necessarily reflect that of the surrounding environment: being organisms in constant movement, they often migrate for feeding or breeding purposes (Oikari, 2006; Selleslagh et al., 2009). Therefore, the developed caging methodology for the European flounder respected specific characteristics such as the cage was made of stainless steel (avoid any plastic contamination) and had a proper length and mesh size (L: 1m; mesh size: 15mm) allowing them space to swim and feed, and to be in contact with both seawater and sediments MPs. The same model was taken into account when developing the cages for the blue mussels but instead they were smaller cages and smaller mesh size (L: 0.5 m; mesh size: 15 mm). In addition, a special stainless-steel grid was inserted in the middle of the cage which served as a fixation substrate for mussels. Both caging models allowed water passage and ensured that the fish and bivalves remained in their deployment site. High survival rates were observed (> 70%) even if the conditions were not optimal (see Chapter 4 Article 5). The

success of such caging experiments is highly related to the acclimatization period to which the individuals would adjust to the transplanted environmental conditions (a period of 1 week, for example) (Oikari, 2006).

Even though for the blue mussels, previous MPs caging experiments have been already tested (Avio et al., 2017; Catarino et al., 2017; Railo et al., 2018) but none of them investigated it further into developing a potential active biomonitoring tool for MPs assessment. In this thesis, the aims were to go further into this active biomonitoring to potentially achieve uniform protocol allowing an accurate assessment of the biological effects of MPs over a predetermined exposure period. Blue mussels have been recently suggested as bioindicators and sentinel species for MPs biomonitoring (Beyer et al., 2017; Bråte et al., 2018; Li et al., 2019). Their characteristics of being sedentary filter-feeder organisms allow them to use waterborne particles as food and, thus, would be able to bioaccumulate high amounts of different contaminants (Beyer et al., 2017; Farrington et al., 2016). So, before caging deployment, all used mussels in this thesis underwent depuration under meticulous laboratory conditions: constant oxygenation, daily feed (live sterile microalgae cultures), daily filtered water (0.1 μm mesh) renewal, thermoregulated temperatures (similar to that of the natural environment), 12 hours light-dark illumination. The success of this depuration process (depurate rates > 95%) indicate that the transplanted mussels were clean and that they would only reflect the number of microplastics existing in the surrounding environment.

Another important factor to be considered is the exposition period of individuals to be able to bioaccumulate and reflect the MPs of their surrounding environment. The period of one-month period for European flounder was suitable to assess MP contamination in estuaries and to a lesser extent their effects on fish condition. Whereas for the mussels, more precise investigations on the necessary deployment time were conducted. An optimal deployment would depend on the necessary time for mussels to accumulate the pollution and reach a steady concentration (Beyer et al., 2017). The deployment time in previous mussels caging for MPs analysis had different exposition periods: 4 weeks (Avio et al., 2017; Railo et al., 2018) and one year (seasonal variation) (Catarino et al., 2018). Thus, in this thesis, several deployment times were tested: 1, 2, 5 and 6 weeks and comparison of MPs between caged mussels and other matrices (water, sediments and native mussels) was realized when possible. The obtained results indicated that clean depurated mussels were able to ingest microplastics with a significant difference after 1

week of deployment. The number of ingested items increased progressively but not significantly for the other weeks. If mussels were considered to reach a steady state while filtering (balance intake and egestion), results indicated that 5 weeks a minimum period for mussels to reach their steady-state microplastics bioaccumulation. An increase of deployment time up to 6 weeks with an additional native mussels MPs number, size and polymer type comparison. In this thesis, we described was the first active biomonitoring for MPs evaluation that did a comparison with the abiotic matrices and native mussels. The results indicated dominance of small size microplastics (<150 µm) and a high index of similarity in MPs abundance between caged and native mussels', and in polymer types between caged mussels and sediments (Chapter 4, Article 4 and 5).

The obtained results indicated that both species are suitable for microplastics active biomonitoring. The first fish caging approach would be useful to study the potential effect of MP ingestion on physiological and toxicological fish responses by measuring different biomarkers in estuarine grounds. Whereas the mussels caging approach included more analyses regarding MPs found in the surrounding matrices and the obtained results highlighted their significant accumulation of MPs during the first week of exposition. At first, a 5 weeks of deployment period seemed to be the minimum exposure period for mussels to reach a bioaccumulation steady-state. But then a longer deployment period of 6 weeks seems to be more suitable into and would, therefore offering a factual assessment of the potential effect of MPs on the organisms. Even if blue mussels caging is a promising tool for MPs biomonitoring in marine coastal environments but several components should be more investigated:

- MPs size, shape and polymer type as well as environmental and biological factors role in the feeding selectivity and behavior of mussels should be investigated.
- Location and depth (with respect to the shore and the depth of their implantation in the water column): a differentiation in MPs ingestion have been seen between 1.5m and 30-45m depth where the latter ingested higher number of MPs (Avio et al., 2017).
- Deployment time: does, for example, deploying during different seasons affect the MPs intake by mussels?
- Blue mussels could be used as coastal MPs bioindicators. Analyzing other aquatic systems (freshwaters, for example) would demand another type of species that can tolerate lower water salinity.

The road to obtaining a uniform active biomonitoring protocol for blue mussels caging is still at its first development stage. Further methodological improvements are still needed.

PERSPECTIVES

In this thesis, we were able to quantify and qualify MPs that are found in different matrices collected in the aquatic environment. We were also able to highlight not only on the importance of WWTP and coastal landfills as MPs sources, but also on the complexity of identifying a precise MPs source. During each sampling, the interaction of another nearby MPs sources as well as the possible effects of abiotic factors (rivers, wind, tides, etc.) were detected. From these results, several approaches could be suggested to have a keener view on the entry and fate of MPs deriving from such a coastal landfill. Previous modelling approaches have been already used to evaluate the pollution degree that river would export microplastics into the marine environment (van Wijnen et al., 2019). A similar approach could be interesting to be tested on coastal landfills. Modelling the number of MPs entering the coastal water with a series of values such as tidal coefficients, the height of the waves, direction of the winds, precipitations and water currents collected on a well-defined period of time. This would help on comprehending the role of such source in MPs entry and try to trace their fate upon their entry in the water. In the same study site, wild mussels were only found during two sampling dates, with one sampling date having normal sized individuals (the other had small mussels not suitable for analyses). Back in 2011, this location had an important aggregation of mussels (Unknown, 2011) that seemed to importantly decreased today. In the obtained results, we could see that the caged blue mussels from this location had a decrease in their condition index that was negatively correlated to the number of ingested items. The harms of MPs ingestion on organisms (such as feeding, growth, weight loss, reproduction, oxidative stress, behavior, etc.) have also signaled a change in community composition and ecological functioning by affecting population size and survival (Besseling et al., 2019; European commission, 2011). Hence, a 6-week blue mussel caging approach should be conducted to investigate the potential effects of MPs discharged by the coastal landfill and to study their ecotoxicological (biomarkers for oxidative stress, for

example). Hence, the importance of standardizing caging tools in the toxicological and physiological assessment of MPs effects on organisms exposed for a specific exposition period.

WWTPs are important MPs sources leading to great MPs amount entry into the aquatic systems (Mintenig et al., 2017; Murphy et al., 2016). But a better WWTPs management and waste collection could lead to a reduction of 50% of global MPs introduced by rivers (van Wijnen et al., 2019). In this thesis, limited number of samples were collected from the influent and the sludge, with no samples collected during treatment (except for the sludge). A wide variety of sampling methods and volumes have been already documented in the literature leading to a unreliability in results between studies (Gatidou et al., 2019; Sun et al., 2019). The need of a standardized sampling and preparations protocol is a must to analyze WWTPs and facilitate study comparison. Future work should consider sampling after each treatment step (coarse screening, fine screening, grit removal, grease removal, primary treatment, secondary treatment and tertiary treatment) and testing various analyses methods (such as direct filtration, or organic material removal, etc.) to finally reach a protocol that could be adapted in all WWTPs. Another important factor that need to be investigated is the seasonal variation: Do WWTPs discharge more MPs during heavy rainfall? and if yes, to what degree. Primary and secondary treatments have been showed to remove the highest amount of MPs but a small amount of MPs in the effluent would lead to a high MPs contamination in the natural environment (Gatidou et al., 2019; Sun et al., 2019). Limiting such discharge should happen at the final step of the treatment before the emission of the effluent. Research on adding a final treatment step consisting of specific Disc filtering system or a density separation basin, for example, would potentially lead to a decrease in the discharged MPs.

The size characteristics of microplastics are the main cause of their transfer through the trophic chain (planktons to secondary, tertiary consumers) to eventually reach the final consumer: humans (Alimba and Faggio, 2019). Ingested microplastics by edible aquatic fauna raises concerns on their potential risk to food and human health (Renzi et al., 2019b, 2018c; Van Cauwenberghe and Janssen, 2014). Implications on human health might be caused by the fact that MPs contain contaminants that are either added during manufacturing or are adsorbed from the natural environment (Fossi et al., 2016; Tanaka et al., 2013). There is a lack of consumer control for MPs intake via seafood since no European Union Regulations exists regarding microplastics as consumer contaminant. The high number of MPs that was found in blue

mussels, spiny oysters and small European anchovies indicate that the consumers are in a high risk of intaking MPs. Small pelagic fish species are highly consumed by the Lebanese population with no degutting. It would be crucial to raise awareness in the number of MPs that could be indirectly consumed and, therefore, encourage the removal of the fish's digestive tube. The work done along the Lebanese coast is considered as preliminary and more detailed data should be collected: an analysis of MPs in a wider range of smaller pelagic (such as the chub mackerel, *Scomber japonicus*, and sardines, *Sardinella aurita* and *pilchardus*, for example) that are wholly eaten under the name of *Bizree* (schools of small pelagic fish); and of benthic fish (such as common pandora, *Pagellus erythrinus*, and sand steenbrass, *Lithognathus mormyrus*). Such analyses must be coupled with deep sea sediments (collected near the fishing sites) and water samples which would give an image on MPs distribution throughout the three different matrices.

Moving to mussels and oysters whose soft tissue is highly consumed by humans. These individuals undergo a phase of depuration to remove any toxic algae prior to their market trade. But no process involving a removal microplastics from the bivalve's tissue has been followed. Due to the attention MPs has been raising, it is necessary to realize a meticulous depuration phase in order to limit the potential risks these MPs could carry. Future approaches to assure the removal of small sized plastics from seafood and processed food (< 10 µm to nanoplastics, ideally) is a must in order to limit their potential effects on the human body tissue (EFSA, 2016; Schirinzi et al., 2017; Wang et al., 2019). To ensure the safety of consumers, a standardized depuration process need to be followed so that mussels would be clean of MPs before their marketing. The depuration process tested in this thesis consisted of various meticulous steps that were, probably, the reason of its success. The high obtained depuration rates were promising and a validation of this experiment should be the next step. A future investigation using the same depuration steps should be tested on several maricultured bivalve species in order to reach a standardized protocol that can be reproduced and repeated with high depuration rates.

The ubiquitous presence of microplastics and their alarming wide range of distribution (water, sediments and biota) should increase the awareness on their negative influences on ecosystems and, thus, drastic measure must be organized. Most of the conducted studies on microplastics effects on organisms were conducted under laboratory conditions. The use of both fish and mussels caging approaches, in this thesis, indicated their promising application in MPs evaluation. These active biomonitoring tools are still in their first phases of development, it is,

therefore, necessary to continue establishing and improving them in order to obtain more relevant data on the potential ecotoxicological and biological effects of MPs to the realistic environment.

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Résumé détaillée de la thèse en français

Ce résumé rédigé en français présente de façon synthétique le travail, les objectifs, les principaux résultats et conclusions obtenues dans cette thèse.

De nos jours et depuis le XXe siècle, les plastiques sont considérés comme des matériaux essentiels dans différents secteurs d'activités (industries, agriculture, transport, électronique et construction) (GESAMP, 2017). Une forte demande mondiale a abouti à une augmentation continue de production de plastiques, plus précisément des plastiques à usage unique, avec une production annuelle de 335 million de tonnes (PlasticsEurope, 2018). Une fois ces matériaux rejeté dans l'environnement, ils sont considéré comme des déchets dangereux et susceptibles de s'accumuler (Andrady, 2011; Thompson et al., 2004). Aujourd'hui, cette pollution est fortement médiatisée et, sur le plan scientifique, elle se focalise plus particulièrement sur la présence dans le milieu marin des microplastiques (MPs; plastiques de taille < 5 mm, (GESAMP, 2017). Moins visibles que les macrodéchets qui flottent à la surface des océans, les MPs sont tout aussi préoccupants. Deux grandes catégories de microplastiques sont définies : les microplastiques d'origine primaires et les microplastiques d'origine secondaire. Les microplastiques primaires sont fabriqués avec une taille microscopique (microbilles, granulé de plastiques industriels GPI) pour un usage direct (cosmétiques, hygiènes) ou indirect (préproduction de plastiques). Alors que les microplastiques secondaires résultent des produits de dégradation des macrodéchets plastiques sous l'action combinée de la lumière (UV), de l'oxydation, de la biodégradation par des microorganismes marins, de l'abrasion mécanique du sable et des marées conduisant à leur fragmentation en fragments de petite taille (Andrady, 2017; Weinstein et al., 2016). Dans le milieu marin, ces MPs sont omniprésents soit au niveau de l'eau, des sédiments ou du biote. Plusieurs sources contribuent à leur présence dans ces trois différentes matrices. Les activités terrestres jouent le rôle le plus important dans leur apport dans l'environnement marin (van Wijnen et al., 2019). Différentes sources d'origine marine ou terrestre sont attribuées à cette répartition des microplastiques. Les stations d'épuration et les décharges côtières sont parmi les sources dont il existe peu informations sur leur rôle comme voie de transfert de microplastiques dans les systèmes aquatiques.

Puisque les microplastiques ont une gamme de taille et une flottabilité souvent comparables à la plupart des organismes planctoniques, ils sont susceptibles d'être ingérés par différents organismes des milieux aquatiques (depuis le plancton jusqu'aux poissons) et peuvent s'accumuler le long de la

chaîne alimentaire (Cole et al., 2011; Lusher et al., 2017). L'analyse des organismes marins indigènes collectés dans leur milieu naturel est considérée comme un outil de biosurveillance passive. La sélection de ces individus est de leur importance et capacité d'être représentative pour un polluant spécifique. Néanmoins, l'espèce choisie n'est pas toujours disponible sur l'ensemble des sites d'étude. Pour éviter ces inconvénients, la biosurveillance active est un outil qui permet d'encager des organismes capables de refléter la pollution de leur environnement (Beyer et al., 2017; Oikari, 2006). Cette technique permet d'utiliser des individus de la même espèce ayant des caractéristiques communes (classe de taille, maturité sexuelle, condition physiologique...).

Dans ce contexte, du fait que la pollution par les microplastiques est en augmentation continue, cette étude vise à mieux étudier les sources de microplastiques et développer et tester la technique d'encagement pour l'évaluation de la pollution par les microplastiques dans les milieux aquatiques. Dans cette thèse, nous nous sommes focalisés sur deux sources de MPs importantes : les stations d'épuration et les décharges côtières, et au niveau de deux environnements aquatiques : l'estuaire de la Seine et la côte libanaise. Le premier objectif sera d'étudier le rôle de la station d'épuration dans l'apport des microplastiques dans l'environnement aquatique et d'utiliser la biosurveillance passive avec des échantillons d'eau et sédiment pour évaluer le devenir des MPs introduit par ces deux sources. Le deuxième objectif était de tester l'efficacité de biosurveillance active pour l'étude des microplastiques et d'essayer plusieurs temps d'exposition pour le 'caging' des bivalves. Aussi, pour valider la méthode de 'caging' des poissons comme un outil de biosurveillance des microplastiques dans les estuaires.

I. Sources de microplastiques et biosurveillance passive:

A. Les sources de microplastiques dans l'environnement aquatique : importance de la station d'épuration et la décharge côtière

Cette première partie présente une étude de la contribution d'une station d'épuration et une décharge côtière dans la pollution des zones côtières par les microplastiques. Un suivi des microplastiques dispersés par la STEP sur un gradient de distance bien défini est réalisé en analysant trois matrices différentes: l'eau, les sédiments et la moule bleue (*Mytilus edulis*) échantillonnées à des distances respectives de 1.1 km, 1.6 km, 5.3 km and 10.3 km de l'effluent. Dans un premier temps, la STEP dans le port du Havre est évaluée par l'analyse d'échantillons de l'influent, de la boue et de

l'effluent. Après, la collecte des échantillons au niveau des sites afin de suivre le devenir des MPs émis par la STEP était réalisé. Des prélèvements d'eau ont été réalisés par pompage via une pompe et un débitmètre pour mesurer le volume d'eau filtrée. Le volume d'eau filtrée varie de 1 à 2 m³ selon le degré de turbidité de l'eau. L'eau est directement filtrée sur une série de tamis en Inox superposés par ordre décroissant de la taille des mailles (500 µm ; 200 µm ; 80 µm et 20 µm). A cause de la turbidité et du colmatage, pour le tamis de 20 µm, un faible volume d'eau était filtré. Les tamis étaient ensuite rincés avec de l'eau Milli-Q à l'intérieur de bouteilles en verre propres. Les sédiments ont été collectés à l'aide d'un carottier au niveau de quatre sites et conservés dans du papier aluminium à -10°C. Pour effectuer une biosurveillance passive, des moules bleues hybrides d'une taille similaire ont été collectées, séparées individuellement dans du papier aluminium et congelées directement à -10°C.

Un pourcentage de rétention des MPs de 98.83% est observé dans la station d'épuration ; ces valeurs étant similaires à celles rapportées par d'autres études (Blair et al., 2019; Carr et al., 2016; Gies et al., 2018; Lares et al., 2018; Magnusson and Norén, 2014). Un grand nombre de MPs observé dans la boue indique l'importance de l'étape du dessablage et de déshuilage (Murphy et al., 2016). La transformation de cette boue en un produit minéral valorisable, via incinération, aboutit à un produit sans microplastiques. Mais lors de l'incinération des boues, des produits toxiques peuvent être émis à cause de la combustion des plastiques.

Les stations d'épuration peuvent contribuer à une quantité significative de MPs dans l'environnement (Mason et al., 2016; Talvitie et al., 2017b; Ziajahromi et al., 2017). La STEP du Havre peut déverser une quantité de l'ordre de 210 x 10⁶ MPs/jour. Une dilution et dispersion des MPs depuis l'effluent de la STEP est observée et une diminution de 96% au niveau du site éloigné de 5.3 km. Alors qu'au niveau du dernier site (10.3 km), une re-augmentation est détectée à proximité de la décharge côtière (6 MPs/L). Au niveau des autres matrices analysées (sédiments et moules), un pattern différent était observé. Cette différence est peut-être due aux conditions météorologiques, la circulation de l'eau et la vitesse de dispersion et la suspension des particules mais aussi dépend de la quantité de MPs rejeté directement par la STEP qui varie temporellement (Talvitie et al., 2017a; Zhang et al., 2017). Cette hétérogénéité dans la distribution et la concentration de MPs entre les matrices est affectée par un complexe d'interaction entre la distance de la source de pollution et les facteurs hydrodynamiques (les courants, les gyres et la forme de la côte) (Schmidt et al., 2018). Les grandes quantités de contamination dans le port du Havre sont liées à la géométrie du port : Les petites structures d'eau ont tendance à avoir une quantité plus élevée de MPs avec un faible débit de

d'eau sortante (Claessens et al., 2011; Luo et al., 2019). Les moules analysées avaient une quantité élevée de MPs dans leur tissus (de 0.41 ± 0.33 items/g jusqu'à 2.75 ± 3.08 items/g) avec une abondance des items de classe de taille $< 200 \mu\text{m}$. Deux formes de microplastiques (fragments et fibres) étaient observées au niveau des trois matrices ; en plus, d'autres formes étaient observées seulement dans des matrices spécifiques : des films pour les moules et des microbilles au niveau de l'eau. L'abondance des fragments, et des polymères comme polystyrène (PS), polyuréthane (PUR) et acrylonitrile butadiène (ABS) est dû au fait que le port du Havre, ayant un tonnage de plus que 73 millions de tons, est localisé au niveau d'une zone très industrialisée, avec de nombreuses raffineries et entreprises de plastiques et il est alors influencé par des grandes quantités de microplastiques affectant les trois matrices.

Dans notre étude, deux sources importantes de microplastiques (la station d'épuration et la décharge côtière) ont été mises en évidence comme des sources très importantes de microplastique et qu'il joue un rôle significatif comme une voie d'entrée de microplastiques dans le milieu aquatique. La taille et caractéristiques des MPs trouvés dans les moules appuie sur le fait que ce sont des individus sentinelles pour les MPs ayant une taille inférieure à $200 \mu\text{m}$. L'apport de MPs émis par la décharge côtière était 2 fois plus important que celui de la STEP, mais la présence de ces particules anthropogénique va par la suite affecter les différentes matrices du milieu. Néanmoins, beaucoup de recherches devraient se concentrer sur les sources de microplastique et leurs variations temporelles en relation avec des différents facteurs tels que les conditions environnementales (saisons, précipitations, vent, courant, marée, etc.) et les facteurs associés au fonctionnement de la station d'épuration (le volume de matières brutes traitées, moment du rejet des effluents ...). Une telle connaissance pourrait aider à contrôler différentes sources de MPs afin de prévenir la pollution liée à ces derniers et devrait donc faire l'objet d'une enquête plus approfondie à l'avenir.

B. La pollution des microplastiques au niveau du littoral Libanais (Basin Levantin) : Occurrence dans l'eau de surface, les sédiments et le biote

Le bassin méditerranéen est considéré comme l'un des environnements marins le plus sensible aux pressions anthropiques et à la pollution par les microplastiques (Cincinelli et al., 2019; Lebreton et al., 2012). La plupart des études publiées ont été menées dans la partie nord-occidentale de la

Méditerranée (ex. Fossi et al., 2016; Lefebvre et al., 2019; Ruiz-Orejón et al., 2016; Schmidt et al., 2018). Alors que sur la globalité des études, une hétérogénéité dans la distribution de MPs a été observée entre les deux parties de la Méditerranée (Cincinelli et al., 2019; Suaria et al., 2016) avec la minorité des études étaient faites sur la partie orientale du bassin. Le littoral libanais situé dans le bassin levantin est fortement affecté par des pressions anthropiques avec d'importantes décharges côtières mal gérées et des petites qui sont sauvages. Aussi, un rejet direct des eaux usées est observé tout au long de la côte et au niveau de plusieurs villes. Pour ces raisons, le but de cette étude était d'étudier la quantité et les types de microplastiques trouvés dans les échantillons d'eau de mer, sédiments et biote marin collecté au niveau de trois sites (Tripoli, Beirut et Sidon) situés le long de la côte libanaise et à proximité des décharges côtières. La caractérisation des MPs était réalisée selon leurs tailles, leurs couleurs et leurs polymères.

L'eau de mer était collectée par un filet Manta avec une taille de maille de 52 μm qui était trainée pendant 10 minutes parallèlement à la côte. Le filet était ensuite rincé avec l'eau distillée filtrée et le contenu des collecteurs était rincé dans des bouteilles en verre préalablement nettoyé. Les premiers deux centimètres des sédiments sublittoraux ont été échantillonnés à l'aide d'un anneau et deux plateaux en Inox et conservés dans des pots de verre à -4°C . Pour le biote, deux espèces ont été choisies selon leur importance socio-économique : une espèce de poisson, l'anchois Européen (*Engraulis encrasicolus*) et une espèce d'huître, le spondyle (*Spondylus spinosus*) qui est une espèce filtreur; ces deux espèces sont consommées entières par la population Libanaise. Dix individus étaient collectés au niveau de chacun des trois sites, disséqués sous une hotte à flux laminaire : le tube digestif des poissons et la chaire des huîtres ont été mis dans du papier d'aluminium et congelé à -4°C .

Après les analyses des échantillons, les résultats obtenus ont souligné le degré élevé de la contamination des microplastiques du littoral libanais. Le nombre de microplastiques trouvé dans les eaux côtières étaient plus élevés que les valeurs trouvées dans les autres de régions de Méditerranée (e.g. Cincinelli et al., 2019; Cózar et al., 2015; Pedrotti et al., 2016) sauf au niveau des eaux côtières israéliennes (van der Hal et al., 2017). Ce qui appuie sur le fait que la morphologie et la courantologie au niveau du bassin levantin est cause de cette pollution très élevée en MPs (Mansui et al., 2015; Menna et al., 2012). De même, un taux très élevé était observé dans nos échantillons de sédiments (2433 ± 2000 MPs/Kg) par rapport à d'autre région. Les microplastiques existant dans l'eau de surface sont susceptibles d'être ramenés sur la côte et intégrer dans les sédiments (Barnes et al., 2009; Poeta et al., 2014). Les MPs trouvés étaient moitiés de macroplastiques visuellement

étaient observés durant l'échantillonnage et qui pourra être dû à une mauvaise gestion des déchets. Différentes formes de MPs étaient détectées dans ces deux matrices parmi lesquels existaient des MPs primaire : des microbilles et des granulés plastiques industriels (GPI) ont été respectivement observés dans l'eau et les sédiments. Ces MPs primaires peuvent être attribués à des rejets directs par les usines de plastiques dans les rivières ou à côté de la côte (Shiber, 1979). Ces particules ayant une taille similaire à celle des proies des organismes marins, sont par la suite ingérés par ces animaux. Les poissons et les huîtres analysés avaient un pourcentage d'ingestion supérieur à 83%. Ces espèces étant des filtreurs ont aussi une tendance à ingérer une quantité de MPs plus importante (Catarino et al., 2018; Collard et al., 2015; Renzi et al., 2019b). Les anchois avaient une quantité de MPs de 2.5 ± 0.3 MPs/individu qui est très élevé par rapport à d'autres régions de la Méditerranée occidentale mais proches à des valeurs trouvés dans l'anchois japonais collectés dans une région très polluée du Japon (2.30 ± 2.50 MP/individu (Tanaka et al., 2013)). Alors que pour le spondyle, les résultats obtenus dans cette thèse sont les premiers réalisés sur cette espèce. Les valeurs obtenues (7.2 ± 1.4 items/individu et 0.45 ± 0.3 items/g ww) étaient comparés avec d'autres espèces d'huîtres et des valeurs similaires ont été trouvés dans *Crassostrea gigas* (Van Cauwenberghe and Janssen, 2014). Par contre, au niveau de la Méditerranée, le nombre de MPs ingérés par nos huîtres était très faible par rapport à ceux trouvés dans les moules méditerranéennes. Cette différence de MPs trouvés par gram de chair est probablement dû à la différence de la masse de chair qui est plus élevé chez les huîtres que chez les moules. Plusieurs classes de taille étaient détectées dans les trois matrices avec une abondance de la taille $< 600 \mu\text{m}$. Les polymères les plus abondants étaient PE, PP et PS. Ces polymères détectés dérivent des microplastiques secondaires résultant de la fragmentation et de la dégradation des macroplastiques (éventuellement d'origine des décharges) et sont plus souvent détectés au niveau des décharges et leur lixiviat (He et al., 2019; Praagh et al., 2018).

La pollution par les microplastiques du littoral libanais était démontrée à proximité des décharges côtières. Ces décharges jouent un rôle très important dans l'apport de plastiques dans le milieu aquatique : des pertes de déchets sont souvent le cas à cause d'une mauvaise gestion des décharges et le manque de traitement adéquat de lixiviat (He et al., 2019; Praagh et al., 2018). Aussi, la contamination par des MPs des deux espèces qui sont complètement consommées, peut augmenter les risques liés à la consommation de produits de la pêche contaminés. Le Bassin Levantin contribue à 2/3 des rejets de plastiques entrant dans l'eau (Advisors and WWF Mediterranean Marine Initiative, 2019). L'ensemble de la Méditerranée doit être considéré comme une étude très importante pour la pollution par les microplastiques. Des initiatives doivent être prises pour assurer une gestion appropriée des déchets et, par conséquent, réduire la pollution par les plastiques.

II. La biosurveillance active comme un outil d'évaluation des microplastiques:

A. L'encagement des poissons juvéniles comme un outil d'évaluation de la contamination par les microplastiques dans les zones estuariennes

Les estuaires sont des habitats essentiels pour des poissons, car ils jouent un rôle de zones de nourricerie pour divers juvéniles de poissons (assurant l'alimentation, l'habitats et le refuge contre les prédateurs) (Selleslagh and Amara, 2008). Mais ils sont aussi parmi les environnements aquatiques les plus modifiés et en danger (Halpern et al., 2008); soumis à plusieurs pressions anthropiques telles que les effluents des industries et les stations d'épuration. Des expériences de transplantation des individus de la même classe de taille et âge ont été effectués dans l'estuaire de La Seine, pour la première fois, pour étudier la contamination des microplastiques par des juvéniles de flet. Les juvéniles de flet juvéniles sont une composante très importante dans l'assemblage des poissons estuariens et a déjà été suggéré dans des étude de biosurveillance dans les estuaires Européens (Amara et al., 2009; Marchand et al., 2003; Selleslagh et al., 2009).

Des flets juvéniles ont été collectés dans La Canche (un estuaire relativement propre (Amara et al., 2009)) et ils ont été acclimatés pendant une semaine dans un aquarium de 500 L alimenté avec l'eau de mer filtrée. Avant la mise des cages, les poissons ont été mesurés, tagués (Alpha tags) et mis dans des cages en Inox qui ont été placées dans 5 sites : Avant-port du Havre, Fosse Nord et Rouen (L'estuaire de la seine), La Canche et La Liane. Ces cages en Inox avaient une longueur de 1 m, tandis que leur largeur et hauteur étaient de 0.6 m ayant la taille de maillage de 15 mm assurant la circulation de l'eau. Les cages ont été récupérées 1 mois après et les poissons ont été identifiés et congelés à -20°C. Des flets sauvages ont été collectés dans La Canche et la Fosse Nord pour avoir une comparaison entre les poissons encagés et ceux vivant dans le milieu naturel.

Après un mois d'encagement, le taux de survie au niveau de chaque site était >70% sauf dans La Canche où la cage était ensablée. L'indice de condition des poissons a varié de 0.55 à 1.39 mg.mm⁻³ avec une moyenne de 0.79 ± 0.11 mg.mm⁻³. Parmi 64 poissons analysés, une moyenne de 75% de

poissons encagés avait la présence d'une fibre et/ou fragment. Les poissons sauvages avaient une moyenne de MPs plus élevée que celle trouvée dans les poissons encagés (2.04 ± 1.93 vs 1.67 ± 1.43) mais un pourcentage d'ingestion plus faible (58%). Pour les poissons encagés, le taux de MPs le plus élevé était observé au niveau de La Liane alors que celui le plus bas était au niveau de La Canche et Fosse Nord. L'ingestion des MPs par les poissons ni le taux de mortalité étaient corrélés avec le nombre de MPs ingéré. Les fibres étant la forme la plus dominante (69%) suivie par les fragments et les films. Les MPs ayant une taille $< 200 \mu\text{m}$ étaient les plus dominants dans le tube digestif des poissons et 3 types de polymères étaient identifiés (PA-6, PET et PUR).

Malgré les études menées sur des poissons sauvages, mais grâce à leur migration et mouvement, leur contamination en MPs ne reflète pas obligatoirement la pollution de MPs existant au niveau du site de capture (Oikari, 2006). Les poissons encagés nous ont permis d'analyser des systèmes estuariens tempérés qui sont peu étudiés. Le pourcentage d'ingestion observé était similaire à celui des flets adultes collectés dans le fleuve de Tamise (70% ; (McGoran et al., 2017)) alors qu'il était supérieur à d'autres espèces marines collectées dans le Méditerranée (Güven et al., 2017; Romeo et al., 2015) ou la mer Nord (Foekema et al., 2013). Ce qui appuie sur le fait que les MPs sont plus communs dans les systèmes estuariens transitoires et peuvent par la suite ramener ces MPs dans le milieu marin (Anderson et al., 2018; Andrady, 2011). La dominance des fibres est observée aussi chez d'autres espèces démersales (Bessa et al., 2018; Lusher et al., 2017; McGoran et al., 2017) ce qui peut être dû au fait que les fibres ressemblent à la nourriture du milieu naturel et les poissons se trompent et les considèrent comme des proies (Ferreira et al., 2018). De plus, la forme la plus abondante dans le fleuve de La Seine était les fibres qui sont souvent rejetées par les STEPs (Dris et al., 2015; Leslie et al., 2017).

La faisabilité d'encager des flets juvéniles dans des zones estuariennes a été testée et confirmée pour la première fois. Ces poissons ont pu ingérer des MPs avec une moyenne plus élevée en comparaison avec d'autres espèces marines indiquant que les MPs sont plus communs dans les estuaires (Horton et al., 2017; Schmidt et al., 2017). Cette technique d'encagement est importante d'évaluer la variabilité spatiale et temporelle du taux de MPs dans les estuaires (zones souvent affectées par le débit des fleuves et la marée). Le nombre élevé de MPs dans les juvéniles pourra causer des effets négatifs pour le recrutement des poissons et le renouvellement des populations. Cette technique d'encagement sera utile pour pouvoir étudier les effets physiologiques et toxicologiques potentiels des MPs ingérés dans le milieu naturel. Néanmoins, des recherches seront nécessaires pour bien comprendre l'impact écologique des MPs au niveau de ces habitats essentiels des poissons.

B. L'effet de l'exposition temporelle des moules bleues (*Mytilus edulis*) sur la bioaccumulation des microplastiques

Une évaluation périodique des moules engagées pour la biosurveillance active des microplastiques a été testée pour la première fois. Les moules bleues sont utilisées comme des bioindicateurs grâce à leur capacité à bioaccumuler dans leurs tissus des substances chimiques (Beyer et al., 2017). Pour chaque substance chimique, une différente durée d'engagement est nécessaire pour que les moules puissent les accumuler. Les engagements réalisés pour l'évaluation des MPs n'ont pas pris en compte que les moules arrivent à un 'steady-state' où il y aura un équilibre ingestion/égestion d'une substance bien définie. Pour cela, trois différents temps d'exposition ont été testés dans le milieu naturel : 1, 2 et 3 semaines.

Malgré la capacité des moules à dépurier les particules sous 72 h après exposition (Ward and Kach, 2009), nous avons décidé de réaliser une dépuration des moules bleues sous des conditions très précises avant de les transplanter dans des cages dans le milieu naturel. Des moules de Bouchot, approximativement de la même taille, ont été collectés et mis dans un aquarium fermé de 160 L nettoyé et l'eau de mer (filtré à 0.1 μm) était changée quotidiennement. Pour assurer une bonne capacité de filtration, les moules étaient nourries avec des microalgues (*Rhodomonas* sp. et *Isochrysis* sp.) et mises dans une salle thermostatée (17 ± 1 °C) avec une illumination 12 h jour-nuit. Pour étudier la cinétique de dépuration, des moules ont été congelés directement avant leur mise en aquarium et après des moules ont été prises chaque 16 h, 24 h, 48 h, 72 h et 7 jours. Après une semaine de dépuration, les moules ont été mises dans les cages au niveau de la plage de Sainte-Adresse à proximité de la décharge. Les moules (150 individus) étaient réparties également dans des cages en Inox ayant une longueur, largeur et hauteur de 0.5 m. Une grille en Inox était mise au milieu des cages comme un support sur lequel les moules peuvent se fixer. Aussi un échantillonnage de l'eau de surface était pris comme décrit dans la partie IA ; pendant la mise des cages et la récupération des cages, des échantillons d'eau ont été collectés.

Durant la dépuration, le nombre de MPs a significativement diminué après 16 h (de 1.82 ± 1.34 items/g jusqu'à 0.28 ± 0.27). Après 72 h, le pourcentage de dépuration était de 98.78% avec juste une fibre observée assurant l'élimination complète des fragments. Le manque d'un protocole homogène

rend la comparaison entre les études difficile. En outre, le pourcentage de dépurabilité obtenu était plus élevé que d'autres études qui ont suivi la dépurabilité des individus (Birnstiel et al., 2019; Fernández and Albentosa, 2019; Van Cauwenberghe and Janssen, 2014). Les fibres ont pris plus de temps pour être éliminées car ils ont un temps de résidence plus important que celui des fragments (Ward et al., 2019). Cette étape est très importante pour avoir des moules propres sans MPs utilisés comme référence et donneront des résultats plus fiables durant la biosurveillance d'un site précis.

Les MPs dans les échantillons d'eau ont varié entre 374 items/m³ et 1380 items/m³. Ces concentrations trouvées sont considérées comme des valeurs importantes et sont liées à la présence de la décharge côtière. Cette décharge, du fait de son effondrement et de son abondance, joue un rôle important comme une route de MPs dans le milieu côtier. Soit par les lixiviats résultant des déchets (He et al., 2019; Praagh et al., 2018) ou des conditions météorologiques, le rôle de la décharge est très important. Selon les résultats obtenus avec ceux obtenus dans la partie IA, la concentration de MPs dans l'eau de surface évolue en fonction du coefficient de marée. Lors des hauts coefficients de marée, la mer montante arrive dans la zone intertidale et serait en contact direct avec la décharge, ce qui aboutit à l'entrée des MPs dégradés et déposés au pied de la décharge. Mais dans la semaine 5, des microplastiques primaires ont été trouvés dans l'échantillon d'eau suggérant que la côte de Sainte-Adresse est affectée par d'autres sources autres que la décharge.

Cette source de MPs est par la suite une raison pour avoir une concentration élevée de MPs au niveau des eaux côtières. Les moules engagées seront alors susceptibles d'ingérer un nombre de MPs plus importants que dans d'autres régions. Des courtes périodes d'exposition ont été déjà testées pour surveiller des contaminants chimiques (Marigómez et al., 2013; Zorita et al., 2006). Après juste 1 semaine d'engagement, les moules ont ingéré un nombre significatif de MPs par comparaison aux moules dépurées. Mais le nombre a continué à augmenter progressivement mais pas significativement durant la deuxième et cinquième semaine d'engagement pour atteindre 1.42 ± 0.76 MPs/g ww. Ceci indique qu'une période d'exposition de 5 semaines semble être le minimum pour que les moules puissent arriver à leur état stable ('steady-state') de bioaccumulation des MPs. De plus, les fragments ingérés par les moules engagées avaient une taille $< 300 \mu\text{m}$ alors que les fibres étaient plus longues avec une taille $> 1000 \mu\text{m}$. Si les moules sont choisies comme des organismes sentinelles des MPs, il y a plusieurs facteurs qui doivent être pris en considération. Les moules ont tendance à ingérer des fragments de petites tailles mais des fibres longues ce qui implique que la forme d'un MPs influence sur son accumulation ou élimination par les organismes (Browne et al., 2013; Ward et al., 2019).

Les résultats soutiennent le fait que les moules bleues engagées peuvent être un outil potentiel pour la biosurveillance des MPs dans les eaux marines côtières (Beyer et al., 2017; Li et al., 2019). Ils indiquent, pour la première fois, la période d'exposition minimale nécessaire pour les moules engagées pour atteindre l'état stable dans la bioaccumulation des MPs. Mais aussi, l'importance de prendre en compte les différentes caractéristiques des MPs pour éviter une sous-représentation de certaines formes, tailles et polymères (Scott et al., 2019). Cependant, la possibilité d'obtenir un protocole de biosurveillance des MPs standardisé est encore dans ses premières étapes et nécessite des études complémentaires. Une période d'exposition plus longue (6 semaines par exemple) sera nécessaire pour développer ce système de biosurveillance

C. La mise en cage des moules bleues est-elle une méthode efficace pour la biosurveillance des microplastiques dans l'environnement ?

Une période d'exposition de moules engagées de 6 semaines a été testée au niveau de Sainte-Adresse mais aussi à côté d'une autre source de MPs : la STEP du Havre. Les différentes caractéristiques des MPs (forme, taille, couleur et polymère) ingérés par les moules engagés étaient comparées avec celles existant dans les moules indigènes mais aussi avec celles qui sont trouvées dans l'eau et les sédiments au niveau des mêmes sites ; afin d'évaluer l'efficacité de la biosurveillance active de MPs.

Cette transplantation était effectuée pour 6 semaines dans des conditions hivernales. Une phase de dépuration des moules de Bouchot était identique à celle décrite en II.B mais la température de la chambre thermorégulée était de $8 \pm 1^\circ\text{C}$ pour être similaires aux températures du milieu naturel. Des moules de référence étaient congelées avant la phase de dépuration et une semaine après (avant la mise des cages). Les caractéristiques des cages étaient aussi identiques à celles utilisées dans II.B et elles étaient mises au niveau de 5 sites différents. Des échantillons des moules indigènes, de l'eau et des sédiments ont été prélevés au niveau du même site pour permettre une comparaison des MPs dans les différentes matrices.

La dépuration des moules a abouti à une diminution de 97.6% des MPs avec 0.05 ± 0.15 fibres/g dans les moules avant la mise des cages. Même si les conditions météorologiques n'étaient pas optimales pour les moules, mais le taux de survie était de 73% et l'indice de condition n'était pas significativement différent entre les sites. Le pourcentage d'ingestion de MPs était approximativement le même entre les moules engagées et indigènes (94.7% et 93%, respectivement).

Les moules encagées avaient des concentrations de MPs égale ou un peu plus élevée que les moules naturelles mais pas significativement différentes (la distribution était différente au niveau du dernier site, car les moules naturelles avaient un poids significativement inférieur à celui des moules encagées). Les MPs ingérés par les moules (encagées et indigènes) avaient une distribution spatiale différente de celles qui sont trouvées dans l'eau et les sédiments. Pour évaluer la similarité entre les différentes matrices, l'indice de similarité de Morisita a été calculé. Une similarité élevée dans la taille des MPs abondants entre les moules encagées et celles du milieu naturel (0.98) et dans le type de polymères entre les moules encagées et ceux des sédiments (0.93).

L'abondance des fragments dans nos moules a été similaire aux autres études conduites sur les moules et les coques (Digka et al., 2018; Hermabessiere et al., 2019; Vandermeersch et al., 2015). La différence dans la distribution spatiale des MPs dans les moules avec les autres matrices peut-être due à une limite d'échantillonnage, aux facteurs hydrodynamiques et à la distribution des MPs tout au long de la colonne d'eau (e.g. densité des polymère, biofilm...) (Li et al., 2018; Scott et al., 2019). Ce travail n'a pas pris en compte les marées au moment de l'échantillonnage ni le temps nécessaire ('time-lag) pour que les MPs dans l'eau sédimentent ou soient ingérés par les moules (Schmidt et al., 2018). Les moules encagées ont ingéré des MPs ayant une taille < 200 µm ce qui est en accord avec d'autres études (Bråte et al., 2018; Naji et al., 2018; Phuong et al., 2018c). Une différence entre la taille et le type de polymère était trouvée entre les trois matrices collectées au niveau de la côte sud-ouest d'Angleterre ; mais les particules trouvées dans les moules ont plus reflété celles trouvées dans le sédiment et pas celles trouvées dans l'eau (Scott et al., 2019). Les moules encagées étant dans la zone intertidale, les polymères trouvés avaient une similarité à ceux trouvés dans les sédiments pris à côté des cages. Cette biodisponibilité dépend de la forme, densité et taille mais aussi du type de polymère des MPs (Carbery et al., 2018).

Des études avancées concernant les facteurs environnementaux et biologiques influençant les concentrations des MPs dans les trois matrices doivent être prises en compte. En partant sur le principe que les moules naturelles atteignent leur état stable, les résultats indiquent qu'une période de 6 semaines semble être adéquate pour être suggérée comme une période nécessaire pour la biosurveillance active des MPs. Les techniques d'encagement rendent la biosurveillance plus fiable en choisissant des individus de même taille, sexe et état physiologique, et peut permettre une évaluation précise des effets biologiques des MPs. Cependant, la biosurveillance active est dans ces premières phases et plusieurs améliorations méthodologiques doivent être envisagées (la profondeur

nécessaire pour la mise des cages par exemple), afin de définir un protocole uniforme pour la biosurveillance active des microplastiques par les moules bleues.

Dans cette thèse, nous avons pu quantifier et qualifier les MPs présents dans différentes matrices collectées dans le milieu aquatique. Nous avons également pu mettre en évidence que d'autres sources autre que de la station d'épuration des eaux usées et les décharges côtières agissent sur la présence importante des microplastiques. Ainsi, lors de l'analyse d'une source MPs, une vue plus globale des sites d'étude doit être prise en compte ainsi que les effets possibles de différents facteurs abiotiques (rivières, vents, marées, etc.). Aussi, la validation des approches de biosurveillance actives a été réalisée. Ces approches seront utilisés comme un outil prometteur pour la biosurveillance des MPs, rendant les résultats plus fiable et permettant une étude précise des effets biologiques des MPs sur une période d'exposition prédéterminée.

Annexes

ANNEX 1: STUDIES ON MICROPLASTICS OCCURRENCE IN THE SURFACE WATER WORLDWIDE.
PES: POLYESTER, PA: POLYAMIDE, PUR: POLYURETHANE, PE: POLYETHYLENE, PP:
POLYPROPYLENE, PET: POLYETHYLENE TEREPHTHALATE, PS: POLYSTYRENE

Location	Sampling Method	Mesh size (μm)	Concentration	Abundant polymer or shape	Reference
Arctic Polar waters	Manta net	333	0.34 ± 0.31 items/ m^3	PES and PA	(Lusher et al., 2015)
Antarctic Peninsula	Manta net	330	1,794 items/ km^2	PUR	(Lacerda et al., 2019)
NE Atlantic Ocean	Water pump	250	2.46 ± 2.43 items/ m^3	PES and PA	(Lusher et al., 2014)
Bohai Sea (China)	Manta net	330	0.60 ± 0.71 items/ m^3	PE	(Zhang et al., 2017)
South China Sea	Pump	44	2569 ± 1770 particles/ m^3	Alkyd resin	(Cai et al., 2018)
Xiangshan Bay (China)	Plankton net	330	8.9 ± 4.7 items/ m^3	PE	(Chen et al., 2018)
Qatar waters	Plankton tow-net	120	0.71 particle/ m^3	PP	(Castillo et al., 2016)
Makran coasts (Gulf of Oman)	Neuston net	333	0.49 ± 0.43 items/ m^3	PP, PE	(Aliabad et al., 2019)
NE Pacific Ocean	Manta net	505	0.004 - 0.19/ m^3	-	(Doyle et al., 2011)
NW Pacific Ocean	Manta net	330	0.13 ± 0.11 items/ m^3	PET	(Mu et al., 2019)
Sri Lanka	Plankton net	80	0 – 29 items/ m^3	PP	(Bimali Koongolla et al., 2018)

Southern North Sea	Pump	40	4 ± 194/L granular particles 88 ± 82 fibres/L	-	(Dubaish and Liebezeit, 2013)
Southern North Sea	Manta net	100	0.1 - 245.4 particles/m ³	PE, PP	(Lorenz et al., 2019)
U.K coastal waters	Bottle	5	3.5 ± 2.0 items/L	PES	(Li et al., 2018)
South West coast of U.K.	Plankton net	53	1.97 to 3.38 items/m ³	PS, PES	(Scott et al., 2019)
Guanabara Bay (Brazil)	Plankton net	300	1.40 - 21.3 particles/m ³	PP	(Olivatto et al., 2019)
Mediterranean sea	Neuston net	200	1.25 ± 1.62 items/m ²	PS, PP	(Suaria et al., 2016)
NW Mediterranean Sea (Ligurian Sea and Sardinian Sea)	WP2 standard net	200	0.62 ± 2 items/m ³	-	(Fossi et al., 2012)
NW Mediterranean Sea	Manta trawl net	333	0.116 particles/m ²	-	(Collignon et al., 2012)
NW Mediterranean Sea (Ligurian Sea)	Manta trawl net	333	158 ± 157, 80 ± 38, 176 ± 216, and 370 ± 378 items/km ²	PP	(Pedrotti et al., 2016)
NW Mediterranean Sea (Gulf of Lion)	Manta trawl net	780 and 330	112 000 items/km ²	Fragments	(Schmidt et al., 2018)
NW Mediterranean (the Rhône River and the Têt River)	Manta trawl net	333	0.19 items/m ³ (The Rhône) and 0.18 items/m ³ (The Têt)	PES and PE	(Constant et al., 2018)

NW Mediterranean Sea (Gulf of Lion)	WP2 plankton net	200	0.23 ± 0.20 items/m ³	PET	(Lefebvre et al., 2019)
Western and Central Mediterranean	Neuston net	200	0.83/m ³	Fragments	(Cózar et al., 2015)
Western and Central Mediterranean	Manta trawl net	333	147,500 items/km ²	Fragments	(Ruiz-Orejón et al., 2016)
Central-Western Mediterranean Sea (Sardinian Sea)	Manta trawl net	500	0.15 items/m ³	Fragments	(de Lucia et al., 2014)
Western Mediterranean Sea (Corsica – Bay of Calvi)	WP2 standard net	200	0.062/m ²	Fragments	(Collignon et al., 2014)
Western Mediterranean Sea (Gulf of Asinara and the Sardinian Sea)	Neuston net	200	0.31 ± 1.17 items/m ³	-	(Fossi et al., 2016)
Western Mediterranean Sea (North Western of Sardinia; Gulf of Asinara)	WP2 standard ring net	200	0.17 ± 0.32 items/m ³	Fragments	(Panti et al., 2015)
Western Mediterranean Sea (Tuscany coastal water)	Manta trawl net	330	0.26/m ³	PE	Baini et al., (2018)
Western Mediterranean Sea (North Catalan coast and the southeastern coast of Spain)	Manta trawl net	335	0.11 ± 0.09 items/m ²	PE	(de Haan et al., 2019)
Western Mediterranean sea (Tyrrhenian Sea)	Bongo net	500	16 items	PE	(Savoca et al., 2019)
Eastern Mediterranean (Turkish	Manta trawl net	333	0.7 items/m ³	Copolymers	(Güven et al., 2017)

Mediterranean Coast)

Eastern Mediterranean (Iskenderun Bay)	Manta trawl net	333	1 067 120 items/km ² (7.26 items/m ³)	Fragments	(Gündoğdu, 2017)
Eastern Mediterranean Sea (Iskenderun Bay and Mersin Bay)	Manta Trawl net	333	0.376 items/m ² 2.73/m ³	Fragments	(Gündoğdu and Çevik, 2017)
South Eastern Mediterranean Sea	Manta Trawl net	333	7.68 ± 2.38 items/m ³	Fragments	(van der Hal et al., 2017)

ANNEX 2: MICROPLASTICS CONTAMINATION IN DIFFERENT MOLLUSKS COLLECTED IN DIFFERENT REGIONS.

Location	Species	Wild/Farmed	Digestion method	[MPs]	Occurrence (%)	Size (µm)	Abundant polymer or shape	Reference
Baie de Saint Brieux (France)	<i>Mytilus edulis</i>	Farmed	Acid mix Method (HNO ₃ + HClO ₄)	0.34 ± 0.33 items/g ww	-	-	Fibers	(Vandermeersch et al., 2015)
Denmark	<i>Mytilus edulis</i>	Farmed	Acid mix Method (HNO ₃ + HClO ₄)	-	-	-	-	(Vandermeersch et al., 2015)
The Netherlands	<i>Mytilus edulis</i>	Farmed	Acid mix Method (HNO ₃ + HClO ₄)	0.32 ± 0.22 items/g ww	-	-	Fibers	(Vandermeersch et al., 2015)
The North Sea (the Dutch coast)	<i>Mytilus edulis</i>	Wild	Powdered, nitric acid destruction, NaOH neutralization and H ₂ O ₂ digestion	105 particles/g dw 19 particles/g dw	-	10 – 5000 (66% 10 – 300)	Fibers	(Leslie et al., 2017)
The North Sea (Germany)	<i>Mytilus edulis</i>	Farmed	Acid: HNO ₃ 69%	0.36 ± 0.07 items/g ww	-	5 - > 25	-	(Van Cauwenbergh and Janssen, 2014)

French, Belgian and Dutch North Sea coast	<i>Mytilus edulis</i>	Wild	Acid: HNO ₃	0.2 ± 0.3		20 - 90	-	
The North Sea (Belgian coast)	<i>Mytilus edulis</i>	Farmed	Acid mix Method (HNO ₃ + HClO ₄)	0.35 fibers/g ww	-	-	Fibers	(De Witte et al., 2014)
The North Sea (Belgian coast)	<i>Mytilus edulis</i>	Wild	Acid mix Method (HNO ₃ + HClO ₄)	0.26 – 0.51 fibers/g ww	-	-	Fibers	(De Witte et al., 2014)
McCormack's Beach and Rainbow Haven Beach (Canada)	<i>Mytilus edulis</i>	Wild	30% H ₂ O ₂ + density separation (NaCl)	126 and 108 items/mussel	100	-	Fibers	(Mathalon and Hill, 2014)
McCormack's Beach and Rainbow Haven Beach (Canada)	<i>Mytilus edulis</i>	Farmed	30% H ₂ O ₂ + density separation (NaCl)	178 items/mussel	100	-	Fibers	(Mathalon and Hill, 2014)
French Atlantic coast	<i>Mytilus edulis</i>	Wild	KOH 10% + density separation (KI 10%)	0.23 ± 0.20 items/g ww	-	30 - 200	PP and PE	(Phuong et al., 2018c)
The English Channel	<i>Mytilus edulis</i>	Wild	KOH 10%	0.25 ± 0.16 and 0.15 ± 0.06 items/g ww	40	15 – 500 (0.9% > 500)	PE and ABS, Fibers	(Hermabessiere et al., 2019)
U.K. coastal waters	<i>Mytilus edulis</i>	Wild	H ₂ O ₂ 30% and density separation (NaCl)	0.7 to 2.9 items/g ww	-	8 – 4700 (abundance of < 500)	PES, Fibers	(Li et al., 2018)

U.K. supermarkets	<i>Mytilus edulis</i>	Farmed	H ₂ O ₂ 30% and density separation (NaCl)	0.9 items/g ww	-	8 – 4700 (abundance of < 500)	PP, Fibers	(Li et al., 2018)
South West coast of U.K.	<i>Mytilus edulis</i>	Wild	KOH 10%	1.43 ± 0.30 items/g to 7.64 ± 1.61 items/mussel	88.5	50 - 1100	Fibers (cellulose, nylon)	(Scott et al., 2019)
West Coast of Scotland	<i>Mytilus edulis</i>	Wild	Enzymatic digestion (trypsin)	1.05 – 4.44 items/g ww	97	200 - 1067	PA	(Courtene-Jones et al., 2017)
Coast of Scotland	<i>Mytilus edulis</i>	Farmed then caged	Enzymatic (Corolease 7089)	0.74 ± 0.125 items/g ww	-	200 - 2000	Fibers	(Catarino et al., 2018)
Norwegian coastal waters	<i>Mytilus galloprovincialis</i>	Wild	KOH 10%	0.97 items/g ww	46.3	70 – 3870 (average 770)	PET, Fibers	(Bråte et al., 2018)
Northern Ionian Sea	<i>Mytilus galloprovincialis</i>	Wild	H ₂ O ₂ 30%	5.3 ± 0.5 items/g	47.5	40 – 737	PE, Fragments	(Digka et al., 2018)
Northern Ionian Sea	<i>Mytilus galloprovincialis</i>	Farmed	H ₂ O ₂ 30%	2.5 ± 0.3 items/g	45	55 – 620	PE, Fragments	(Digka et al., 2018)

Portugal	<i>Mytilus galloprovincialis</i>	wild	Acid mix Method (HNO ₃ + HClO ₄)	0.34 ± 0.33 items/g ww	-	-	Fibers	(Vandermeersch et al., 2015)
Italy	<i>Mytilus galloprovincialis</i>	wild	Acid mix Method (HNO ₃ + HClO ₄)	0.05 ± 0.11 items/g ww	-	-	Fragments	(Vandermeersch et al., 2015)
Italy	<i>Mytilus galloprovincialis</i>	Farmed	Acid mix Method (HNO ₃ + HClO ₄)	0.25 ± 0.26 items/g ww	-	-	Fragments	(Vandermeersch et al., 2015)
Spain	<i>Mytilus galloprovincialis</i>	Wild	Acid mix Method (HNO ₃ + HClO ₄)	0.15 ± 0.33 items/g ww	-	-	Fibers	(Vandermeersch et al., 2015)
Spain	<i>Mytilus galloprovincialis</i>	Farmed	Acid mix Method (HNO ₃ + HClO ₄)	0.04 ± 0.09 items/g ww	-	-	Fibers	(Vandermeersch et al., 2015)
Ariatic Sea	<i>Mytilus galloprovincialis</i>	Wild	H ₂ O ₂ 15% and density separation	1.54 ± 0,93 items/individual (surface) 1.66 ± 0.57 items/individual (bottom)	36 (surface) 10 (bottom)	-	Fibers	(Avio et al., 2017)

Ariatic Sea	<i>Mytilus galloprovincialis</i>	Wild then caged	H ₂ O ₂ 15% and density separation	2 ± 1 (surface) 1 ± 0 (bottom)	27 (surface) 12 (bottom)	100 – 5000 (abundance of 500 – 5000)	Fibers	(Avio et al., 2017)
Italian coast	<i>Mytilus galloprovincialis</i>	Farmed	H ₂ O ₂ 30%	4.2 – 9.2 - 11.4 items/g ww	-	750 - 6000	Fibers	(Renzi et al., 2018c)
Italian coast	<i>Mytilus galloprovincialis</i>	Wild	H ₂ O ₂ 30%	7.2 items/g ww	-	750 - 6000	Fibers	(Renzi et al., 2018c)
Adriatic Sea	<i>Mytilus galloprovincialis</i>	Wild	Tween 20 5%, Enzyme (protease) and KOH 10% , then density separation (NaI)	1.06–1.33 fragments/g and 0.62–0.63 fibers/g	-	< 100 (fragments) <300 (Fibers)	PE, Fragments	(Gomiero et al., 2019)
Scottish coast	<i>Mytilus spp.</i>	Wild	Enzymatic (Corolase 7089)	3 ± 0.9 items/g ww	-	200 - 2000	Fibers	(Catarino et al., 2018)
Scottish coast	<i>Modiolus modiolus</i>	wild	Enzymatic (Corolase 7089)	0.086 ± 0.031 items/g ww	-	200 - 2000	Fibers	(Catarino et al., 2018)
Coast of Hanko (Baltic Sea)	<i>Mytilus trossolus</i>	Wild	Enzymatic (Biozym F and SE)	0.26 ± 1.3 items/g	-	-	Fibers	(Railo et al., 2018)

The North Sea (the Dutch coast)	<i>Crassostrea gigas</i>	wild	Powdered, nitric acid destruction, NaOH neutralization and H ₂ O ₂ digestion	87 particles/g dw 30 particles/g dw	-	10 – 5000 (73% 10 – 300)	Fibers	(Leslie et al., 2017)
Brittany, France	<i>Crassostrea gigas</i>	Farmed	Acid: HNO ₃ 69%	0.47 ± 0.16 items/g ww	-	5 - > 25	-	(Van Cauwenbergh and Janssen, 2014)
French Atlantic coast	<i>Crassostrea gigas</i>	Wild	KOH 10% + density separation (KI 10%)	0.18 ± 0.16 items/g ww	-	50 – 1300 (53% < 100)	PE, Fragments	(Phuong et al., 2018b)
Offshore central California	<i>Crassostrea gigas</i>	Wild	KOH 10%	0.6 ± 0.9 items/individual	33.33	5471 ± 5200	Fibers	(Rochman et al., 2015)
New South Wales coast (Australia)	<i>Saccostrea glomerata</i>	Wild	KOH 10% + density separation (NaI)	0.15 to 0.83 particle/g ww	-	50 – 3000 (Abundance of 50 – 1000)	PET, Fibers	(Jahan et al., 2019)
North and South of China	<i>Crassostrea gigas</i> , <i>Crassostrea angulate</i> , <i>Crassostrea hongkongensis</i> and <i>Crassostrea sikamea</i>	Farmed	H ₂ O ₂ 30% and KOH 10%	0.62 items/g	84	20.34 – 4807.22 (902.82 ± 782.99)	CP and PE, Fibers	(Teng et al., 2019)

