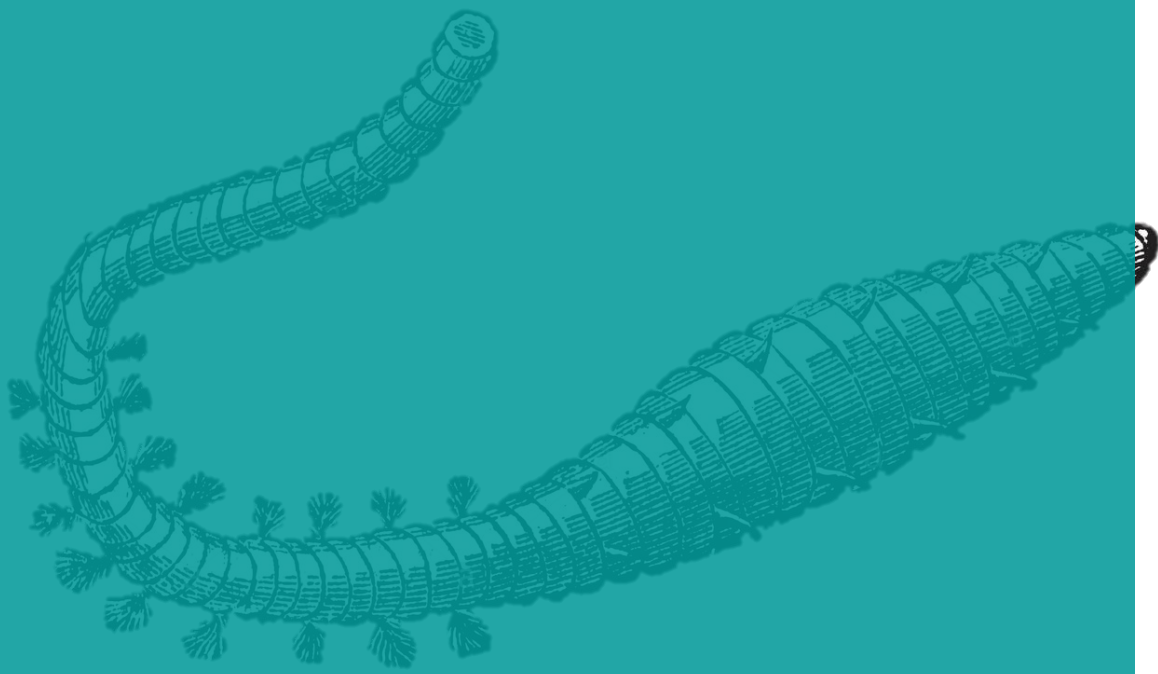
Impact assessment of mechanical harvesting *Arenicola marina* on macrobenthic communities

and the potential of DNA metabarcoding to replace traditional methods



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1. Abstract

Anthropogenic disturbances negatively affect marine ecosystems and biodiversity. Macrobenthic communities are a good indicator for marine ecosystem health, as they are known to respond fast under a range of anthropogenic and natural pressures. The traditional macrobenthic biomonitoring based on morphological identification is labour intensive and costly. DNA-based identification is a possible solution to overcome these challenges. Currently, methodologies differ substantially between studies. This study aims to assess the impact of mechanically harvesting *Arenicola marina* on macrobenthic communities. This impact assessment is performed on a morphological, quantitative dataset. For future comparison of both methods, this study aims to determine optimal methods for DNA metabarcoding of macrobenthos based on a literature study. Furthermore, the potential of DNA metabarcoding replacing traditional methods is explored using a presence absence dataset. It was hypothesised that mechanically harvesting would have a negative effect on macrobenthos and cause a shift towards more opportunistic species in the macrobenthic communities. However, this was not found. Mechanically harvested *Arenicola marina* were quickly replaced by juvenile recruits. There was no significant effect found of mechanically harvesting on macrobenthic communities, both over the entire sampling period and within the first three months. Macrobenthic community difference was explained by two other factors: time and location. This is likely due to natural variation in populations and the heterogenous sediment properties of the Wadden Sea. Interestingly, using presence-absence data led to similar outcomes as the abundance data. This implies that metabarcoding might replace traditional morphological identification in the future. Despite this promising outcome, the effects of using DNA metabarcoding data rather than morphological identified data on the outcome of biomonitoring analysis remains to be tested more elaborately. Therefore, it is recommended to use DNA metabarcoding approaches complementary to the traditional methods until the consequences of using presence-absence data instead of quantitative data are better understood.

2. Content

3. Introduction

3.1 Macrobenthos as indicator for marine ecosystem health

Anthropogenic disturbances such as oil drilling, mining, aqua farming, wind farms or trawling are affecting marine ecosystems and biodiversity [1]–[4]. Marine ecosystems are also affected by climate change effects, such as increasing ocean temperatures, deoxygenation and acidification [5], [6]. These findings stress the need for marine biodiversity monitoring and protection. A good indicator for marine ecosystem health are the macroinvertebrate communities [7].

Macroinvertebrate communities are used worldwide to assess the ecological status of aquatic ecosystems as they demonstrated to respond fast under a range of anthropogenic and natural pressures [8]–[10]. This has resulted in in the development of Biotic indices such as the AZTI's Marine Biotic Index [11] and environmental directives as the European Union Marine Strategy Framework Directive (MSFD 2008/56/EC) [12]. Specifically, for the Netherlands, two indices using benthos species were developed. First for the MSFD the Benthic Indicator Species Index (BISI, Bentische Indicator Soorten Index) [13] is developen. This index focusses on the presence and abundance of specific indicator species. Second the Benthic Ecosystem Quality Index 2 (BEQI2) [14], which is developed for the Water Framework Directive (WFD) and assesses the benthic communities in transitional and coastal waters.

Traditional biomonitoring is based on morphological identification of individual specimen. This method is highly time consuming, labour intensive and is associated with high costs [15], [16]. It requires extensive expertise of taxonomists, a specialism rapidly declining in numbers [16], [17]. Misidentification can happen if the organisms´ body is damaged, the organism is in immature stage which makes keys are inapplicable, or the characteristics used for identification are subject to genetic or phenotypic variation [17], [18].

3.2 DNA-based biomonitoring

DNA-based identification is a possible solution to overcome the challenges in traditional biomonitoring. One of these methods is called DNA metabarcoding. A DNA barcode is a genetic marker which is used to identify a species [17]. Metabarcoding is referring to the taxonomic identification of multiple species simultaneously in a sample using DNA barcodes [3], [19]. The cytochrome oxidase c subunit 1 gene (CO1) located on the mitochondrial genome is a widely used barcode for identification of animals [17], [20]. It is the most represented marker in reference databases, it is highly variable between species and there are already many studies showing its applicability for macrobenthic biodiversity assessment [21]–[24].

DNA metabarcoding is a relatively new method of surveying biodiversity, nevertheless it shows great potential [3], [19]. Its seems to be faster and cheaper, as assessments of benthic communities using metabarcoding can result in a 73% time reduction and can save up to 55% of the costs according to Aylagas *et al.* 2018 [23], [25]. Metabarcoding can lead to higher resolution information as more organisms can be identified to species level [3], [15], [17], [26]. Furthermore, eDNA metabarcoding has the potential to assess entire benthic communities and disturbance gradients, as it can process macrofauna, meiofauna and bacteria at once [16], [27]–[31]. Lobo *et al.* (2017) [15] stated that species richness would be underestimated using solely morphological methods, as they identified 27 species (and 28 organisms up to higher taxonomic levels) using morphology versus 61 species using DNA metabarcoding.

However, eDNA metabarcoding is not yet applicable on a large scale as the method needs to overcome some challenges first. When Lobo *et al.* (2017) [15] tested in the same study bulk samples of known assembly, only 78-83% of the species was recovered using DNA metabarcoding. Aylagas *et al.* (2018) [23] sampled 18 monitoring stations used by the Basque Water Agency. Using DNA metabarcoding, they retrieved an average of only 20% of the 206 taxa found by morphology. In total

they found 112 taxa using metabarcoding. Therefore using a combination of the two methods is recommended by Cahill *et al.* (2018) [22], after finding significant differences in taxonomic composition of the same samples using the two different methods.

Returning explanations for the lower recovery rates using metabarcoding, are incomprehensive reference databases [32] and varying primer amplification success. If species are not present in the database, DNA reads from the environmental samples cannot be assigned to species [16], [22], [33], [34]. For example, Cahill *et al.* (2018) [22] found that 15,1% of the DNA reads could not be classified at the Barcode Of Life Database (BOLD). Cahill *et al.* (2018) [22] also demonstrated the effect of primer choice. Bivalves accounted for a large part of the individuals and biomass in a sample. However, due to low amplification success using universal CO1 primers, they were nearly absent in the DNA metabarcoding results. Other disadvantages of DNA metabarcoding include the inability to gather information on species abundance (numbers and biomass) and population structure (age and sex ratios)[3], [19], [35]. Nonetheless, an increasing number of studies [3], [19] demonstrate that eDNA metabarcoding can be used reliably to measure relative abundance. This is promising for future application of eDNA metabarcoding for biodiversity monitoring.

Currently, it is not yet possible to compare eDNA studies as methodologies differ substantially. Standardization is expected to remain a challenge due to the uniqueness of each study site and each group of target species [19], [36]. However, there is an increasing request for standardised methodologies for eDNA metabarcoding, to fulfil the need for time and cost reduced biodiversity monitoring.

3.3 Research aims

An international initiative to develop and test a standard methodology for monitoring macroinvertebrate communities using DNA metabarcoding is GEANS - Genetic tools for Ecosystem health Assessment in the North Sea [38]. Part of their aims are to harmonise DNA-based protocols and apply those through pilot studies. One of their pilot studies focuses on the assessment of the effects of mechanical harvesting of lugworms *Arenicola marina* on macroinvertebrate communities. Both traditional morphological identification and DNA metabarcoding methods are applied. This will create the opportunity to validate the DNA metabarcoding methods with traditional methods.

In this subpart of that study, an impact assessment of harvesting *A. marina* on macrobenthos is performed, using a morphological dataset. It is aimed to repeat this assessment with DNA metabarcoding data for future comparison of both methods. Therefore, the most promising method for DNA metabarcoding is selected based on a literature research. In the current absence of DNA based data, the effect of DNA metabarcoding data on the impact assessment is explored by transforming the morphological data to presence-absence data.

Finding a macroinvertebrate community change after mechanically harvesting *A. marina* is hypothesised. First, because the fishing boats remove the top layer of the soil and discards it back into the gully after filtering the sediment, turning the seabed. It might leave macrobenthos vulnerable for predators or buries them under 20-30 cm of sediment. This is lethal for species as *Cerastoderma edule*, a cockle that only survives in the upper 10 cm of the sediment [39]. Second, previous studies have shown changes in macroinvertebrate communities in both species abundance and species composition after physical disturbance [1], [8], [25], [40]–[42]. From dredging studies, which effects have been extensively studied, it is known that dredging is followed by a decline in species numbers, population density and biomass of macrobenthos [43]. Shortly after the dredging event, the more opportunistic species with a relatively fast reproduction cycle and growth are prevalent. The species with a longer lifespan and slow growth need more time for recovery. Recolonization in areas of low current velocity can take up to 5-10 years [43], [44]. Dredging removes the soil including benthic organisms. Although mechanical harvesting of *A. marine* causes less disturbance, a noticeable effect is expected.

4. Materials and methods

This chapter describes the methods to obtain the morphological data and perform the impact assessment. It also describes the outlines of optimal methods for assessing the samples using DNA metabarcoding. The information concerning the sample collection is provided by Lise Klunder (Royal Netherlands Institute for Sea Research, NIOZ, unpublished data). A more detailed description for the creation of the reference database and metabarcoding protocol can be found in the report 'Potentie van DNA metabarcoding voor Biomonitoring van macrobenthos' by Bas Vooijs (2020, unpublised data).

4.1 Ecological impact assessment

4.1.1 Mechanical harvesting

In this study, the impact of mechanical harvesting of *A. marina* on benthic macrofauna is assessed. Lugworms can only be harvested during high tide. The trawler releases an anchor and pulls itself slowly towards the anchor using a winch. While moving forward, the top layer of the sediment is removed and sieved. Sediment and macrobenthos is transferred on a conveyor-belt and transported onto the ship. The *A. marina* are selected by hand and the remaining organisms and sediment are discarded back into the dug trench [45], [46].

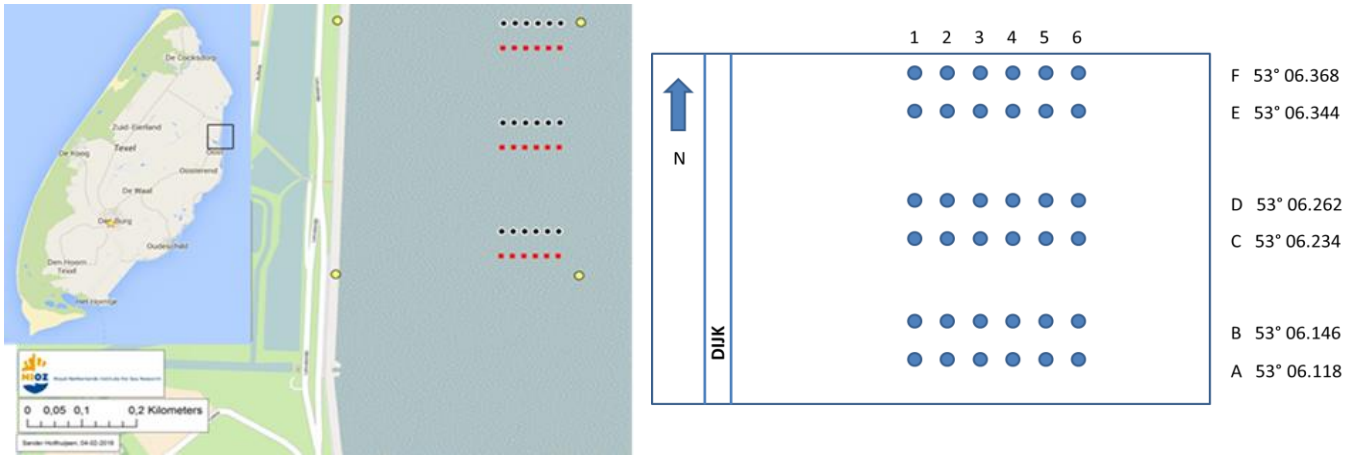

Figure 1: The map on the left shows the locations of the sample transects. The black dots represent the control transects, the red dots the disturbed transects. The dotted lines correspond to the dots on B. The schematic depiction on the right is a graphical representation of the sample transects including the latitudes of the transect. For this study only data from the even sites 2, 4 and 6 was used.

4.1.2 Study area and sample collection

The study area is located in the Dutch Wadden Sea area, east of the island Texel (figure 1a). The Wadden Sea is a protected area because it is the world's largest uninterrupted intertidal system consisting of sand and mudflats. Throughout most of the area, the natural processes are undisturbed resulting in a species composition consisting of species particularly adapted to the challenging environmental conditions [47].

A total of 36 sampling locations divided along six transects were sampled (figure 1b). Each transect contained 6 randomly divided sampling locations, different for each sampling event. The coordinates of these locations can be found in appendix II. Transects A, C and E were mechanically harvested for Arenicola (in red), transect B, D and F served as control site, they are undisturbed. The morning before harvesting (20-03-2016), all transect were sampled. This was done according to the Before-After-Control-Impact (BACI) approach [48], to assess the short-term effects. The transects were sampled on 17 occasions between 21st of March 2016 and 26th of June 2017. Exact dates can be found in appendix IV.

The samples were taken using a macrofauna core (NIOZ). In total 177m2 sediment is sampled up to 25-30cm depth. All cores were sieved over a 1mm sieve. Samples from the even locations (2, 4 and 6) of each transect were morphologically identified. All samples were freeze dried and stored by -80°C. The morphological data was obtained by Lise Klunder et al. (2019, NIOZ, unpublished data) and shared for this study.

4.1.3 Statistics

All statistics and data handling were performed in R version 3.6.1 and R Studio version 1.2.5033 [49]. The morphological identified dataset obtained by Klunder *et al.* (2019, NIOZ, unpublished data) was used for the ecological impact assessment. The dataset contained data from the 18 stations divided over six transects for 17 sampling events, 306 samples in total. Each sample contained a taxa list identified to the highest possible taxonomic level and their abundance. Per transect were 3 replicates available, these were combined resulting in a final 102 samples.

Adult and juvenile *Arenicola marina* were counted separately. This data was used to indicate the effects of mechanical harvesting on the lugworm itself. The difference between the juvenile number of the disturbed and undisturbed areas is tested using a Mann Whitney U test. For further analyses, the number of juveniles and adults were combined.

For each statistical test, the samples were analysed on two levels. For the first distinctive level, the transects were combined into two groups: disturbed and undisturbed. The second level was an analysis per transect. The latter one was done to check if the differences between the transects influenced the differences between the disturbed or undisturbed situation. As different locations could account for a larger difference on species composition than the impact of mechanically harvesting.

The dataset was tested for the biodiversity indices species richness and Shannon diversity index (H) between statuses and transects. The latter one using the Vegan package [50], [51]. Both patterns were graphically visualised over time applying the smooth curve (geom_smooth) from the Ggplot package [52]. The difference between the disturbed and undisturbed areas for the entire sampling period was tested using an independent samples t-test for the richness and a Mann Whithey U test for H. Abundance of all organisms was plotted for both the disturbed area and undisturbed area to indicate disturbance effects on the total number of organisms. Moreover, abundance was used to indicate seasonal change as H is affected by the evenness of the species, not only the number of species. The dataset contained species and aggregates. Some species were only identified up to order or family level. To indicate the effect of aggregates on the analysis, both richness and H, were compared to the pattern found when only species were included in the analysis. The R scripts are included in appendix V for repetition and comparison with the metabarcoding dataset.

The non-metric multidimensional scaling (NMDS) function from the Vegan package[51] was used to visualise the differences in macrobenthic community composition. This ordination method uses the Bray-Curtis distance to order the datapoints in a multidimensional space (k=2) based on its (dis)similarity. Similar datapoints cluster, and the higher the dissimilarity, the further they were spaced apart. This method is applied in exploratory data analysis as its axis are non-explanatory, they are solely an expression of (dis)similarity.

The data is not normally distributed, hence the analysis of similarity (ANOSIM) [53] from the Vegan package [50], [51] is applied to test if there was a significant difference between the disturbed and undisturbed data (T1-T17). The test was also pairwise applied between all transects. This non-parametric test assigned ranks to the dissimilarity matrix and used these ranks to test if there was a higher similarity between the ranks of the tested groups than within the tested groups[54].

The ANOSIM provided us with information whether the difference in community composition between two or more areas is significant. However, this test was not able analyse the effect of other variables such as time on the community composition. Therefore, the effects of status, time and

location (transect) on the community composition is tested using the permutational analysis of variance (PERMANOVA) [55], [56] from the Vegan package [50], [51]. The PERMANOVA is a non-parametric multivariate statistical test that also uses the (dis)similarity matrix created with the Bray-Curtis method to test for differences. Unlike ANOSIM, PERMANOVA was able to calculate differences between object classes instead of only between objects [54].

Furthermore, the dataset is analysed for indicator species using the indicspecies package [57], [58] in R. Indicator species are used to assess the quality of an ecosystem [13], [59], these species provided us with information on the status of the sampling locations since they are known to react to environmental changes. For future studies, comparing the indicator species from the morphological dataset with the metabarcoding dataset would also be interesting. If different indicator species are identified, this might imply different patterns found using metabarcoding compared to morphological identification.

4.2 DNA metabarcoding

4.2.1 Reference Database

To genetically identify the organisms in the bulk samples provided by Klunder *et al.* (2019, NIOZ, unpublished data), it is important to have a complete and reliable reference DNA database [22], [32], [60]. The most commonly used databases for the CO1 marker such as Barcode Of Life Database and Genbank [61], [62] are extensive but have been found to be incomplete [63]. To create a complete reference database, non-characteristic body fragments of morphologically identified organisms (provided by Klunder *et al.*) will be subsampled, DNA will be extracted, amplified, and sequenced.

From the fragments, DNA will be extracted using the KingFisher extraction robot as described in Vooijs (2020). The 'Machery_Nagel_Tissue96 KingFisher Flex' program will be applied in combination with the NucleoMag Tissue kit. The Dropsense 96 (Trinan) will be used to determine the concentration of the DNA and the DNA will be diluted to a concentration between 2-30ng/uL. The primers jgLCO1490 and jgHCO2198 [24], with an M13 tail for sequencing, will be used to amplify the CO1 region using PCR. The PCR product will be tested for a successful amplification on an E-gel 96 Agarose Gel (2%). When successfully amplified, it will be sent to Baseclear for Sanger-sequencing.

4.2.2 Metabarcoding protocol

The sediment samples were provided in freeze dried state and will be grinded before DNA extraction. The DNA will be extracted in triplo using the Powersoil Kingfisher kit and the Kingfisher extraction robot, according to the provided protocol. This method was shown to be successful by previous DNA metabarcoding studies performed by NBC (unpublished data). The DNA will be eluted and cleaned using the OneStep-96^{tm} PCR Inhibitor Removal kit. The three replicates will be pooled, and the DNA concentration will be measured using the Dropsense 96.

The next primers will be used: mlCOIintFNXT (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC 3') [21] and jhHCO2198NXT (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTANACYTCNGGRTGNCCRAARAAYCA 3') [24]. Both primers produced reliable results in previous studies performed by the NBC (unpublished data). The primers can be used with three different PCR mixes; KAPA HiFi HotStart ReadyMix, Taqman Environmental Master Mix 2.0 and Phire Hot Start II. After PCR, the results will be tested on an E-gel 96 Agarose Gel (2%).

Thus far, most metabarcoding studies preserve their samples successful in ethanol [15], [16], [22], [23], [64], [65]. Therefore, it might be preferred over freeze drying because the organisms can be preserved in the field immediately after capturing, resulting in less DNA degradation. However, based

on a literature study performed by Van der Loos & Nijland (2020) [37], the use of DESS is recommended.

4.2.3 Bioinformatics

After isolation and amplification of the CO1 marker, it will be sequenced using Next Generation Sequencing methods (e.g. Illumina). For processing the output of the illumina, bioinformatic tools and packages are developed. Depending on the parameter settings of these tools, higher or lower number of Operational Taxonomic Units (OTU) will be retrieved. OTU's are clusters of highly similar reads that can be assigned to a species using the previously discussed reference database. An example of a bioinformatic pipeline can be found in Van der Hoorn (2019) [60], and a detailed protocol for examining Illumina MiSeq metabarcoding data is written by Aylagas and Rodriguez-Ezpeleta (2016) [66].

5. Results

After processing all samples and morphologically identifying the macrobenthos, a total of 9634 organisms were found in the 306 samples, divided over 40 taxa (species or species-aggregates). 27 (67,5%) of these taxa could be identified to species level, accounting for 8550 (88,7%) of all specimen found. Species that could not be identified to species level were combined into aggregates. These aggregates were identified to genus or family level. 25 of the 40 taxa belong to the order Annelida (62,5%), accounting for 71,2% of the total number of organisms found. 8 out of the 40 taxa are Arthropoda (20%), accounting for 28,1% of the total number of organisms found and 7 are Mollusca (17,5%), accounting for 0.7% of the total number of organisms found. The complete species list can be found in appendix I.

5.1 Impact on *Arenicola marina* abundance

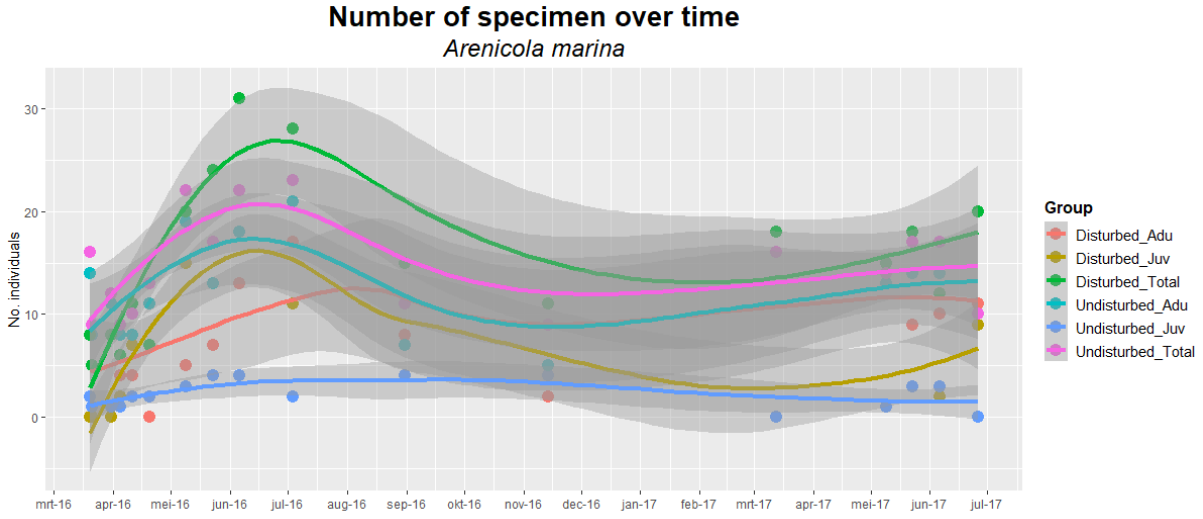

Figure 2: The number of A. marina *over time for adults (adu), juveniles (juv) and total number of* A. marina *for both disturbed and undisturbed areas. The disturbed situation has been mechanically harvested for* A. marina.

Numbers of adult and juvenile *A. marina* in undisturbed areas peak in late spring and summer (figure 2). *A. marina* in disturbed areas follow a similar but delayed pattern compared to adult *A. marina* in undisturbed areas. Remarkably, throughout the entire sampling period, the number of juveniles in undisturbed areas is lower than juveniles in disturbed areas. Especially during the summer all three disturbed transects show increased numbers of juveniles, whereas the trend of juvenile numbers in undisturbed transects is constant (figure 3). However, the difference in juvenile numbers between the disturbed and undisturbed areas for the entire sampling period was just not significant (W = 194, p-value = 0.08869).

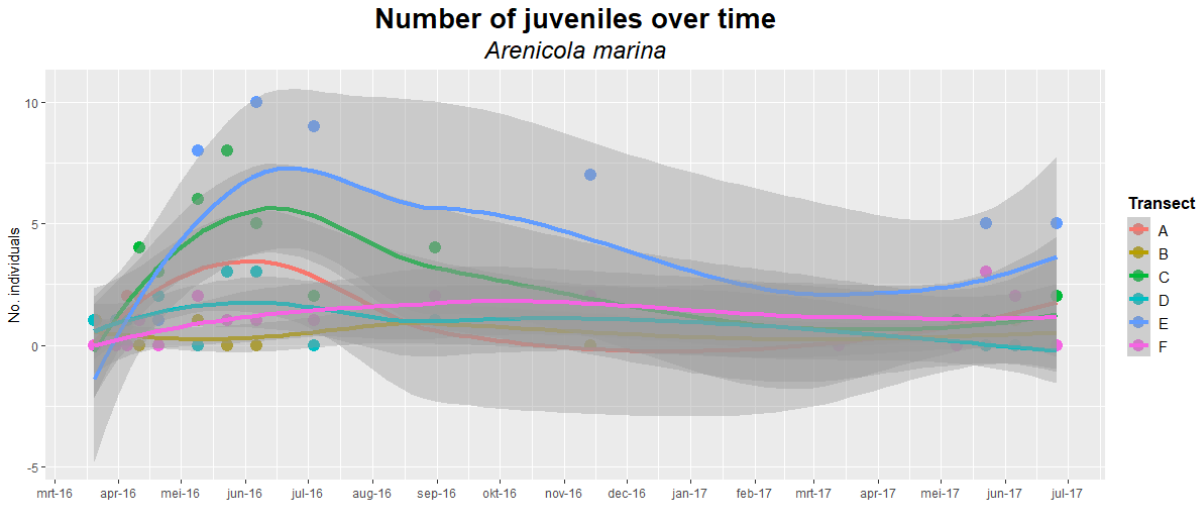

Figure 3: The number of juveniles of A. marina *over time per transect. Transect A, C and E are disturbed. Transects B, D and F are undisturbed. The numbers of juveniles in undisturbed areas are constantly low, whereas the disturbed transects show an increase in numbers during the summer months of the first year.*

5.2 Macrobenthos biodiversity indices

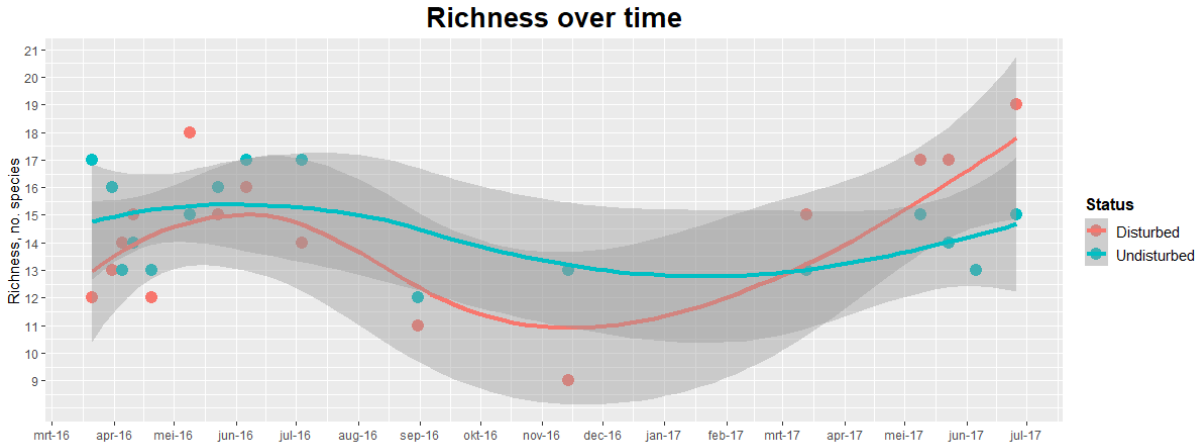

Figure 4: The richness expressed in number of species for both the disturbed and undisturbed areas plotted from timepoint 1, after mechanically harvesting A. marina.

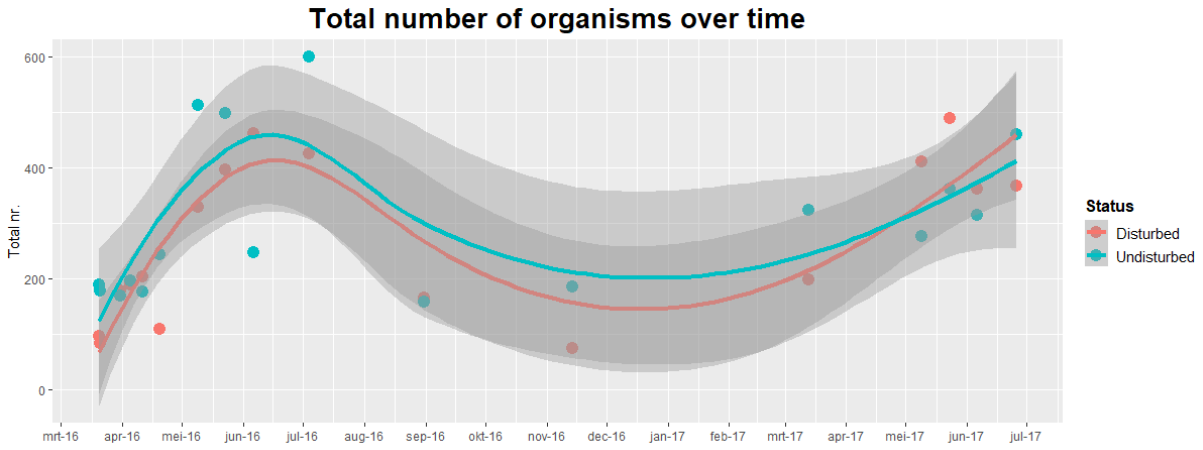

Figure 5: The total number of organisms plotted over time separated on status. The disturbed area is harvested for A. marina.

Exclusively trends are observed for the univariate diversity indices. For richness (expressed in number of species) a trend of a higher number of species is observed for the undisturbed areas in the first year after harvesting *A. marina* (figure 4). However, for the entire sampling period there is no significant difference found (t(30)=-0.24, P=0.81) between the richness of the disturbed and undisturbed area. There is no difference observed for total number of organisms between the disturbed and undisturbed area (figure 5). And the trend observed for the Shannon index (H) indicates a strong decrease in the undisturbed area's during the winter. However, no significant difference is found between the disturbed and undisturbed area (W=130, P=0.96). When plotting the transects separately, a trend indicating that richness and H can be connected to location is observed (appendix III supplementary figure 1 & 2).

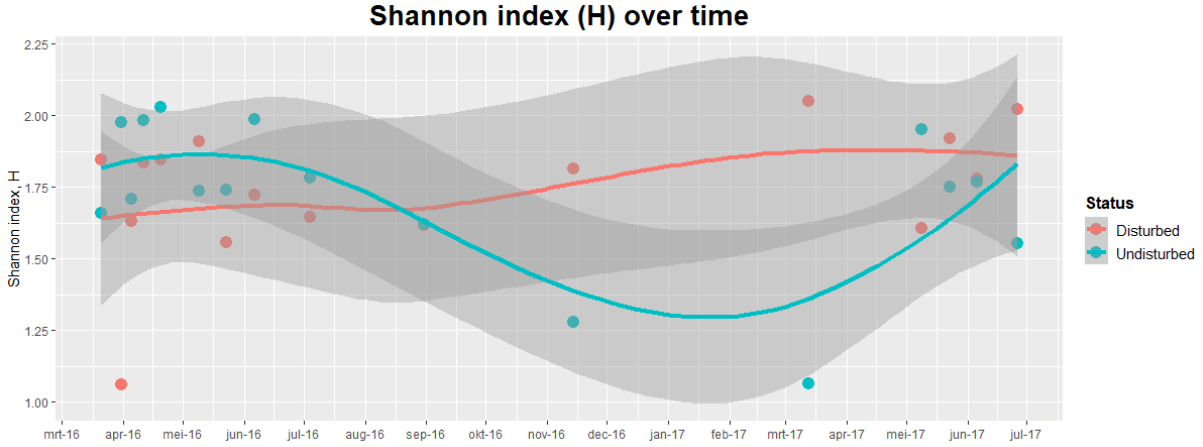

Figure 6: The Shannon index (H) expressed in number of species for both the disturbed and undisturbed areas plotted from timepoint 1, after mechanically harvesting A. marina

5.3 Impact on macrobenthic community composition

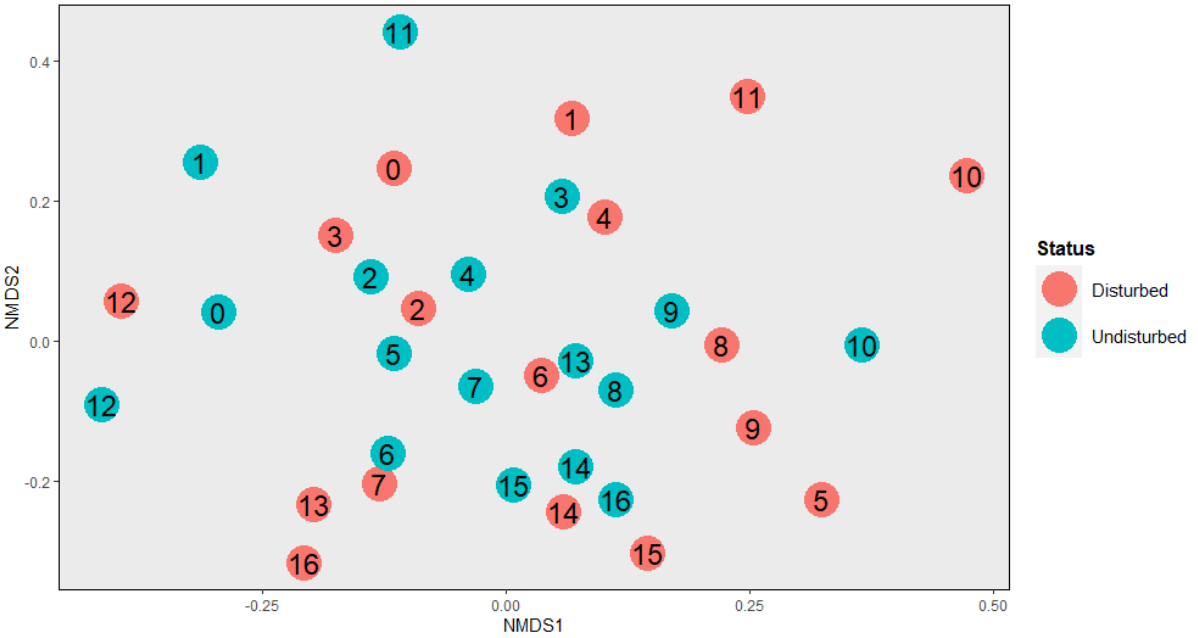

Figure 7: Non-metric multidimensional scaling of the transects mechanically harvested for A. marina *(disturbed in orange) and the control transects (undisturbed in blue). The number in the circles correspond to the timepoints, with 0 being the day before harvesting. The intervals are irregular. Stress=0.253*

No difference was found between the macrobenthos communities of disturbed and undisturbed areas (figure 7, ANOSIM R=-0.008, P=0.652). There is clustering on time and location when plotting the transects separately (appendix III, supplementary figures 3 and 4). This is confirmed by a PERMANOVA, both factors explaining respectively 7,86% (R2=0.079, P=0.001) and 21,3% (R2=0.213, P=0.001) of the variation in differences (table 1). The PERMANOVA confirms the lack of macrobenthic community difference between the disturbed and undisturbed areas (PERMANOVA R2 = 0.008, P=0.361). Both the factors time and location remain significantly explanatory for transformed and presence-absence data (table 1). Furthermore, when comparing the first three months after harvesting, there is no significant difference found between the disturbed and undisturbed areas in both untransformed and transformed data (appendix III, supplementary table 3).

*Table 1: Outcomes of the PERMANOVA for untransformed and transformed abundance data, obtained through traditional morphological identification, and presence-absence data. *=significant.*

	Status (dist./undist.)		**Time**		**Location**	
	R2	**P**	**R2**	**P**	**R2**	**P**
Untransformed data	0.00825	0.370	0.07860	0.001*	0.21316	0.001*
Transformed data (√)	0.00594	0.615	0.08446	0.001*	0.21140	0.001*
Presence-Absence data	0.00099	0.966	0.03869	0.003*	0.13546	0.001*

Pairwise analysis shows a significant community composition difference between transect A and its control site B, and between transect E and its control site F (appendix III, supplementary figure 5 and ANOSIM, table 2). Status is only explaining difference in community compositions between transect A and its control site B (PERMANOVA, table 2). Time remains an explaining factor for difference in macro benthic communities for all transects and their controls separately.

Table 2: Pairwise analysis of similarity and permutation of analysis for all transects. ANOSIM: The R is a measure for difference between the two dissimilarity matrices. In this case the R is very low, meaning small differences between the transects. P stands for p-value (significance). PERMANOVA: R2 is a measure for the partition explained by the variable. P stands for p-value (significance). The P with (*) sign indicates significance (p<0.05).

	ANOSIM		**PERMANOVA**			
			Time		**Status/Location**	
Transect pair	R	P	R2	P	R2	P
A-B	0.09671	0.037*	0.105	0.014*	0.083	0.032*
C-D	0.02345	0.2362	0.139	0.002*	0.038	0.225
E-F	0.01271	0.022*	0.121	0.004*	0.016	0.777

5.4 Indicator species

No species were found to be specific for the disturbed or undisturbed areas. Location might be a larger predictor for species, as *Urothoe poseidonis* is an indicator species for the northern transects only and the Oligochaeta species in the southern transects (table 3).

Table 3: Indicator species calculated between the transects. Stat is a measure of association of the species with the transect, higher meaning a stronger association.

Transect	**Species**	**Stat.**	**P-value**
E	*Lanice conchilega*	0.423	0.0311
D+E+F	Oligochaeta_sp	0.866	1e-04
A+B+C+D	*Urothoe poseidonis*	0.942	1e-04
A+C+D+E+F	*Heteromastum filiformis*	0.76	7e-04

6. Discussion and conclusion

The aim of this study was to assess the impact of mechanically harvesting of *Arenicola marina* on the macrobenthos communities in the Wadden Sea and to explore if metabarcoding can replace traditional morphological identification. The data were collected between March 2016 and June 2017 along six transects in the North Sea and consists of three disturbed transects with their controls.

6.1 Impact assessment

6.1.1 *Arenicola marina*

Findings of Van den Heiligenberg (1987), Beukema (1995) and Winkelman (1999) [46], [67], [68] demonstrate that only 1% of the *A. marina* population is harvested on a yearly basis in the Western Wadden Sea, hardly posing any thread to the species. This is in line with the absence of any strong trends in our data from harvested sites. Long term negative effects, such as an average lower biomass, are found in previous studies after repeatedly harvesting. These effects could not be observed in the current study as there is only harvested once.

In this study, mechanically harvesting *A. marina* was found to reduce the number of adult lugworms in the first few months after harvesting. This is in line with previous studies [67] and can be explained by the active removal of adult *A. marina*. Within four to five months the adult *A. marina* numbers are similar to the control areas again. There is a strong trend of increased juvenile numbers in the disturbed areas observed, especially in the first months after harvesting. The increase in numbers in disturbed areas has also been observed in previous studies [67], and might be explained by the biology of the lugworm. Juvenile lugworms live closer to the coast in areas with relatively low adult densities. These areas are muddy and have a relatively high detritus content, therefore they are not suitable for adult lugworms [69], [70]. When the juveniles reach an age of 1-1,5 years old, they become recruits. Between January and March these recruits migrate to the sandier areas where they occupy locations with relatively low adult densities and thus less competition [71], [72]. The harvesting took place halfway March, during the migration period of the juveniles. Therefore, the increase in juvenile densities might be explained by the recruits occupying the space left by adult lugworms that were harvested. From August until October the reproduction cycle of *A. marina* takes place. The recruits mature over summer and increase in weight until they weight as much as the adults. Once they reach a similar weight as the adults, they will be counted as adults. This might explain the decrease in juvenile lugworms during the summer months June and July [69], [70]. The separate disturbed transects start with similar numbers of juveniles compared to the undisturbed transects. When combining the transects the numbers of individuals are added, amplifying the difference between disturbed and undisturbed. Making the undisturbed area look deserted of juveniles, whereas in reality they also show small increases during spring, albeit not as strong as the disturbed area.

During the summer months of the consecutive year, the juveniles in the disturbed areas again increase in numbers. However, in this case it is not trend shared by all disturbed transects. One of the transects is causing it. A local disturbance might have taken place and in combination with lower sampling occasions during that period. During these months, the *A. marina* adult numbers were stagnating. This is very likely caused by natural variation in population numbers as *A. marina* population composition and numbers can differ substantially from year to year [72], [73].

6.1.2 Macrobenthos

In this study, no significant difference between the macrobenthic communities of disturbed and undisturbed areas was found. Previous studies have demonstrated that mechanically harvesting *A. marina* can both cause a decrease in abundance and an increase in richness (expressed in number of species). Harvesting *A. marina* can cause a reduction in numbers of at least the two species *Mya*

areniaria and *Heteromastus filimormis* [68]. Findings from Cadée (1977) [67] show that the latter one suffered a reduction of 85%. This reduction might in turn negatively affect the species predators. Van den Heiligenberg (1987)[67] found that richness increased after harvesting, due to a fast immigration of species like *Macoma balthica* and *Scoloplos armiger.* Furthermore, they observed a larger recruitment of juveniles of the species *A. marina, Nereis diversicolor, Heteromastum filiformis* and *Scoloplos arminger* in disturbed areas compared to undisturbed areas. The observed trends in this study are line with these previous findings. The studies mentioned in this paragraph were performed before the newly implemented legislation in 1990. This legislation is aiming to reduce the effects of mechanically harvesting *A. marina* on macrobenthos and making it more sustainable [46]. Therefore, the negative trends found after mechanically harvesting *A. marina* are likely to be less strong than those mentioned in the studies performed before 1990.

A low Shannon index (H) was expected for the disturbed areas as environmental stress is known to result in less complex communities [10]. However, the trend found for H shows a higher value for disturbed areas throughout the winter months after harvesting. A possible explanation could be that less species are living in the disturbed transects, but those remaining are evenly distributed. For example *M. balthica* and *Hydrobia ulvae* are remaining species which increase in numbers after harvesting [68], [74]. This could be due to reduced survival or reduction in numbers of its competitors after harvesting *A. marina*. The other remaining species might show a similar pattern.

The impact of mechanically harvesting *A. marina* did not have a significant effect on macrobenthic communities. However, the results indicate two other factors affecting community composition. The first factor explaining macrobenthic community difference is time. Communities show natural dynamic behaviour due to seasonal and annual variation. This includes long-term cycles in total abundance and biomass differences, and population variation [73], [75], [76]. Macrobenthos abundance in this study does not differ substantially between the disturbed and undisturbed area. The observed trends in abundance follow a seasonal pattern with higher mortality (or migration) during winter compared to the spring and summer. The higher macrobenthos abundance in the second year at comparable points in time might imply a mild winter [77], but is more likely to be caused by the applied smooth curve in combination with a low number of sampling occasions.

The second factor explaining macrobenthos community differences is location. Macrobenthic species are not homogeneously distributed in the Wadden Sea, as species distribution is highly related to sediment composition [73], [78]. Sediment composition is inextricable from tidal zone, elevation, and tidal currents [76], [77]. As transect combinations are approximately 200 meters apart, the sediment can differ substantially. This is supported by the richness and Shannon index patterns of the separate disturbed transect with their undisturbed. Transect pairs (joining disturbed and undisturbed transects) follow more similar patterns than transects belonging to the same impact. The difference in location between transects is also supported by the indicator species analysis. The outcome implies a gradient in species composition as adjacent transects share indicator species. However, none of these indicator species are specific for disturbed or undisturbed areas. It is recommended to collect information on the sediment composition, which has not been done in this study. It might be possible to compare transects based on their sediment similarity, rather than combine them based on treatment. Since the strong effect of location on macrobenthos community composition has been shown in this study.

Pairwise analysis (ANOSIM) between the transect of a pair implies a significant macrobenthic community difference between transects A-B and E-F. However, this is nullified by the low R. A low R implies as much statistical dissimilarity within groups as between groups. Only the transect combination A and B show significant influence of location on species composition when applying a PERMANOVA. However, transect B is a very deviant transect, compared to the other transects. It has similar species richness but shows an increased trend in macrobenthos abundance and decreased

trend of H over winter. Suggesting a strong increase of a few specific species. When looking at the raw dataset, high peaks of *Urothoe poseidonis,* might explain this phenomenon. This shrimp is found to be inhabiting burrows of *A. marina* in large numbers [79] in the Wadden Sea in Denmark. This implies a close association between those two species. However, this cannot be concluded from our data. *A. marina* numbers are relatively low in transect B.

The lack of impact found in this assessment does not exclude an effect of mechanically harvesting *A. marina*. Effects might be clearer on individual species level. Van den Heiligenberg (1987) [67] observed a severe short term impact of mechanical harvesting *A. marina* on other macrobenthic animals. He sampled several species individually over a time span of 180 days for both biomass and numbers in disturbed areas and corresponding undisturbed areas at 4-10m distance of the disturbed area. He observed strong differences between disturbed and undisturbed areas in terms of species abundance and total biomass. To compare the results, the same species as Van den Heiligenberg (1987) should be extracted from our data and analysed for patterns. This could also lead to interesting conclusions regarding the effectiveness of the improved and more sustainable legislation for lugworm harvesting since 1990[46] as Van den Heiligenberg (1987) studied the effects before the new legislations.

Harvesting frequency might be an explanation for the negative effects found in other studies. The dataset used for this study is collected in an area impacted once in a time span of 15 months. Whereas the areas allocated for harvesting *A. marina* in the Wadden Sea*,* are impacted yearly during the timespan of a few months. For example, Tulp et al. (2020) [80] studied the effects of brown shrimp (Crangon crangon) trawling on macrobenthic communities and showed negative effects of increased fishing pressure on macro benthic communities. Despite the difference in fishing method, they emphasize the effect of increased harvesting frequency. Neto *et al. (2010)* found that continuous impact gradually decreases ecosystems, whereas short impacts might show stronger effects, but allow for a faster recovery of macrobenthic communities[81]. Furthermore, there might be indirect effects of an increased harvesting frequencies such as sediment homogenization [82]. Due to repeatedly turning the seabed over, the sediment layers mix and can negatively affect the settlement of larvae of macrobenthos species.

To evaluate whether mechanically harvesting *A. marina* negatively affects the macrobenthic communities using the BACI method [48], it would be highly recommended to collect more samples before the harvesting event [83]. In this study there is information available of only one sampling occasion before harvesting. Hence, it is difficult to differ between natural dynamics and effects of harvesting. Moreover, Beukema (1995) and Smokorowski & Randall (2017) [68], [83] emphasize the long term effects of disturbance, especially on species with a life-cycle longer than the current 15 months of observation. Furthermore, the data is gathered irregularly over the 15-month time span. The interval between two sampling moments varies from days to months. This could have influenced the observed trends, as substantial variation between two close sampling moments is seen in the first few months. This information is lost towards the end of the time span when there are months between two consecutive sampling moments.

6.2 DNA Metabarcoding

DNA metabarcoding data, consisting of presence-absence data, could not have created the same insight in the effects of harvesting on the population of *A. marina* as morphological identified abundance information. The recovery of adults in harvested areas, with its accompanying recruitment of juveniles, would not have been visible, since age cannot be determined using metabarcoding. Instead, it would have only detected a higher number of *A. marina* sequencing reads in the disturbed area, implying a positive effect of harvesting on the presence of *A. marina*. Whereas this study shows

that this higher number of reads is likely due to a combination of increased recruitment and an amplified effect of combining multiple transects.

The impact assessment would have likely led to similar conclusions. Both ANOSIM and PERMANOVA produce comparable results using presence-absence data. All variables were significant or non-significant according to the same pattern as the abundance data. This is in line with promising outcomes from studies performed by Aylagas *et al.* (2018) and Lejzerowicsz *et al.* (2015) [23], [60], [84]. They found positive correlations between the metabarcoding derived biotic indices and their morphology based biotic indices (the AZTI Marine Biotic indices and the Infaunal Thropic Index). Which leads, according to Aylagas *et al.* (2018), to comparable biomonitoring conclusions for metabarcoding based approaches and morphological based methods. Cahill *et al.* (2018) [22] conclude from their comparative analysis that metabarcoding using the CO1 gene is very promising, but thus far only supplementary to a morphological analysis. They were only able to classify a part of their species found using metabarcoding. Nonetheless, they found comparable diversity and composition metrics for both methods [22]. Furthermore, Guardiola *et al.* (2016) [85] characterised deep-sea sediment communities using a DNA metabarcoding approach and successfully detected spatial patterns. Based on their findings, a strong link between location and macrobenthic community composition is expected to be found using a DNA metabarcoding approach. Similar to the link found using the morphological identified dataset.

It will not be possible to calculate all univariate diversity indices for macrobenthos communities using DNA metabarcoding data. Because the univariate diversity indices applied in this study, are calculated using the species richness and abundance [86], [87]. Thus far quantification for bulk samples is not possible [22]. However, these univariate statistics are exploratory and indicate differences between communities. They are generally not appropriate to calculate significant differences between communities handling a multi-species matrix [86]. Furthermore, the NMDS patterns will change when they are based on presence-absence information. In this study community compositions are relatively similar, making it difficult to demonstrate differences between them using the ordination method. Without abundance information, communities become more similar and small differences between sites will be stressed. Nonetheless, with larger differences between the communities or sites, similar clustering patterns are expected for both types of data.

Based on studies by Aylagas *et al.* (2018) [23] and Lobo *et al.* (2017) [15], one might expect to identify less species using DNA metabarcoding compared to the morphological identification. Aylagas *et al.* (2018) identified 0-66,6% (average 20%) species from bulk samples of known composition using the CO1 marker. Lobo *et al.* (2017) was able to identify 78-83% of the species in known bulk samples using multiple primer pairs for the CO1 marker. However, only 88,7% of the specimen provided by Klunder *et al.* (2019, NIOZ, unpublished data) could be morphologically identified up to species level. Therefore, it is expected that using a DNA metabarcoding approach to identify the organisms in the samples, will result in a different species list. Because DNA metabarcoding might not recover all morphological identified species, it will be able to identify at least part of the 11,3% unidentified organisms. Which are accounting for 32,5% of the total number of found species. Furthermore, there might be a possibility that metabarcoding recovers DNA of small organisms from the guts larger species, resulting in a higher total number of species using DNA metabarcoding [22]. Therefore, it is highly recommended to repeat the analysis performed in this study using the species list resulting from the real DNA metabarcoding data. Until this effect is studied more elaborately, it recommended to use DNA metabarcoding approaches as a complementary method to the traditional methods.

6.3 Conclusion

In this study an impact assessment is performed to assess the effects of mechanically harvesting *Arenicola marina* on macrobenthos communities. This was accomplished by comparing communities from sample stations along three disturbed and three undisturbed transects. Furthermore, the traditional morphological identified, quantitative, data was replaced by DNA metabarcoding data (presence-absence). The effect of this replacement on the outcomes of this impact assessment were studied.

Based on the impact assessment no significant effect of mechanically harvesting *A. marina* on the macrobenthic communities was found, the communities recover within months. Strongly influencing the macrobenthos communities are the factors time and location. The effect of time is likely due to natural variation in populations over time and season. The heterogeneously distributed sediment of the Wadden Sea could be and explanation for the effect of location. Sediment composition is inextricably connected to elevation and tidal currents and determines the macrobenthic community composition.

When replacing the abundance data with presence-absence data the same factors remain of significant or insignificant influence on macrobenthic community composition. This implies that, although population information such as age ratios are lost, DNA metabarcoding data might be sufficient to draw comparable conclusions. However, the identified species composition is expected to change when using real DNA metabarcoding data.

DNA metabarcoding might replace traditional morphological identification of macrobenthos in the future. Under the current biomonitoring management programs, the conclusions drawn from DNA metabarcoding based approaches are already comparable to those drawn from traditional identification methods. However, the effects of using DNA metabarcoding data rather than morphological identified data on the outcome of biomonitoring analysis remains to be tested more elaborately. Therefore, it is recommended to use DNA metabarcoding approaches complementary to the traditional methods until the consequences of using presence-absence data instead of quantitative data are better understood.

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9. Appendices

Appendix I: Full Species list

The full species list is composed by Klunder *et al.* (2019, NIOZ, unpublished data). Macrobenthos specimen from the samples were morphologically identified. Some organisms could not be identified to species name, they were included as aggregates and can be recognised by '_sp' (supplementary table 1).

Supplementary table 1: Full species list based on morphological identification. The list is composed by Klunder et al. *(2019, NIOZ, unpublished data).*

Phylum	Class	Order	Family	Genus	Species
Annelida	Haplotaxida	Oligochaeta	-	-	Oligochaeta_sp
Annelida	Polychaeta	Phyllodocida	Glyceridae	Glycera	Glycera_sp
Annelida	Polychaeta	Phyllodocida	Nephtyidae	Nephtys	Nephtys hombergii
Annelida	Polychaeta	Phyllodocida	Nereididae	Hediste	Hediste diversicolor
Annelida	Polychaeta	Phyllodocida	Nereididae	-	Nereididae_sp
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	Eteone	Eteone longa
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	Phyllodoce	Phyllodoce mucosa
Annelida	Polychaeta	Phyllodocida	Polynoidae	Bylgides	Bylgides sarsi
Annelida	Polychaeta	Phyllodocida	Polynoidae	Malmgrenia	Malmgrenia darbouxi
Annelida	Polychaeta	Phyllodocida	Polynoidae	-	Polynoidae_sp
Annelida	Polychaeta	Scolecida	Arenicolidae	Arenicola	Arenicola_marine_adult
Annelida	Polychaeta	Scolecida	Arenicolidae	Arenicola	Arenicola_marine_juvenile
Annelida	Polychaeta	Scolecida	Capitellidae	Capitella	Capitella capitata
Annelida	Polychaeta	Scolecida	Capitellidae	Heteromastus	Heteromastus filiformis
Annelida	Polychaeta	Scolecida	Orbiniidae	Scoloplos	Scoloplos armiger
Annelida	Polychaeta	Scolecida	Paraonidae	Aricidea	Aricidea minuta
Annelida	Polychaeta	Spionida	Spionidae	Marenzelleria	Marenzelleria viridis
Annelida	Polychaeta	Spionida	Spionidae	Polydora	Polydora cornuta
Annelida	Polychaeta	Spionida	Spionidae	Pygospio	Pygospio elegans
Annelida	Polychaeta	Spionida	Spionidae	Spio	Spio_sp
Annelida	Polychaeta	Spionida	Spionidae	Spiophanes	Spiophanes bombyx
Annelida	Polychaeta	Spionida	Spionidae	-	Spionidae_sp
Annelida	Polychaeta	Terebellida	Cirratulidae	Aphelochaeta	Aphelochaeta marioni
Annelida	Polychaeta	Terebellida	Terebellidae	Lanice	Lanice conchilega
Annelida	Polychaeta	-	Magelonidae	Magelona	Magelona_sp
Arthropoda	Malacostraca	Amphipoda	Bathyporiidae	Bathyporeia	Bathyporeia saris
Arthropoda	Malacostraca	Amphipoda	Gammaridae	Gammarus	Gammarus_sp
Arthropoda	Malacostraca	Amphipoda	Urothoidae	Urothoe	Urothoe poseidonis
Arthropoda	Malacostraca	Cumacea	-	-	Cumacea_sp
Arthropoda	Malacostraca	Decapoda	Brachyura	-	Brachyura_sp
Arthropoda	Malacostraca	Decapoda	Carcinidae	Carcinus	Carcinus maenas
Arthropoda	Malacostraca	Decapoda	Crangonidae	Crangon	Crangon crangon
Arthropoda	Malacostraca	Mysida	-	-	Mysida_sp
Mollusca	Bivalvia	Adapedonta	Pharidae	Ensis	Ensis directus
Mollusca	Bivalvia	Cardiida	Cardiidae	Cerastoderma	Cerastoderma edule
Mollusca	Bivalvia	Cardiida	Tellinidae	Limecola	Limecola balthica

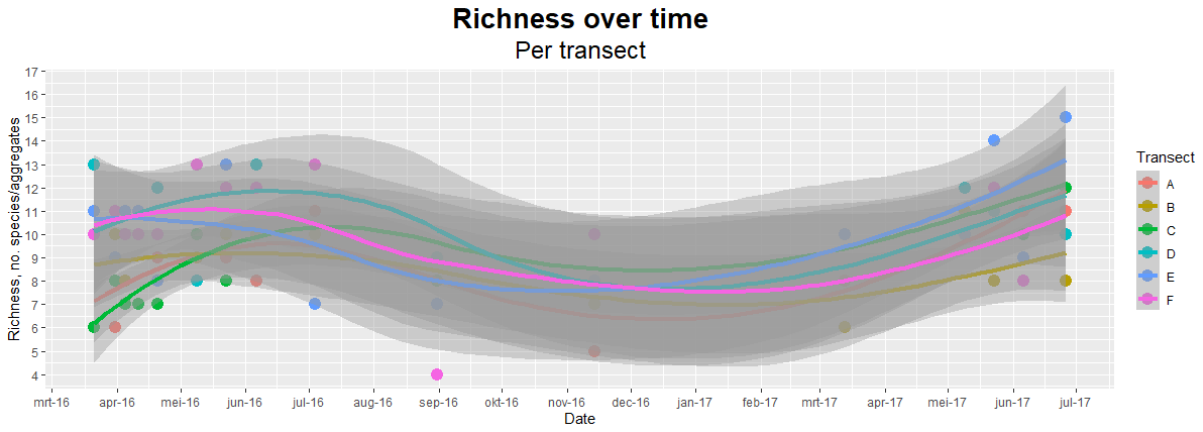
Mollusca	Bivalvia	Cardiida	Tellinidae	Tellina	Tellina_sp
Mollusca	Bivalvia	Galeommatida	Lasaeidae	Kurtiella	Kurtiella bidentata
Mollusca	Bivalvia	Myida	Myidae	Mya	Mya arenaria
Mollusca	Gastropoda	Littorinimorpha	Hydrobiidae	Peringia	Peringia ulvae

Appendix II: Sampling locations and information

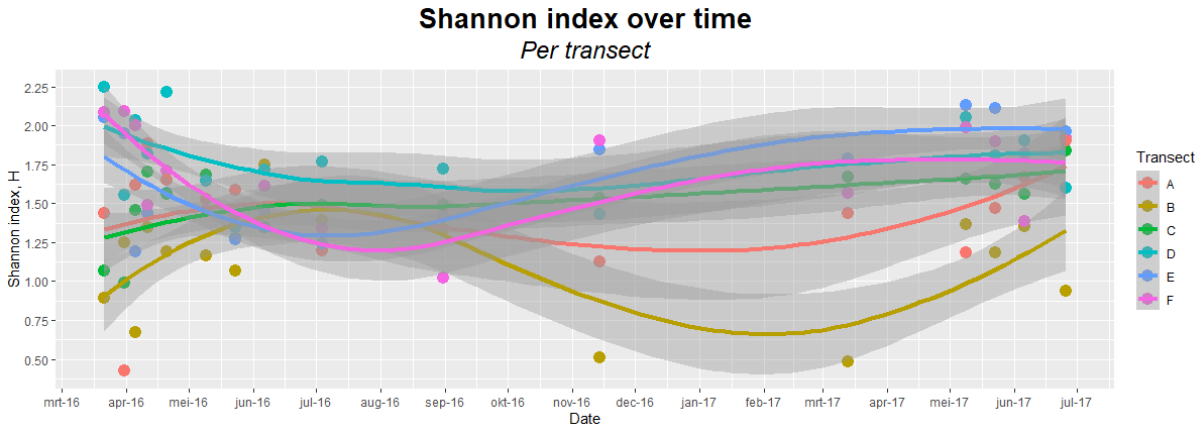
The six sampling locations along the transects differed between two sampling occasions. Each exact location was sampled once, to exclude the effect of sampling on the impact assessment. The coordinates of each sampling occasion can be found in 'AppendixV_datasheets_Lvs.xlsx', in the sheet 'Coordinates_sampling_locations'.

Appendix III: Supplementary results

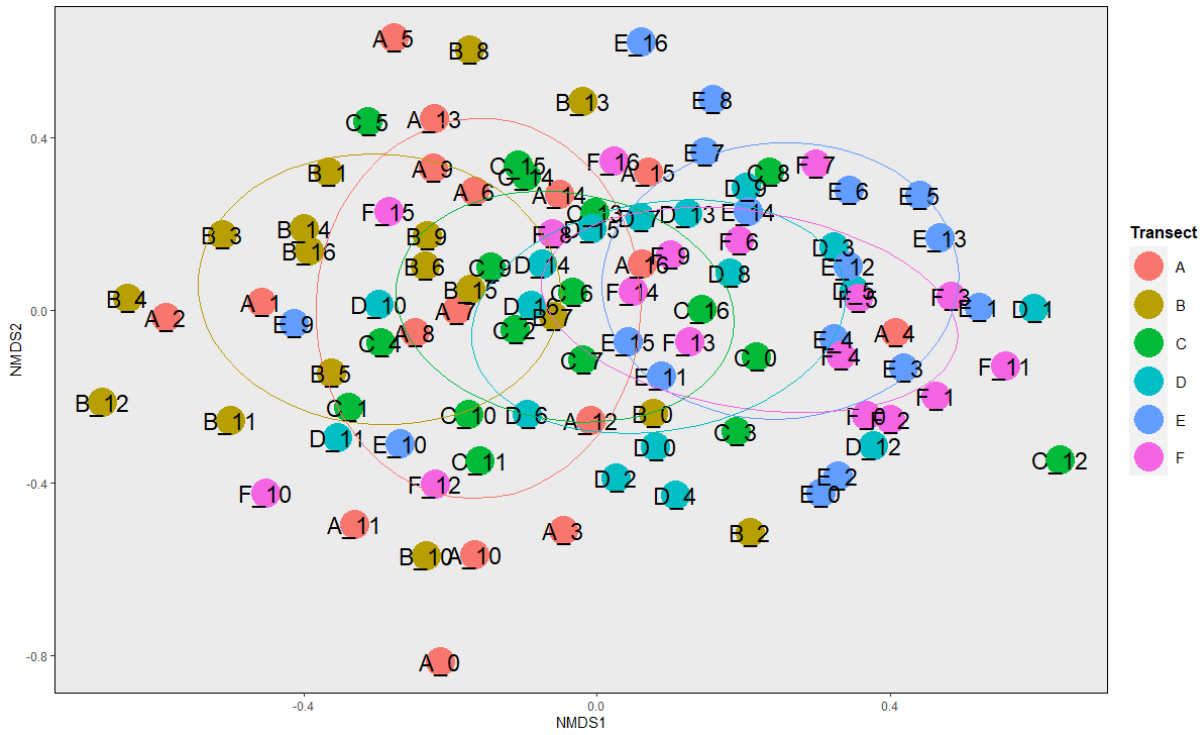
This appendix contains supplementary results. The figures include univariate diversity indices such as richness (expressed in number of species) and the Shannon index (supplementary figures 1 &2). The NMDS coloured on transect and time (supplementary figures 3&4), a pairwise NMDS (supplementary figure 5, supplementary table 2) and the ANOSIM results for the first three months after harvesting *A. marina* (supplementary table 3). Transec



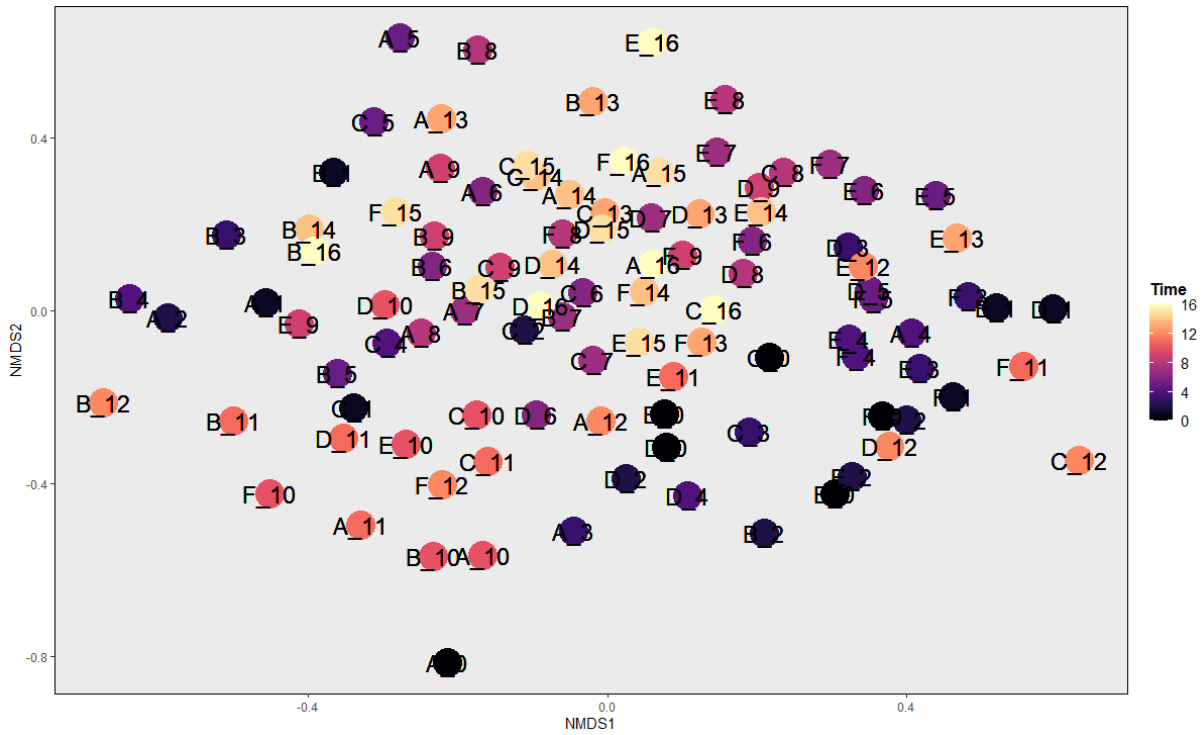
Supplementary figure 1: Richness per transect, each disturbed transect (A, C and E) follows a similar pattern as its paired undisturbed transect (B, D and F).



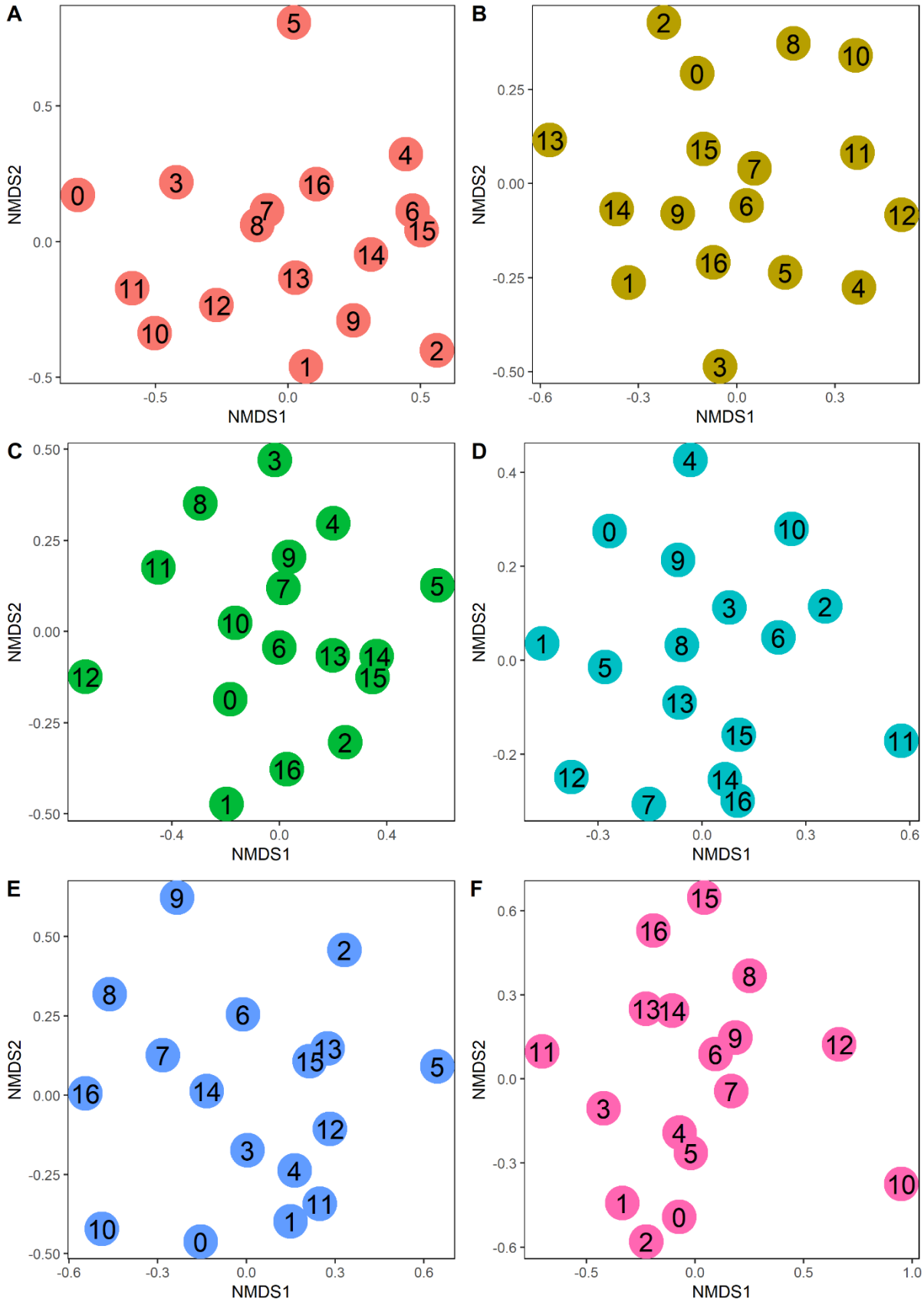
Supplementary figure 2: Shannon index per transect, each disturbed transect (A, C and E) follows a similar pattern as its paired undisturbed transect (B, D and F).



Supplementary figure 3: Per transect, all 17 sampling occasions are plotted in this NMDS. The plot is coloured on transect and includes ellipses (level=0.5), to visualise the different species compositions between the transects. Stress = 0.2903.



Supplementary figure 4: Per transect, all 17 sampling occasions are plotted in this NMDS. The plot is coloured on time. The plot is implying a larger difference in species composition in the beginning, the darkest spots are furthest apart. However, over time they move closer together, implying a smaller difference in species composition. The datapoints cluster on time. Stress =0.2903.



Supplementary figure 5: Non-metric multidimensional scaling for all six transects separately. Along the transects A, C, E is harvested for A. marina, transects B, D and F are undisturbed. *No clear conclusions can be drawn from these graphs.*

Supplementary table 2: Summary of the non-metric multidimensional scaling per transect in supplementary figure 5.

Transect	**Nr. of runs solution reached**	**Stress**
A	20	0.151
B	171	0.220
C	47	0.219
D	20	0.214
E	81	0.214
F	20	0.150

Supplementary table 3: There are no significant differences between the disturbed and the undisturbed areas for the for the entire sampling period and the first three months after harvesting A. marina *(ANOSIM).*

	Untransformed data	Transformed data (√)	Presence-Absence data
Entire sampling period	R=-0.00762 P= 0.6515	R=-0.01045 P=0.7523	R=-0.01664 P=0.91
First three months	R=-0.00156 P= 0.4164	R=0.01111 P=0.2904	R=0.01938 P=0.228

Appendix IV: Timepoints and their exact data

The intervals between sampling occasions are irregular (supplementary table 4). There is one sampling occasion before harvesting *A. marina*, T0, on the 20th of March 2016. And there are 16 occasions after harvesting between 21-03-2016 and 26-06-2017. In the first few months after harvesting, there is a sampling occasion every few weeks, and towards the winter this interval prolongs towards months.

Supplementary table 4: Exact dates of sampling occasions with their corresponding time code.

T_code	Date
T0	20-3-2016
T1	21-3-2016
T2	31-3-2016
T3	5-4-2016
T4	11-4-2016
T5	20-4-2016
T6	9-5-2016
T7	23-5-2016
T8	6-6-2016
T9	4-7-2016
T10	31-8-2016
T11	14-11-2016
T12	13-3-2017
T13	9-5-2017
T14	23-5-2017
T15	6-6-2017
T16	26-6-2017

Appendix V: R-script and datafiles

The R-script for the impact analysis, containing all summarized scripts, can be found in the additional file called 'AppendixV_impact_assessment_complete_Lvs.R'. The datasheets for the impact assessment can be found in 'AppendixV_datasheets_Lvs.xlsx'. They are named 'Arenicola_Data_R', 'Arenicola_location_R', 'Arenicola_Taxonomy_R' and 'Arenicola_Time_R'. Make sure to save the separate datasheets as .csv format before usage.

The script is subdivided into chapters similarly named as the chapters in the results section. After importing the data, variables and datasheets are reoccurring throughout the entire analysis. Therefore, it is important to check the previous chapter for data manipulation if the console shows an error message.