

Benthic biodiversity near brine discharge sites in the Port of Rotterdam

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ABSTRACT

The Port of Rotterdam is a heavily man-modified estuarine system, Europe's major seaport and home to a large number of chemical and petrochemical plants, many of which are supplied with distilled water from two demineralized water plants in Botlek and Maasvlakte. In this study, we assessed the ecological conditions near the brine outfalls and at a reference site unimpacted by brine, focusing on the benthic macrofaunal composition. Overall, the analysis of the communities and species revealed a similar macrobenthic composition, although with lower abundance and diversity compared to the nearby North Sea environment. The outfall area of the Botlek demineralized water plant, which is a dead-end entirely marine waterway with no to little currents in contrast to the well flushed brackish environment of the outfall of the Maasvlakte demineralized water plant, was characterized by the lowest abundance of benthic macroinvertebrates, species richness and Shannon diversity index. Higher biodiversity values at the Maasvlakte demineralized water plant compared to the reference site were also associated with the presence of a large biogenic reef of the Pacific oyster *Crassostrea gigas* with specimens of over 8 years estimated age being observed, however with many dead individuals recorded at the time of surveys. The outfall area of the Botlek demineralized water plant also had the highest disturbance levels according to the results of AZTI's Marine Biotic Index (AMBI) in conjunction with Benthos Ecosystem Quality Index (BEQI). Its community was dominated by the opportunistic native species *Capitella capitata* and *Varicorbula gibba*, and species typical for organic matter enrichment such as *Alitta succinea* (native), *Streblospio cf shrubsolei* (native) and *Theora lubrica* (invasive), which correlates well with higher levels of anthropogenic pollution in this area. Generalized linear model (GLM) and distance-based multivariate linear model (DistLM) showed that the distance from the outfalls was an important factor in terms of brine impacts however explaining a small part of the variation observed. Similarity percentage analysis (SIMPER), multivariate analysis, and permutational multivariate analysis (PERMANOVA) showed low similarity between and within study sites, confirming that high levels of heterogeneity exist within the estuarine system in terms of natural conditions and anthropogenic stress.

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Abbreviations

AIC	Akaike Information Criterion
AMBI	AZTI's Marine Biotic Index
BEQI	Benthos Ecosystem Quality Index
DistLM	Distance-based multivariate linear model
DWP	Demineralized Water Plant
EF	Enrichment factor
EQR	Ecological Quality Ratio
GLM	Generalized Linear Model
IEX	Ion Exchange Technology
MPC	Maximum Permissible Concentration
nMDS	non-metric Multi-Dimensional Scaling
PERMANOVA	Permutational Multivariate Analysis Off Variance
RO	Reverse Osmosis
SIMPER	Similarity Percentage
WFD	Water Framework Directive

1. Introduction

The world's population is growing, and global water demand is increasing. Climate change is threatening global access to clean water and many areas are exposed to water-related risks (drought or flooding), while the marine environment is facing multiple man-made stressors including warming and acidification of ocean waters, alien species, and plastic pollution [1]. Natural resource crises are among the top risks by impact facing the planet [2]. Desalination is considered a feasible and increasingly common method to meet the water demand for drinking water purposes as well as industrial and agricultural uses. However, currently desalination is far from being sustainable. Main environmental challenges relate to the high energy consumption needed to drive the process and subsequent greenhouse gas emissions, as well as the marine environmental impacts associated with the salty wastewater effluent discharged called brine [3–6].

Impacts of reverse osmosis (RO) brine on the marine environment are mainly associated with the high concentration of salts, the release of chemicals used during the seawater pretreatment stage (such as antiscalants), and cleaning of the RO membranes [5,7]. As brine has a higher density than seawater, it sinks to the seabed, extends horizontally following the slope of the sea bottom bathymetry [8] and therefore benthic communities are affected. The magnitude of the brine impact on the aquatic environment depends on the physicochemical characteristics of the desalination brine, the discharge method, the hydrogeological factors such as bathymetry, waves, currents, depth of the water column [7], and the ecological conditions of the ecosystem that receives the brine. The hydrogeological factors determine the extent of the mixing of the brine and therefore the geographical range of the impact [9]. Thus, high energy oceanic coasts with parallel coastal currents have lower sensitivity to the effects of a desalination plant in comparison to poorly flushed environments especially when hosting sensitive benthic communities [10].

The sensitivity of benthic macroinvertebrates to an increase in salinity levels depends on the tolerance of the given species. On high-energy open coast near Kurnell, New South Wales, Australia, with mainly rocky reef substrate, the cover by polychaetes, bryozoans and sponges reduced as far as 100 m distance from desalination outfall discharging $250,000 \text{ m}^3 \text{ d}^{-1}$, while barnacles proliferated and dominated in the communities [11]. However, the study mentioned that this was the result of the increased flow created by the high-pressure diffusers rather than hypersalinity or other potential stressors. A detrimental effect on both the abundance and diversity of benthic communities has been mentioned in a study [12] close to the outfalls of seawater RO desalination plants with a brine discharge volume of $245,000 \text{ m}^3 \text{ d}^{-1}$ per plant on the west coast of Algeria at depths of 18 m and 16.5 m, and at distances of 1.4 km and 2.5 km from the coast, respectively. Only some organisms were capable of surviving near the discharge (*Spionidae*, *Urothoe grimaldi*, *Paraonidae*, *Synchelidium haplocheles*, *Perioculodes longimanus*, *Chamelea gallina*, *Nemertea*), but in very small abundances compared to control areas. In a study for the San Pedro (southeast Spain) desalination outfall with a discharge flow of $150,000 \text{ m}^3 \text{ d}^{-1}$ through a 5 km pipeline at 33 m depth, species of amphipods showed some sensitivity to abrupt changes in salinity produced by concentrated brine effluent [13]. This study also recorded the presence of *Ampelisca diadema*, *Ampelisca typica* and *Photis longipes* at a distance of 250 m from the outfall which indicated certain tolerance of these species to increased salinity. Surveys in the seawater RO brine discharge of $132,000 \text{ m}^3 \text{ d}^{-1}$ on the shoreline south of Alicante city (southeast Spain) at 16 m depth with periodic dilution of the brine demonstrated that echinoderms are organisms extremely sensitive to salinity increments [14]. Marine environmental surveys at the Alicante seawater RO brine discharge of $65,000 \text{ m}^3 \text{ d}^{-1}$ demonstrated a substitution of a community characterized by the presence of Polychaeta, Crustacea and Mollusca, for another one dominated by nematodes at a distance of 400 m from the outfall [15]. A follow-up study at the same site [16] examined the effect especially on a soft bottom polychaete assemblage, and showed different sensitivity of polychaete families at a distance of 400 m from the outfall. More specifically, Ampharetidae were the most sensitive, followed by Neptydiidae and Spynidae, while Syllidae and Capitellidae showed some resistance initially, and Paraonidae were the most tolerant. Einav et al. [9] mentioned that benthic species which have originated in the Pacific in comparison to Atlantic species can cope more easily with an increase in salinity. Moreover, the same study mentioned that certain species are able to tolerate higher salinities after a

period of acclimation. An experiment [17,18] in which echinoderms, ascidians, gorgonian corals, and stone crabs were transplanted to site receiving effluents showed that the echinoderms were the most sensitive dying within 2–3 days exposure to low concentrations of brines. Survival improved when copper concentrations in effluents were reduced. Apart from the direct effects on the physiology of microbenthic organisms, indirect effects are also found. For example, pathogenic fungal infection appeared to be enhanced in brine-exposed oysters [19].

Efforts to minimize both environmental impacts associated with the brine discharge and the carbon footprint of the desalination process are currently in progress. For example, within the EU project called SOL-BRINE, a Zero Liquid Discharge pilot system was developed [20] that was powered completely by solar energy to reduce GHG emissions [21,22]. This project was then followed up by the EU project ZERO BRINE which aimed to facilitate the implementation of the Circular Economy package and the SPIRE Roadmap in various process industries to reduce industrial saline wastewater streams by recovering and reusing the minerals and water from the brine in other industries, thus ‘closing the loop’ and improving the environmental impacts of production. One out of 4 pilot projects was in the Port of Rotterdam where two demonstration plants were developed to treat part of the brine generated by DWP Botlek. In the framework of this pilot project, the current work was carried out in order to assess the ecological quality status of the Port of Rotterdam and in particular on the areas of DWPs Botlek and Maasvlakte due to brine discharge-related activities.

The port of Rotterdam, currently the biggest port in Europe, is one of the largest chemical and petrochemical clusters in Europe whose supply of distilled/deionized water is sourced from the above mentioned DWPs. It is located along Nieuwe Waterweg and Nieuwe Maas distributaries of the Rhine-Meuse estuary and has an open link with the heavily navigated North Sea. The Rhine and Meuse rivers have a long history of serious pollution that began around 1930 and increased significantly after World War II with the discharge of sewage and chemical compounds from industrial facilities and agriculture. Two chemical accidents with insecticides in the River Rhine, i.e. the endosulfan accident in 1969 and the Sandoz accident in 1986, had tremendously impacted the aquatic environment in terms of water quality and macrofauna abundance. In 1970 water pollution was at its peak [23,24] reducing biodiversity to a low number of pollution-tolerant species [25] while after 1990 there has been a substantial improvement in the concentrations of eutrophication parameters, organic compounds, and heavy metals [23] as a result of national laws against pollution and protection of the environment, and the Rhine Action Programme [26] introduced by the International Commission for the Protection of the Rhine in 1987. In 2000, the European Parliament and the European Council adopted the Water Framework Directive 2000/60/EC [27], with the purpose to establish a framework for the protection of European waters. For transitional¹ artificial² sea water bodies, like the Port of Rotterdam, the WFD stipulates that Member States shall protect and achieve good ecological potential and good chemical status by 2015, with extensions to 2021 and 2027, respectively. According to the results of the Rhine River Basin Management Plan 2015 [28], the ecological potential of the area was characterized as “moderate”³ and the chemical status [29,30] as “not good”⁴. Regarding the biological quality elements, phytoplankton, phytobenthos, and macrophytes and macrozoobenthos were characterized as indicating “good” ecological potential and the fish fauna with “moderate” ecological potential. For the area, the most significant pressure is introduced species and diseases (invasive alien species), and the most significant impact is the elevated temperature together with organic, nutrient, chemical pollution, saline pollution or intrusion, altered habitats due to hydrological or morphological changes [31]. These conditions have a strong impact on the composition of the fauna, allowing the establishment of thermophilic, salt-tolerant, and opportunistic species [32,33].

Remarkably, there are hardly any peer-reviewed academic publications about the biodiversity and ecology of the Port of Rotterdam which is a major shortcoming given the socioeconomic importance of the port and the scale of environmental impacts of the activities there. This study aims to address this shortfall and, specifically, to assess the benthic biodiversity of the Port of Rotterdam near two DWPs which combine ion exchange technology (IEX) and membrane technology (RO) for the purification process. The choice of sampling sites was imposed by the location of the DWPs investigated in the ZERO BRINE project (cf. the other articles in this special issue). In this study, we focused on benthic biodiversity since these are mostly sessile or burrowing animals of limited horizontal mobility, i.e. they cannot evade a local environmental stressor and their community composition and number is likely to reflect the environmental conditions at a given place. In addition, benthic macroinvertebrates constitute a biological quality element in the European umbrella regulations for water systems for the assessment of the ecological quality status of a water body [34,35]. In the future, brine discharge at these sites may be strongly reduced or disappear if ZEROBRINE technology was to be installed at industrial scale at these two DWPs, allowing to test the hypothesis that reduced brine discharge will increase benthic biodiversity and especially of taxa indicative of good environmental quality.

¹ “Transitional” waters are bodies of surface water in the vicinity of river mouths which are partly saline in character as a result of their proximity to coastal waters, but which are substantially influenced by freshwater flows.

² “Artificial” water body means a body of surface water created by human activity.

³ “Moderate” ecological potential in terms of biological quality elements means that there are moderate changes in the values of the relevant biological quality elements as compared to the values found at maximum ecological potential. Whereas “Good” ecological potential of biological indicators means that there are slight changes in the values of the relevant biological quality elements as compared to the values found at maximum ecological potential.

⁴ “Not good” chemical status means that concentrations of priority substances exceed the relevant EQS established in the Environmental Quality Standards Directive 2008/105/EC (as amended by the Priority Substances Directive 2013/39/EU). EQS aim to protect the most sensitive species from direct toxicity, including predators and humans via secondary poisoning. A smaller group of priority hazardous substances were identified in the Priority Substances Directive as uPBT (ubiquitous (present, appearing or found everywhere), persistent, bioaccumulative and toxic). The uPBTs are mercury, brominated diphenyl ethers (pBDE), tributyltin and certain polyaromatic hydrocarbons (PAHs).

2. Methods

2.1. Description of demineralized water plants and brine discharge

The Dutch industrial water company EVIDES supplies high-quality demi water from its DWP Botlek in the Brittaniëhaven area and DWP Maasvlakte in Hartelkanaal to a large number of chemical and petrochemical companies in the Port of Rotterdam. Both DWPs are fed with freshwater from Brielse Meer. DWP Botlek has been operating since December 2009, with a maximum production capacity of $1400 \text{ m}^3 \text{ h}^{-1}$ demineralized water. It discharges the brine streams into the Brittaniëhaven by pipelines (A & B, Fig. 1) on the slopes of the river and above the water level at low tide. DWP Maasvlakte has been operating since January 2018 and has a maximum production capacity of 800 m^3 demi water per hour and discharges the brine into the Hartelkanaal through two headwalls (C & D, Fig. 1) on the slopes of the river and above the water level at low tide. In both DWPs, two effluent streams are generated from IEX and RO process which are discharged separately in compliance to the term set by the Dutch Water Authority Rijkswaterstaat. DWP Maasvlakte discharges $400\text{--}500 \text{ m}^3 \text{ d}^{-1}$ IEX brine and $100\text{--}120 \text{ m}^3 \text{ h}^{-1}$ RO brine. DWP Botlek discharges $1200 \text{ m}^3 \text{ d}^{-1}$ IEX brine and $300 \text{ m}^3 \text{ h}^{-1}$ RO Brine.

Suppl. Table S1 depicts the average concentration of ions and organic matter at RO and IEX streams of the DWP Botlek [36]. It can be assumed that the effluent characteristics of DWP Maasvlakte will be similar to those of DWP Botlek as the same IEX and RO process is applied in both plants except from total organic carbon (TOC) that is expected to be about 1 ppm higher than at DWP Botlek because DWP Maasvlakte is fed with water from the same system however from a location that contains more phytoplankton. The IEX and RO effluent is characterized by its high salinity and density (negatively buoyant), with a temperature of $17\text{--}19 \text{ }^\circ\text{C}$, low nutrient content, and elevated levels of chloride (Cl^-), total dissolved solids (TDS), bicarbonate (HCO_3^-) and sulfate (SO_4^{2-}). Generally, and also in the case of the two DWPs investigated here, RO effluent also contains high concentrations of dissolved organic content compared to the intake water and the water bodies it is discharged into. As for heavy metal concentrations, the IEX effluent contains elevated levels of chromium (Cr), aluminium (Al), copper (Cu), zinc (Zn), barium (Ba) and lead (Pb). The RO effluent contains elevated levels of lithium (Li) and boron (B) compared to the intake water and the water bodies it is discharged into [36] which may be significant for marine life [37].

2.2. Study sites and sampling stations

Three sites were sampled within the framework of this study (Fig. 2): one in the vicinity of DWP Masvlakte in the Hartelkanaal area (Site A), one in the vicinity of the DWP Botlek in the Brittaniëhaven area (Site B), and one in the Elbeweg area of Hartelkanaal that was



Fig. 1. Brine discharge points (A & B: IEX and RO effluents from DWP Botlek, C: IEX effluent from DWP Maasvlakte, D: RO effluent from DWP Maasvlakte).

designated as Reference site. Due to being located on a waterway directly connected with the open North Sea, Site A has inherently variable salinities due to tides (range 1.2–1.6 m) (Table 1) and strong tidal currents. Thus, this site is a well flushed environment that may dilute and disperse the brine discharge. Annually, ~2,500 tons of chloride (Cl^-) yr^{-1} (estimation) are discharged from the Evides DWP Maasvlakte. The seabed consists predominantly of a *Crassostrea gigas* reef. Site B is a dead-end poorly flushed entirely marine waterway with no to little currents and constant salinities. Apart from the brine effluents from the Evides DWP Botlek, this area receives effluent from chemical industry, Huntsman Holland BV, which manufactures synthetic resins, plastics materials, and non-vulcanizable elastomers. Annually, ~6,000 tons Cl^- yr^{-1} (estimation) are discharged from Evides DWP Botlek while a significant amount of 22,460 tons Cl^- yr^{-1} (emission data for year: 2019) as well as 25 tons of iron (Fe) yr^{-1} and 74 tons of Total Organic Carbon (TOC) yr^{-1} are discharged by chemical industry, i.e. Huntsman [38]. The Reference site has naturally changing salinities due to tidal influence. In this study, this site was selected following the advice of the Port of Rotterdam Authority as a less-polluted site in the port unimpacted by brine effluent.

In total, 4 sampling surveys were performed, in September 2019, January 2020, July 2020, and September 2021. A spring sampling event was planned for April 2020 but became impossible due to the COVID19 pandemic. An alternative sampling in April 2021 was also cancelled due to the pandemic. The sampling survey in September 2019 was a reconnaissance survey for the design of the subsequent surveys. For this reason, there are some differences in the sampling setup applied in this survey in comparison to the subsequent surveys. A priority in the present surveys has been given to the Site A compared to Site B as it is surrounded by less intense industrial activity, received effluent discharge solely from the DWP and therefore, any impacts directly from the brine could be identified more better. However, Site A lacks historical data for benthic communities in comparison to Site B which is regularly monitored from 2004 for the needs of Helsinki Commission (Baltic Marine Environment Protection Commission – HELCOM)/Oslo Paris convention (for the Protection of the Marine Environment of the North-East Atlantic - OSPAR) reporting [32]. A total of 6 stations (Fig. 1) were selected and established for benthic macroinvertebrate surveys (Table 1). The survey and sampling scheme of the present study was defined based on the wider ZEROBRINE project with its installations in the Port, as well as the project's available resources and time plan. Hydrodynamic conditions were assessed based on visual observations as budget constraints did not allow measurements through appropriate equipment. The stations were sampled aboard MV Tender (small vessel) during the 1st field survey and MV Surveyor 2 of the Port Authority of the Port of Rotterdam during the 2nd, 3rd, and 4th field surveys. It is noted that sampling in the Britanniëhaven was particularly difficult because of the busy shipping traffic in this area, also observed during the surveys of this study. Sampling in S1 of Hartelkanaal was successfully performed even though the area consists of a thick layer of pacific oyster *Crassostrea gigas* that made the sampling difficult.

2.3. Field sampling and laboratory analysis

2.3.1. Benthic macroinvertebrates

Three replicates were collected at each sampling station using a Van Veen grab of 2 L capacity. At each replicate, the Van Veen grab collected sediment twice, and the total volume per replicate was 4 L, thus the surface area sampled per replicate was 0.052 m². The sediment samples were sieved through a 1.0 mm mesh, stained with Rose Bengal (Sigma-Aldrich) and preserved in ethanol. In the laboratory, benthic macroinvertebrates were sorted, identified to the lowest taxonomic level possible [39–48], and counted.

2.3.2. Water and sediment analysis

Water samples from S1, S4, and R were collected using an on-board pump from a depth of 1.5 m from the water surface. Water samples were analysed for pH, electrical conductivity (EC), total dissolved solids (TDS), nutrients, sulfate (SO_4^{2-}), Total Organic Carbon (TOC), heavy metals (Cd, Cr, Fe, Cu, Pb, Ni, Zn), and the 16 polycyclic aromatic hydrocarbons (PAHs) listed as high priority pollutants by the Environmental Protection Agency (EPA) (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-c,d]pyrene). All samples were analysed at an external laboratory (1st field survey: C-MARK B.V.; last 3 field surveys (SGS Environmental Analytics B.V., Netherlands) following standard methods (see notes in Suppl. Table S2). Sediment samples were collected from S3, S4, and R with a van Veen grab and analysed for Total Organic Carbon (TOC) analysis, Kjeldahl nitrogen, granulometric analysis, heavy metals (Cd, Cr, Cu, Fe, Hg, Ni, Pb, Zn), 16 EPA-listed PAHs, as well as benzene, toluene, ethylbenzene and xylenes (BTEX) at an external laboratory (1st field survey: SGS Environmental Analytics B.V.; last 3 surveys: Eurofins, Netherlands) following standard methods (see notes in Suppl. Table S3). Budget limitations determined the parameters examined. It is noted that at Site A, sediment from S3 instead of S1 was collected as it was impossible to collect sediment at S1, as well as S2, due to the presence of a biogenic reef of *Crassostrea gigas*.

Concentrations of the variables were assessed, where possible, in relation to the environmental quality standards [27]⁵ set out in the Rhine River Basin Management Plan 2015 [28], the target values [49]⁶ and the Maximum Permissible Concentrations (MPC) [49]

⁵ Environmental quality standard means the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment.

⁶ Target values are derived from MPC and negligible concentrations (NC). NC is defined as MPC/100 and takes into account possible effects of combination of toxicity due to the presence of other substances. In general, target values may ensure protection of river ecosystems to mixture toxicity. In Dutch water pollution control, target values have a more or less symbolic value for long term measures.

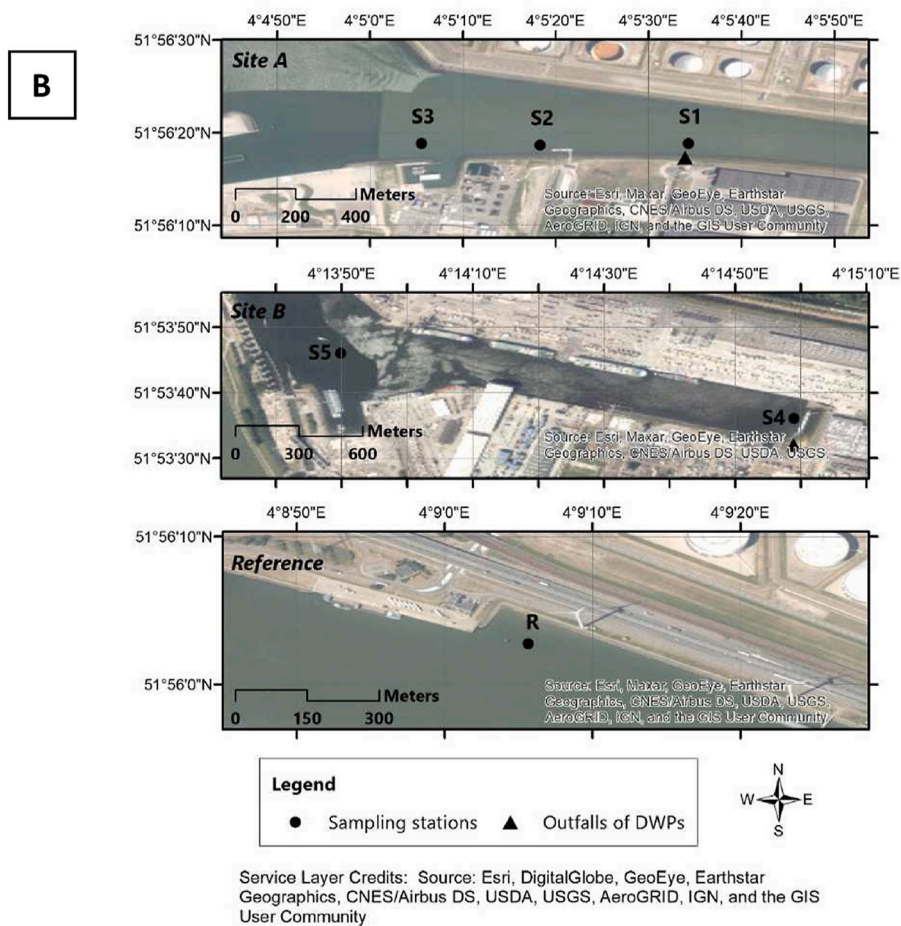
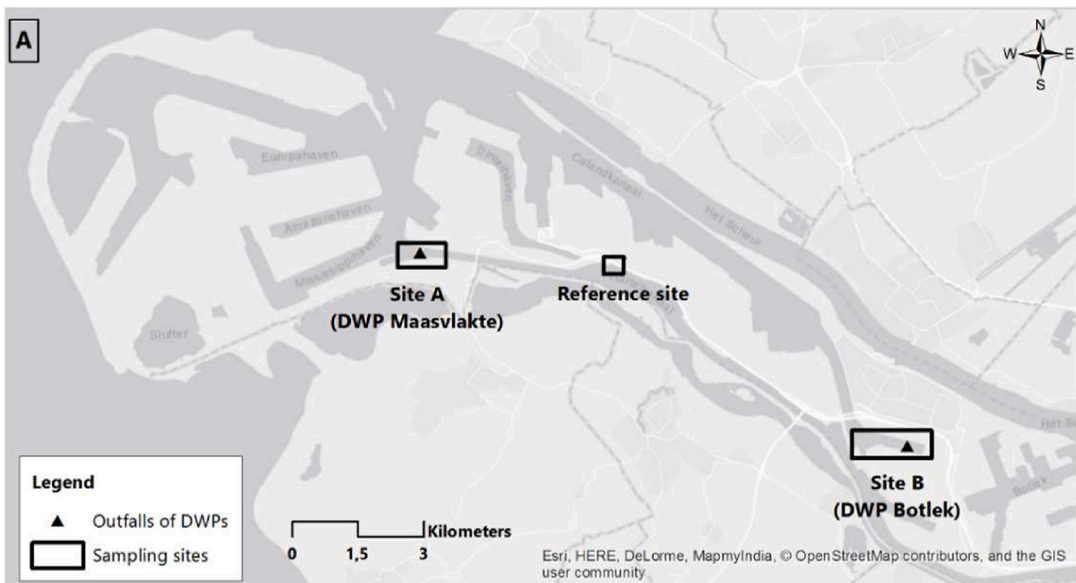


Fig. 2. a) Map of the Port of Rotterdam indicating the sampling sites and the DWPs. b) Satellite photos indicating the location of each sampling station at each of the three sampling sites.

Table 1

Main characteristics of the sampled stations at the three sites (A, B and Ref). The number of the survey each station was sampled is also indicated. 1st: September 2019, 2nd: January 2020, 3rd: April 2020, 4th: September 2021.

Site	Station	Coordinates (latitude/longitude) WGS 84	Port sector	Tide range (m)	Depth at low tide (m)	Water environment	Stream velocity (visual observations)	Water exchange (visual observations)	Bottom sediment (see Suppl. Table S3)	Surveys in which the stations were investigated
A	S1	51°56'18.84"N, 4°5'34.32"E	Hartelkanaal	1.2–1.6	6	Brackish water with strong tidal currents	Very fast flowing	High water exchange	<i>Crassostrea gigas</i> reef	1 st , 2 nd , 3 rd , 4 th
	S2	51°56'18.66"N, 4°5'18.34"E	Hartelkanaal	1.2–1.6	6	Brackish water with strong tidal currents	Very fast flowing	High water exchange	Hard substrate of <i>Crassostrea gigas</i> mixed with gravelly muddy sand (estimation)	1 st , 2 nd , 3 rd , 4 th
	S3	51°56'18.84"N, 4°5'5.54"E	Hartelkanaal	1.2–1.6	6	Brackish water with strong tidal currents	Very fast flowing	High water exchange	Gravelly muddy sand	1 st , 2 nd , 3 rd , 4 th
B	S4	51°53'36.12"N, 4°14'59.04"E	Brittaniëhaven	0.5	6	Marine waterway with no to little currents	Slow flowing	Limited water exchange	Muddy sandy gravel	2 nd , 3 rd , 4 th
	S5	51°53'46.08"N, 4°13'49.86"E	Brittaniëhaven	0.5	8	Marine waterway with no to little currents	Slow flowing	Limited water exchange	Muddy sandy gravel (estimation)	3 rd , 4 th
Ref	R	51°56'1.72"N, 4°8'30.93"E	Hartelkanaal – Dolfijnweg	1.2–1.6	8	Brackish water with tidal currents	Fast flowing	Water exchange intermediate between the other two sites	Gravelly sand	1 st , 2 nd , 3 rd , 4 th

⁷, set out in the Staatcourant, The Netherlands, June 2000 [50].

We applied the metal enrichment factor (EF) approach to evaluate the degree of metal pollution of anthropogenic origin in the sediment.

$$EF = (\text{Metal}_{\text{sample}} / \text{Fe}_{\text{sample}}) / (\text{Metal}_{\text{background}} / \text{Fe}_{\text{background}})$$

As background metal concentration data were not available for the Port of Rotterdam, and following [51] approach, the background values were obtained from Ref. [52] (Earth's crust average values, sedimentary rocks, sandstone: Cd: 0.02; Cr: 35; Cu: 2; Fe: 9800; Hg: 0.03; Ni: 2; Pb: 7; and Zn: 16). Following Schintu [51] and according to Ref. [53], $EF < 1$ indicates no enrichment, $1 \leq EF < 3$ is minor, $3 \leq EF < 5$ is moderate, $5 \leq EF < 10$ is moderate to severe, while $10 \leq EF < 25$ constitutes severe enrichment.

2.4. Statistical analysis and biotic indices

The AZTI's Marine Biotic Index (AMBI) [54] was applied to qualify the ecological status of the study area based on the AMBI values and classification estimated from the relative abundance of the different ecological groups (EG) sensu Grall and Glemarec [55], where the EGI, EGII, EGIII, and EGIV are sensitive, indifferent, tolerant, and opportunistic species, respectively. For the calculations of AMBI, hard substrate and epifaunal taxa were removed [56]. In a few samples due to the low number of taxa (1–3) or individuals (less than 3 per replicate), the results should be considered with caution as the robustness of the assessment is significantly reduced [56]. Additionally, the Benthos Ecosystem Quality Index BEQI, developed at the Netherlands Institute of Ecology (NIOO), was applied to evaluate the benthic macrofauna community at the ecotope⁸ [57] level (Level 3) [58].

Generalized Linear Model (GLM) analysis was used to assess potential effects of site, seasonality and distance from the outfall on Species richness (S), Abundance (N), Shannon diversity (H') index, and AMBI. Data for N, H' and AMBI were fitted to a linear model

⁷ MPC is the concentration above which the risk of adverse impacts is considered to be unacceptable.

⁸ Ecotopes are the smallest ecologically distinct landscape features in a landscape mapping and classification system.

with an identity link. A $\log_{10}(x+1)$ transformation was employed for N and AMBI in order to normalise the data distribution. Data for S were fitted to a Poisson model with an identity link since S is count data and it also gave better model fit based on the Akaike information criterion (AIC) values. Analyses were performed in software package Statistical Package for the Social Sciences (SPSS version 27).

In order to observe the differences of the benthic community composition between stations across locations and time, a multivariate analysis based on the complete dataset was performed. Differences between the multivariate species data set of each station were determined using the Bray-Curtis similarity measure on square root transformed abundance data to reduce the weight of the highly abundant taxa. Community patterns were then visualised by non-metric multi-dimensional scaling (nMDS). Group average cluster analysis results on the same data were also overlaid on the nMDS ordination diagram. Permutational multivariate analysis of variance (PERMANOVA) using the dissimilarity matrix described previously was performed to assess the potential effects of site and seasonality. The effect of the distance from the outfall on the community structure was tested using a distance-based multivariate linear model (DistLM). The significance of the model was tested using the Akaike Information Criterion (AIC). The contribution of individual species to the similarity within and dissimilarity across stations or sites was tested with Similarity Percentage Analysis (SIMPER). Multivariate statistical analyses were run using PRIMER 6.0 (PRIMER-e).

3. Results and discussion

3.1. Water and sediment quality

Comparison of the obtained analysis results in water (Suppl. Table S2) and sediment (Suppl. Table S3) showed that overall Site A had a low pollution impact while Site B and Reference site showed an environment with higher influence from anthropogenic activities. The conditions at the latter were similar to those reported in other areas of the port of Rotterdam which are monitored regularly by Rijkswaterstaat [33].

For the water, conductivity values recorded showed, as expected, a more brackish character for S1 and Ref - conductivity varied depending on the tidal stage when the sampling was performed and the season - than the more marine S4. This was also confirmed by sulfate concentrations, with the highest ones at S1 while S4 and R had similar lower concentrations. The pH values ranged from 7.1 to 8.5 which are within the typical range. Nutrients appeared to be high at all stations in comparison to North Sea values [59]. Cd, Cr, and Ni concentrations in water column were lower than the environmental quality standards, MPC and target values. The mean Cu concentration exceeded the MPC, and the target values with higher values recorded in S1 and R compared to S4. Pb and Zn concentrations exceeded target values at S4. Concentrations of PAHs in seawater were below the detection limits of the method. S4 had higher TDS values than S1 and R water which indicates impact of port activities on this enclosed water environment.

TOC and Kjeldahl nitrogen values in the sediment showed a typical range for the type of sediment found. Values were 2–5 times higher at S4 in comparison to S3 and R due to the finer grain size found in the former (30% silt and clay at S4 vs 5–13% at the other two stations). However, Huntsman discharge of 74 tons of TOC yr^{-1} at S4 may have contributed to TOC concentration in the sediment. The lower hydrodynamic conditions at Site B favour the accumulation of finer particles, which tend to be enriched in organic matter due to the higher surface area available for absorption [60]. The organic matter in the sediment was relatively fresh, more so at stations S4 and R, as indicated by the C:N ratio (C:N ~9) suggesting that the organic matter sediments rapidly from the water column or is produced *in situ* due to the shallow depth. Slightly higher values for the heavy metals Cd, Cr, Hg, Ni, Pb, however below the limit values, were found in the sediment at S4 in the 3rd survey and for PAHs, specifically fluoranthene and pyrene compounds, in R in the 4th survey compared to the rest. Zn mean concentration was above the target value for S4. BTEX contamination of seabed sediment was observed in S4 due to toluene and o-xylene which can be associated with oil spills and industrial emissions.

The enrichment factor results (Table 2) of sediment samples showed moderate or moderate to severe enrichment for Cu, Hg, Ni, Zn with maximum values at S4 and R followed by S3. Very severe enrichment of Cd was indicated at S4, while severe enrichment was indicated for Cd at S3 and R. Low EF values were observed for Cr and Pb indicating no to minor enrichment.

The less polluted conditions at S3 are a result of lower industrial pollution, discharges, and maritime traffic as well as natural factors such as strong tidal currents that make this area a well-flushed environment in contrast to the Britanniëhaven's strongly industrial character with low water exchange which was indicated as the most polluted environment between the three sites. However, dredging activities may have contributed to the reduction of sediment heavy metal concentrations at this site.

In this study, water and sediment quality were below the ranges found in other ports worldwide where much higher concentrations of nutrients, metals, and PAHs have been measured recently [61–64] while similar conditions have been observed in Australian ports [65] and in Port of Koper in the Northern Adriatic Sea [66]. It is worth noting that there are almost no recent scientific studies on the water and sediment conditions in terms of pollution in the ports of the North Sea. Of course, large differences are expected due to differences in the port location, water exchange rates, activities carried out within the port, and environmental quality measures. In that sense, the Port of Rotterdam is located at the mouth of the estuary with significant water exchange. In addition, environmental conditions in the Port of Rotterdam have significantly improved over the years as a result of the implementation of national and international laws on the protection of the water environment.

Table 2

Enrichment factors results for metals (EF < 1 indicates no enrichment, 1 ≤ EF < 3 is minor; 3 ≤ EF < 5 is moderate; 5 ≤ EF < 10 is moderate to severe; 10 ≤ EF < 25 is severe enrichment, 25 ≤ EF < 50 very severe enrichment).

Metal	EF results		
	S3	S4	R
Cadmium (Cd)	15.19	27.48	17.07
Chromium (Cr)	0.58	0.48	0.70
Copper (Cu)	3.52	8.55	5.45
Mercury (Hg)	3.11	3.08	3.50
Nickel (Ni)	4.94	4.05	5.46
Lead (Pb)	1.87	1.74	2.13
Zinc (Zn)	4.12	6.75	4.24

3.2. Benthic macroinvertebrate communities

Species richness and abundance of macrofauna in the samples were relatively low (see Suppl. Table S4 for list of species). In many replicates less than 6 species (26 out of 63 replicates) or less than 500 ind m⁻² (36 out of 63 replicates) were recorded. The maximum number of species found in a single replicate was 15, whereas the maximum number of individuals was 162 (3100 ind m⁻²). Shannon diversity values followed these trends being overall noticeably low, always below 2 and closer to 1 on many occasions, with higher values at Stations 1–3 (Fig. 3). Considerable variability was recorded between stations and sites, even among replicates, as indicated by

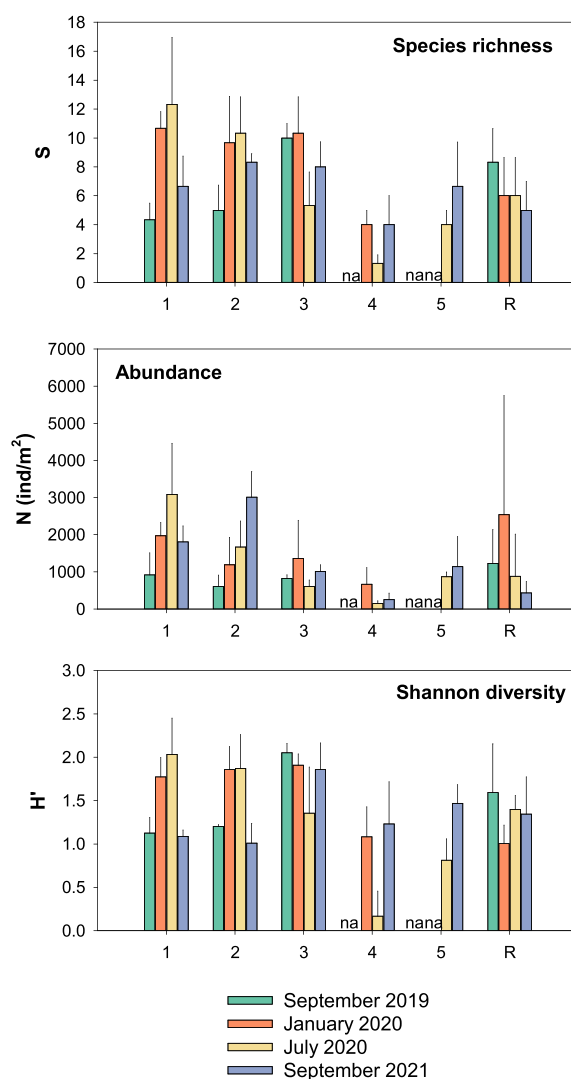


Fig. 3. Species richness, abundance, and Shannon diversity index for each station on each sampling. S1–S3 belong to Site A (Hartelkanaal), S4 & S5 to Site B (Brittaniëhaven), and station R is the Reference station. Values represent means ± SD (n = 3). na: no samples available.

the standard deviations (Fig. 3), indicating the patchy distribution of the macrobenthic community. The highest species richness, abundance and diversity were recorded at stations in Hartelkanaal (Stations 1–3, Fig. 3). In fact, Generalized Linear Model analysis showed that Site A has over 4.5–4.8 times higher S and H' values compared to the reference site and 3.4 times higher N values compared to the reference. No differences were found between Site B and Ref in the univariate measures.

The multivariate analysis based on the complete dataset revealed that the similarity between replicates from each sample was quite low, usually between 20% and 40%, confirming the high heterogeneity of the system (Fig. 4). Only S1, S4 and S5 showed on few occasions similarities among replicates of >60%. At Site A, variability within each station increased from S1 to S3 with S1 being the most homogeneous group regardless of season. Communities were more similar between S1 and S2. For Sampling Site B, communities across stations are quite distinct, forming clearly different clusters probably due to the high degree of human activities in this restricted environment (Fig. 4). PERMANOVA analysis at the site level confirmed the low similarity between sites with communities at the three sites which were significantly different (Suppl. Table S5). In terms of temporal changes, the September samples were different from the January and July ones.

Here, the comparatively more diverse area was the brackish water site (S1, S2 and S3), probably due to the position of the more marine sampling points (S4 and S5) in our study in an enclosed area with no water renewal and within the estuarine section of the port. In addition, the micro-ecosystem associated to the *Crassostrea gigas* and *Mytilus edulis* reefs - both these species were found at S1, S2 and S3 - increased the habitat diversity and the existence of microniches available for colonization, such as Cirripedia (barnacles) and other hard bottom species, a typical case of ecosystem engineering [67]. Higher diversities in association with this type of reefs in the area, similar to the ones found here, have been described previously [68,69].

More than half of the individuals found at Site A were related with sessile animals from hard substrate (barnacles, mussels) and are the ones that contributed most to the average similarity within samples of each station and dissimilarity across stations at this site (SIMPER, Suppl. Table S6). There, a reef formed by the non-native oyster *C. gigas* was present, on which the hard substrate organisms probably grew. The reef was most developed at S1 and was shared with *Mytilus edulis*. During the samplings, many of the *C. gigas* oysters collected were dead and mostly old individuals, while most individuals of *M. edulis* attached on them were alive. Based on the oysters' shell size and according to the *C. gigas* growth rate increment of $26 \pm 3 \text{ mm yr}^{-1}$ [70] resulting from observations at three intertidal oyster reefs in the Oosterschelde estuary in Netherlands, most of the oysters collected in our study were more than 4 years old while some of them were over 8 years old at time of collection. This means that the establishment of the reef occurred before the initiation of the operation of the DWP Maasvlakte. However, there is no indication as to when and to what extent mortalities occurred. Possible explanations of mass mortalities are high water temperature, unsuitable salinities, low water exchange and decreased oxygen concentrations, low food availability (phytoplankton concentration), and infective agents.

Studies on the competition between the two bivalve species have shown that *C. gigas* benefits from higher temperatures compared to *M. edulis* [71]. Although no direct measurements were made *in situ*, DWP brine outflow has a temperature of 17–19 °C, and given the relatively small volumes involved, no significant thermal contamination is expected. During summer months, the temperatures recorded in the wider area are higher than 20 °C which is considered necessary for *C. gigas* for the recruitment of its larvae [71]. However, a wide range of temperature characteristics for different stages of gametogenesis have been found in different geographical areas, including the North Atlantic region, indicating the high adaptability of the species [72]. *C. gigas* is also able to reproduce and grow in salinities of 10–42 psu with 23–36 psu optimum range for fertilization [73]. It is necessary to study the reason behind the larger abundances of *C. gigas* at Site 1, near one of the outfalls. More data on the current velocities, winds and turbulence, vertical temperature and salinities profiles, and chlorophyll *a* at S1 would also help explaining the presence of *C. gigas* as they have shown to

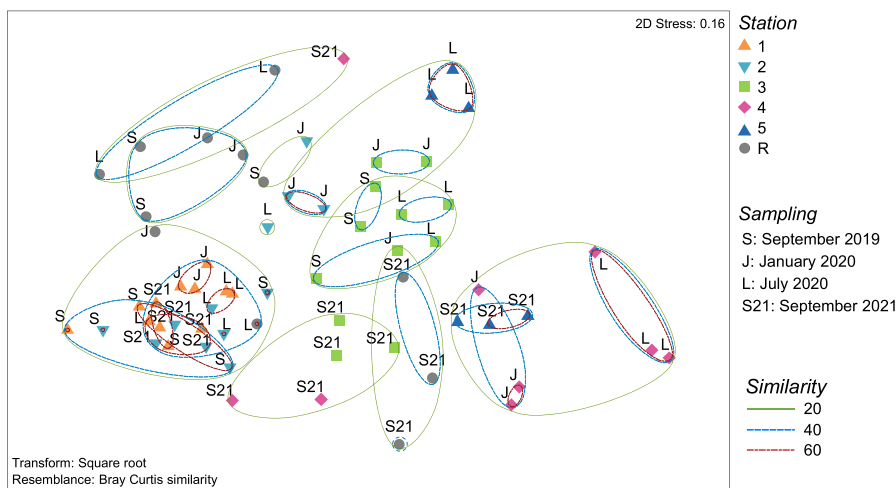


Fig. 4. nMDS ordination plot of the sampling stations in Site A (S1–S3), Site B (S4 & S5), and the Reference site (R). nMDS is based on a Bray Curtis similarity index matrix between stations on square root transformed abundance data. Lines indicate the similarity between samples based on a group average linkage cluster analysis using the same matrix.

determine the conditions necessary for its initial establishment and growth to a full-scale reef [67,74]. It is also important to examine whether oysters' mortality is the result of a pathogenic fungal infection that according to the study of Mandelli [19] is enhanced in oysters exposed to brine or due to another factor.

In the calculations of AZTI's Marine Biotic Index (AMBI) [56], in the majority of cases, the results showed a slightly disturbed system and only S4 and S5 were characterised on occasions as moderately or heavily disturbed (Figs. 5 and 6). With regards to AMBI, only seasonality was observed to be an important factor in Generalized Linear Model (GLM) analysis with 1.1 times higher AMBI in January compared to September. In the results of Benthos Ecosystem Quality Index (BEQI), even though both sampling sites would be characterised as a sub-littoral transitional water, they were analysed separately given the difference in the sediment substrate. However, only one reference site from within the disturbed environment of the harbour was available and this was more similar to Site A. The results for both locations assessed the ecological status of the system as Good (Table 3). Therefore, the overall disturbance status of the sites according to indices AMBI and BEQI is characterised as slightly to moderately disturbed and the ecological status generally as Good, with the exception of S4 that showed the lowest values and highest disturbance levels. Although these indices show that the ecological status of most of the stations is Good, some important points have to be considered. First, these indices are designed for soft substrate systems and thus sessile macrofauna, epi- and hyper-fauna have to be excluded [56,75]. When this was done here, both the species richness and abundance decreased due to the high abundance of barnacles and bivalves in the samples. Despite this, the disturbance levels in the samples did not change indicating the robustness of the indices. Second, the reference site is not a truly undisturbed and pristine site, a general problem for transitional waters even for sites unimpacted from anthropogenic activities. Finally, it is interesting to note that the reference site was the site that showed the largest variability between replicates in terms of

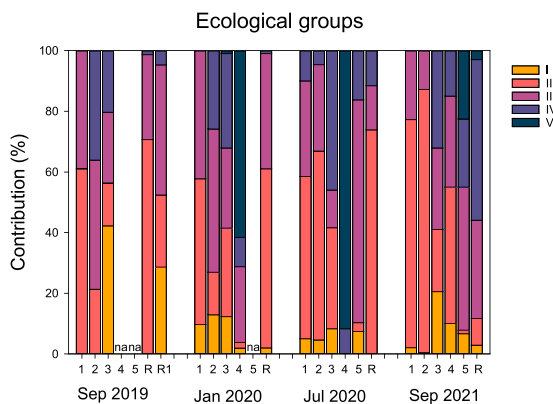


Fig. 5. Contribution of the AMBI distinct ecological groups for the stations sampled on each sampling occasion. S1–S3 (Site A, Hartelkanaal), S4 & S5 (Site B, Britanniëhaven), R (Reference site). Ecological groups I, II, III, IV, V. The contribution of groups IV and V, more tolerant to perturbations, only dominate in a few samples. na: no samples available.

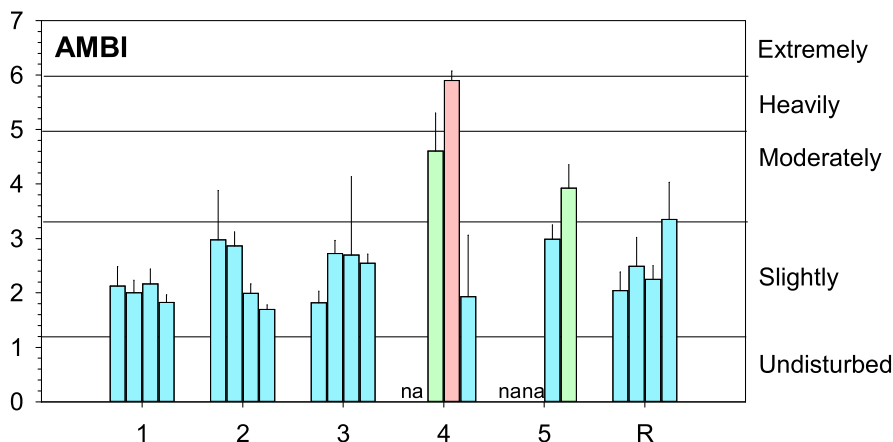


Fig. 6. AMBI index for the sampled stations in the three surveys. S1–S3 (Site A, Hartelkanaal), S4 & S5 (Site B, Britanniëhaven), R (Reference site). The classification of disturbance level for the sampling points according to the AMBI index is indicated on the right side. Values represent mean \pm SD (n = 3). na: no samples available.

Table 3
BEQI index results.

Sampling site	Similarity Ecological Quality Ratio (EQR)	No. of species EQR	Density EQR	Final EQR	EQR Netherlands	Ecological status
A (S1, S2, S3) Hartelkanaal	Moderate (0.49)	High (1)	Good (0.669)	0.72	0.766	Good
B (S4, S5) Brittaniëhaven	Poor (0.39)	Good (0.633)	High (0.824)	0.616	0.661	Good

community composition both within and between sampling cruises.

Previous studies have shown variable effects in terms of the impacts of brine discharge on benthic communities, often not being distinguishable from other environmental factors which confound any interpretations such as grain size distribution of the substrate, distance from brine source etc. [76–78]. Here, although sediment type was different between sites, GLM analysis revealed that all univariate measures increased for every metre increase in distance from the outfall (by 0.04% for H', 0.03% for N and 0.2% for S) (Suppl. Table S7). Similarly, the DistLM analysis using distance from the outfall as an explanatory variable also showed that the distance from the outfall was significant, explained however also a small part of the total variation (4.8%) (DistLM, Pseudo-F: 3.0721, $p < 0.001$, proportion: 0.04795).

Overall, this study recorded a macrobenthic composition similar to nearby environments of the North Sea, although with a lower abundance and diversity in comparison. Several studies have found a higher complexity and biodiversity in polyhaline ecotopes than for mesohaline ecotopes [79,80]. This has been attributed to the higher primary production usually available in the eu- and polyhaline sections of the estuaries, resulting in a higher biomass. In addition, a higher seasonal variability in river flow rate and associated disturbance induces physiological stress for benthic macrofauna in the reduced salinity zones decreasing the biomass. Thirdly, in the maximum turbidity zone, situated in the oligohaline zone, near the freshwater–seawater salt wedge, the microbial activity is usually high due to the accumulation and flocculation of organic matter resulting in oxygen depletion and reduced macrobenthic abundance.

Thus, de Jong et al. [81] found higher species richness along the Dutch coastal zone in front of the Port of Rotterdam. In contrast, Ysebaert et al. [82,83] found lower richness but much higher abundances in the adjacent Schelde estuary. Wijnhoven et al. [84], in a historical study of the inner Rhine-Meuse estuary, found similar abundances and species richness and diversity as those found here; however, the sampling zone and community was more meso- to oligohaline. A study in the Danish Wadden Sea [85] found results similar to the Højer tidal flat and a similar species list to what was found in the samples of the present study. Most of those studies were focused on the *Corophium* bed community, represented with species such as *Corophium volutator*, *Peringia (Hydrobia) ulvae*, *Macoma balthica*, *Mya arenaria*, *Hediste diversicolor* or *Heteromastus filiformis*. Most of these species, which essentially form part of the *Macoma balthica* community, were found in our samples. Although the *M. balthica* community is obviously characterized by the presence of *that species*, this can occasionally functionally be replaced by another bivalve, *Scrobicularia plana*, as was observed in some samples of our study. This is a community typical of areas with a different grain size to the one found here, with a tendency towards fine and muddy sediments. This type of sediments is often related to increased organic matter and to low oxygen and high hydrogen sulfide concentrations in the sediment. However, this community is important to improve and maintain healthy sediment conditions as many of its members (e.g. *C. volutator*, *M. balthica*, *M. arenaria*, *H. diversicolor*) are important bioturbators reworking and oxygenating the sediment in the process [86].

Other species typical of this community, such as members of the Spionidae family and the polychaete *Capitella capitata*, were also found in the samples at Site B in the present study. *C. capitata* is widely cited as an indicator species of pollution related to high concentrations of organic matter and environments contaminated with polycyclic aromatic hydrocarbons (PAHs) [87]. However, according to Warren [88] and Gray [89], its presence in these areas is due to the opportunistic characteristic of the species which allows *C. capitata* to continuously re-populate disturbed areas rather than its tolerance to anoxia and hydrogen sulfide. In terms of a possible effect of the brine, our data confirm the findings of Belatoui et al. [12] with regards to salinity sensitivity with Nephtyidae and Spionidae found furthest from the brine outfalls whereas Capitellidae were present at S4. In addition, in estuaries, *C. capitata* tends to show higher abundances in the areas with a stronger marine influence, rather than the brackish ones [82], which agrees with what we found here; highest abundances were recorded at S4 which together with S5 are influenced only by marine water. Thus, the distribution of the intertidal macrozoobenthic species, like *C. capitata*, seems to be controlled mainly by salinity rather than sediment organic matter. This pattern has been observed in similar systems elsewhere [25,90–92], although it should be taken into account that estuaries and port areas have their own particular physico-chemical conditions. On the other hand, Ysebaert et al. [82] related the absence of typical species of this community, such as *Streblospio shrubsolii* in the brackish waters of the Schelde, with pollution and anthropogenic disturbance. The same reasons have been used to explain the low penetration in the estuary of euryhaline species, such as *H. diversicolor* or *C. volutator* [82]. In our case, however, we found high abundances of *S. shrubsolii* at S2 and S3, as well as S5, which could indicate lower levels of contamination compared to historical data.

Finally, the presence of *Theora lubrica* found here agrees with its co-occurrence with *Hydrooides* sp., specifically *Hydrooides ezoensis*, which is also an invasive species coming from Pacific Ocean. *T. lubrica* can be very easily confused with *Abra nitida* so most likely records of the latter in the area would have to be reclassified as suggested previously [93,94]. *H. ezoensis* and *T. lubrica* are usually

found sharing habitat with other invasive species such as *Mulinia lateralis*, *Ruditapes philippinarum* or the polychaete *Pseudopolydora paucibranchiata*. Reish [95] pointed out the resistance of *P. paucibranchiata* to enrichments of organic matter and contaminated conditions. *T. lubrica* shows a similar behaviour, with a high capacity to tolerate low oxygen concentrations, showing a high fecundity and establishing itself in the community rapidly [96]. Studies in areas nearby [97] have established the presence of *M. lateralis* in coexistence with other species identified in this study such as *Alitta succinea*, *Tharyx* sp., *Heteromastus filiformis*, or *C. volutator*.

4. Conclusions

This study has established an understanding of the aquatic environmental conditions close to the brine outfall of DWP Botlek and DWP Maasvlakte in the Port of Rotterdam with focus on benthic macroinvertebrates. The results of this study provide essential information for the assessment of the environmental benefits from the implementation of a large-scale zero brine technology in the future in terms of aquatic environmental impacts. We observed a remarkable diversity of taxa, enabling a detailed characterization of biological communities, which constitutes a significant asset considering how little published literature exists about the unique system of the Port of Rotterdam. Even though environmental conditions have been improved over the years due to management and environmental protection policies, the Port of Rotterdam remains an impacted area being exposed to multiple stressors such as chemical and organic compounds discharged or deposited, nuisance of shipping traffic, dredging and sediment disposal to maintain navigability in channels, river engineering etc. In this study, we recorded a similar macrobenthic composition, although with a lower abundance and diversity in comparison with nearby environments of the North Sea. Even when considering the invasive species present and the taxa Spionidae and Capitellidae indicating poor environmental quality, the present data confirm that the community established in our study area is comparable to similarly impacted areas investigated previously.

Long-term monitoring of physicochemical variables and hydrodynamic conditions is required to understand the patterns influencing species composition, however, distinguishing possible responses of benthic organisms to the brine does not seem practically feasible due to the low volume flow rate of the brine discharge in study sites which are simultaneously impacted by multiple anthropogenic stressors (especially at Site B) and natural stressors (especially at Site A). Regarding the *C. gigas* reef at the outflow of the DWP Maasvlakte, it is expected that a number of conditions have co-occurred for its successful establishment before the operation of the DWP. However, the occurrence of many dead oysters during the field surveys needs further investigation in terms of mortality scale and key environmental variables such as temperature, salinity, and chlorophyll a, as well as toxicological investigations.

Author contributions

Eleni Avramidi: Investigation; Methodology; Project administration; Resources; Writing – review & editing; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Sergio Carlos García Gómez: Investigation; Formal analysis (expert in the identification of benthic macroinvertebrates); Visualization; Writing - review & editing.

Sokratis Papaspyrou: Investigation; Formal analysis; Visualization; Writing - review & editing.

Vasilis Louca: Conceptualization; Formal analysis; Investigation; Supervision; Writing - review & editing.

Dimitrios Xevgenos: Funding acquisition; Project administration; Resources; Writing - review & editing.

Frithjof C. Küpper: Funding acquisition; Investigation; Methodology; Project administration; Resources; Writing – review & editing; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.wri.2022.100173>.

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