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Ecological Condition of the Benthos in Milford Haven Waterway: the Centre of the UK's Oil and Gas Industry in an Area of High Conservation Value

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Abstract: This study determined the environmental condition of the benthos of Milford Haven Waterway, an area that is arguably the most vulnerable in the UK to anthropogenic activities, including the potential effects of a major oil spill in 1996, using historical data on the macrobenthos more than a decade later in 2008, 2010 and 2013. These data show a gradual decline in numerous univariate diversity measures from the outer (marine) to inner (estuarine) stations. Taxonomic distinctness generally falls within the expected range, and most stations have above-average values compared with other monitoring stations around the UK. The *W*-statistics for Abundance/Biomass Comparison (ABC) plots are usually strongly positive and never negative. There was a sequential change in community composition from the outer to inner stations, which was strongly related to salinity, and, to a lesser extent, sediment granulometry. None of the species regarded as indicators of organic pollution were prominent in the macrobenthic community of Milford Haven Waterway. On this basis, although there are some slight indications of environmental perturbation at particular sites in certain years, it can be concluded that the benthic communities of Milford Haven Waterway are in a healthy state. This study provides a baseline against which the potential effects of any future environmental accidents and/or the increased industrial development can be assessed.

Keywords: biodiversity; community composition; environmental assessment; estuary; macrobenthos; petroleum industry

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1. Introduction

The Milford Haven Waterway, Pembrokeshire, Wales, UK, at 55 km^2 is the largest ria-estuary complex in the UK and comprises a central waterway (maximum depth 27.5 m) with numerous shallow embayments, tributaries and pills [1]. The maximum tidal range is 7.76 m, and 30% of the area is intertidal. The waterway has been industrialised in some form since at least the medieval period, albeit at a limited scale [2,3]. However, this changed in the 1960s following a government decision to make it the major deep-water oil port in the country [4,5]. Currently, there are two large Liquefied Natural Gas (LNG) plants, jetties and pipelines, as well as one of the UK's largest storage terminals for bulk petroleum products and the port handling the most shipments of these products of any port in the

UK. Together, these facilities are capable of supplying nearly a third of the UK's gas needs alone [1,6,7]. This infrastructure is economically important, supporting over 3800 full-time equivalent jobs, equating to ~40% of total employment in the local economy and 7% across the wider region [6].

The transport of large quantities of hydrocarbons renders the waterway vulnerable to environmental accidents, and there have been some comparatively minor pipe leakages and refinery explosions over the years. Since the 1960s, the waters of the Haven have received chronic inputs of hydrocarbons from the refineries and the oil-fired power station sited on its banks, as well as from small oil spills and domestic inputs [8,9], including the *Chryssi P. Goulandris* spill of >250 t in 1967 [3,10]. However, Little et al. [11] estimated that <240 t of oil enters Milford Haven annually, most of it well dispersed in water and already associated with suspended particles. A major environmental catastrophe did occur when the *Sea Empress* oil tanker became grounded on mid-channel rocks at the entrance to the waterway on 15 February 1996 en route to the Texaco oil refinery. Over a week, ~72,000 t of its cargo of 131,000 t of light crude oil (Forties Blend) and 480 t of heavy fuel oil were released into the surrounding waters, resulting in the contamination of 200 km of the Pembrokeshire coastline [8,12]. It was Britain's third-largest oil spill and the twelfth-largest in the world at the time [13]. The cost of the clean-up operation was estimated to be £60 m at the time (£115 m in 2020) and involved the 446 t of chemical oil dispersant, which enhanced the rate of natural dispersion of the oil and prevented an additional 57,000 to 110,000 t of emulsified oil from impacting the beaches [12,14].

Despite the industrial infrastructure, Milford Haven is regarded as having a high conservation value, being designated by the European Habitats Directive as a component of the Pembrokeshire Marine Special Area of Conservation, and is also a Site of Special Scientific Interest [1,15]. The area contaminated by the oil spill in 1996 is well known for its natural beauty and is utilised for various purposes such as tourism, fisheries (e.g., herring; *Clupea harengus*) and aquaculture. The value of the ecosystem and its history of anthropogenic use have led to numerous monitoring projects since the 1960s (see summary in [3]). More contemporary monitoring has revealed that heavy fuel oil was still detectable in the sediments of Milford Haven in 2010 [9] as were metals, organotins, polyaromatic hydrocarbons and polychlorinated biphenyls in a range of algae and benthic invertebrates [16].

Macrobenthic invertebrates, in particular annelids, molluscs and crustaceans, are major components of estuarine fauna in the UK and globally [17,18] and play vital structural and functional roles in the benthic environment. Through their movements and behaviour, e.g., respiration, bioirrigation and bioturbation, they influence carbon, nitrogen and sulphur cycling and help oxygenate sediments [19,20]. Moreover, by ingesting detritus and becoming prey for many higher trophic organisms such as fish and birds, they are crucial links in the food web [21,22]. Changes in the diversity and community composition of the macrobenthos have been detected along natural environmental gradients, e.g., salinity and sediment grain size, but also in response to a range of deleterious anthropogenic influences, e.g., eutrophication and hypoxia [23–26]. These predictable and reliable changes in the macrobenthos have resulted in these taxa becoming one of the mainstays of monitoring the ecological condition of waterways [17,27,28]. Many studies have demonstrated the deleterious effects of hydrocarbon spills on invertebrate communities [29–31].

The objective of this paper is to establish the status quo with regard to the condition of the macrobenthos in Milford Haven, against which the potential effects of any future environmental accidents, or the increased development of the port to meet the UK's growing energy needs, can be assessed. More specifically, the aims are: (i) to determine the extent to which attributes of the macrobenthic communities were indicative of environmental perturbation, both immediately after the *Sea Empress* oil spill in 1996 using historical data, and more than a decade later in 2008, 2010 and 2013 using data collected in this study. And (ii), if possible, to correlate these attributes with environmental variables that might imply cause and effect.

2. Materials and Methods

2.1. Sampling Methodology

All historical subtidal and intertidal macrobenthic datasets in Milford Haven from 1974 onwards were reviewed by Warwick [32], who made recommendations for a future cost-effective and ecologically meaningful macrobenthic surveillance programme for the waterway. In order to capitalise on the data from previous surveys, surveillance focused on a subset of stations for which sufficient high-quality data were already available. Eight stations, designated 1–8 in sequence from the outer to inner waterway (Figure 1), were selected that had good representative geographical coverage, i.e., the lower, middle and upper Haven sensu [8], and that encompass the range of benthic habitat types [1,3].

Figure 1. Map of the eight stations sampled in the Milford Haven Waterway (51°42′0″ N, 5°6′46.8″ W). The star symbol marks the *Sea Empress* grounding site at St. Ann's Head. Satellite image provided by Google, TerraMetrics, CNES/Airbus and Maxar Technologies. Black circle on the inset denotes the location of the Milford Haven Waterway within the British Isles.

Between three and five sediment samples were taken with a 0.1 m^2 Day grab and sieved through a 0.5 mm mesh at each station in 2008, 2010 and 2013, except for stations 1 and 6 in 2008 and 6 in 2010 (Table A1). The abundance and biomass of each soft sediment macrobenthic species were determined in each sample. Colonial hard bottom species such as sponges, hydroids, bryozoans and tunicates that would have been attached to larger pebbles and stones and were recorded as present or absent in the samples have not been included in the analyses. To ensure comparability with the more recent data, historical samples taken immediately following the *Sea Empress* oil spill were chosen from the same year and with the same level of replication (3 per station) from two separate studies by Hobbs and Smith [33] (stations 1–7 taken in March 1996), and Levell et al. [34] (station 8 taken in October 1966). Note, however, that these authors did not measure biomass.

As the composition and distribution of macrofaunal communities in Milford Haven and in other estuaries are influenced by sediment granulometry and salinity [32,35], these variables were measured. However, the collection of sediment samples for particle size analysis was not conducted each year and was often not replicated. Since data for a single replicate are not representative of the range of particle size distributions, data for the mean values of five replicates at each station in 2013 (the most robust dataset) are assumed to be similar for each sampling year. Sediment particles were classified using the Wentworth scale [36], i.e., medium pebble (>8 mm), small pebble (4–8 mm), granule (2–4 mm), very coarse sand (1–2 mm), coarse sand (500–1000 μm), medium sand (250–500 μm), fine sand (125–250 μm), very fine sand (63–125 μm) and silt and clay (<63 μm), and the percentage contribution was calculated. GRADISTAT Version 8.0 [37] was used to analyse the granulometric properties of the sediments. Salinity was not directly measured at the time of sampling, as, given the large tidal range (up to 7.76 m), the instantaneous value would be meaningless in terms of the range of values that would be experienced at that station over diurnal and lunar tidal cycles. However, this information has been determined for each station by visual interpolation from the isohaline maps at high and low water on Spring and Neap tides given by Hobbs and Morgan [38].

2.2. Data Analyses

All statistical analyses have been undertaken using the PRIMER v7 software package and the PERMANOVA+ add-on [39,40].

2.2.1. Univariate Indices of the Macrofauna of Milford Haven

Since this is essentially a baseline study, a range of univariate metrics (means and 95% confidence intervals) for each of the eight stations in each year were calculated using the DIVERSE routine so that they can be compared with studies elsewhere in which various metrics may have been reported. These were: the number of species or species richness (S), number of individuals (0.1 m^{-2}; N), Margalef's species richness (d), Pielou's evenness (J′), the estimated number of species for 20 individuals, i.e., rarefaction, (ES(20))), Shannon diversity ($\log_{e;}$ H′), Simpson's index ($1 - \lambda$), taxonomic diversity (Δ), quantitative taxonomic distinctness (Δ^*), average qualitative taxonomic distinctness (Δ^+) and variation in taxonomic distinctness (Λ^+). These variables were examined visually in a Draftsman plot to determine whether any required transformation and the number of individuals was $\log_e$ (x + 1) transformed. Data for each variable was then used to create a separate Euclidean distance matrix and subjected to two-way univariate PERMANOVA [39] testing for differences ($p \leq 0.05$) between Year (four levels, i.e., 1996, 2008, 2010, 2013) and Station (eight levels, i.e., 1–8). The percentage contribution made by the mean square for each factor and interaction to the corresponding total mean square in each PERMANOVA test was calculated to estimate the relative importance of each factor and interaction in that test. Any significant differences were explored using line graphs with 95% confidence limits.

A data matrix was constructed from the presence or absence of each species in each Year and Station combination and subjected to TAXDTEST to determine the 'expected' value and 95% probability limits for Δ^+ and Λ^+ in random subsamples of different numbers of species drawn from the full suite of 707 taxa found in the waterway. These data were used to construct funnel plots onto which the measured values of Δ^+ and Λ^+ were superimposed, both to compare values and to test for any significant departures from expectation [41].

Abundance Biomass Comparison (ABC) curves, i.e., separate dominance curves for abundance and biomass on a cumulative scale (y-axis) against the species ranked, on a logarithmic scale (x-axis), were plotted using averaged data for each station in each year. This analysis was not performed using data for each sample because the largest possible sample is needed to determine the abundance of rare large-sized individuals [42]. The degree of separation of the abundance and biomass curves on the dominance plot was summarised using the W statistic, which is strongly positive in the unperturbed case (abundance < biomass), approaches zero when the curves are closely coincident and is strongly negative when they are transposed abundance > biomass [43].

2.2.2. Multivariate Analyses of the Macrofauna of Milford Haven

The abundance of each species in each sample was dispersion-weighted and square-root transformed to down-weight the abundance of those species which varied far more markedly among replicate samples than those that were more consistent and to balance the contributions of abundant and rare species [44]. The resultant data were used to construct a Bray–Curtis resemblance matrix and subjected to the same two-way PERMANOVA design as in univariate analyses. A non-metric multidimensional scaling (nMDS) ordination plot was used to visualise the degree of similarity in species composition among stations in each year. The species-level data were aggregated to family and phylum levels and subjected to the same PERMANOVA test and used to construct nMDS ordination plots. The RELATE procedure [40] was used to identify whether the pattern of rank orders of resemblance among replicate samples at each of the three taxonomic levels was statistically similar in each year ($p < 0.05$). The extent of any relationship was measured by the size of the test statistic (ρ), which ranges from~0 to 1.

The dispersion-weighted and square-root transformed concentrations for the 50 most abundant macrobenthic species in each year were used to construct a separate shade plot sensu Clarke et al. [45]. The range of shading from grey to black for a species represents increasing concentrations of that species, while a white space denotes that the species was absent. Species (y-axis) were clustered using the Index of Association [46] and thus aligned in their optimum serial order, while samples (x-axis) followed the order stations from the outer to the inner waterway. CLUSTER-SIMPROF analyses are added to these plots to indicate groups of species that were coherent, i.e., with no significant difference in their distribution pattern across stations.

The BEST (BIOENV) routine [40] was used to determine if spatial patterns in macrofaunal composition in each year were significantly related ($p < 0.05$) to one or more of the environmental variables, i.e., salinities in various tides and sediment particle size. Prior to these analyses, a Draftsman plot was used to determine if any of the 13 environmental variables were skewed and/or if any pair was highly correlated (i.e., Pearson's correlation > 0.95). Following this, salinity at neap high and low water were removed as they were correlated with salinity at spring high water and the percentage contribution of each of the nine sediment particle size categories was square-root transformed. These data were then normalised to place values for all variables on a common scale. These data were subjected to BEST together with a separate Bray–Curtis resemblance matrix from each year, constructed using the average transformed density of each macrofaunal species at each site.

2.2.3. Comparison of the Severity of Disturbance with Other Northern European Areas

Phylum-level meta-analysis [47] has been used to compare the potential degree of disturbance at the eight stations in Milford Haven on a continuous comparative scale. Macrobenthic data aggregated to 20 phyla from nine studies from the northern European shelf, representing different types and severities of disturbance ranging from sites regarded as unaffected by disturbance to severely disturbed sites, were combined giving a total of 50 samples. Proportional production (P) was approximated by the allometric equation $P = (B/A)^{0.73} \times A$, where B is the biomass of a phylum in a sample, A is the abundance of organisms making up that biomass and B/A is the mean body size. The exponent 0.73 is the average exponent of the regression of annual production on body size for macrobenthic invertebrates [48].

For each sample, production data were standardised (expressed as a proportion of the sample total) and fourth-root transformed and used to construct a Bray–Curtis similarity matrix. nMDS ordination of this 'training data' matrix produces a 'wedge'-shaped plot which clearly separates samples along a common axis of 'disturbance'. The average abundance and biomass data for each station in Milford Haven in each year were used to calculate the proportional production from each phylum in each sample. The resulting data were fourth-root transformed, combined with the 'training matrix' and used to produce a separate nMDS plot for the Milford Haven data in each year. To aid visual interpretation of the nMDS plots, vectors were added showing the direction in which the various phyla increase in abundance and with the length of the vector reflecting the strength of the pattern in those abundances along that direction. Moreover, the Bray–Curtis similarity matrix was subjected to Principal Component Analysis to create a PC score for each site along the axis of 'disturbance' [17]. These scores were then grouped into quintiles and the sample was colour-coded from dark green (best) to red (worst).

Finally, the means and 95% confidence intervals of the macrobenthic univariate metrics defined above and determined for MHWESG stations in each year were viewed in a national context by comparison to data collected as part of the Clean Safe Seas Environmental Monitoring Program (CSEMP), formerly the National Marine Monitoring Program NMMP [17]. Species abundance data have been averaged over five replicate samples and three years (1999, 2000 and 2001) for each sampling station for the 28 estuarine and 33 coastal CSEMP stations around the UK. A separate taxonomic aggregation file has been used for those species. The data for each variable were used to create a Euclidean distance matrix and subjected to one-way univariate PERMANOVA, to test for differences between the eight stations in Milford Haven and those of the estuarine and coastal CSEMP stations. Data for the number of individuals was $\log_e$ (x + 1) transformed.

3. Results

3.1. Environmental Variables

Stations 1 and 2 are fully marine (salinity~33), with no tidal variation in salinity, but thereafter the maximum tidal variation in salinity increases sequentially in an upstream direction, with salinity reaching a minimum of 14 at station 8 on a neap low water (Figure 2a). All sediments were classified as "very poorly sorted", except for station 2 which is "poorly sorted", and all have bimodal, trimodal or polymodal particle size distributions. Thus, univariate measures such as mean or median particle size are uninformative in terms of the relationship between sediment granulometry and macrobenthos. For example, station 7 has a very high percentage by weight (41.6%) of medium pebbles but also a relatively high silt and clay ("mud") percentage (10%; Figure 2b). The silt and clay size fraction is perhaps the most ecologically relevant, and from the outermost station 1, it increases to a maximum of 60.4% at station 5 and then decreases to the inner stations 7 and 8.

3.2. Macrobenthos Data

3.2.1. Univariate Analyses of the Macrofauna of Milford Haven

The values for each of the 11 univariate metrics differed significantly with Year, Station and Year × Station, except for Λ^+ where only the latter two terms were significant (Table A2). Patterns of diversity changed across the sequence of the eight stations and were not consistent among years (Figures 3 and 4). Mean values for the number of species and Marfalef's species richness were greatest at stations 5–7 in 1996; however, in the three subsequent years, there was a gradual decline in these measures from the outermost to innermost sites (i.e., 1 to 8). Comparing the patterns among years, mean values for the above two metrics were similar at sites 5 to 8; however, those at sites 1 to 4 were far lower in 1996 than in 2008, 2010 and 2013. In 1996, the high value for the number of individuals at station 3 was mirrored by low values of Pielou's evenness, ES(20), Shannon diversity, Simpson's index and Δ, reflecting the very high abundance and dominance of several species (i.e., the polychaetes *Chaetozone gibber* and *Phoronis* spp.; see Section 3.2.3). In 2008, 2010 and 2013, a similar situation occurred for station 5, where a high number of individuals corresponded with low values for Pielou's evenness, ES(20), Shannon diversity, Simpson's index and Δ. With the exceptions of these two sites in particular years, values for the latter five metrics were relatively similar among sites (Figures 3 and 4). The trends for the various taxonomic distinctness indices among sites were different in 1996 compared to each of the three more recent years. In 1996 Δ^* and Δ^+ had a sinusoidal pattern of change, decreasing from stations 1–3, then increasing to station 6 and decreasing again until station 8. In 2008, 2010 and 2013 there was a more linear trend of decreasing Δ^* and Δ^+ from stations 1 to 8.

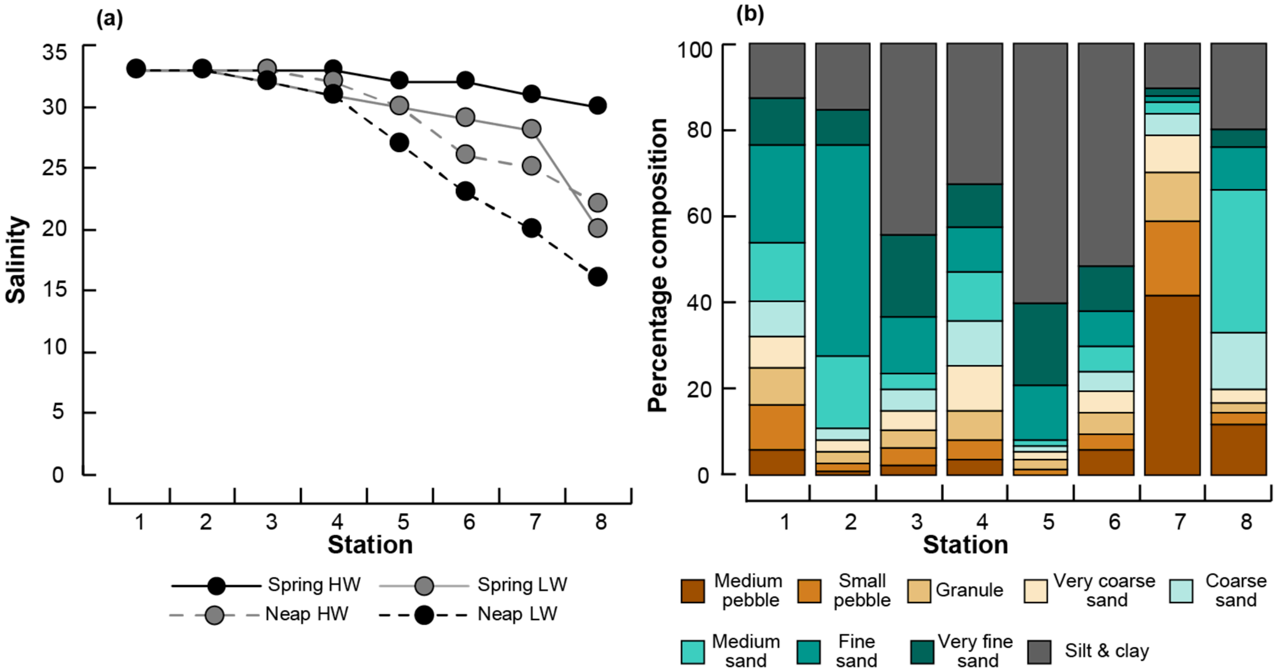

Figure 2. (**a**) Water column salinity at Spring and Neap Tides and High and Low Water (HW, LW) and (**b**) sediment particle size distribution at the eight stations in Milford Haven.

On frequency-based funnel plots for Δ^+ and Λ^+ using the total species inventory for all stations in Milford Haven as the regional species pool, with values for each station in each year superimposed, all samples for stations 1 to 6 fall within the 95% probability intervals for both indices (Figure 5). This indicates that there was no significant departure from the expectation and that they constitute a random selection of species from the regional pool. However, the Δ^+ value for station 8 falls below the probability interval of the funnel in all three years, as does station 7 in 2010, implying that biodiversity in this respect is significantly lower than expected here. For Λ^+, station 7 in 2010 falls above the upper interval of the funnel, again indicating a significant departure from expectation. Note that values of Δ^+ at stations 2 and 3 are consistently above the average (Figure 5).

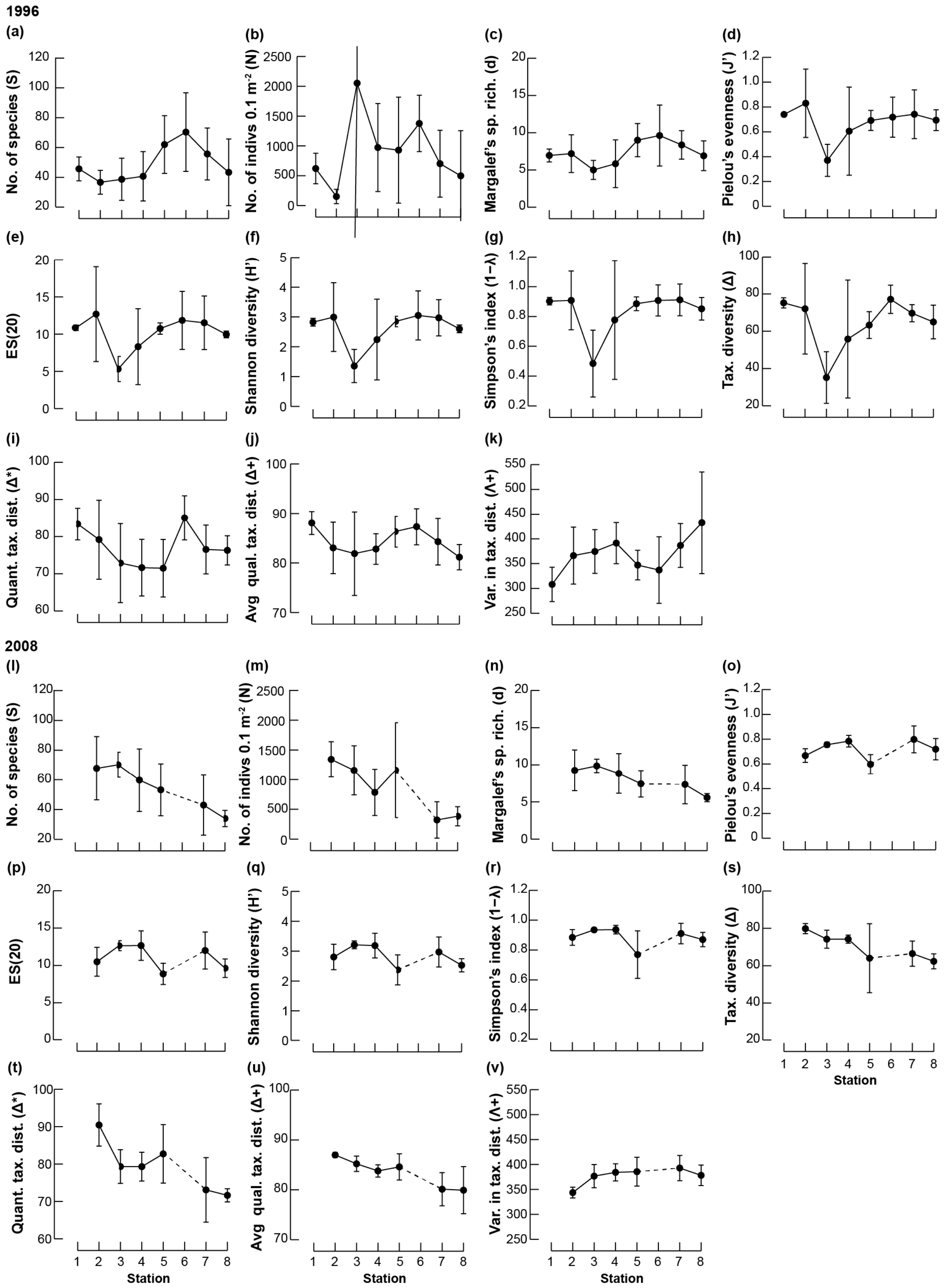

Figure 3. Mean values (±95 confidence intervals) for each of the 11 univariate metrics at each of the eight stations in Milford Haven in (**a**–**k**) 1996 and (**l**–**v**) 2008.

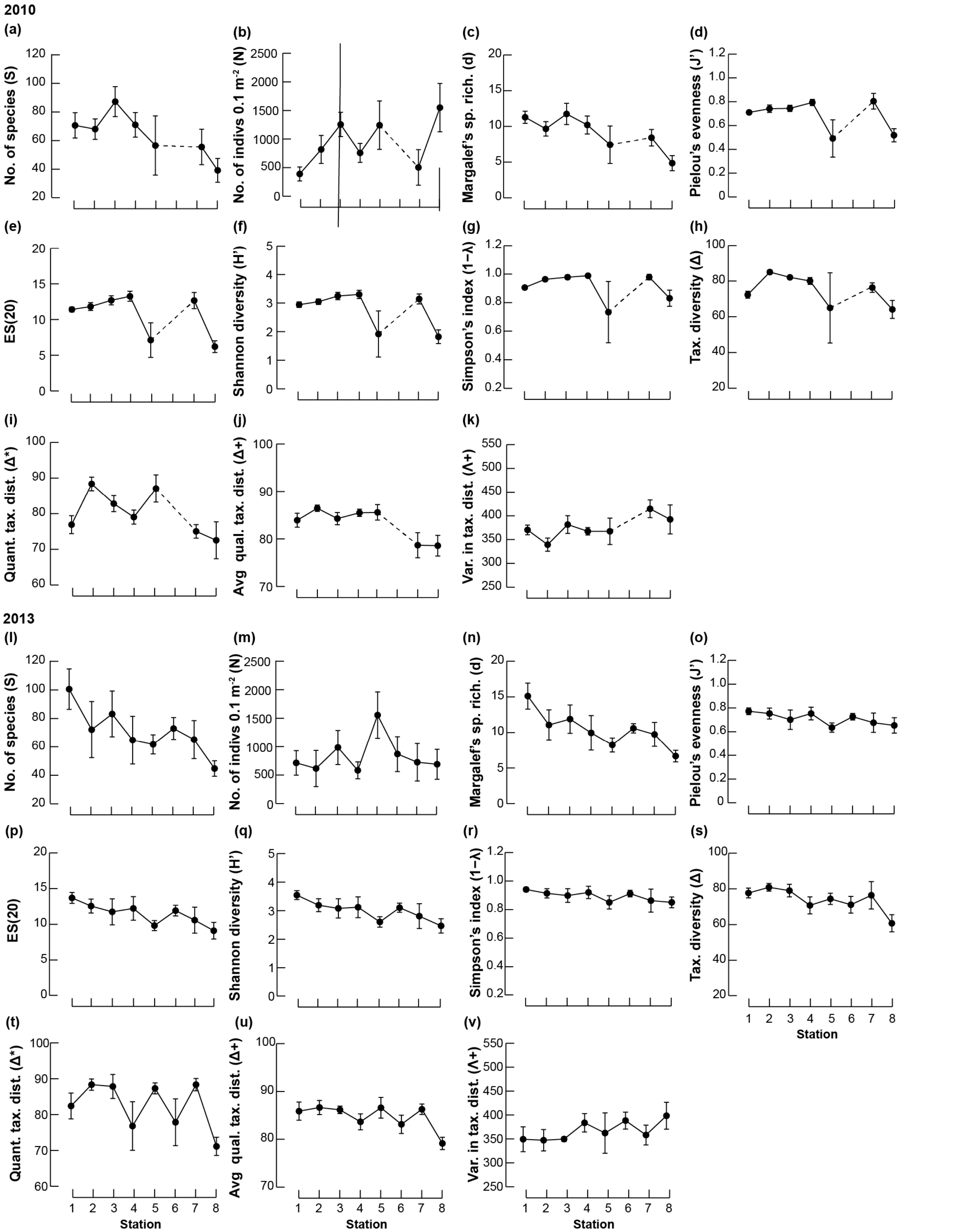

Figure 4. Mean values (±95 confidence intervals) for each of the 11 univariate metrics at each of the eight stations in Milford Haven in (**a**–**k**) 2010 and (**l**–**v**) 2013.

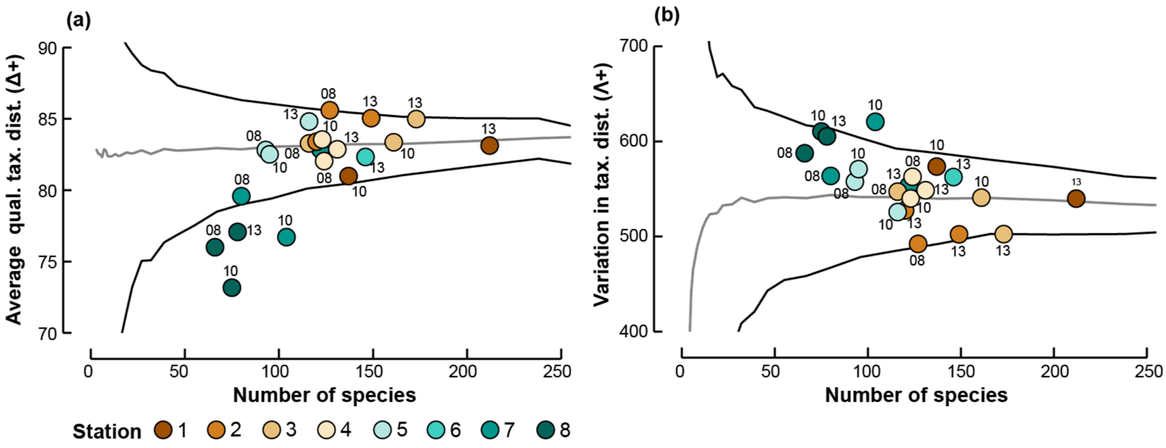

Figure 5. Frequency-based funnel plots for (**a**) average taxonomic distinctness (Δ^+) and (**b**) variation in taxonomic distinctness (Λ^+) based on total species for all replicates at the eight stations in Milford Haven in 1996, 2008, 2010 and 2013. The grey line denotes the 'expected' value derived from data for random subsamples of species found in the waterway and the black lines the upper and lower 95% probability limits.

3.2.2. Abundance/Biomass Comparison (ABC) Curves of the Macrofauna of Milford Haven

In 2008, station 2 had a moderately disturbed configuration, with the abundance and biomass curves virtually coincident throughout their length (W = 0.036). The remaining stations had an undisturbed configuration, with the biomass curve above the abundance curve throughout (W = 0.073–0.233; Figure 6a–f). In 2010, stations 1 and 2 were borderline between undisturbed and moderately disturbed, station 8 was moderately disturbed and the remainder undisturbed (Figure 6g–m). In 2013 all eight stations were undisturbed (Figure 6n–q). Although W statistic values are close to zero at some stations in some years, which would be indicative of moderate disturbance, they are usually strongly positive and never negative.

3.2.3. Multivariate Analyses of the Macrofauna of Milford Haven

Macrofaunal composition at the species, family and phylum levels differed significantly with Year, Station and Year × Station (all p = 0.001; Table A3). In all years, the points on the nMDS ordination plots formed a sequence, progressing from the outer stations on the left to the inner stations on the right, although the positions of stations 1 and 2 were reversed in 2010 compared with 1996 (Figure 7). The positions of the points representing each site on the plots are very similar when the species data are aggregated to the family level. RELATEs test detected a significant correlation between the data at the species and family levels (all $p < 0.001$), with correlation coefficients of 0.867, 0.942, 0.960 and 0.929 for the data in 1996, 2008, 2010 and 2013. While the pattern of distribution of the sites was less clear at the phylum level, the left-right sequence of stations 1–8 on the nMDS plots was still evident (Figure 7). Moreover, there was a significant correlation between the spatial pattern of sites at the phylum and species levels (all $p < 0.001$), albeit with lower correlation coefficients of 0.423, 0.668, 0.573 and 0.375 for each of the four years.

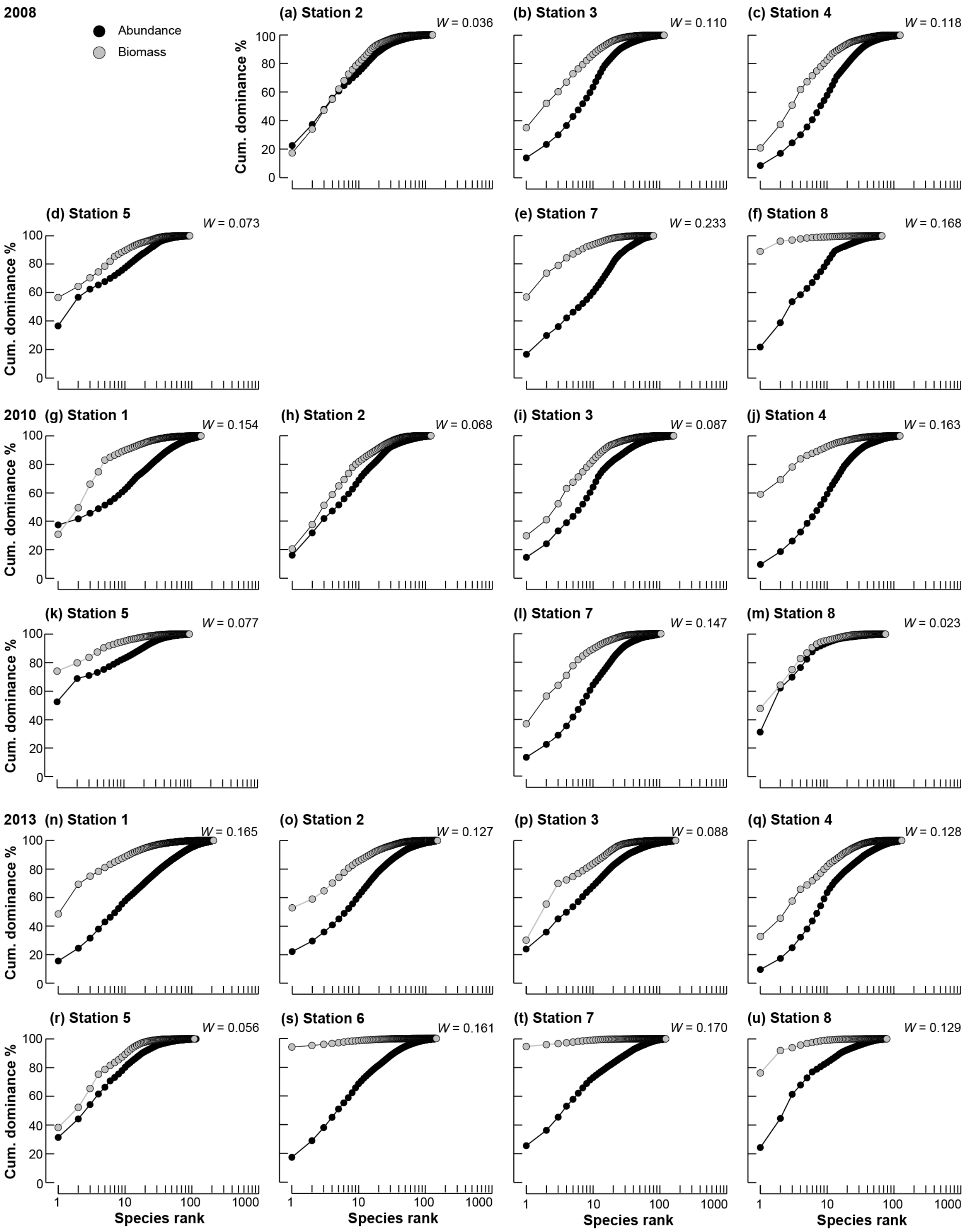

Figure 6. Abundance Biomass Comparison (ABC) plots and W-statistics for each of the eight stations in Milford Haven in (**a**–**f**) 2008, (**g**–**m**) 2010 and (**n**–**u**) 2013.

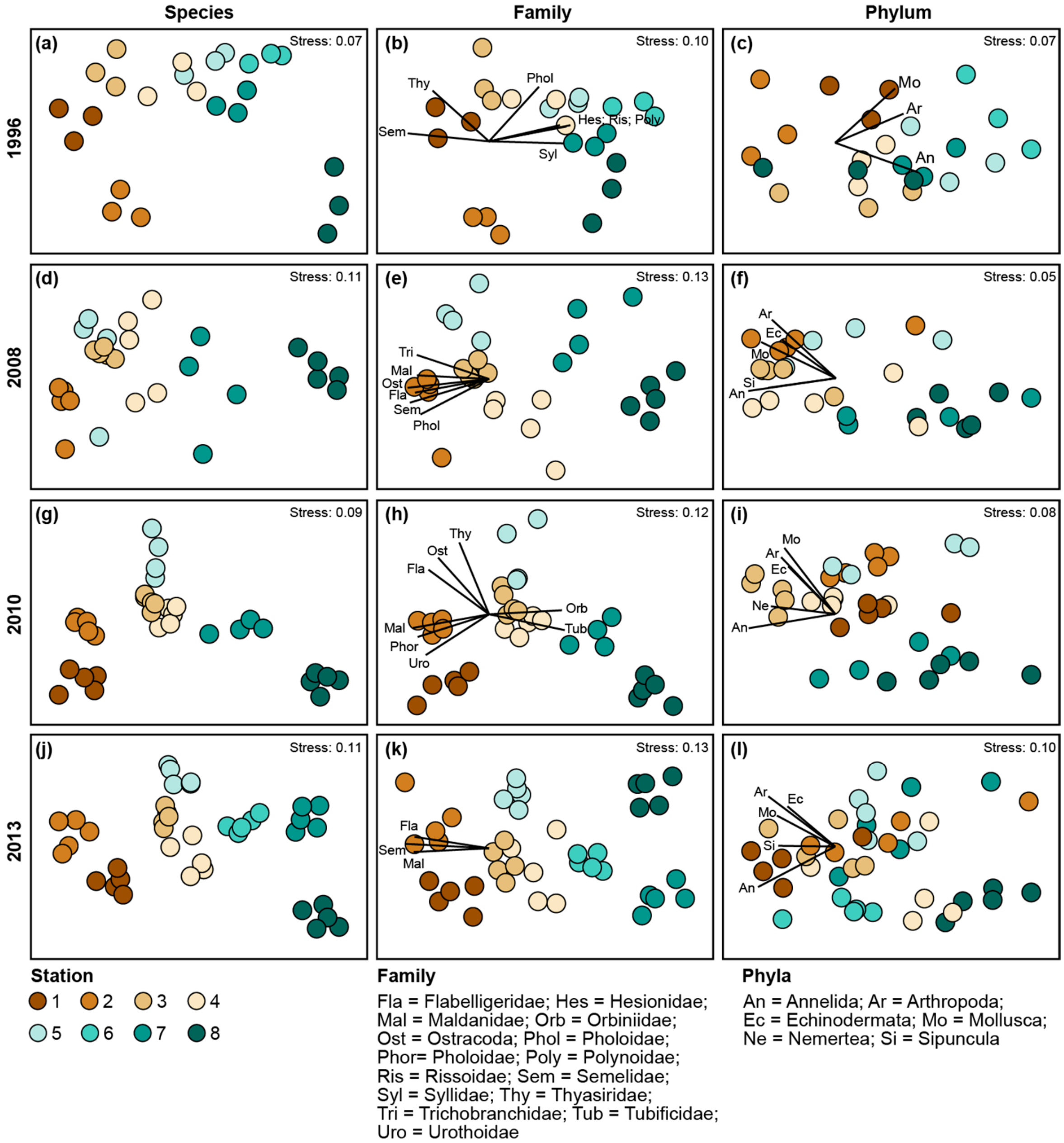

Figure 7. nMDS plots of the transformed density of each benthic macroinvertebrate species, family and phylum at each site in (**a**–**c**) 1996, (**d**–**f**) 2008, (**g**–**i**) 2010 and (**j**–**l**) 2013. Vectors provided for families and phyla whose density changed in a linear direction (Pearson correlation >0.8 and >0.5, respectively) relative to the nMDS axes.

Shade plots show clearly in every year a diagonal pattern of abundance of individual species along the sequence of stations from the outer (1) to inner (8) parts of the waterway that give rise to the sequential change in community composition on the MDS plots (Figure 8). In all years, some coherent groups of species sequentially decreased in abundance, some increased and others had modes of abundance at intermediate stations along the sequence. However, inspection of the species composition of groups of species that have coherent patterns of distribution shows that this varied considerably among years.

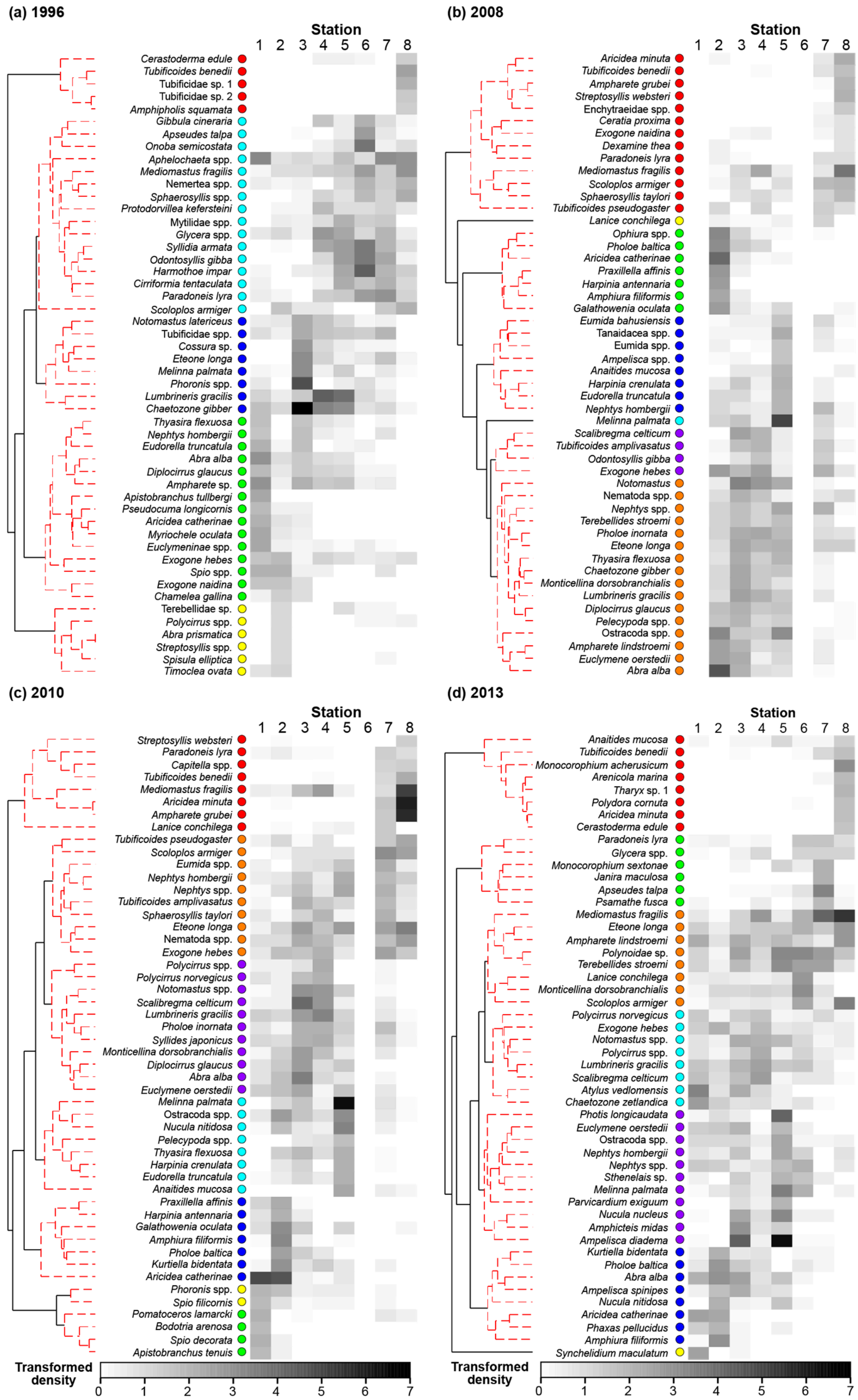

Figure 8. Shade plots showing the transformed abundance of each of the 50 most important species at each station in Milford Haven in (**a**) 1996, (**b**) 2008, (**c**) 2010 and (**d**) 2013. Coherent groups of species, i.e., those with a statistically indistinguishable pattern of abundance across stations, are denoted by a red dashed line and the same-coloured circle.

At the family level, in 1996, greater densities of thyasirid and semelid bivalves were found at sites 1 and 2, as were pholoid polychaetes at sites 3 and 4, polynoid and hesionid polychaetes and rissoid gastropods at sites 4, 5 and 6 and syllids at sites 7 and 8 (Figure 7). In each of the three more recent years, flabelligerid and maldanid polychaetes were abundant in sites 1 to 3, as were ostracods, pholoids and semelids in two of the years. Greater densities of orbiniid polychaetes and tubificid oligochaetes were recorded in sites 7 and 8 in 2010. Annelids, followed by molluscs and crustaceans dominated the densities of macrofauna at all stations in 1996; however, some spatial trends were evident. Sipunculids were generally only recorded at site 1. Molluscs and arthropods were most abundant in sites 5 to 7, as were annelids and chelicerates at sites 5 to 8. Nemerteans and echinoderms were present both at site 1 (outermost) and the innermost four sites (Figure 7). In the other three years, densities of most phyla, particularly arthropods, molluscs, annelids, echinoderms and sipunculids were greater at the outermost sites (excluding site 1 in 2010).

The sequential changes in community composition in each year were strongly correlated with the measured environmental conditions (Table 1). In 1996, the spatial pattern of community changes was significantly related to salinity at spring water (p = 0.001; ρ = 0.827), with the addition of other salinity or sediment variables, lowering the strength of the correlation (Table 1). In the three other years, this variable individually had the strongest correlation to community composition (i.e., ρ = 0.809–0.913), followed by salinity at high water. The proportion of medium sand in 2008 was the only sediment variable to have a ρ value > 0.500. However, the addition of several sediment particle sizes did improve the extent of the correlation in 2008, 2010 and 2013 by between 0.02 and 0.07, with the proportions of fine sand selected in each year and silt and clay twice.

Table 1. BEST results showing the influence of environmental variables (Var) individually and in combination on the composition of the macrobenthos among sites for each year of sampling. Individual variables are ranked according to the strength of their correlation (ρ). Significance test only conducted on the best combination (p).

1996		**2008**		**2010**		**2013**	
Single variable							
ρ	Var	ρ	Var	ρ	Var	ρ	Var
0.827	Sal_LW	0.913	Sal_LW	0.809	Sal_LW	0.846	Sal_LW
0.573	Sal_HW	0.816	Sal_HW	0.673	Sal_HW	0.736	Sal_HW
0.404	MS	0.568	MP	0.347	MS	0.455	MS
0.135	FS	0.354	VFS	0.257	VFS	0.424	VFS
−0.013	VFS	0.350	MS	0.231	MP	0.403	MP
−0.022	CS	0.236	CS	0.086	FS	0.375	FS
−0.105	SC	0.079	FS	0.047	CS	0.082	CS
−0.136	MP	0.007	SP	−0.082	SC	0.053	SP
−0.274	G	−0.068	SC	−0.134	SP	0.052	SC
−0.328	SP	−0.075	G	−0.197	G	0.011	G
−0.402	VCS	−0.236	VCS	−0.273	VCS	−0.212	VCS
Combination of variables							
ρ	p	**ρ**	p	**ρ**	p	**ρ**	p
0.827	0.010	0.950	0.013	0.829	0.040	0.911	0.001
Sal_LW		Sal_HW, Sal_LW, MS, FS, VFS		Sal_HW, Sal_LW, FS, SC		Sal_HW, Sal_LW, FS, SC	

Sal_HW = salinity at spring high water; sal_LW, salinity at spring low water; MP = medium pebble (>8 mm); SP = small pebble (4–8 mm); G = granule (2–4 mm); VCS = very coarse sand (1–2 mm); CS = coarse sand (500–1000 µm); MS = medium sand (250–500 µm); FS = fine sand (125–250 µm); VFS = very fine sand (63–125 µm) and SC = silt and clay (<63 µm).

3.2.4. Meta-Analysis Comparing Milford Haven to Other Northern European Sites

In the phylum-level meta-analysis, the training data were divided into quintiles represented by coloured symbols indicating where on the disturbance gradient they lie, with the eight stations for 2008, 2010 and 2013 numbered. Unperturbed samples are on the left side of the plot (wider end of the wedge-shaped configuration), and grossly perturbed samples are on the right side (pointed end; Figure 9). The Milford Haven stations run along the top edge of this configuration, and most of them are clustered at the unperturbed left-hand end of it. Stations furthest to the right, and thus the most perturbed, are station 7 in 2008 and 2010, which had improved in condition from being the furthest to the left in 2013, and station 5 in 2008 and 2010, which had also improved in condition by 2013. Station 8 was also moderately disturbed in 2010 but in better condition in 2008 and 2013. The remaining stations were in good condition throughout the five-year sampling period. Vectors on these plots indicate the phyla mainly responsible for the MDS configuration and help to explain the reason for the placement of the Milford Haven stations along the top edge of the training data. Arthropods are proportionally more important along the top and echinoderms along the bottom of the configuration. Indeed, echinoderms contribute 10.24% of total phylum production in the training data but only 1.61% in the Milford Haven data, whereas the corresponding percentages for arthropods are 3.58 and 6.87%.

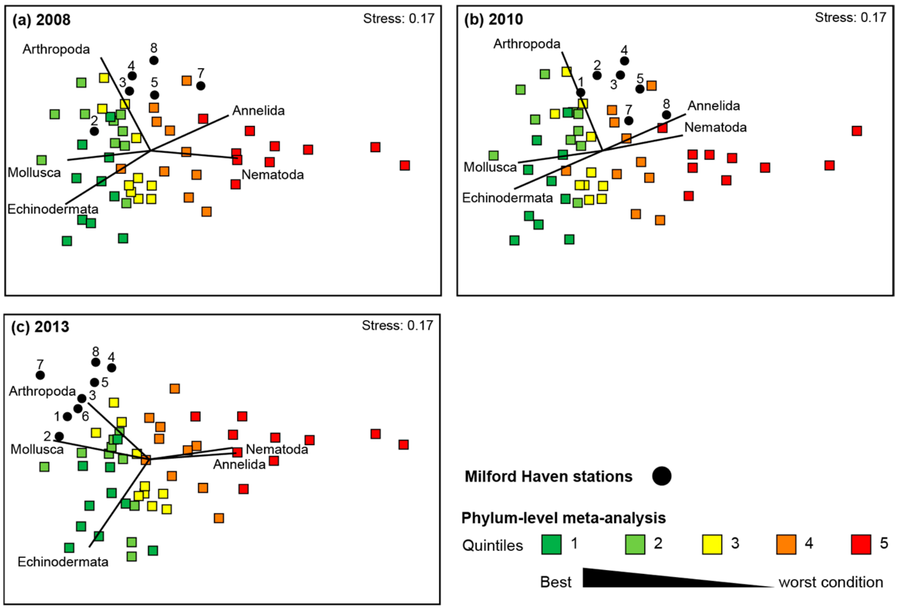

Figure 9. nMDs plots of the phylum-level meta-analysis, including data for the eight stations in Milford Haven in (**a**) 2008, (**b**) 2010 and (**c**) 2013 (numbered black circles), with the training data divided into quintiles (coloured squares).

3.2.5. Comparison of the Milford Haven and CSEMP Data

Values for each of the 11 univariate metrics differed significantly among the comparison group (i.e., the eight sites in Milford Haven and CSEMP estuaries and coastal waters (all $p = 0.001$; Table A4). The mean values for the number of species, Pielou's evenness, Shannon diversity, Simpson's index, Δ and Δ^* were all significantly lower in the CSEMP estuarine sites than the coastal sites, the exception being the number of individuals, which was significantly higher (Figure 10). For the taxonomic distinctness indices based on the presence and absence of species, i.e., Δ^+ and Λ^+, the 95% confidence intervals are overlapping, indicating no significant difference between the coastal and estuarine sites.

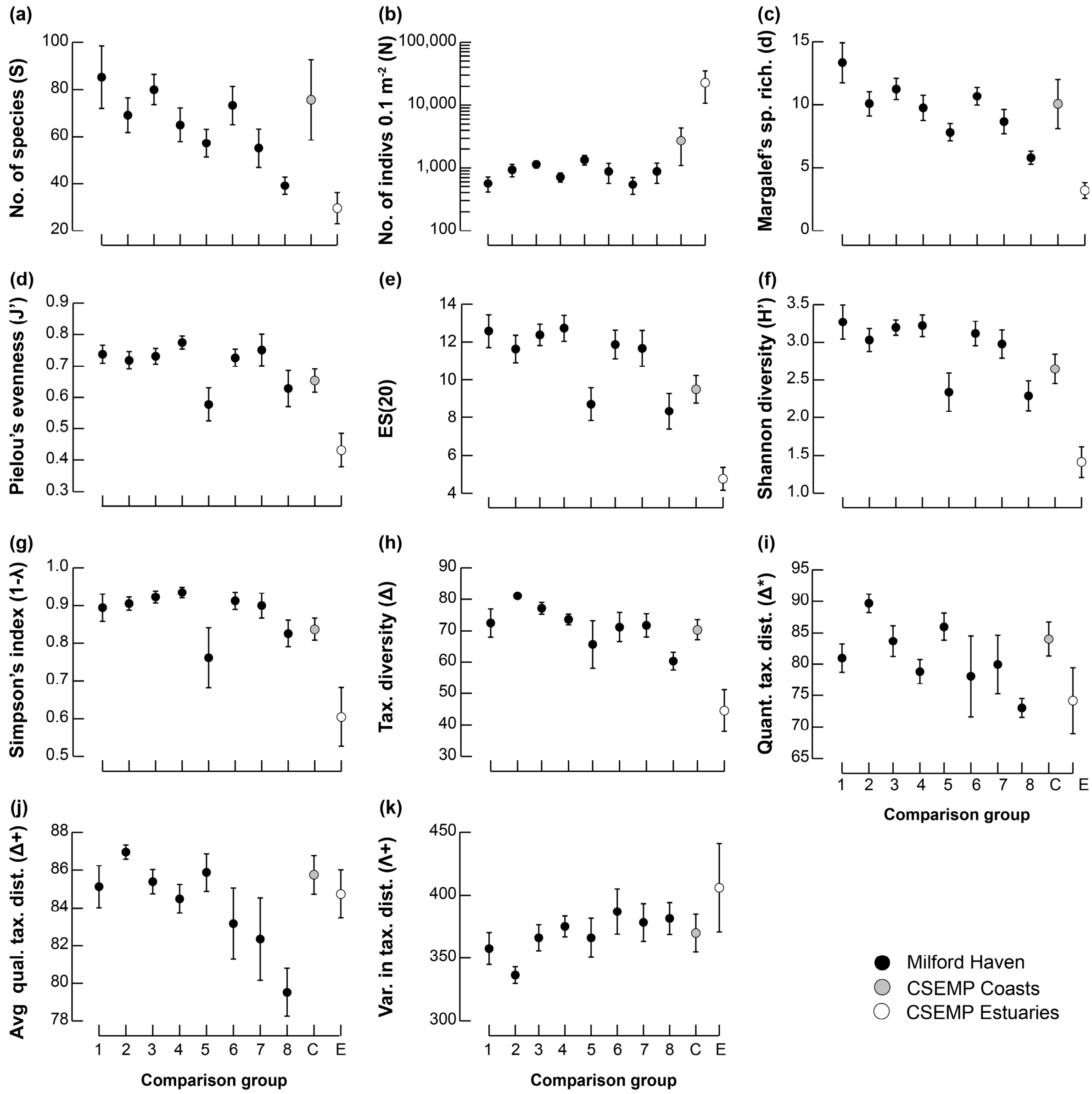

Figure 10. Mean values (±95 confidence intervals) for each of the 11 univariate metrics (**a**–**k**) at each of the eight stations in Milford Haven (pooled across years) and all coastal (C) and estuarine (E) CSEMP stations.

While a wide range of metrics are included in Figure 10 for completeness, we must bear in mind that there are large differences in sample sizes within and between the two studies (i.e., the number of samples in each comparison group vary from 28 to 110 in Milford Haven and 9 to 209 in the CSEMP study), and many of these indices are dependent on sample size. Simpson's index is the best species diversity metric for comparative purposes, as is the quantitative measure of taxonomic distinctness (Δ*), because of their independence from the sample size. Values for Simpson's index at all stations in Milford Haven are comparable to the coastal CSEMP sites (Figure 10g), while the estuarine CSEMP sites are substantially lower. Station 5 has a significantly lower value and much greater variability among replicates than the other stations, a feature that remains unexplained. Values of Δ* exhibit a gradual decline from the mouth to the head of Milford Haven, although this decline is not linear (Figure 10i). The outermost stations have comparable values to the coastal CSEMP sites, with the innermost station 8 being similar to the mean of the estuarine CSEMP sites.

4. Discussion

The main objective of this study was to use data from the surveillance program in Milford Haven to determine the extent to which attributes of the macrobenthic communities in this waterway are indicative of environmental perturbation and, if possible, to correlate these attributes with environmental variables that might imply cause and effect. A problem with such an exercise is establishing reference conditions against which the status of these communities can be evaluated in the future [49]. Usually, the best-scoring samples, indicating the most pristine state, are used to establish local reference conditions that act as a baseline against which spatial differences and/or temporal changes can be assessed. Several authors have argued against the use of a pristine state as a reference point against which potentially impacted sites or systems can be evaluated [50,51], and this is particularly relevant in the case of estuaries, where all sites might be impacted to some degree and no appropriate reference sites may be available. Furthermore, environmental conditions in macrotidal estuaries are highly dynamic and can vary dramatically and unpredictably at different times of the year [18].

Three of the indices used here, i.e., ABC curves, taxonomic distinctness and the phylum-level meta-analysis, adopt different approaches to setting reference conditions that do not require extensive temporal or spatial data to establish a baseline. The ABC method exploits the fact that when an assemblage is perturbed, the conservative species are less favoured in comparison with the opportunists, and the distribution of biomass among species behaves differently from the distribution of numbers of individuals among species. The three conditions (unperturbed, moderately perturbed or grossly perturbed) are recognisable without reference control samples in time or space, the two curves acting as an internal control against each other and providing a snapshot of the condition of the assemblage at any one time or place. With average taxonomic distinctness (Δ^+) and variation in taxonomic distinctness (Λ^+), the permutation test determines the significance of departure from expectation under specific null hypothesis conditions, i.e., that the species present are a random selection from the regional species pool, and these conditions act as a reference against which the status of samples can be assessed. In this case, the concept of spatial or temporal reference *sites* is replaced by the concept of a reference condition. With the phylum-level meta-analysis, the scale of perturbation is determined by comparison with the 50 samples in the training data which represent a range of types and severity of perturbation, none of which is of necessity pristine. Although all three of these methods provide some slight indications of environmental perturbation in certain years, the overriding conclusion is that the communities are in a healthy state, even in 1996, immediately after the *Sea Empress* oil spill. Taxonomic distinctness indices do indicate significant community stress at stations 7 and 8, both in comparison with other stations in Milford Haven and with the more extensive CSEMP data at other coastal sites in the UK, as well as in the phylum-level meta-analysis. However, reduced salinity is likely to be implicated here. For example, echinoderms, which are very sensitive to reduced salinity [52], are virtually absent from station 8. ABC curves, which are not taxonomically based, have strongly positive W-statistic values at stations 7 and 8 in all years, except for station 8 in 2010, which is still slightly positive.

The dominance or prominence of benthic "indicator species", i.e., those that have been recorded in areas polluted or enriched by organic material such as the small opportunistic polychaetes *Capitella capitata* and certain spionids [25], have been widely used in the assessment of ecological condition. This principle was elevated to a much more sophisticated level by Borja et al. [53] in AZTI's Marine Biotic Index AMBI by classifying the benthic macroinvertebrate species present into five ecological groups based on their implied sensitivity to environmental stress. However, AMBI is essentially an indicator of organic enrichment and associated reduction in the oxygenation of the sediments, properties that vary naturally and potentially confound any biotic responses to anthropogenic contamination or disturbance [54]. This is a particular problem in estuaries, where reduced and fluctuating salinities and tidal water movement scours the sediment also impose natural stresses on the fauna. Thus, Tweedley, Warwick and Potter [17] found that AMBI did not reflect levels of contamination at the 61 estuarine and coastal sites in the CSEMP study, whereas taxonomic distinctness indices were significantly correlated with contaminant loadings. Similarly, Muxika et al. [55] found that AMBI was a poor indicator for detecting the physical impacts of sediment disturbance, which might be associated with the oil and gas industry, there being no increased abundance of opportunistic species as a result, and was similarly not useful in other naturally stressed communities. Therefore, because of the estuarine environment present at the inner stations in Milford Haven, AMBI was not applied in this study, but suffice to say, none of the species listed by Pearson and Rosenberg [25] as indicators of excessive organic pollution are prominent at any station.

The disappearance of taxa considered sensitive to the effects of environmental perturbation has also been regarded as symptomatic of anthropogenic disturbance, especially oil pollution. For example, Hobbs and Smith [33] reported that the immediate effect of the *Sea Empress* oil spill on the benthic assemblage of the waterway was a decrease in amphipods, a common feature noted in other European coastal waters. For example, after the 1978 *Amoco Cadiz* oil spill in the Bay of Morlaix in the western English Channel and the 1992 *Aegean Sea* oil spill in the Ria de Ares and Betanzos in the northwestern Iberian Peninsula, amphipods disappeared [56,57]. In Milford Haven, amphipod populations showed clear signs of recovery within five years of the oil spill [8]. The Milford Haven data are confusing in this respect. In 1996, stations 1–7 were sampled in March, immediately following the *Sea Empress* oil spill on 15 February, while station 8 was sampled the following October. Amphipods comprised 6.2% of the total abundance of macrobenthos in that year, followed by 3.5% in 2008, 2.1% in 2010 and 19.3% in 2013. This suggests that amphipod abundance may be an unreliable symptom of perturbation in situations where other indicators imply quite minimal effects.

Relating community composition to potentially causal environmental variables poses a greater problem since no contemporaneous measurements of contaminants such as hydrocarbons or heavy metals were made at the faunal sampling stations. However, it is relevant to note that, in the more extensive survey of 36 stations in October 1996 by Levell, Hobbs, Smith and Law [34], salinity is the overriding factor in determining the community patterns and that sediment granulometry, polycyclic aromatic hydrocarbons and total hydrocarbons were of little importance [32]. Salinity and sediment granulometry in the present study were taken to be temporally stable and thus could not be invoked to explain any differences between the years in which the macrobenthic samples were collected. The sequential change in community composition from the outer station (1) to the inner station (8), resulting from the distributions of individual species along this transect, can, however, be accounted for in terms of these two suites of variables. The strong correlations between community composition and the environmental variables in each of the four years support the conclusion of Levell, Hobbs, Smith and Law [34] that salinity is the major driver, with sediment granulometry being of overall secondary importance. Thus, species gradually declining or increasing in abundance can be related to the gradient of reducing salinity, while those peaking at the intermediate stations may be favoured by the higher silt/clay content there. The decreasing number of species recorded in an upstream direction overall in Milford Haven matches that recorded by Ysebaert, Herman, Meire, Craeymeersch, Verbeek and Heip [23] in the macrotidal Schelde Estuary in the Netherlands and Belgium.

Although the number and total volume of oil spilled annually from ships decreased over the past four decades, catastrophic releases continue to occur from the production and transport of petroleum [29,58]. As such, the data in the current study provided a comprehensive spatial and temporal baseline against which the effects of any future environmental accidents or industrial developments can be assessed.

5. Conclusions

The results of ABC curves, taxonomic distinctness and the phylum-level meta-analysis provide multiple lines of evidence that, while there are some slight indications of environmental perturbation in certain years, the benthic communities of Milford Haven Waterway are in a healthy state, even in 1996 immediately after the *Sea Empress* oil spill. There is a general trend for numbers of species and taxonomic distinctness to decrease in an upstream direction, which was reflected in a shift in species composition. Densities of most phyla, particularly arthropods, molluscs, annelids, echinoderms and sipunculids were greater at the outermost sites. These changes were primarily related to changes in salinity and, to a lesser extent, sediment granularity.

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Appendix A

Table A1. Number of replicate 0.1 m^2 Day grab samples of the macrobenthos collected at each of the eight stations in Milford Haven in each year. - denotes that no samples were collected from that station in that year.

	Station							
Year	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1996	3	3	3	3	3	3	3	3
2008	-	5	5	5	4	-	4	5
2010	5	5	5	5	4	-	4	5
2013	5	5	5	5	5	5	5	5

Table A2. Mean squares (MS), contribution of mean squares to total mean squares (%MS), Pseudo (*pF*) and significance levels (*p*) from two-way PERMANOVA tests on separate Euclidean distance matrices constructed from the data for 11 univariate matrices calculated from the densities of the various benthic macroinvertebrate taxa in replicate samples collected from the eight sites in Milford Haven Waterway in 1996, 2008, 2010 and 2013. df = degrees of freedom.

		Number of Species (S)				**Number of Individuals (N)**				**Margalef's Species Richness (d)**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Year	3	2492	52.57	24.79	0.001	0.34	9.53	3.03	0.032	51.34	53.40	32.09	0.001
Station	7	1617	34.11	16.08	0.001	2.06	57.08	18.13	0.001	32.61	33.92	20.38	0.001
Year × Station	18	531	11.20	5.28	0.001	1.09	30.24	9.61	0.001	10.59	11.01	6.62	0.001
Residual	96	101	2.12			0.11	3.15			1.60	1.66		
		Pielou's evenness (J′)				**ES (20)**				**Shannon diversity (H′)**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Year	3	0.019	16.86	7.03	0.001	9.26	17.74	7.75	0.001	0.81	27.39	13.14	0.001
Station	7	0.056	48.57	20.24	0.001	27.42	52.50	22.94	0.001	1.31	44.48	21.34	0.001
Year × Station	18	0.037	32.27	13.45	0.001	14.34	27.46	12.00	0.001	0.77	26.05	12.50	0.001
Residual	96	0.003	2.40			1.20	2.29			0.06	2.08		
		Simpson's index (1 − λ)				**Delta (Δ)**				**Delta* (Δ*)**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Year	3	0.029	26.93	10.09	0.001	494	36.54	18.79	0.001	161.63	29.24	16.19	0.001
Station	7	0.038	35.52	13.31	0.001	518	38.29	19.69	0.001	277.55	50.22	27.80	0.001
Year × Station	18	0.038	34.87	13.06	0.001	314	23.23	11.95	0.001	103.50	18.73	10.37	0.001
Residual	96	0.003	2.67			26	1.94			9.98	1.81		
		Delta+ (Δ^+)				**Lambda+ (Λ^+)**							
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*				
Year	3	7.00	7.08	2.89	0.044	256	3.22	0.77	0.521				
Station	7	72.54	73.36	30.00	0.001	5594	70.23	16.79	0.001				
Year × Station	18	16.93	17.12	7.00	0.001	1781	22.36	5.35	0.001				
Residual	96	2.42	2.45			333	4.18						

Table A3. Mean squares (MS), contribution of mean squares to total mean squares (%MS), Pseudo (*pF*) and significance levels (*p*) from two-way PERMANOVA tests on separate Bray–Curtis similarity matrices constructed from the pre-treated densities of the various benthic macroinvertebrate species, families and phyla in replicate samples collected from the eight sites in Milford Haven Waterway in 1996, 2008, 2010 and 2013. df = degrees of freedom.

		Species-Level				**Family-Level**				**Phylum-Level**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Year	3	19,528	46.31	22.66	0.001	8466	36.52	15.18	0.001	1797	40.85	10.88	0.001
Station	7	17,327	41.09	20.11	0.001	11,594	50.01	20.78	0.001	1773	40.31	10.73	0.001
Year × Station	18	4447	10.55	5.16	0.001	2563	11.06	4.59	0.001	664	15.09	4.02	0.001
Residual	96	862	2.04			558	2.41			165	3.75		

Table A4. Mean squares (MS), contribution of mean squares to total mean squares (%MS), Pseudo (*pF*) and significance levels (*p*) from one-way PERMANOVA tests on separate Euclidean distance matrices constructed from the data for 11 univariate matrices calculated from the densities of the various benthic macroinvertebrate taxa in replicate samples collected from the eight sites in Milford Haven Waterway (pool across years) and from the Clean Safe Seas Environmental Monitoring Program in UK estuarine and coastal waters. df = degrees of freedom.

		Number of Species (S)				**Number of Individuals (N)**				**Margalef's Species Richness (d)**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Comparison group	9	6502	89.83	8.83	0.001	14.5	92.84	12.96	0.001	12,684	86.38	6.3402	0.001
Residual	158	736	10.17			1.1	7.16			2001	13.62		
		Pielou's evenness (J')				**ES (20)**				**Shannon diversity (H')**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Comparison group	9	0.24	96.27	25.81	0.001	136.87	98.11	51.791	0.001	7.38	97.52	39.35	0.001
Residual	158	0.01	3.73			2.64	1.89			0.19	2.48		
		Simpson's index (1 − λ)				**Delta (Δ)**				**Delta* (Δ*)**			
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*
Comparison group	9	0.23	95.10	19.41	0.001	2457	96.06	24.37	0.001	486.3	88.59	7.77	0.001
Residual	158	0.01	4.90			101	3.94			62.6	11.41		
		Delta+ (Δ^+)				**Lambda+ (Λ^+)**							
Source	df	MS	%MS	*pF*	*p*	MS	%MS	*pF*	*p*				
Comparison group	9	70.11	91.55	10.84	0.001	6547	73.82	2.82	0.008				
Residual	158	6.47	8.45			2322	26.18						

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