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Quality assessment of the blue mussel (*Mytilus edulis*): Comparison between commercial and wild types

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ABSTRACT

This study compared species identity, microplastics, chemical and microbial contamination between consumption mussels and wild type mussels, collected at Belgian department stores and Belgian groynes and quaysides, respectively. Species identification based on genetic analysis showed a high number of *Mytilus (M.) edulis* compared to *M. galloprovincialis* and *M. edulis/galloprovincialis* hybrid mussels. The number of total microplastics varied from 2.6 to 5.1 fibres/10 g of mussel. A higher prevalence of orange fibres at quaysides is related to fisheries activities. Chemical contamination of polycyclic aromatic hydrocarbons and polychlorobiphenyls could be related to industrial activities and water turbidity, with maximum concentrations at the quayside of port Zeebrugge. The inverse was noted for *Escherichia coli* contamination, which was relatively low at Zeebrugge quayside with a total count of 3.9×10^2 CFU/100 g tissue, due to limited agricultural effluents. Results of this complementary analysis stress the importance of integrated monitoring and quality assessment.

1. Introduction

In response to the increasing impact of human activities on the marine environment, different monitoring tools for ecological integrity and marine health status assessment have been implemented in legislation programs worldwide. These tools consist of systematic measurements of biotic and abiotic parameters of the marine environment within a predefined spatial and temporal schedule.

Organisms that have been extensively used in marine monitoring programmes, are bivalve mollusc shellfish such as mussels. Due to their sessile lifestyle, they accurately reflect local environmental conditions (Barker Jørgensen, 1990) and because of their wide geographic distribution, easy sampling, tolerance to a considerable range of salinity, resistance to stress and high accumulation of a wide range of pollutants, they are the ideal test organisms for environmental monitoring (Tanabea et al., 2000). Mussels are benthic filter feeders with a selective mechanism of suspension feeding. They process relatively large amounts of water during feeding, maximizing their exposure to any harmful material within the water column. This can result in the accumulation of microplastics, chemical pollutants and microorganisms (Barker Jørgensen, 1990), which are the marine health status parameters selected within this publication. The use of mussels to evaluate different types of pollution, however, may be affected by existing genetic differences (Bourlat et al., 2013). Molecular techniques such as DNA-barcoding, metagenomics and transcriptomics may help in normalizing measurements by making correct species identification possible, by revealing differences in population structures or dynamics or by unravelling distributional range and pattern variations. The presence and quantification of particularly sensitive and/or tolerant species may also indicate the degree of stress exposure (Bourlat et al., 2013).

Within this study, molecular tools were primarily used for a correct identification of the sampled *Mytilus sp.* individuals (Inoue et al., 1995; Dias et al., 2008). Biologists today recognize, although not generally accepted, the presence of three different species in the genus *Mytilus*, a Southern variant, *Mytilus galloprovincialis*, a central variant, *Mytilus edulis,* and a Northern brackish water variant, *Mytilus trossulus*, in European waters and the existence of mussel hybrids and hybrid zones (Innes and Bates, 1999; Kijewski et al., 2011). Consequently, this existence of different species within the genus *Mytilus sp.* leads to differences in genomic composition and gene expression profiles that can alter the way in which individuals deal with stress (Negri et al., 2013).

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The occurrence of microplastics in mussel is the first selected marine health status parameter. The environmental impact of marine plastic litter and microplastics has already been indicated as an area of global concern. This anthropogenic contaminant is included as descriptor 10 within the EU Marine Strategy Framework Directive to protect and restore the marine environment across Europe and to obtain a Good Environmental Status (GES) by 2020 (Anon., 2008; Zarfl et al., 2011). Research exploring the occurrence of microplastics in the marine environment and ecosystem is clearly expanding (Ivar do Sul and Costa, 2014). Microplastics (plastic particles smaller than 5 mm) enter the environment from both primary and secondary sources. Primary sources include particulates which are produced either for direct use, such as for industrial abrasives, exfoliants and cosmetics, or as precursors (resin pellets) for the production of consumer products. Secondary sources relate to the formation of microplastics in the environment due to the degradation of larger plastic material (Piha Henna et al., 2011). Marine microdebris can be described based on criteria such as colour, stage of erosion, shape (fibre, film, spherule or fragment) or polymer type (polyethylene, polystyrene, nylon, etc.) (Hidalgo-Ruz et al., 2012). Synthetic fibres, mainly originating from degradation of plastic debris such as rope and packaging materials, and washing of synthetic clothing, are the most abundant type of microplastics in marine sediments (Browne et al., 2011; Claessens et al., 2011; Cole et al., 2011). Ingestion and accumulation of microscopic synthetic particles were demonstrated and translocation was demonstrated for diverse filter feeders such as eastern oyster (*Crassostrea virginica*), zebra mussel (*Dreissena polymorpha*) and blue mussel (*M. edulis*) (Browne et al., 2008; Farrell and Nelson, 2013; Lei et al., 1996; von Moos et al., 2012; Ward and Kach, 2009; Wegner et al., 2012). Mussels ingest and accumulate microplastics and smaller nanoplastics in the gut, after which accumulation of plastic beads (<10 μm) into the digestive system and the tissues of the haemolymph might occur. Microbeads were also seen on the gills of the mussels after microplastics exposure through the water, indicating that not only sediment particles but also plastic beads could be trapped directly from the water column (von Moos et al., 2012). The localization of plastic in the mussel tissues and organs suggests the potential ability of microplastics to accumulate in higher trophic levels, as observed for the littoral crab after feeding on plastic exposed mussels (Farrell and Nelson, 2013). Bivalves are also able to expel foreign particles such as microplastics through egestion without them passing through the digestive tract (Beecham, 2008; Beninger et al., 1999; Lei et al., 1996; Levinton et al., 1996; Wegner et al., 2012; Wright et al., 2013).

A second marine health status parameter is chemical contamination. Within this work, the chemical assessment was done by polychlorinated biphenyl (PCBs) and polycyclic aromatic hydrocarbons (PAHs) analysis. PCBs and PAHs are organic pollutants, strongly related to industrialization and obliged to be monitored by all countries who subscribed the OSPAR convention (OSPAR, 2010).

Although PCBs were banned in the mid-1980s, sources of PCBs still remain present, e.g. within waste disposal, PCB-containing equipment, by remobilisation from sediments or by formation of by-products in thermal and chemical processes (OSPAR, 2012). For environmental monitoring and evaluation of PCBs, OSPAR has determined marine monitoring to be done on a set of seven PCBs (CB28, CB52, CB101, CB118, CB138, CB153 and CB180), which can be compared to environmental assessment criteria (EAC). EAC values were used as an evaluation tool in the latest OSPAR Quality report (OSPAR, 2010) but were also subject to debate and adjustments in subsequent working group meetings (ICES, 2012). The same set of PCBs, with exception of CB118, can also be used for food safety evaluation, according to EU regulation (EC) 1259/2011 (Anon., 2011b).

PAHs are widespread pollutants entering the marine environment by offshore activities, oil spills, river discharges and atmospheric transport. Five PAHs were selected for OSPAR marine monitoring: anthracene, fluoranthene, phenanthrene, benzo(a)pyrene and benzo(ghi)perylene (OSPAR, 2012). In scientific literature, comparisons are frequently made by summation of 16 priority PAHs, defined by the US Environmental Protection Agency (Donata, 2010), often reduced to 15 when naphthalene is excluded. For food safety issues, focus was on the most toxic PAH, i.e. benzo(a)pyrene, until 2012. Since then, food safety analysis is broadened, incorporating benzo(a)anthracene, benzo(b)fluoranthene and chrysene in the evaluation according to EU regulation (EC) 835/2011 (Anon., 2011a).

As third parameter, microbial pollution of the mussel at sampling locations is studied. Causes for faecal pollution are multiple, e.g. wildlife, sewage effluents, industrial discharges, run-off from farm animals and agricultural lands (Campos et al., 2013). The consumption of contaminated bivalves may result in consumer illness through the ingestion of faecally borne pathogens. Shellfish are often consumed raw or lightly cooked, which makes them highly ranked on the list of foods transmitting diseases (Huss et al., 2000; Feldhusen, 2000). In the EU, *Escherichia coli* is used as an indicator organism for the potential health hazard to consumers of shellfish and to assess the sanitary quality of shellfish. European regulations (EC) 854/2004 (Anon., 2004) and 2073/2005 (Anon., 2005) set out the criteria for microbiological standards based upon *E. coli* levels in bivalves. In order to be fit for human consumption, legislation/guidelines require that mussels should contain less than 5×10^5 colony forming units (CFU) per g total heterotrophic bacteria and less than 230 *E. coli* per 100 g flesh and intervalve water (ICMSF, 1986; Vernocchi et al., 2007; Kacar, 2011).

This research work is set up as a comparative assessment study between consumption and two types of wild type mussels. Consumption mussels were confirmed as on-bottom cultures from the Eastern Scheldt (The Netherlands), bought at Belgian department stores, and can be considered as environmental samples from this region. Wild type mussels came from groynes (Nieuwpoort, Oostende, Knokke) and quaysides (Nieuwpoort, Oostende, Zeebrugge) along the Belgian coastline. Groynes are situated in open sea while quaysides are sheltered and encounter an influence of harbours (Nieuwpoort en Oostende) or ports (Zeebrugge).

The aim of this publication was therefore fourfold: a limited genetic screening to identify the mussels coupled to the evaluation of three marine health status parameters: (1) uptake of microplastics, (2) determination of chemical contamination by PAH and PCB analysis and (3) evaluation of microbial characteristics for mussels. This was performed on mussels from different origin (consumption mussels, groyne and quayside) with three main locations for each group. To our knowledge, this is the first study comparing these different forms of contamination for commercial and wild type blue mussels. We will assess and compare the quality of the mussel samples based on existing environmental guidelines and food safety legislation.

2. Materials and methods

2.1. Sample collection

Consumption mussels were purchased at three different Belgian department stores whereas mussels from groyne and quayside were sampled manually at the specific locations (Fig. 1). All samples for genetic, microplastic and chemical analysis were taken in the first two weeks of March, 2013. For microbial analysis, all samples were taken during the last two weeks of October 2013 on exactly the same sampling locations. Mussels for genetic,

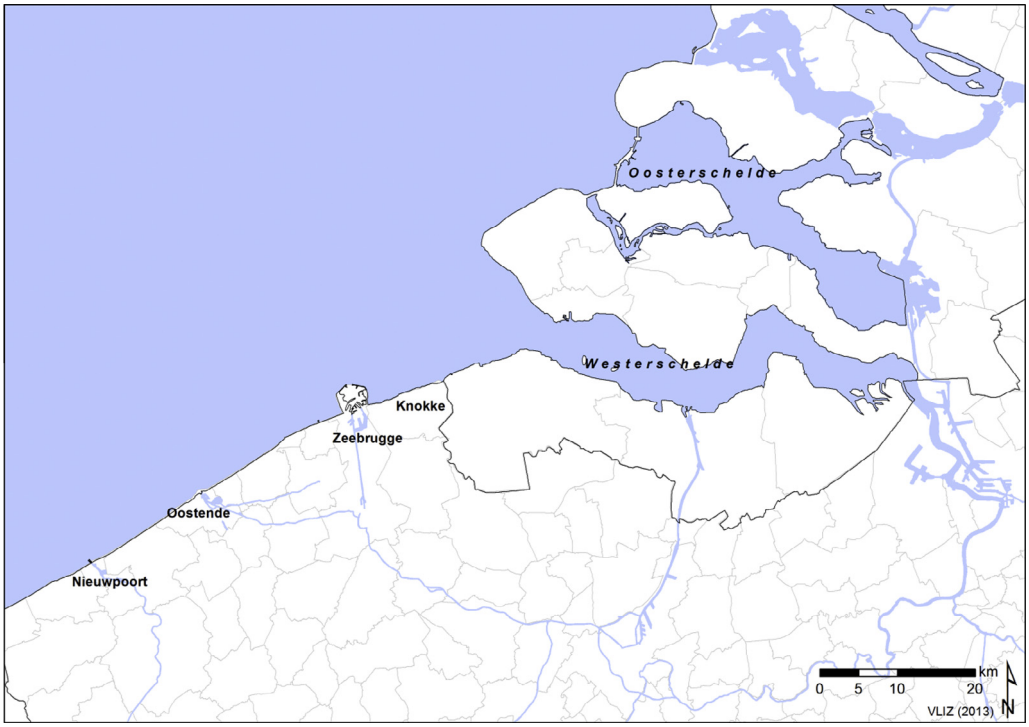

Fig. 1. Map of Belgian coastline and Eastern Scheldt (The Netherlands) including the geographic location of Nieuwpoort, Oostende, Zeebrugge and Knokke.

microplastic and chemical analysis were taken with bare hands whereas samples for microbial testing were taken with gloves and immediately stored within sterile bags for transport to the lab. Shell length and mussel tissue weight were measured. A random selection was made for genetic, microplastic and microbial analysis while mussels between 40 and 50 mm shell length were selected for chemical analysis. After subdivision between the different disciplines, samples for genetic analysis were stored in a plastic bag in the freezer (−20 ± 4 °C). The mussels for chemical analysis and microplastics assays were stored for 24 h in salt water to remove sediments and to depurate the gut. For microplastics analysis, mussels were stored in aluminium foil in the freezer (−20 °C ± 4 °C) whereas for chemical analysis, mussels were stored in aluminium foil in the freezer (−20 ± 4 °C) after mixing. Mussels for microbial analysis were stored in the refrigerator and analysis was performed within 24 h after sampling.

2.2. Genetic identification

For each group, identification was performed on a set of 30 individual mussels, equally divided over the 3 locations. Herefore, species identity and hybridization status of all samples were analysed by genotyping technology. DNA was extracted from 40 mg of muscle tissue using the Invisorb Spin Tissue Mini Kit (Invitek). DNA extracts were checked for fragmentation by means of gel electrophoresis on a 0.8% agarose gel and concentration and purity were analysed on a microvolume spectrophotometer (Nanodrop).

Following DNA extraction, species identification and hybridization status were determined through a combined approach of classical PCR and real-time PCR. In the classical PCR, primers Me15 (5′-CCAGTATACAAACCTGTGAAGACAAGTTA-3′) and Me16 (5′-TGTTGTCTTAATAGGTTTGTAAGATGACAG-3′) were used in 20 μl reaction volume combining 10 μl Red Taq DNA Polymerase mastermix 2.0 (1.5 mM $MgCl_2$) (VWR), 1 μl of forward and reversed primer (in a final concentration of 0.5 μM), 2 μl DNA template (5–10 ng) and 6 μl sterile PCR water. The primers amplify a small part of the byssus protein coding gene that shows a deletion characteristic for the three different species and results in fragment of 180 bp, 168 bp and 126 bp for *M. edulis*, *M. trossulus* and *M. galloprovincialis*, respectively. PCR conditions consisted of 1 cycle of 10 min at 95 °C to allow complete denaturation of the DNA and activation of the hot-start DNA polymerase, 45 cycles of 30s at 95 °C, 30s at 56 °C and 1.5 min at 72 °C, followed by 1 cycle of 5 min at 72 °C for a final elongation and a hold step at 16 °C. Species identification was scored based on fragment length and hybridization status by the presence of one or two bands.

The real-time PCR confirmation approach was run at the same conditions of the classical PCR but the reaction mix was completed by the addition of a species specific fluorescently labelled TaqMan-MGB probe in a final concentration of 1 μM. Three probes, *M. edulis*, 5′-6-FAM-AAGAAAGTGGACTATCGTC-MGB-3′, *M. trossulus*, 5′-6-FAM-AAGAAACCAATGGACTATAA-MGB-3′ and *M. galloprovincialis*, 5′-VIC-CTATTCGTAGGATGATAACTT-MGB-3′ (Applied Biosystems Custom TaqMan probe) were used in three separate reactions. Species identification and hybridization status were scored by presence or absence of an amplification profile after thermal cycling.

2.3. Microplastic analysis

All required solutions were filtered with a qualitative filter with particle retention 10–20 μm (VWR, Grade 310) before starting the destruction protocol. All laboratory glassware was cleaned with acetone and filtered type 1 water before use, as recommended by Claessens et al. (2013). After opening the shells, the mussel body was rinsed with filtered type 1 water to remove the intervalve water. Extraction of microplastics from the mussel bodies was performed using an acid destruction with a mixture of nitric acid (VWR, 65%) and perchloric acid (VWR, 68%), HNO_3:$HClO_4$ (4:1 v:v). For an optimal digestion of the mussel bodies, 500 ml acid solution was used per 100 g tissue. The stronger perchloric acid helps to reduce the remaining greasy tissue fraction after destruction. The mussel body was digested overnight at room temperature

in a closed fume hood. The solution was covered with a clock glass to avoid contamination by air. The digest was boiled during 10 min, followed by a dilution of the digest with 500 ml heated type 1 water. The solution was boiled a second time until the tissue was completely digested as observed by visual inspection, followed by a cool down period of 30 min. The acid digest was filtered over a qualitative filter (VWR, Grade 310) and the filter was transferred on a glass Petri dish for transport and visualization of microplastics under a stereo microscope (Leica M 20:5:1 or M 16:5:1 zoom). Observed microplastics were classified by category (fibre – film – spherule – fragment) and colour for each assessed mussel or blank sample. Each plastic fragment was verified as plastic with a hot needle. Synthetic polymer types were not identified. One destruction batch was performed for each location, which consisted of 5 mussels and 3 blank analyses. For the blank analysis, the entire procedure was performed without mussel tissue. Results were evaluated after blank subtraction. One destruction batch was executed for each sampling location.

2.4. Chemical analysis

For each chemical parameter, three independent samples were analysed for each sampling location. The amount of total lipids was determined based on the Bligh and Dyer method (Hanson and Olley, 1963). Herefore, 10 g of wet sample was mixed with 20 ml of methanol absolute (Biosolve, ⩾99.9%) and 2 × 10 ml of chloroform (Merck, Suprasolv, ⩾99.8%). After addition of 10 ml type 1 water, the mixture was filtered over a Whatman quantitative filter (GE healthcare, Grade 589/3). The filter was mixed in 30 ml of chloroform and filtered over a second Whatman quantitative filter. After phase separation for 2 h, the solvent phase was evaporated by means of a rotavapor at 70 °C (Büchi, R-114) and subsequent drying in an oven at 50 °C for 30 min. The total lipid amount was determined gravimetrically.

For PCB analysis, 80–100 mg of lipid was purified with 2 × 25 ml of hexane on a glass column filled with 10 g of aluminium oxide (Merck, Aluminium oxide 90 active basic), deactivated with 5% of type 1 water. The extract was evaporated to 1.0 ml by a Turbovap II evaporator (Zymark) and eluted with 12 ml of hexane (Merck, Suprasolv, ⩾98.0%) on a glass column filled with 3 g of silicon oxide (Merck, Silica gel 60) deactivated with 5% of type 1 water. After evaporation and reconstitution to 1.0 ml iso-octane (Merck, Lichrosolv, ⩾99.0%), 3.5 μl of sample solution was injected on a Varian 450-GC with a split/splitless injector at 210 °C. Separation was done by a HT-8 column (SGE, 50 m, 0.22 mm, 0.25 μm). Hydrogen (Parker hydrogen generator) at a flow rate of 0.8 ml/min was used as carrier gas. A temperature programme from 90 °C to 285 °C was programmed for 54 min. The GC was equipped with an ECD-detector. Detection was done at 300 °C with 25 ml/min 95% Ar – 5% methane as make-up gas. Tetrachloronaphtalene (TCN) was used as internal standard and for calibration, dilutions were made out of 100 ppm solutions of CB28, CB52, CB101, CB118, CB138, CB153 and CB180 in iso-octane purchased from LGC standards.

Sample preparation of mussels for PAH analysis was started with chemical drying of 3 g sample with ca. 9 g of Na_2SO_4 (Merck, ⩾99.0%). The samples were extracted by accelerated solvent extraction (Dionex, ASE350). Cells of 22 ml were filled with the dried sample, 2.5 g of florisil (Merck, 0.150–0.250 mm) and diatomaceous earth (Sigma Aldrich, Celite 545) and extracted with hexane (Merck, Suprasolv, ⩾98.0%):acetone (Biosolve, Pesti-S, ⩾99.9%) 3:1 at 100 °C. For the extraction, 3 cycles of 5 min static time each were programmed. The extract was evaporated to 1.0 ml by a Turbovap II evaporator (Zymark) and eluted with 15 ml of hexane on a glass column filled with 2.0 g of aluminium oxide (Merck, Aluminium oxide 90 active basic), deactivated with 10% of type 1 water. A second evaporation step to 1.0 ml was performed, followed by the extract elution with 10 ml of hexane on a glass column filled with 1.0 g of silicon oxide (Merck, Silica gel 60). After evaporation and reconstitution to 0.5 ml iso-octane (Merck, Lichrosolv, ⩾99.0%), 5 μl of sample was injected on an Agilent 7890A GC equipped with a PTV-injector with glass bead liner (Gerstel, 6495-U), temperature programmed from 80 °C to 320 °C. Separation was done by a select PAH column (Agilent, 30 m, 0.25 mm, 0.25 μm) with helium (Air Liquide, Alphagaz 2) as carrier gas at a constant flow of 2.2 ml/min. The oven temperature was programmed at 50 °C for 1.05 min, followed by an increase of 40 °C/min to 70 °C, 35.5 °C/min to 180 °C/min, 3.55 °C/min to 230 °C hold for 13.8 min, 25.36 °C/min to 280 °C hold for 13.8 min and an final 15.22 °C/min increase to 325 °C which was hold for 2 min. Detection was done with an Agilent 5975C MS-detector with electron impact ionisation in single ion mode with transfer line temperature at 340 °C, ion source temperature at 300 °C and quadrupole temperature at 150 °C. As internal standard, chrysene d12 in toluene was added to the vial. Quantification was performed against the following list of recovery standards, added to the extraction cells: acenaphthene d10, anthracene d10, pyrene d10, benzo(a)-anthracene d12, benzo(a) pyrene d12 and indeno(123cd)pyrene d12, all dissolved in iso-octane and purchased from LGC-standards. The following compounds were determined: acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, indeno(123 cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene. All compounds were present in a PAH-mix 27 (LGC-standards, PAH-mix 27 in toluene).

The dry matter content was determined gravimetrically by mixing 1–2 g sea sand (Merck, Sea Sand extra pure) per g of sample and drying at 105 °C for at least 4 h.

2.5. Microbial analysis

Three independent samples were analysed for each location. The mussels were aseptically opened with a sterile knife. The meat was removed aseptically and 10 g was transferred into a stomacher bag containing an appropriate volume (1/9) (w/v) of sterile Maximum Recovery Diluent (MRD, Oxoid). Samples were homogenized during 60 s with a microbiological homogenizer (BagMixer, Interscience). Further appropriate 10-fold dilutions of the homogenates were made in chilled MRD. To get an overall picture of the microbiological quality, samples of 0.1 ml of the dilutions were spread in duplicate on marine agar (MA, Gentaur) plates for enumeration of total heterotrophic bacteria. The plates were incubated for 72 h at 20 °C and colony forming units (CFU) were counted. Results were normalized by conversion into logarithmic values (log CFU/g).

E. coli were analysed as faecal indicators by the colony-count technique (based on ISO 16649-2) as an alternative to the MPN method (ISO 16649-3). The method was validated for shellfish by CEFAS, the European Union Reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs (Anon., 2011c). For each sample, 5 tryptone bile X-glucuronide agar (TBX) plates were inoculated with 2 ml of a 1:1 mussel dilution. The plates were incubated for 4 h at 37 °C, followed by 18–24 h at 44 °C. Colony forming units were counted and expressed as CFU/100 g.

3. Results

3.1. Weight/length identification

The weight/length ratio for each location is given in Fig. 2. No significant difference was noted between consumption, groyne

and quayside mussels, considered as group (ANOVA, $\alpha = 0.05$). Within the group of groyne mussels, a significant higher weight/length ratio of 1.59 ± 0.26 at Nieuwpoort and a significant lower ratio at Knokke of 0.77 ± 0.13 is recorded.

3.2. Genetic identification

The genetic species composition is given in Fig. 3 for each group, based on the analysis of 30 mussels. Based on our limited dataset, the results clearly show that the majority of the tested individuals were identified as *M. edulis*. Results also show the presence, albeit in low numbers, of *M. galloprovincialis*, the *M. edulis/galloprovincialis* hybrid form and the absence of *M. trossulus*. A higher presence of the *M. edulis/galloprovincialis* hybrid form could be noted within groyne and quayside mussels compared to consumption mussels.

3.3. Microplastic analysis

The optimization of the acid destruction revealed that the extraction of microplastics from mussel bodies was subjected to background contamination by microscopic synthetic fibres. The main sources of microplastic contamination during the protocol were assigned to unfiltered solutions, the use of plastic recipients such as a wash bottle, the use of synthetic clothing and nevertheless the surrounding air. To overcome these limitations, strict measures were taken in the laboratory for these tissue destructions: all glassware was rinsed with acetone and filtered type 1 water, and all solutions were filtered before use, no synthetic clothing was allowed in the lab and the digest/blank was covered from air. Due to the abundant airborne contamination of synthetic fibres, it is recommended to use blank samples and to consider limit of detection (LOD) values for the most abundant airborne fibres.

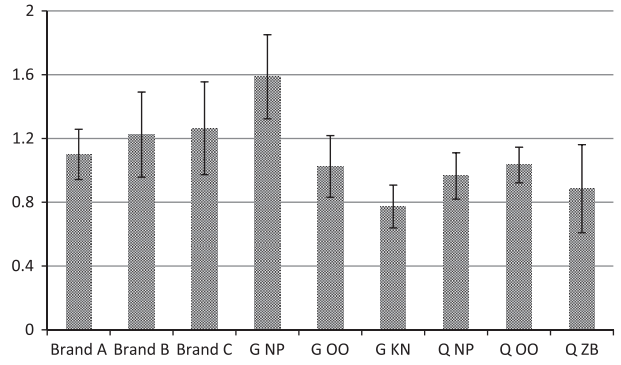

Fig. 2. Weight/length ratio per location (G: groyne, Q: quayside; OO: Oostende; KN: Knokke; NP: Nieuwpoort; ZB: Zeebrugge).

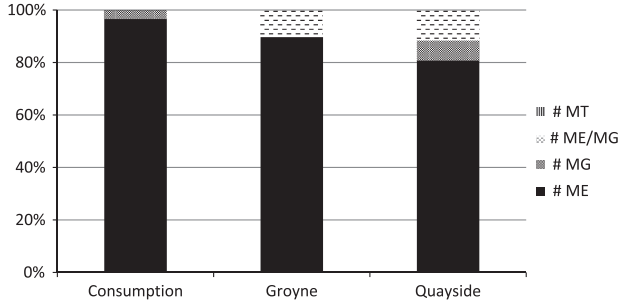

Fig. 3. *Mytilus* sp. species composition per group. (# ME:% *Mytilus edulis*, # MG:% *Mytilus galloprovincialis*, #MT:%*Mytilus trossulus*, #ME/MG:% *Mytilus edulis/galloprovincialis* hybrids).

Not only the environmental contamination of natural and synthetic fibres in honey and sugar samples was reported by Liebezeit and Liebezeit (2013), but also the contamination of laboratory air by microparticulate material was mentioned, though more information is needed to identify the nature and origin of these fibres.

A LOD (average + 3* standard deviation) was obtained by analysing all blank samples for microplastics by type and colour. Only black, blue and red fibres (Fig. 4) were observed frequently on the filters of the blank samples. For the determination of the LOD values, 30 blank samples were assessed. LOD values of 2.3, 4.7 and 1.5 fibres/analysis were established for black, blue and red fibres, respectively. For the other types of plastic microdebris and for the other coloured synthetic fibres, a LOD <1 fibre/analysis was

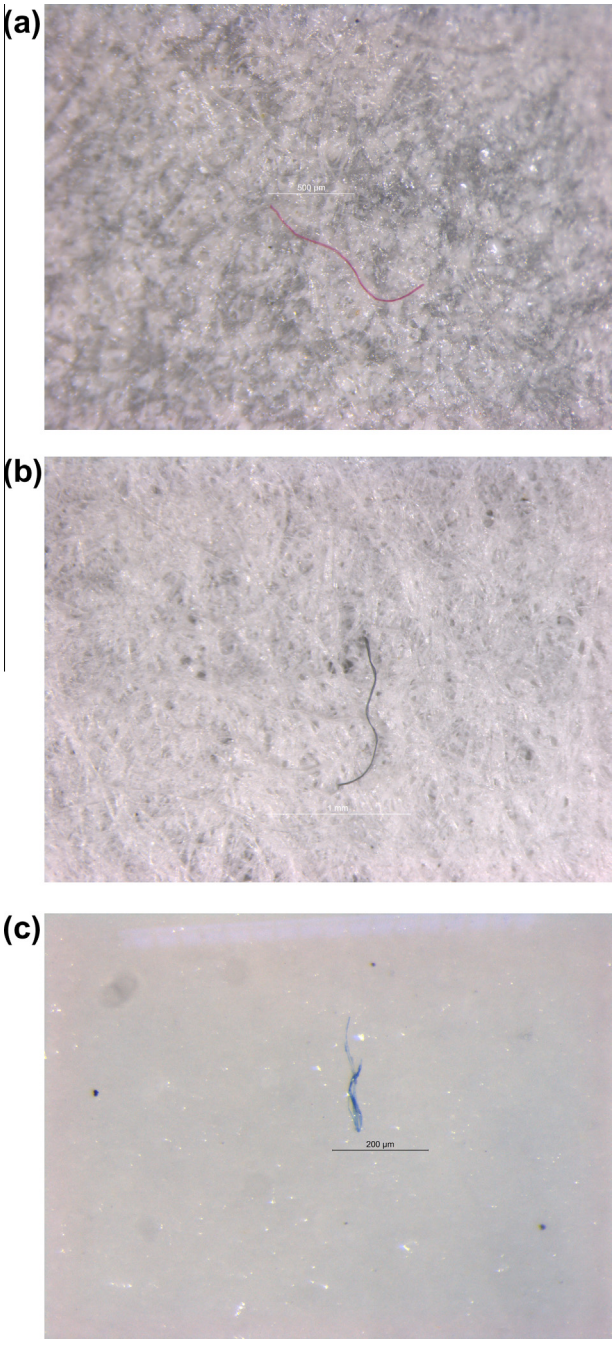

Fig. 4. Synthetic fibres observed in mussel bodies. (a) Black fibre, (b) blue fibre and (c) red fibre.

defined. The LOD values are applicable for the digestion of bodies of small organisms <15 g.

Microscopic synthetic fibres ranging from 200 μm up to 1500 μm size were detected in the bodies of the examined mussels, including black, red, blue, purple, translucent, transparent, orange, green and yellow fibres, with the most common size class being 1000–1500 μm. Although the ability of mussels to accumulate spherical plastic particles in the haemolymph after controlled lab exposure was reported (von Moos et al., 2012; Browne et al., 2008), the uptake of plastic films and spherical or granular microplastics from their natural environment was not observed for the assessed mussels. The spherical and granular particles were clearly removed from the digestive tract during the gut depuration period, while the egestion of synthetic fibres appeared to be delayed. Due to the particle retention of 10–20 μm on the qualitative filters, nano and microparticles smaller than the filter limit were not considered. The occurrence of the blue, black and red fibres in mussel bodies was below the LOD value in all examined samples.

For the determination of 'total microplastics', all fibres were included with exception of blue/black/red fibres (<LOD). No significant difference (ANOVA, $\alpha = 0.05$) in total microplastics content was observed between the consumption, groyne and quayside mussels and a mean prevalence of 3.5 fibres/10 g wet weight (w.w.) was established for consumption mussels, 2.6 fibres/10 g w.w. for the groyne mussels and 5.1 fibres/10 g w.w. for the quayside mussels as shown in Table 1.

Although orange fibres were observed frequently in the major part of the sampled mussels, no orange fibres were ever discovered in the blank filters. As a case study, the uptake of orange synthetic fibres was evaluated in each mussel. A significantly higher level (ANOVA, $\alpha = 0.05$) of orange fibres was observed in the mussels from the quaysides compared to groyne mussels and consumption mussels of the Eastern Scheldt (Table 1). A mean prevalence of 1.5 orange fibres/10 g w.w. was observed for the quayside mussels, while a clearly lower incidence was calculated for the consumption mussels (0.7 orange fibres/10 g w.w.) and groyne mussels (0.2 orange fibres/10 g w.w).

3.4. Chemical analysis

The results of the chemical analyses for the parameters lipid content, dry matter, PCB and PAH are given in Table 2. For evaluation of the chemical environmental quality of the mussel samples, the sum of OSPAR-7 PCBs (CB28, CB52, CB101, CB118, CB138, CB153 and CB180) and 15 EPA-priority PAHs (acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(123 cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene) were evaluated on a lipid and wet weight basis, respectively. Values were also compared to food legislation criteria (Anon., 2011a,b). Therefore, the sum of ICES-6 PCBs was taken (CB28, CB52, CB101, CB138, CB153 and CB180), benzo(a)pyrene concentrations were looked at as well as the sum of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene. Calculation of the PAH and PCB values on a dry weight basis was performed to allow comparison of the data with OSPAR environmental assessment criteria (OSPAR EAC).

For lipid content, there was no significant difference between the consumption, groyne and quayside mussel groups. Focusing on the individual locations, a significantly lower amount of total lipids was found in mussels at quayside Zeebrugge and a significantly higher amount in mussels at quayside Nieuwpoort and groyne Knokke (ANOVA, $\alpha = 0.05$, post hoc analysis Tukey).

PCB analysis revealed a significantly lower OSPAR-7 PCB concentration (357 ± 52 μg/kg lipid weight) in consumption mussels (ANOVA, $\alpha = 0.05$, post hoc analysis Tukey) whereas open sea groyne mussels equalized the PCB concentration of quayside mussels within enclosed harbours (928 ± 156 and 937 ± 372 μg/kg lipid weight, respectively). The samples from quayside Zeebrugge revealed a significantly higher PCB-concentration (1404 ± 129 μg/kg lipid weight) than all other samples (ANOVA, $\alpha = 0.05$). Within the group of groyne mussels, a PCB concentration gradient is noticed from west to east (direction Nieuwpoort–Oostende–Knokke). No significant difference in PCB concentration was noticed between the different brands of the consumption mussels.

Assessment against OSPAR EAC reveals an exceedance of the CB118 threshold of 1.2 μg/kg dry weight for all samples and an exceedance of the 5.4 μg/kg dry weight and 6.0 μg/kg dry weight threshold for CB52 and CB101, respectively, in all groyne and quayside samples.

Remarkably, food quality standards are met for all samples, even the most polluted samples, i.e. quayside Zeebrugge. These mussels have a high PCB concentration on lipid basis, indicating a more polluted area. However, due to their very low fat content, food legislation criteria on a wet weight basis are met (<75 μg/kg ICES-6 PCBs).

PAH concentrations at quayside Zeebrugge reached 719.2 ± 137.2 μg/kg wet weight, which is up to 15 times higher than at other sampling locations (Kruskal–Wallis, $\alpha = 0.05$). PAH concentrations in mussels at quayside Oostende (116.0 ± 8.8 μg/kg wet weight) and to a lesser extent at quayside Nieuwpoort (85.2 ± 0.4 μg/kg wet weight) were also higher than in groyne and consumption mussels. Remarkably, PAH concentrations based on the 15 EPA priority PAHs were not significantly different for

Table 1
Overview of the data of the microplastics and microbial analysis.

		Total microplastics	Orange fibres	Total viable count	Min. total *E. coli*	Max. total *E. coli*	Average total *E. coli*	Coeff. of variation
		Number of fibres/10 g	Number of fibres/10 g	Log CFU/g	CFU/100 g	CFU/100 g	CFU/100 g	%
Consumption	Brand A	5.3	1.3	5.6 ± 0.6	<10	4.5×10^1		
	Brand B	1.6	0.7	6.1 ± 0.3	<10	3.2×10^2		
	Brand C	3.6	0.0	5.5 ± 0.5	<10	<10		
	Average	3.5	0.7	5.7 ± 0.5				
Groyne	Nieuwpoort	3.9	0.0	5.4 ± 0.0	1.2×10^3	1.8×10^3	1.6×10^3	22.2
	Oostende	0.4	0.0	4.4 ± 0.3	1.6×10^3	1.7×10^4	8.3×10^3	94.8
	Knokke	3.5	0.6	5.1 ± 0.2	7.2×10^2	7.6×10^2	7.4×10^2	3.3
	Average	2.6	0.2	5.0 ± 0.5			3.5×10^3	150.4
Quayside	Nieuwpoort	2.9	1.2	5.2 ± 0.5	3.5×10^3	1.1×10^4	7.3×10^3	49.4
	Oostende	8.1	1.4	4.3 ± 0.1	8.7×10^2	5.4×10^3	2.5×10^3	99.6
	Zeebrugge	4.2	1.8	5.1 ± 0.4	2.5×10^2	4.6×10^2	3.9×10^2	31.0
	Average	5.1	1.5	4.8 ± 0.6			3.4×10^3	110.8

Table 2
Overview of the data of the chemical analysis.

		Lipid content %	PCB OSPAR-7 µg/kg Lipid weight	PCB ICES-6 µg/kg Wet weight	EPA-PAH[1] µg/kg Wet weight	Benzo(a)pyrene µg/kg Wet weight	4-PAH[2] µg/kg Wet weight
Consumption	Brand A	1.35 ± 0.19	365 ± 18	4.35 ± 0.64	59.2 ± 8.7	0.85 ± 0.14	9.5 ± 1.3
	Brand B	1.71 ± 0.10	295 ± 12	4.45 ± 0.12	47.6 ± 0.1	1.30 ± 0.09	12.3 ± 0.4
	Brand C	1.82 ± 0.14	410 ± 11	6.67 ± 0.37	62.3 ± 6.8	0.68 ± 0.13	8.8 ± 1.3
	Average	1.63 ± 0.25	357 ± 52	5.16 ± 1.20	56.4 ± 8.7	0.95 ± 0.29	10.2 ± 1.9
Groyne	Nieuwpoort	1.54 ± 0.06	747 ± 35	10.20 ± 0.77	68.3 ± 6.1	2.23 ± 0.31	18.2 ± 1.8
	Oostende	1.34 ± 0.08	953 ± 9	11.35 ± 0.66	66.2 ± 8.9	2.76 ± 0.56	19.8 ± 2.7
	Knokke	2.08 ± 0.06	1084 ± 95	20.30 ± 1.84	57.7 ± 6.1	2.02 ± 0.20	16.7 ± 1.7
	Average	1.65 ± 0.34	928 ± 156	13.95 ± 4.90	64.1 ± 7.9	2.34 ± 0.47	18.3 ± 2.3
Quayside	Nieuwpoort	2.31 ± 0.31	581 ± 34	11.82 ± 2.15	85.2 ± 0.4	1.93 ± 0.11	18.1 ± 0.3
	Oostende	1.35 ± 0.01	826 ± 20	9.75 ± 0.16	116.0 ± 8.8	3.48 ± 0.21	32.9 ± 2.7
	Zeebrugge	0.84 ± 0.07	1404 ± 129	9.96 ± 0.15	719.2 ± 137.2	42.3 ± 13.3	177.9 ± 41.1
	Average	1.50 ± 0.67	937 ± 372	10.51 ± 1.46	306.8 ± 317.1	15.9 ± 20.9	76.3 ± 79.2

consumption mussels compared to groyne mussels (56.4 ± 8.7 and 64.1 ± 7.9 µg/kg weight, respectively). On the contrary, a significant lower concentration is measured for consumption mussels when only food legislation PAHs are taken into account, with the sum of the 4 PAHs equal to 10.2 ± 1.9 µg/kg wet weight compared to 18.3 ± 2.3 µg/kg weight at groynes. Fig. 5 shows the relative amounts of 3, 4, 5 and 6-ring PAHs for consumption, groyne and quayside mussels. The 4, 5 and 6-ring PAHs are known to be especially sediment related while 3-ring PAHs are more water-column related (Webster et al., 2006). Groyne mussels have a relatively low amount of 3-ring PAHs whereas consumption mussels and mussels at quayside Zeebrugge have a high percentage of 3-ring PAHs.

Regarding environmental criteria, a good ecological status is met for PAHs for all consumption and groyne mussels samples while exceedances of OSPAR EAC values are noted for fluoranthene in all quayside samples, pyrene in the quayside Oostende and Zeebrugge samples and benzo(a)anthracene and benzo(ghi)perylene in the quayside Zeebrugge samples. Food criteria were met for consumption mussels, the groyne mussels and mussels from quayside Nieuwpoort.

3.5. Microbial analysis

Consumption mussels show a significantly higher total viable count (TVC) than mussels picked on the quaysides and the groynes (Table 1). None of the mussels reached the upper limit of acceptability (5.7 log CFU/g) in the environment (ICMSF, 1986).

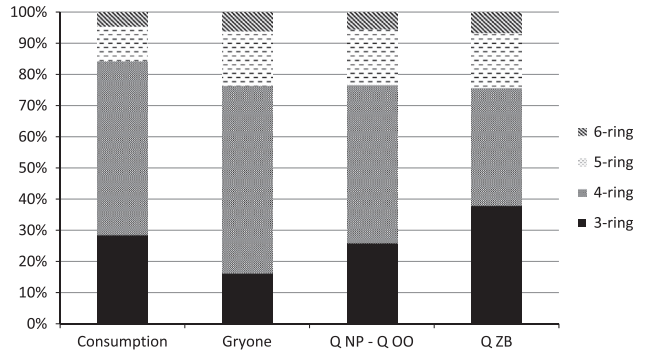

Fig. 5. Relative amount of 3, 4, 5, and 6-ring PAHs to the total amount of 15 EPA-priority PAHs (naphthalene excluded) for the different sampling groups (Q: quayside, OO: Oostende; NP: Nieuwpoort; ZB: Zeebrugge).

No statistical differences in TVC values could be found between the quaysides and groynes (ANOVA, $\alpha = 0.05$).

In order to determine the degree of faecal pollution, the number of *E. coli* was determined (Table 1). The mean number of *E. coli* bacteria varied between the detection level and 8.3×10^3 CFU/100 g. The mean values of the commercial samples were all below the European legal limit of 230 CFU/100 g (Anon., 2004, 2005). Noteworthy, one consumption mussel's subsample did not comply the legal limit for consumption and would therefore be discarded for human consumption. The level of *E. coli* at quaysides and groynes was determined to be higher than the allowed limit in all the stations. The highest concentrations of *E. coli* were obtained on the groyne of Oostende and the quayside of Nieuwpoort with respectively 1.7×10^4 and 1.1×10^4 CFU/100 g. On the other hand, the groyne of Knokke and quayside Zeebrugge displayed similar low concentrations of *E. coli* between 2.5×10^2 CFU/100 g and 7.6×10^2 CFU/100 g. The results of Kruskal–Wallis ANOVA variations of levels of *E. coli* showed a significant difference ($\alpha = 0.05$) between the harbours.

4. Discussion

4.1. Genetic identification

The blue mussel (*M. edulis*) is a key model organism that is frequently used in monitoring programs for assessment of the marine environmental quality status. Morphological variations due to habitat difference are common within the genus Mytilus (Groenenberg et al., 2011) while the systematic status of the different forms is still subject of debate (Martínez-Lage et al., 1995). Species identification solely based on morphometrics is therefore uncertain and time consuming (Beamont et al., 1989). The identification problem was solved by the application of a previously validated technique using specific primers for the *Glu* gene (polyphenolic adhesive protein) (Westfall and Gardner, 2010).

This study therefore follows new trends in biological effects monitoring using mussels by actually checking local differences in species composition within mussel populations used in a stress monitoring study (Brooks and Farmen, 2013). The results of this limited genetic screening indicate that there indeed might be differences in mussel species or genotype composition between the different groups. Since *M. edulis/galloprovincialis* hybrids will have different levels of stress proteins compared to *M. edulis* or *M. galloprovincialis* (Fuentes et al., 2002) resulting in different mortality (Fuentes et al., 2002; Wilhelm and Hilbish, 1998), a different

response on chemicals, microplastics or bacterial contamination may occur. However, results of this limited genetic screening are not conclusive and effects of these stressors on different species or genotypes should be subject to further investigation. Within this publication, the presence of different stressors is studied in detail.

4.2. Evaluation of quayside mussels

Mussel samples from quaysides were all taken within an enclosed harbour or port, limiting mixing of water with the open sea. The port of Zeebrugge is a large, industrialized port with total cargo traffic of 43.5 million tons in 2012. The harbour of Oostende is much smaller with total cargo traffic in 2012 of 3.2 million tons whereas Nieuwpoort is especially focused on leisure boats (Merckx and Neyts, 2013). These differences in port activities and harbour size are reflected in the degree of chemical contamination. The most heavily polluted mussels, as indicated by PCB and PAH contamination, are found at the largest port, i.e. at Zeebrugge quayside, which results in a negative effect on the total lipid content. Oostende quayside mussels are relatively more polluted than Nieuwpoort quayside mussels, were industrialization is less. The high degree of chemical contamination for Zeebrugge quayside mussels does not directly affect the total viable count of bacteria. Moreover, the lowest count of *E. coli* for all quayside samples was noted at Zeebrugge. This can be explained by a lower degree of runoff from animal farms and agricultural lands as Zeebrugge is surrounded by industry. Research showed that the port of Zeebrugge also has a better water quality in terms of agricultural effluents as ammonium, nitrite and total phosphorous than Nieuwpoort and Oostende (Boets et al., 2012). For all quayside samples, the legal limit of *E. coli* for human consumption is exceeded, indicating that the marine environment surrounding the shellfish is polluted by faecal microorganisms for all locations.

While no differences in total numbers of microplastics were found, it was remarkable that quayside mussels contained a significantly larger number of orange fibres compared to consumption and groyne mussels. These orange synthetic fibres mainly originates from polyethylene dolly rope at fishing nets and can be related to fisheries related activities at ports and harbours such as fishing net repair and dumping of old nets. The port and harbours of Zeebrugge, Oostende and Nieuwpoort had a total landing from Belgian fisheries vessels in 2012 of 11,230, 6170 and 184 ton, respectively (Anon., 2013).

Considering the rather high portion of orange fibres in quayside mussels (30%) in relation to the total amount of microscopic fibres, it could be conceptualized that orange coloured fibres could be popular as food and could be favoured above other colours of microplastics. However, the idea of colour preferable ingestion is still controversial between researchers and this hypothesis has not been empirically tested (Lusher et al., 2012; Boerger et al., 2010; Verlis et al., 2013; Schuyler et al., 2012; Kawamura et al., 2010). It was suggested for several other species that micro-debris could be ingested due to food/prey resemblance, for example be mistaken for floating fish eggs or small zooplankton, and that the colour could influence the uptake of the microplastics (Wright et al., 2013; Boerger et al., 2010; Verlis et al., 2013; Schuyler et al., 2012; Kawamura et al., 2010). On the other hand, Boerger et al. (2010) also mentioned the colour similarity between the ingested plastics and the environmental plastics in the seawater, which indicates that the ingested plastic may reflect the abundance of plastic in the water column. For benthic filter feeders such as the blue mussel, it must be considered that not only the resemblance of debris to natural food is important for ingestion, but also the feeding behaviour of the organism. It was already suggested that factors additional to the particle size (e.g. temporal variations in feeding, fluorescence of particles, environmental factors) could influence the feeding selectivity response in *M. edulis* and it was demonstrated that small particles (1–4 μm) occasionally serve as dietary compound (Strohmeier et al., 2012; Ward and Shumway, 2004; Newell et al., 1989). Due to the complexity of the particle capture mechanism, no comparison can be made between the amount of ingested spherical or granular particles and the occurrence of environmental plastic particles. The uptake mechanism for synthetic fibres is more ambiguous and expecting that mussels possess no colour preference for food, it could be assumed that the synthetic fibres ingested by the mussel will reflect the abundance of these microplastics in the water column. However, no environmental assumptions could be made based on the ratio of fibres and spherical synthetic particles.

4.3. Evaluation of groyne mussels

Mussel samples taken at the groynes were exposed to the open sea, which may explain the limited number of orange synthetic fibres compared to quayside mussels. Chemical data, however, shows ambiguous results. The PCB load equalizes the high level of quayside mussels whereas the PAH load is at the level of commercial samples. This cannot be solely explained by industrialization differences. Fig. 5 shows the relative amount of 3, 4, 5 and 6-ring PAHs for Eastern Scheldt mussels, groyne mussels and quayside mussels. Groyne mussels have the highest relative amount of 4,5 and 6-ring PAHs. Since PCBs and 4, 5 and 6-ring PAHs are sediment-related contaminants, the results indicate a high level of sediment contact caused by a high degree of turbidity.

Within the group of groyne mussels, a PCB concentration gradient is noticed from west (Nieuwpoort) over Oostende to East (Knokke). This is consistent with yearly monitoring data on mussels for OSPAR, reported to the public available ICES data portal (ecosystemdata.ices.dk). At the eastside of the Belgian coast, there is a larger degree of industrialization nearby the port of Zeebrugge together with a high degree of sedimentation compared to the west side (Fetweiss et al., 2009) which results in less chemical polluted mussels with a better weight/length ratio. Analogous to the quayside mussels, the lowest degree of *E. coli* contamination was noted at the more industrialized east side of the Belgian coast line, less subjected to agricultural effluents while the influx of nutrients of the Scheldt are mainly northward driven by North Sea currents (Passy et al., 2013).

4.4. Evaluation of consumption mussels

Consumption mussels mainly originate from on-bottom culture mussel farms at the Eastern Scheldt. The chemical PCB and 4, 5 and 6-ring PAH contamination is low compared to groyne and quayside mussels and may be due to by several factors. Due to the construction of a storm-surge barrier and two compartment dams, the estuarine has changed into a tidal basin without river influx. This will reduce input of chemical contamination from inland. Schipper et al. (2009) mentions a low degree of pollution at the Eastern Scheldt, taking into account OSPAR-7 PCB concentrations and PAH concentrations in sediment. Reported concentrations are, however, still higher than own reported concentrations for open sea sediment data nearby the Belgian coastline and reported to the ICES data portal. This indicates that other factors play a role to explain the difference in concentrations with groyne mussels. At the Eastern Scheldt, an increased accumulation of fine sediments is reported (ten Brinke et al., 1994) which favours PCB and PAH adsorption. This may however be counteracted by a decreased turbidity (ten Brinke et al., 1994). The low concentrations of PCBs and heavier 4, 5 and 6-ring PAHs at Eastern Scheldt mussels is probably related to limited sediment contact due to low water turbidity (Fig. 5). Remarkably, those effects did not result in a significant

lower amount of total microplastics in consumption mussels. With the exception of one subsample, all *E. coli* bacterial counts of the commercial samples were below the legal limit of 230 CFU/100 g. Higher TVC concentrations for consumption mussels can be explained by the fact that they were obtained from supermarkets. Therefore, more time elapsed between the picking of the mussels and the analysis in the laboratory (varying between 3 and 4 days after packaging of the mussels) and spoilage bacteria could develop in the MAP packaged mussels. The initial TVC of mussels was 4–5 log CFU/g which corresponds to the values found by Goulas et al. (2005). During storage, TVC raises steadily to finally reach the upper limit of acceptability.

5. Conclusion

A comparison of groyne and quayside mussels from the Belgian coastline and consumption mussels from the Eastern Scheldt, bought at Belgian department stores, was made, taking into account genetic differences as well as microplastic uptake, chemical and microbial contamination. Although genetic analysis showed small species composition differences between the different sources of origin, biological effect responses on external factors cannot be excluded.

The pollution of PCBs and PAHs in the mussel samples was found to be affected by the degree of industrialization along the Belgian coastline and by the degree of turbidity. For consumption mussels, the latter was affected by the embankment of the Eastern Scheldt. In contrast to chemical pollution, the *E. coli* contamination revealed an inverse correlation with industrialization as it is related to agricultural activities which were less pronounced in the more industrialized regions. Remarkably, no significant differences in total microplastics were found between the samples. Occurrence of orange synthetic microfibres, however, can be linked to fisheries related activities at the harbours and port, such as fishing net repair and dumping of old nets.

Within this study, common environmental and consumption evaluation of chemical and microbial pollution was combined with the introduction of microplastic evaluation. This appears to be valuable when procedures can be standardized, including blank control and LOD determination. The introduction of genetic analysis for monitoring purposes was limited in this study to species identification. Results indicate that it would be valuable to further investigate the genetic identification in comparative monitoring studies. The outcome from the different disciplines revealed different conclusions in evaluating "the most polluted" samples. This stresses the importance of balancing monitoring efforts between different disciplines in order to get an overall picture.

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