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Stress resistance for unraveling potential biopollutants. Insights from ballast water community analysis through DNA

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Abstract

In marine settings, anthropogenic disturbances and climate change increase the rate of biological invasions. Predicting still undescribed invasive alien species (IAS) is needed for preparing timely management responses. We tested a strategy for discovering new potential IAS using DNA in a trans-equatorial expedition onboard RV Polarstern. During one-month travel, species inside ballast water experienced oxygen depletion, warming, darkness and ammonium stress. Many organisms died but several phytoplankton and zooplankton survivors resisted and were detected through a robust combination of individual sampling, DNA barcoding and metabarcoding, new in ballast water studies. Ammonium was identified as an important influential factor to explain diversity changes in phytoplankton and zooplankton. Some species reproduced until the end of the travel. These species tolerant to travel stress could be targeted as potential IAS and prioritized for designing control measures. Introducing resistance to travel stress in biosecurity risk analysis would be recommended.

Key words: Ballast water; DNA barcoding; Invasive species; IAS prediction; Metabarcoding.

1. Introduction

Chan and Brisky (2017) highlighted the need of more research efforts for understanding and preventing marine biological invasions, since the majority of studies are conducted in terrestrial ecosystems. In marine settings the identification of potential invasive species is becoming urgent (e.g. Della Venezia and Leung, 2020), for planning early detection tools and apply different management measures (Giakoumi et al., 2019). Introductions of biological pollutants i.e. non-indigenous and invasive species - invasive alien species (IAS), are often the result of small-scale events like ballast water discharges, but adding up all those events across the world it becomes a wide-scale problem in seas and estuaries (Elliott, 2003). Many of those IAS species are cryptic for their biology, scarcity or appearance, and remain undetected and undescribed; to know their real proportion is the first outstanding question that should guide future research efforts (Jarić et al., 2019). This study aims at providing a strategic framework for answering that question, unraveling undescribed (potential) IAS considering their survival capacity to hard environmental changes, and undetected species through DNA.

Different types of environmental disturbances (Osborne & Poynton, 2019; Novoa et al., 2020), including climate warming (Hulme, 2017), increase the rate of biological pollutants introductions in marine areas. This happens because successful IAS typically exhibit a high tolerance to environmental stress (Lenz et al., 2011), being able to adapt to wide thermal and salinity ranges (e.g. Bates et al., 2013; Bollen et al., 2016; Galil et al., 2019). Grounded in the idea "the more tolerant the more invasive", we develop here a proof of concept for exploration of potential marine IAS based on their resistance to long travels.

Ballast water (BW thereafter) i.e. water loaded in ballast tanks for stabilizing cargos, is a main vector of aquatic biological invasions worldwide (Carlton and Geller, 1993; Molnar et al., 2008). Plankton communities are taken with the water; inside the tank they experience abiotic changes that are common on any voyage, like light deprivation. In a voyage that crosses latitudes, other factors will vary rapidly: temperature oscillations up to 18°C or more, and associated oxygen depletion or oversaturation in a few weeks (Gollasch et al., 2000; Zaiko et al., 2015). A number of species survives: BW-associated invasions have been reported worldwide (Bax et al., 2003; Molnar et al., 2008). Miralles et al. (2018) suggested selection for wider tolerance ranges as a cause of high rate of introduction of Antipode species to Europe. Although BW seems to be a non-selective transport vector (Carlton and Geller, 1993), resistant species within different phylogenetic and ecological groups will be selected in long travels (Miralles et al., 2018). More functional studies of BW communities are needed (Darling et al., 2018); we need to understand the resistance of each functional group to voyage-associated environmental stress, for several reasons. First, travel stress seems to be associated with invasiveness capacity (Ardura et al., 2018). Second, because exotics of different trophic groups may impact differently on native species (Anton et al., 2019). Darling et al. (2018) and others identified BW-transported species of concern based on their known history of invasiveness and impacts, but to our knowledge differential resistance to travel stress has not been taken into account yet for IAS identification.

In this proof of concept we have analyzed the plankton community of a real ship ballast tank during a long trans-Equatorial voyage. Studies of BW arriving in different ports generally report more zooplankton than phytoplankton taxa (Carlton and Geller, 1993; Smith et al., 1999; Zaiko et al., 2015); thus we expected a higher risk of heterotrophic

(versus autotrophic) potential IAS. Species were detected using a combination of NGS metabarcoding, visual survey (recognizing species from their morphology) and classic DNA barcoding.

2. Material and methods

2.1. Experimental BW

Data were obtained during RV Polarstern PS102 expedition Bremerhaven (12 November 2016) - Cape Town (12 December 2016), crossing the Equator on the 16th travel day. The route and cruise details are described in Wiltshire et al. (2017), see Supplementary Table 1. Ballast tank #7 (aft tank, 70m^3) was filled off Bremerhaven port, water temperature was 9ºC and salinity 36.6psu. Untreated during the trip, BW was treated after the experiment following German regulations.

2.2. Work at sea: samples and environmental data

Abiotic parameters were monitored with YSI Professional Plus Multimeter at 13:45 daily: pH, salinity, oxygen concentration, temperature, ammonium. On even days (#2 to #28; Suppl. Table 1) BW samples were taken via the sounding pipe from approximately 1.5 m depth, through a build-in ballast pump (operational pressure up to 6 bar, loading capacity ca. 20L/min). Three 2L samples of ballast water were collected per day and vacuum-filtered through a 0.2µm Nucleopore^{TM} membrane, then preserved in 96% ethanol until eDNA extraction.

For individual biota analysis, 100L were filtered through a 20µm mesh plankton net, concentrated in 50ml. Organisms visible at 50x magnification, moving or at least integer and normally coloured if immobile –assumed to be viable or recently alive- were counted and identified *de visu* down to the lowest taxonomic level possible with taxonomic guides. They were sorted by taxon, some individuals were picked each sampling day and stored in 96% ethanol for DNA barcoding.

2.3. DNA Barcoding

Total DNA was extracted using silica gel columns (QIAmp DNA Mini Kit, Qiagen). Aliquots were frozen at -20ºC for long-time preservation. A 400-600 nucleotide (bp) fragment within the nuclear small subunit ribosomal DNA (18S rRNA gene, 18S thereafter) was amplified by polymerase chain reaction (PCR) with Uni18SF and Uni18SR primers (Zhan et al., 2013). PCR was performed in a total volume of 20µl with Promega (Madison, WI) Buffer 1x, 2.5mM MgCl2, 0.25mM dNTPs, 20pmol of each primer, 20ng of template DNA, 1U of DNA Taq polymerase (Promega), and the following conditions: initial denaturing at 95ºC for 5 min, 25 cycles of denaturing at 95ºC for 30s, annealing at 50ºC for 30s, extension at 72ºC for 90s, final elongation at 72ºC for 10 min.

In phytoplankton a sequence of 312 bp within the chloroplast gene RuBisCo (rbcL) was amplified using Rivera et al. (2017) primers. PCR was performed with the mix described above and the following conditions: initial denaturing at 95ºC for 15 min, 30 cycles of denaturing at 95ºC for 45s, annealing at 55ºC for 45s, extension at 72ºC for 45s, final elongation at 72ºC for 5min.

PCR products were visualized in 2% agarose gel dyed with SimplySafe (EURx® Ltd). Purification and sequencing were performed in Macrogen (Spain). Sequences were

BLASTed against GenBank database within NCBI (https://blast.ncbi.nlm.nih.gov) using best match for species assignment, >97% identity for rbcL and >80% for 18S (Fernandez et al., 2018). Taxonomic information was checked in World Register of Marine Species (WORMS; http://www.marinespecies.org/) and AlgaeBase (http://www.algaebase.org/), and invasive status in IUCN Globally Invasive Species Database (http://www.iucngisd.org/gisd/, Pagad et al., 2015).

2.4. Environmental DNA and bioinformatics analysis

DNA was extracted from filters and pellets with PowerWater® DNA Isolation Kit (QIAGEN) under sterile conditions inside a laminar flow PCR-cabinet. Negative controls of pure water were added to monitor contamination during extraction and PCR processes. From the extracted DNA, 18S and a fragment of the mitochondrial cytochrome oxidase subunit I gene (COI) were PCR amplified using, respectively, Uni18SF and Uni18SR (Zhan et al., 2013), and m1COIintF and jgHCO2198 (Leray et al., 2013) primers. Bovine Serum Albumin (BSA) was added to increase PCR yields and avoid the effect of possible inhibitors. For multiplexing, one PGM sequencing 3´adaptor per sample and a ‘‘GAT” spacer were added to the original primers before PCR. Amplicons were purified with GeneMATRIX BASIC DNA Purification Kit (EURx® Ltd). PCR products were quantified using a Qubit 2.0 fluorimeter and double-checked in a Bioanalyser 2100 (Agilient Technologies, USA) to confirm the fragment size.

Next-generation sequencing (NGS) was performed in the Sequencing Unit of Oviedo University (Spain). Samples were diluted down to 26 pmol for preparing an equimolar pool that was processed by liquid emulsion PCR in One Touch System using Ion PGMTM Template OT2 200 Kit. Then samples were loaded in Ion ‘‘314” Chip (Life Technologies) and sequenced employing the Ion Torrent Personal Genome Machine (PGM Life Technologies) following the protocol Ion PGMTM Sequencing 200 Kit v2 for 500 flows (Life Technologies). Low quality and polyclonal sequences were filtered automatically and the PGM adaptor was trimmed within the PGM software. One ⁄. fastq file per sample (different barcode) was obtained. Reads were quality-filtered by length (400-600 bp) and Phred score (>20). For taxonomic assignation, best hit was employed (max e value = 0.001; min percent identity = 80.0 for 18S and 97.0 for COI). Sequences were BLAST-aligned against NCBI database using QIIME platform (Caporaso et al., 2011). A partial NCBI reference database was built for 18S using Baker (2017) algorithm. Assignment was done employing ‘‘assign_taxonomy. py” python script. Sequence reads were clustered into OTUs (Operational Taxonomic Units) at 100% similarity threshold. Finally, a list of OTUs and the number of reads assigned to them in each sample was constructed with the ‘fromTaxassignments2OtuMap.py’ algorithm.

After initial inspection, sequences assigned to organisms unlikely to be present in ballast water (e.g. lung vertebrates and non-aquatic species) were eliminated from the dataset (expert check). Most probably, these sequences come from remains like scales or feathers with no biological significance in plankton – except as a substrate for saprophytes. Although singletons are often removed to decrease false positives (Scott et al., 2018), in marine biosecurity a false negative could be costly (Von Ammon et al., 2018), thus singletons were retained. For 18S, 43% of the quality reads obtained were assigned to an OTU (Suppl. Table 2); COI yielded more quality reads but fewer assigned to a species level, 3.4%, with the thresholds employed. Untransformed data (number of reads per OTU per day) were used to generate OTU rarefaction curves using

Vegan package in R software (Oksanen et al., 2013). Most samples reached a plateau or were close to it for the two barcodes (Suppl. Fig. 1A). Species richness was adequately covered from the two barcodes being close to 1 in all the samples (Suppl. Fig. 1B). Observed OTU coverage was also close to 1 for the two markers (Suppl. Fig 1C, 1D).

2.5. Statistical analysis

Metabarcoding results were presence/absence because for eukaryotes the number of reads does not reflect accurately the number of individuals (Kelly et al., 2014; Bonk et al., 2018). Since the ballast tank is a closed system, the presence of a species' DNA over a day implies that it was present in the tank the previous days. Species identified from NGS were conservatively analysed at genera level; for individual barcoding a species level was used. Functional groups considered were autotrophs, myxotrophic, phagotrophic, parasite, saprotrophic, symbiotic and unknown/other, following Ortiz-Alvarez et al. (2018). Their diversity was measured from the number of species or genera (OTUs).

To visualize the relationships among the environmental parameters and the diversity of functional groups along the travel, non-metric multidimensional scaling (NMDS) was done on two dimensions on diversity of functional groups (as number of genera genetically identified in each group) with Bray-Curtis distance, including the five environmental variables considered. They were represented as diagonals with the length proportional to the relative weight of each variable. Differences in the community functional composition between the beginning and the end of the travel were tested using Monte-Carlo (9999 permutations) and contingency Chi-square, assuming equal distribution if the null hypothesis is true.

Trends of functional groups during the travel were tested for best-fit models (linear, polynomial, exponential, quadratic) from Akaike information criteria (AIC), and adjusted r^2 for the statistical significance of the regression over the number of days. The association between environmental variables and the proportion of resistant species of each functional group was explored through multiple regression model, testing its fit from adjusted multiple r^2 and significance from ANOVA. Homoscedasticity and normality were checked using Breusch-Pagan and Shapiro-Wilk tests respectively. Statistics was done with the free software PAST (Hammer et al., 2001).

3. Results

3.1. Variation of environmental parameters and living organisms

BW temperature increased over the first 15 travel days up to 28.9 ºC (Table 1), then dropped gradually. Dissolved oxygen decreased to 2.5 mg/L, ammonium (NH_4^+) increased to 106.2 mg/L, acidity increased slightly and salinity did not change significantly (Table 1). The density of living organisms observed *de visu* increased between days 2 and 4, probably due to reproduction of some species because many larvae and copepodites (copepod juveniles) were observed; then it dropped to 5.7% of the initial value on day 24, for increasing on the two last days (Table 1). Best-fit trend was polynomial (Figure 1). Phytoplankton organisms observed *de visu* dominated the living community at the beginning and decreased pronouncedly and significantly towards the end of the travel (best-fit to polynomial model with AIC = 8.485, $r^2 = 0.85$, $p < 0.001$).

Table 1. Evolution of ballast water physic-chemical factors (above) and biota, observed de visu (middle) and detected from DNA (below). Temperature (°C), oxygen (mg/L), NH4 (mg/L), biota density (number of organisms per 100 L), proportion of phytoplankton organisms (% phyto). Biota DNA is the number of genera identified from barcodes and/or metabarcodes (OTUs) of each functional group considered. O/U: Other/Unkown.

		Travel days													
		2	4	6	8	10	12	14	16	18	20	22	24	26	28
Abiotic factors	Temperature	15	16	18.8	21.2	22.5	24.2	27.8	28.7	27.1	25.8	23.9	21.4	20.6	19.9
	Oxygen	5.1	4.9	4.1	3.8	4.1	3.7	3	2.9	2.5	2.6	2.6	2.9	2.7	2.8
	Salinity	36.7	36.4	36.6	36.7	36.8	36.7	36.7	36.8	36.7	36.64	36.8	36.7	36.7	36.7
	pH	7.99	8.03	8.01	7.97	7.97	7.91	7.89	7.86	7.88	7.85	7.84	7.86	7.82	7.87
	NH_4	43.23	44.35	45.81	55.75	51.7	60	68.9	78.73	79.2	81.6	86.6	89.65	98.1	106.24
De visu biota	Biota density	1136	2693	2584	2190	1019	387	327	131	199	127	80	65	146	130
	% phyto	0.99	0.99	0.99	0.99	0.99	0.97	0.98	0.89	0.95	0.858	0.538	0.554	0.448	0.585
Biota DNA	Autotroph	101	96	79	77	72	60	39	27	24	20	19	18	17	8
	Mixotroph	10	9	9	9	9	8	6	6	5	5	4	4	4	0
	O/U	6	6	6	6	5	5	4	4	3	3	3	3	2	0
	Parasite	18	18	18	16	16	16	14	12	11	9	9	7	7	2
	Phagotroph	199	188	180	171	162	150	138	127	106	84	75	66	58	26
	Saprotroph	4	4	4	3	3	3	3	0	0	0	0	0	0	0
	Symbiont	4	4	4	3	3	3	2	1	1	1	0	0	0	0

3.2. Community evolution from DNA

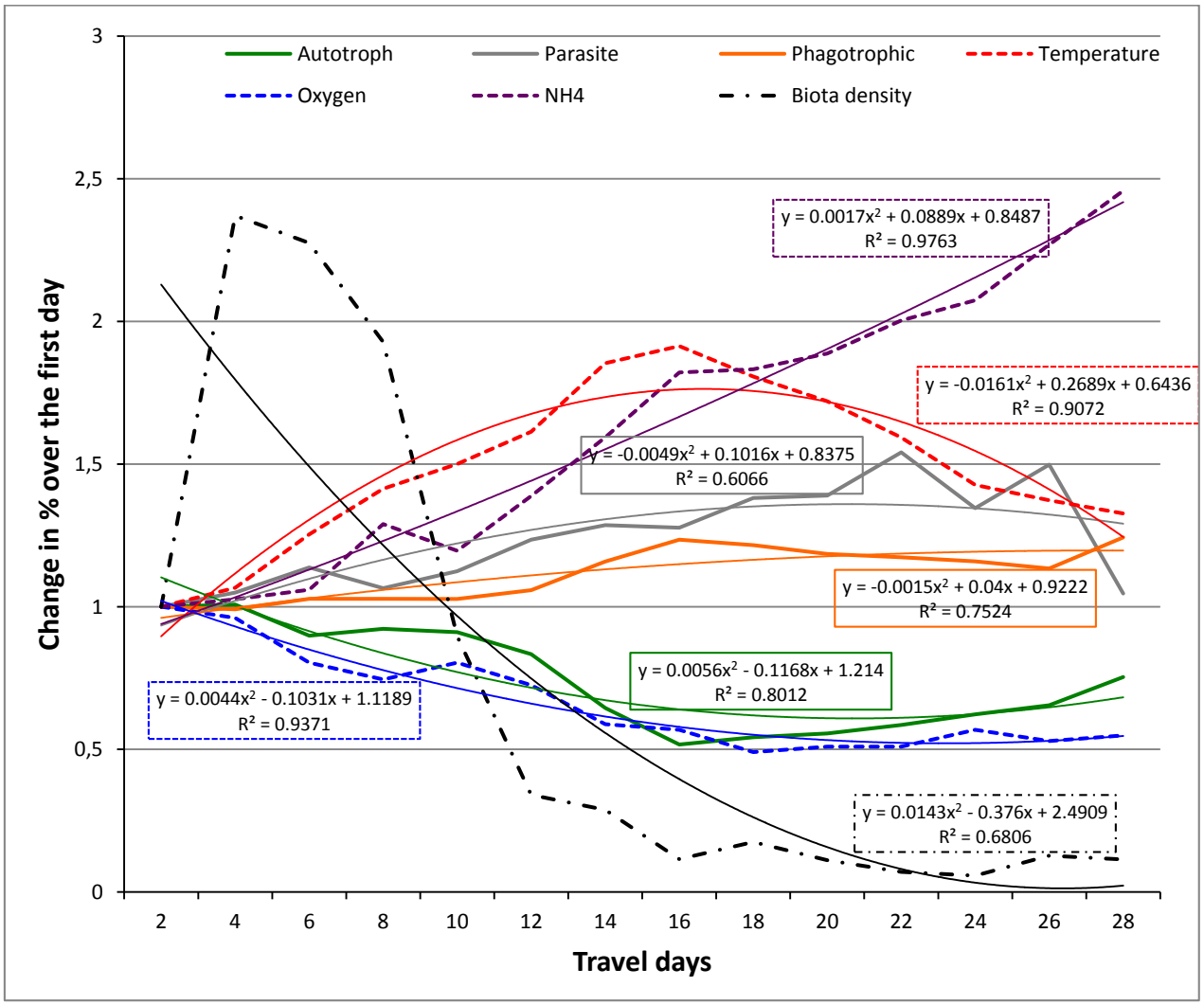
All the species presented in this study were identified from DNA, using barcodes and/or metabarcodes that are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Individual barcodes have accession numbers MK295012-MK295026 for 18S and MK314115-16 for rbcL. Metabarcode Fastaq files are in PRJNAS510002 (https://www.ncbi.nlm.nih.gov/sra/PRJNA510002), references SAMN10592608-SAMN10592648 (18S rDNA) and SAMN10592566-SAMN10592607 (COI gene).

Metabarcoding provided species from a wide taxonomic spectrum. Combining the two metabarcodes a total of 631 OTUs (562 for 18S and 69 for COI) were obtained, that were assigned to 339 genera of 34 Phyla (Suppl. Table 3). The decrease observed *de visu* in the density of organisms was significantly correlated with a sustained decrease in the number of DNA barcoded genera (Table 1 below), $r^2 = 0.792$ with $p = 0.0007$. The six functional groups described by Ortiz-Alvarez et al. (2018) in phytoplankton were found, and a few taxa of unknown classification were categorized as Other/Unknown.

The community detected from DNA changed along the voyage. At the end DNA of only 36 taxa (10.6%) was found, representing resistant species' DNA. By functional group, the initial community detected from DNA was 29.5% autotrophs, 58.1% phagotrophic and 12.3% of other groups - parasites the most abundant of them, 5.3% (Table 1). The trend of resistant autotrophs during the voyage was polynomial and significantly negative ($r^2 = 0.75$, $p < 0.001$), like in visual observations. Phagotrophs and parasites

also decreased in absolute number of genera, but the slope was less steep than that of autotrophs. Moreover, the proportion of resistant parasites and phagotrophs over the total number of resistant species *increased* significantly (Figure 1), best-fit polynomial regressions on voyage days having respectively $r^2 = 0.67$ and $r^2 = 0.71$ (both with $p < 0.001$). At the end of the voyage the community had a functional composition of 22.2% autotrophs, 72.2% phagotrophs, and 5.6% parasites (no other groups). The final functional composition was not significantly different from that of the beginning ($\chi^{7,2} = 4.21$, $p = 0.65$, not significant; Monte Carlo $p = 0.59$), although indeed with much fewer taxa (putative resistant species).

Figure 1. Variation of environmental parameters and functional groups in Polarstern PS102 expedition, relative to the initial level measured on day 2. DO, oxygen density. Best-fit trend lines, their equations and r^2 values are given framed with variable's colour and style codes.



DNA barcodes of individual samples confirmed taxa assigned from NGS metabarcoding and unambiguously identified 34 down to a species level: 22 of phytoplankton, 11 of zooplankton and one parasite/saprophyte (Table 2, Supp. Table 3).

Four of these species (11.7%) were seen alive on the two last sampling days. In general the days when individuals of a species were sampled coincided with the presence of the species' DNA in NGS dataset, with a few exceptions. In phytoplankton, *Chaetoceros* individuals were observed in samples until day 4, although their DNA was found on day 18 (Suppl. Table 3); *Ditylum* DNA was found until the end of the travel but individuals only until day 4; *Tripos horridus* was physically seen until day#26 and its DNA on day 28. In zooplankton, the copepod *Thompsonula hyaenae* was seen by day#8 and DNA found by day 14; the bivalve *Xenostrobus securis* was seen until day#10 while DNA detected until day 12.

Table 2. Species resistance in Polarstern BW measured from DNA-barcoded individuals. Days when individuals were seen are marked in black (1 = presence). The percentage of species surviving across the Equator in different groups is given in parenthesis.

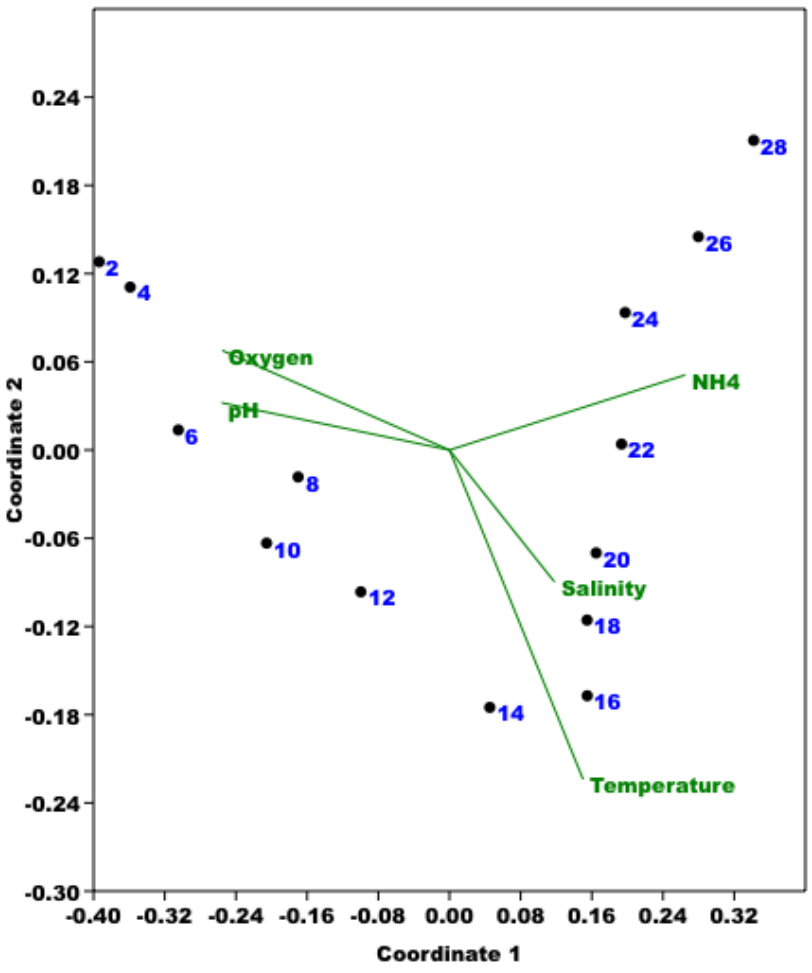
			Travel days													
	Group (% survivors)	Species	2	4	6	8	10	12	14	16	18	20	22	24	26	28
Phytoplankton	Diatoms (6.25%)	*Actinopthychus octonarius*	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Bacillaria paxillifer	1	1												
		Bacteriastrum hyalinum	1	1												
		Chaetoceros densus	1	1												
		Chaetoceros diadema	1	1												
		Chaetoceros decipiens	1	1												
		Chaetoceros affinis	1	1												
		Chaetoceros debilis	1	1												
		Chaetoceros curvisetus	1	1												
		Ditylum brightwelli	1	1												
		Eucampia zodiacus	1	1	1	1										
		Pseudo-nitzschia delicatissima	1	1	1	1	1									
		Rhizosolenia setigea	1	1												
		Thalassionema frauenfeldii	1	1												
		Thalassiosira punctigera	1	1	1	1	1	1								
	Dinoflagellates (20%)	*Ceratium tripos*	1	1	1	1	1									
		Lepododinium viride	1	1	1	1	1	1								
		Neoceratium platycorne	1	1	1	1	1	1								
		Protoperidinium punctulatum	1	1	1	1	1	1	1							
		Tripos horridus	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Silicoflagellates (0%)	*Octactis speculum*	1	1												
Zooplankton	Arthropods (50%)	*Acartia clausii*	1	1	1	1	1	1	1	1	1	1	1	1		
		Lucicutia flavicornis	1	1	1	1	1	1	1	1	1	1				
		Nitokra spinipes	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Paracalanus parvus	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Parvocalanus crassirostris	1	1	1	1	1	1								
		Sewelliapusia tropica	1	1	1	1										
		Thompsonula hyaenae	1	1	1	1	1									
		Undinula vulgaris	1	1	1	1	1									
	Bryozoans (0%)	*Anguinella palmata*	1	1	1											

Enteroprocta (0%)	*Loxosomella varians*	1	1	1	1	1
Molluscs (0%)	*Phaxas pellucidus*	1	1			
	Xenostrobus securis	1	1	1	1	1
Fungi (0%)	*Dothiora pyrenophora*	1	1	1		

3.3. Co-variation of environmental parameters and functional groups

NMDS plots created using diversity of the functional groups at genus level (stress 0.006, r^2 of axis 1 = 0.99, axis 2 = 0.019) showed the gradual change of the BW community (Figure 2), with the 14 studied days arranged almost consecutively left to right. After crossing the Equator the days were arranged a shorter distances to each other and aligned in a steep line. In the first part of the travel oxygen and pH variation dominated the environmental changes while NH_4 and temperature changes were more pronounced after the Equator.

Figure 2. Scatter plot of coordinates 1 and 2 visualizing non-metric multidimensional scaling of functional diversity in ballast water along the trans-Equatorial travel. Environmental variables are represented as diagonals, their length proportional to their relative loading. Travel days are marked as dots (red coloured after crossing the Equator) and numbered.



Although direct pairwise correlations between functional groups and pH, oxygen and ammonium were all significant (Supplementary Table 4), multiple regression models suggested that ammonium was probably the most influential factor on the proportion of resistant OTUs in the four main groups (with at least 10 OTUs) (Table 3). It was significantly associated with them, while none of the other four abiotic variables alone reached statistical significance in partial correlations.

Table 3. Multiple Regression Model constructed from the proportion of resistant species of each functional group. SE, standard error.

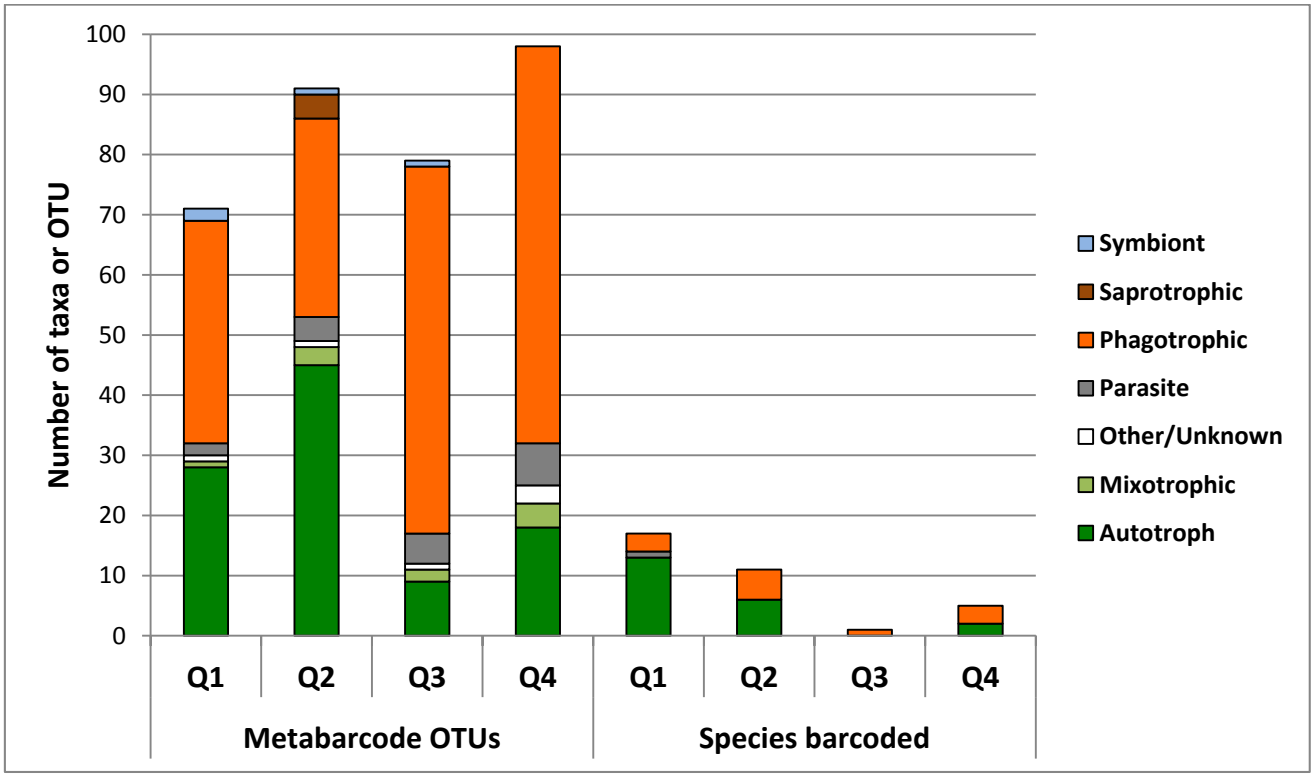
		Coefficient	SE	t	p	R^2
Autotroph	Constant	-375.96	885.9	-0.424	0.682	
vs	Temperature	-0.843	0.762	-1.106	0.301	0.360
vs	Oxygen	12.058	6.348	1.899	0.094	0.929
vs	pH	27.923	63.888	0.437	0.674	0.900
vs	NH_4	-0.911	0.249	-3.665	0.006	0.921
vs	Salinity	6.653	15.945	0.417	0.687	0.192
Multiple adjusted $r^2 = 0.98$, $F_{5,8} = 133.42$, $p < 0.001$						
Mixotrophic	Constant	126.85	134.49	0.943	0.373	
vs	Temperature	-0.089	0.116	-0.777	0.459	0.103
vs	Oxygen	-0.162	0.964	-0.168	0.870	0.675
vs	pH	-20.006	9.699	-2.063	0.073	0.665
vs	NH_4	-0.194	0.038	-5.137	0.001	0.934
vs	Salinity	1.471	2.421	0.608	0.560	0.071
Multiple adjusted $r^2 = 0.94$, $F_{5,8} = 43.25$, $p < 0.001$						
Parasite	Constant	244.17	174.82	1.397	0.20	
vs	Temperature	0.092	0.15	0.613	0.557	0.067
vs	Oxygen	0.069	1.253	0.055	0.957	0.645
vs	pH	-19.772	12.607	-1.568	0.155	0.69
vs	NH4	-0.297	0.049	-6.059	0.0003	0.957
vs	Salinity	-1.545	3.146	-0.491	0.636	0.099
Multiple adjusted $r^2 = 0.97$, $F_{5,8} = 81.24$, $p < 0.001$						
Phagotrophic	Constant	446.17	2098.2	0.213	0.837	
vs	Temperature	2.776	1.806	1.537	0.163	0.096
vs	Oxygen	19.469	15.035	1.295	0.231	0.716
vs	pH	-34.827	151.31	-0.23	0.824	0.753
vs	NH4	-2.147	0.589	-3.649	0.006	0.964
vs	Salinity	-0.664	37.764	-0.017	0.986	0.104
Multiple adjusted $r^2 = 0.96$, $F_{5,8} = 65.05$, $p < 0.001$						

3.4. Inference and analysis of potential IAS

Species observed until the end of the voyage survived a suite of changes that made the BW environment very different to that of the North Sea where the BW was taken from. They have therefore potential as IAS. Species were thus classified according to their presence over travel quartiles. From the NMDS plots we observed oscillations of abiotic factors and biota density and classified the travel quartiles as such: Q1 – days 1-8, Q2 - days 9-14, Q3 – 15-22, Q4 – 16-28 (see data in Table 1). The species lost in Q1 would

be the most sensitive and those present in Q4 the most resistant ones. While the majority of individually barcoded species were no longer seen after the Equator (Q1 and Q2), the quartile with most metabarcoding OTUs was Q4, that contained 28.9% of total OTUs while only 14.7% of the individually barcoded species (Figure 3). In the two datasets most autotrophs were lost earlier than phagotrophs, consistently with more resistant zooplankton. The difference between the two datasets in the distribution of species by quartile was highly significant ($\chi^{2,4}$ = 19.63, p = 0.0002; Monte Carlo p = 0.0003). This could be explained from hypoxic BW, since environmental DNA can last very long in anoxic conditions (e.g. Borin et al., 2008; Carinesaldi et al., 2011). Since the mere presence of its DNA in water does not ensure that a species is alive (Barnes et al., 2014), we retained conservatively as potential IAS only species with DNA seen on day 28 from NGS dataset, and Q4 species from physical samples.

Figure 3. Functional diversity and species resistance in ballast water. The number of species or OTUs lost in each functional group and quartile of the travel in BW is presented. Resistant species (OTUs) are those of Q4 (last three sampling days).



As many as 56% of the 36 species found the last day from metabarcoding dataset exhibit traits typical of IAS or are from distant regions (Table 4); seven (19.4%) species are already considered globally invasive or regional IAS. The six species individually sampled on Q3 and Q4 days (one diatom, one dinoflagellate and four copepods), not listed as invasive yet, could be IAS too.

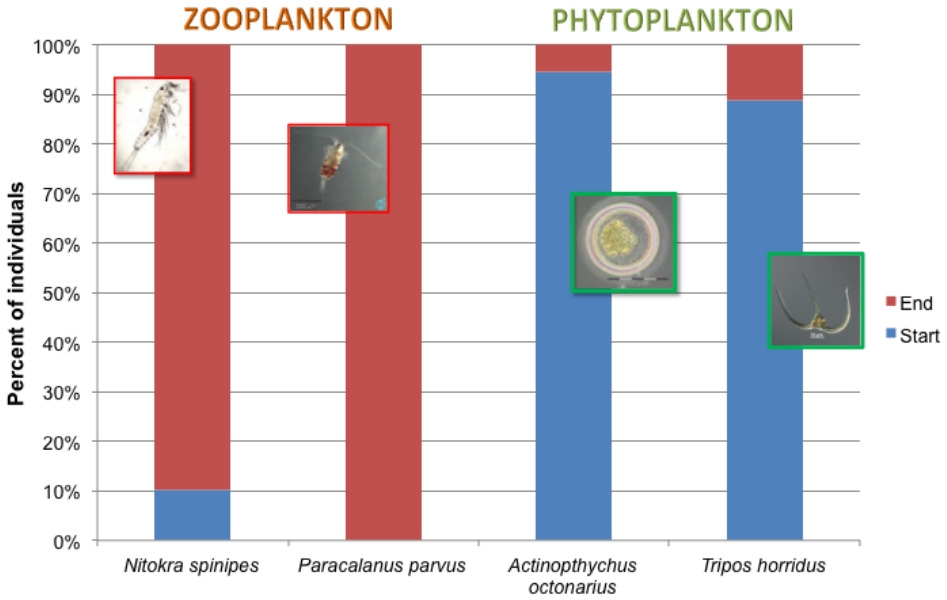
Table 4. Species found on day 28 from NGS metabarcoding. Voyage quartile if individuals were barcoded is indicated, and any traits indicators of invasive capacity. Distribution as in WORMS if not otherwise indicated. 1, Arnott and Hussainy (1972); 2, Lavigne (2020); 3, López-Fuerte and Siquieros–Beltrones (2016); 4, Fernandes and Ramaiah (2019); 5, Dahl and Breitholtz (2008); 6, Dudakova (2012); 7, Ardura et al. (2015b); 8, Chang et al. (2012); 9, Felder and Camps (2009).

Phylum	Group	Species	Individuals	Traits
Arthropoda	Phagotrophic	*Acartia clausii*	Q3	Tolerance (1), wide distribution
Ochrophyta	Autotroph	*Actinoptychus octonarius*	Q4	Wide distribution (2, 3)
Myzozoa	Autotroph	*Amphidoma languida*		Wide distribution
Apicomplexa	Parasites	*Babesia sp.*		
Choanozoa	Phagotrophic	*Choanoeca perplexa*		
Cnidaria	Phagotrophic	*Crambionella sp.*		
Mollusca	Phagotrophic	*Magallana gigas*		Global IAS
Arthropoda	Phagotrophic	*Cyclops cf. kikuchii*		
Myzozoa	Autotroph	*Dinophysis tripos*		Wide distribution
Ochrophyta	Autotroph	*Ditylum brightwelli*		IAS in South America
Arthropoda	Phagotrophic	*Gammarus crinicordis*		Paneuropean distribution
Gastrotricha	Phagotrophic	*Halichaetonotus cf. atlanticus*		Wide distribution
Ciliophora	Phagotrophic	*Haptoria sp.*		
Arthropoda	Phagotrophic	*Idotea metallica*		Wide distribution
Choanozoa	Phagotrophic	*Lagenoeca sp.*		
Ciliophora	Phagotrophic	*Loxophyllum shini*		
Arthropoda	Phagotrophic	*Lucicutia flavicornis*	Q3	Tolerance (4), wide distribution
Cercozoa	Phagotrophic	*Massisteria cf. diva*		
Ciliophora	Parasites	*Metaracoelophrya sp.*		
Ciliophora	Phagotrophic	*Metaurostylopsis marina*		From Gulf of Mexico
Mollusca	Phagotrophic	*Mytilus galloprovincialis*		Global IAS
Arthropoda	Phagotrophic	*Nitokra spinipes*	Q4	Tolerance (5), invasion in Russia (6)
Ciliophora	Phagotrophic	*Opisthonecta minima*		
Arthropoda	Phagotrophic	*Paracalanus parvus*	Q4	Near cosmopolitan
Nematoda	Phagotrophic	*Pellioditis (Rhabditis) mediterranea*		From New Zealand
Myzozoa	Autotroph	*Prorocentrum triestinum*		IAS in Mexico
Heliozoa	Phagotrophic	*Pterocystis cf. foliacea*		
Chlorophyta	Autotroph	*Pterosperma cristatum*		From Black Sea to Sweden
Heliozoa	Phagotrophic	*Raineriophrys erynaceoides*		
Mollusca	Phagotrophic	*Saccostrea cucullata*		IAS in French Polynesia (7)
Choanozoa	Phagotrophic	*Salpingoeca tuba*		
Choanozoa	Phagotrophic	*Savillea micropora*		
Mollusca	Phagotrophic	*Sepia aculeata*		From Pacific Ocean
Choanozoa	Phagotrophic	*Stephanoeca cauliculata*		
Ciliophora	Phagotrophic	*Stichotrichia sp.*		
Myzozoa	Autotroph	*Symbiodinium sp.*		
Myzozoa	Autotroph	*Tripos horridus*	Q4	Wide distribution (8, 9)
Phaeophyceae	Autotroph	*Undaria pinnatifida*		Global IAS
Ciliophora	Phagotrophic	*Vorticella cf. campanula*		

Focusing on the four species found alive in Q4 (Figure 4), from their relative counts at the beginning and end of the voyage the phytoplankton species decreased in abundance while the two animals increased. *Nitokra spinipes* females carrying eggs were observed and many copepodites were barcoded, demonstrating that the species reproduced *en*

route. The other resistant copepod *Paracalanus parvus* was less abundant and copepodites were not observed, but its frequency increased during the voyage so it likely reproduced –no one individual of this species was sampled on days 2 and 4 so its frequency was surely low, see Figure 4.

Figure 4. Count distribution of the most resistant species found alive until Q4 in the first (2 and 4) and last (26 and 28) days in Polarstern ballast water, represented as "Start" and "End". Images under Creative Commons - 3.0: *Actinoptychus octonarius*, *Paracalanus parvus* and *Tripos horridus*, AWI Plankton net (https://planktonnet.awi.de/, accessed April 2020); BY 4.0: *Nitokra spinipes*, Inta Dimante-Deimantovica, Norsk Institutt for Naturforskning.



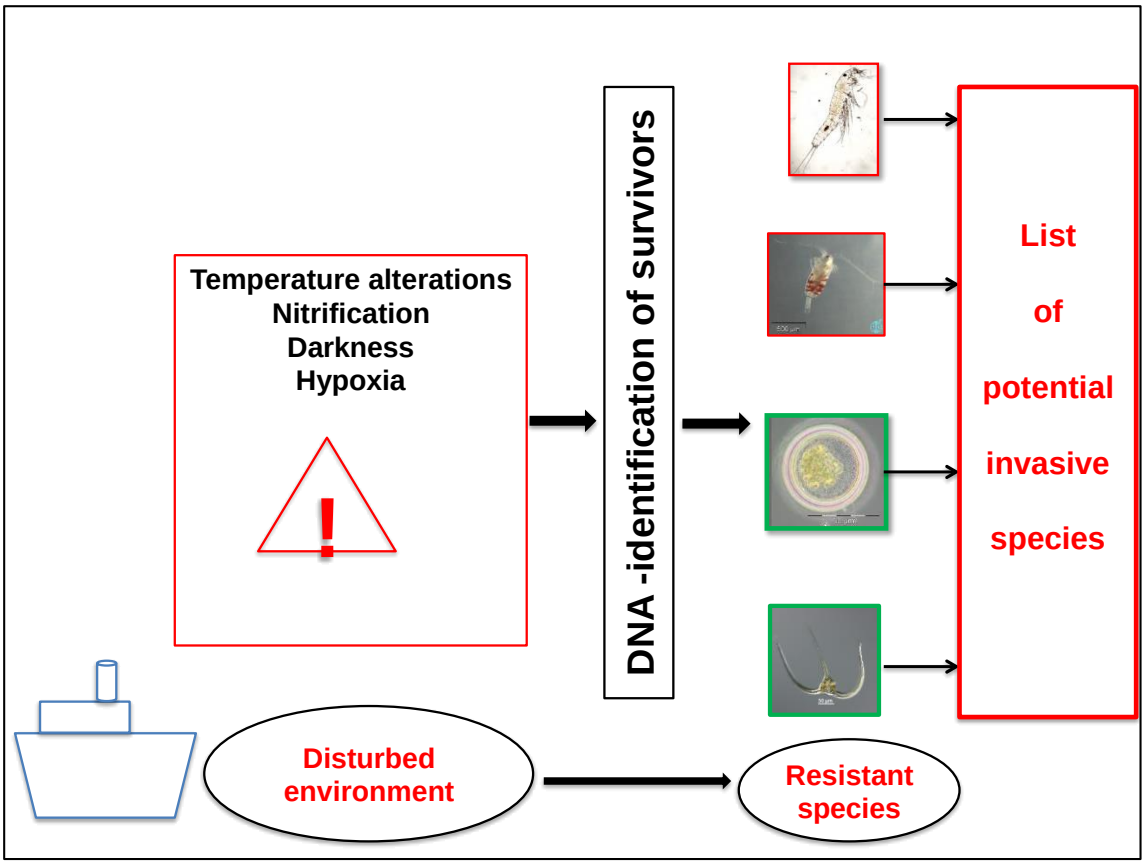
Out of the list of most resistant species, *Xenostrobus securis* (seen until day 10 and its DNA until day 12; Table 2) is an IAS prioritized for inclusion in the list of concern of the European Union, with hull fouling as a main vector of introduction (Tsiamis et al., 2020). We showed here that their larvae are transported in BW and have reached North Sea waters already. This is the northernmost record of this species in European waters to date (WORMS, http://www.marinespecies.org/aphia.php?p=taxdetails&id=140485, accessed April 2020).

4. Discussion

This proof of concept, summarized in Figure 5, demonstrates the power of analyzing altered small-scale ecosystems for IAS prediction, using a very robust combination of DNA methodologies for unambiguous identification of plankton at a species level. We have introduced the temporal duration in BW as an indicator of species resistance. From just one voyage we could describe and genetically authenticate several species with typical IAS traits: highly tolerant, of wide distribution, able to resist environmental stress, and some capable to reproduce *en route*. They could be potential IAS. The search strategy employed seems to be adequate for answering the question posed by Jarić et al. (2019) about how to unravel undescribed and undetected marine IAS. In a previous

study Ardura et al. (2015) suggested *Peringia ulvae* (found in BW along the same route in 2012), as a potential invader. It has been recently described from Mauritanian waters (Wilke and Delicado, 2019), far south from their European distribution recognized today (MolluscaBase, 2020). Not listed as an invader yet, but at least able to arriving in far regions, this example supports our idea.

Figure 5. Graphical summary of this study.



The deep functional analysis of en route BW is another novelty of our study, and has importance in marine invasion science. Gollasch et al. (2000) found 0-10% phytoplankton species surviving cross-latitudinal voyages, and 17-29% zooplankton species. Our results fit well in that range. We found here parasites and phagotrophs to be similarly resistant (Figure 1), perhaps because parasites survive as long as their hosts do and may even switch to a different host when the original disappears (Strona, 2015). Diatoms and dinoflagellates managed to survive too, as they do in most voyages (Hallegraeff and Bolch, 1992; Hallegraeff, 1998; McCarthy and Crowder, 2000; McCollin et al., 2007; Brisky et al., 2014; Steichen and Quigg, 2015). Resistant phytoplankton could be prioritized in IAS search for diverse reasons. For example, the relationships between propagule pressure (number of individuals) and colonization pressure (number of species) over long voyages are still unclear in diatoms (Brisky et al., 2012, 2014). On the other hand, exotic primary producers and marine predators are the only trophic groups that exert significant decreases on marine ecological properties (Anton et al., 2019); this emphasizes the need of more research on primary producer IAS.

Results also suggested that ammonium can be a factor of stress in BW. Although scarcely considered in other studies, ammonium is toxic for most marine eukaryotes and may cause mass extinctions (e.g. Sun et al., 2019). It could be explored in further research as a factor of selection of resistant species.

In metabarcoding data we have conservatively considered only the last travel day for IAS exploration because DNA lasts in water after individuals die. An alternative to DNA metabarcoding could be RNA metabarcoding, since RNA degrades more rapidly than DNA and would represent living species better. However, technical issues like post-transcription molecular edition and altered gene expression under stress pose challenges for finding primers and interpreting RNA metabarcodes (e.g. Zaiko et al., 2018).

A limitation here was the use of only water samples in Metabarcoding. Koziol et al. (2019) demonstrated that a single substrate underestimates the total eukaryotic diversity. We aimed at illustrating the proposed framework and not at estimating true biodiversity, but systematic surveys for prediction of IAS should include also surface samples (for example biofilm taken from tank walls, where precursors for colonization of larger IAS can be detected from DNA, Pochon et al., 2015), sediments and water from different depths. Since the communities are not homogenous within a ballast tank (Rey et al., 2019), for representativeness samples should be taken from different access locations of the same tank.

Using real systems like true BW adds ecological validity to the proposed strategy for finding undescribed IAS. Since current procedures for treating BW (BW exchange, physical or chemical treatments) are not fully efficient for preventing the introduction of IAS (e.g. Grob and Pollet, 2016; Carney et al., 2017; Darling et al., 2018), this strategy has still some years ahead, until 100% fully safe methods are developed for the accomplishment of the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM). Investigating survivors of other anthropogenic stresses progressing rapidly over time, like the construction of marine wind farms or periodical discharges of new thermal power plants, could help to discover potential IAS capable to resist adverse, stressful abiotic conditions.

5. Conclusions and implications

This study tests a strategy for predicting marine invasive species based on surveying real ecosystems where plankton is temporarily subjected to very harsh conditions. Our ecosystem model was ballast water crossing the Atlantic Ocean from the North Sea to South Africa. During one month plankton experienced big temperature changes, quadruplicate ammonium levels and reduction by half of dissolved oxygen, but phytoplankton and zooplankton species survived and even reproduced *en route*. Individual DNA barcoding and NGS metabarcoding served to identify the biota. Species resisting by the end of the voyage, still not listed as a risk but capable of crossing the planet in ballast water, were considered potential invasive species. Similar approaches could be followed in ballast water and other anthropogenic systems for identifying highly tolerant species thus predicting future invaders.

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Additional Information

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Supplementary Tables

Supplementary Table 1. Ship location (coordinates) by date; temperature in °C registered overboard during Bremerhaven - Cape Town voyage (overboard T); density (number of individuals/100L) of >20µm organisms observed at x50 magnification.

	Date	Latitude	Longitude	Overboard T	Density of organisms visually observed						
					Diatoms	Dinoflagellates	Other phyto	Arthropods	Molluscs	Other zoo	Total N
Day 2	14/11/2016	50.284	1.465	9	765	357	0	4	0	10	1136
Day 4	16/11/2016	45.367	-10.399	17	1807	855	5	6	2	18	2693
Day 6	18/11/2016	38.279	-13.11	20.1	1939	633	7	2	1	2	2584
Day 8	20/11/2016	30.97	-14.84	22	1990	197	0	2	0	1	2190
Day 10	22/11/2016	26.12	-17.53	22.8	944	71	1	2	0	1	1019
Day 12	24/11/2016	18.6	-21.48	24.4	370	6	0	10	0	1	387
Day 14	26/11/2016	10.27	-20.1	28.6	319	3	0	5	0	0	327
Day 16	28/11/2016	3.3	-16.73	28.2	116	1	0	14	0	0	131
Day 18	30/11/2016	-2.15	-11.79	26.8	188	1	0	10	0	0	199
Day 20	2/12/2016	-6.94	-4.62	25.3	102	7	0	18	0	0	127
Day 22	4/12/2016	-11.71	-1.03	22.5	40	3	0	37	0	0	80
Day 24	6/12/2016	-18.63	5.32	20.1	34	2	0	28	1	0	65
Day 26	8/12/2016	-25.58	9.55	18.6	65	1	0	79	1	0	146
Day 28	10/12/2016	-31.42	14.45	19	76	0	0	54	0	0	130

Supplementary Table 2. NGS results per sample and sampling day, given as: raw (number of reads that passed the first quality control), assigned (sequences assigned to an OTU with the required thresholds), assigned after expert check (final number of sequences assigned after expert quality control, discarding non-marine taxa and ambiguous or unclear references).

		18S rDNA					COI		
Day	**Sample**	**QC reads**	**Assigned**	**After expert check**	**Day**	**Sample**	**QC reads**	**Assigned**	**After expert check**
DAY 2	1	20721	16359	15852	DAY 2	1	50431	4816	4775
	2	248	99	97		2	195785	10932	9941
	3	133	84	83		3	75913	3145	2787
DAY 4	1	9608	5829	5666	DAY 4	1	8203	5678	4733
	2	300	105	92		2	258724	26131	24774
	3	201	79	77		3	3078	515	1
DAY 6	1	2443	1302	1285	DAY 6	1	36948	701	428
	2	8829	545	5312		2	186	0	0
	3	117	42	41		3	7385	361	115
DAY 8	1	-	-	-	DAY 8	1	25006	181	97
	2	9616	512	4957		2	70611	465	11
	3	320	116	110		3	118522	246	87
DAY 10	1	3979	1408	1346	DAY 10	1	58276	167	15
	2	10813	4472	4249		2	5682	213	32
	3	27500	13030	12540		3	6785	497	448
DAY 12	1	56931	30024	27888	DAY 12	1	38016	1182	1099
	2	5047	2036	1905		2	24769	921	855
	3	22806	9338	8302		3	7111	300	280
DAY 14	1	8898	3650	3493	DAY 14	1	7043	211	171
	2	5565	2923	2868		2	28971	473	377
	3	17701	10308	9964		3	233528	3536	2052
DAY 16	1	894	703	703	DAY 16	1	2622	12	5
	2	87764	28383	26334		2	30594	137	77
	3	2240	1602	1598		3	1913	72	11
DAY 18	1	53648	14808	14589	DAY 18	1	245392	1049	52
	2	484	330	328		2	119918	87	46
	3	9167	3632	3518		3	78742	177	36
DAY 20	1	21729	6268	6115	DAY 20	1	33098	107	1
	2	3992	3031	3016		2	25154	10570	10515
	3	3866	1489	1426		3	182888	3902	3628

DAY 22	1	4072	2404	2392	DAY 22	1	135	10	9
	2	16244	11209	8920		2	27297	9719	8328
	3	457	251	250		3	79504	12987	3659
DAY 24	1	2725	1394	1375	DAY 24	1	495402	7728	3643
	2	683	383	379		2	499391	3716	294
	3	293	139	137		3	166726	700	456
DAY 26	1	1257	802	798	DAY 26	1	1054	0	0
	2	319	174	172		2	53971	646	393
	3	18132	7789	7199		3	4119	109	37
DAY 28	1	1105	906	904	DAY 28	1	4053	470	3
	2	576	125	121		2	306698	2916	120
	3	4967	3727	3578		3	73139	10479	44

Supplementary Table 3. Functional analysis of the taxa found in RV Polarstern ballast water on voyage days 2 - 28, identified from meta-barcoding and barcoding individual samples (IB, "Individual barcodes"). Results are given as presence (1) / absence (0) .

Phylum	Genus	IB	Functional group	D2	D4	D6	D8	D10	D12	D14	D16	D18	D20	D22	D24	D26	D28
Amoebozoa	*Nematostelium*		Other/Unknown	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Amoebozoa	*Squamamoeba*		Other/Unknown	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Amoebozoa	*Lingulamoeba*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Amoebozoa	*Platyamoeba*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Amoebozoa	*Vannella*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Amoebozoa	*Lycogala*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Annelida	*Exogone*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Annelida	*Pomatoceros*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Annelida	*Serpula*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Annelida	*Spirobranchus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Aphelida	*Amoeboaphelidium*		Parasites	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Aphelida	*Aphelidium*		Parasites	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Aphelida	*Paraphelidium*		Parasites	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Apicomplexa	*Babesia*		Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Apicomplexa	*Theileria*		Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Apicomplexa	*Cryptosporidium*		Parasites	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Apusozoa	*Amastigomonas*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Apusozoa	*Chelonemonas*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Apusozoa	*Multimonas*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Arthropoda	*Gammarus*	*Gammarus crinicaudatus*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arthropoda	*Idotea*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arthropoda	*Paracalanus*	*Paracalanus parvus*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arthropoda	*Cyclops*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arthropoda	*Nitokra*	*Nitokra spinipes*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arthropoda	*Sewelliapusa*	*Sewelliapusia tropica*	Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Neocrangon*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Acartia*	*Acartia clausii*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Arthropoda	*Undinula*	*Undinula vulgaris*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Arthropoda	*Centropages*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Arthropoda	*Clausocalanus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Arthropoda	*Lucicutia*	*Lucicutia flavicornis*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Arthropoda	*Parvocalanus*	*Parvocalanus crassirostris*	Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Arthropoda	*Temora*		Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Oithona*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Arthropoda	*Pontostratiotes*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Amphiascoides*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Paramphiascella*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Arthropoda	*Parameiropsis*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Thompsonula*	*Thompsonula hyaenae*	Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Tisbe*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0

Arthropoda	*Zosime*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Arthropoda	*Dosima*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Arthropoda	*Lepas*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Arthropoda	*Octolasmis*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Arthropoda	*Poecilasma*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Arthropoda	*Lichomolgus*		Symbiont	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	*Austrominius*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Arthropoda	*Yamaguchiella*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Arthropoda	*Candona*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Ascomycota	*Dothiora*	*Dothiora pyrenophora*	Parasite/Saprotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Ascomycota	*Penicillium*		Saprotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ascomycota	*Cordyceps*		Saprotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Ascomycota	*Lecanicillium*		Saprotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Bryozoa	*Watersipora*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Bryozoa	*Alcyonidium*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Bryozoa	*Anguinella*	*Anguinella palmata*	Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Cercozoa	*Massisteria*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cercozoa	*Chlorarachnion*		Mixotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cercozoa	*Reticulamoeba*		Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Cercozoa	*Pseudodifflugia*		Other/Unknown	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cercozoa	*Paulinella*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cercozoa	*Pseudopirsonia*		Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cercozoa	*Cyranomonas*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Cercozoa	*Spongospora*		Parasites	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cercozoa	*Paracercomonas*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Cercozoa	*Viridiraptor*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cercozoa	*Protaspa*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cercozoa	*Reckertia*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Cercozoa	*Thaumatomastix*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Cercozoa	*Thaumatomonas*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cercozoa	*Mataza*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Chlorophyta	*Pterosperma*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorophyta	*Chlamydomonas*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Chlorophyta	*Polytoma*		Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Chlorophyta	*Scenedesmus*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Chlorophyta	*Cymbomonas*		Autotroph	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Chlorophyta	*Pyramimonas*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Chlorophyta	*Nannochloris*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Chlorophyta	*Picochlorum*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Choanozoa	*Savillea*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Choanozoa	*Stephanoeca*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Choanozoa	*Choanoeca*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Choanozoa	*Lagenoeca*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Choanozoa	*Salpingoeca*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Choanozoa	*Acanthoeca*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Choanozoa	*Bicosta*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0

Choanozoa	*Calliacantha*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Choanozoa	*Diaphanoeca*	Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Choanozoa	*Helgoeca*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Choanozoa	*Parvicorbicula*	Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Choanozoa	*Didymoeca*	Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Choanozoa	*Monosiga*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Choanozoa	*Hartaetosiga*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Chordata	*Anguilla*	Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Chordata	*Amniataba*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Chordata	*Oikopleura*	Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Ecteinascidia*	Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Chordata	*Branchiostoma*	Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Haptoria*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Loxophyllum*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Metaracoelophrya*	Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Opisthonecta*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Vorticella*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Stichotrichia*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Metaurostylopsis*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ciliophora	*Platyophrya*	Symbiont	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Woodruffides*	Other/Unknown	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ciliophora	*Sorogena*	Other/Unknown	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Askenasia*	Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ciliophora	*Mesodinium*	Symbiont	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ciliophora	*Lacrymaria*	Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Spathidium*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ciliophora	*Chaenea*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ciliophora	*Trachelophyllum*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ciliophora	*Kentrophyllum*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Ciliophora	*Litonotus*	Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Ciliophora	*Leptopharynx*	Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Nassula*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ciliophora	*Pseudocollinia*	Symbiont	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Colpidium*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Ciliophora	*Lembadion*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Porpostoma*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Paratetrahymena*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Ciliophora	*Parauronema*	Parasites	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Ciliophora	*Philaster*	Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Uronemella*	Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ciliophora	*Cyclidium*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Vorticellides*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Dysteria*	Other/Unknown	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Ciliophora	*Epalxella*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Ciliophora	*Tiarina*	Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Spathidiopsis*	Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0

Ciliophora	*Urotricha*		Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Pelagostrobilidium*		Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Strobilidium*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Euplotidium*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Uronychia*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Choreotrichia*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Oligotrichia*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Strombidium*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ciliophora	*Pseudoamphisiella*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Gonostomum*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Halteria*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Meseres*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Hemigastrostyla*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Oxytricha*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Pleurotricha*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Sterkiella*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Trachelostyla*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Ciliophora	*Amphisiella*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Kahliella*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Urospinula*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Ciliophora	*Tintinnopsis*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Ciliophora	*Metacylis*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Ciliophora	*Rhabdonella*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Ciliophora	*Amphorellopsis*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ciliophora	*Amphorides*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ciliophora	*Eutintinnus*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Ciliophora	*Salpingella*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Ciliophora	*Tintinnidium*	*Tintinnidium sp.*	Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Undella*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ciliophora	*Paraholosticha*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Birojimia*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Ciliophora	*Hemicycliostyla*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Cnidaria	*Crambionella*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cnidaria	*Cricophorus*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cnidaria	*Stephanogorgia*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cnidaria	*Paragorgia*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cnidaria	*Hydractinia*		Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Cnidaria	*Schuchertinia*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Cnidaria	*Clytia*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Cnidaria	*Eucheilota*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Cnidaria	*Obelia*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Cnidaria	*Rhopilema*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cnidaria	*Sanderia*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cnidaria	*Haliclystus*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cryptophyta	*Goniomonas*		Mixotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cryptophyta	*Falcomonas*		Mixotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Cryptophyta	*Geminigera*		Mixotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Cryptophyta	*Teleaulax*		Mixotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Cryptophyta	*Rhodomonas*		Mixotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Cryptophyta	*Katablepharis*		Mixotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Cryptophyta	*Leucocryptos*		Mixotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Cryptophyta	*Telonema*		Mixotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Ctenophora	*Velamen*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ctenophora	*Mnemiopsis*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ctenophora	*Thalassocalyce*		Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Enteroprocta	*Loxosomella*	*Loxosomella varians*	Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Euglenozoa	*Cercomonas*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Gastrotricha	*Halichaetonotus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gastrotricha	*Chaetonotus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Haptophyta	*Gladiolithus*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Haptophyta	*Ochrosphaera*		Autotroph	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Haptophyta	*Reticulofenestra*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Haptophyta	*Phaeocystis*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Haptophyta	*Chrysochromulina*		Autotroph	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Haptophyta	*Haptolina*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Haptophyta	*Prymnesium*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Haptophyta	*Algirosphaera*		Autotroph	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Haptophyta	*Syracosphaera*		Autotroph	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Heliozoa	*Pterocystis*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Heliozoa	*Raineriophrys*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mollusca	*Mytilus*	*Mytilus sp.*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mollusca	*Crassostrea*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mollusca	*Saccostrea*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mollusca	*Sepia*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mollusca	*Phaxas*	*Phaxas pellucidus*	Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	*Xenostrobus*	*Xenostrobus securis*	Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Mollusca	*Donax*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	*Mytilopsis*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Mollusca	*Spisula*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Mollusca	*Tagelus*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	*Scissula*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	*Octopus*		Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Mollusca	*Sepiella*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Mollusca	*Larochella*		Parasites	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Mollusca	*Gibbula*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Mollusca	*Umbonium*	*Umbonium sp.*	Phagotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Mollusca	*Doto*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Dinophysis*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Myzozoa	*Amphidoma*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Myzozoa	*Tripos*	*Tripos horridus*	Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Myzozoa	*Prorocentrum*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Myzozoa	*Symbiodinium*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Myzozoa	*Blastodinium*		Parasites	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Triposolenia*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Ornithocercus*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Phalacroma*		Autotroph	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Myzozoa	*Sinophysis*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Azadinium*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Ceratium*	*Ceratium tripos*	Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Neoceratium*	*Neoceratium platycorne*	Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Gambierdiscus*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Alexandrium*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Protoceratium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Heterodinium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Lingulodinium*		Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Myzozoa	*Brachidinium*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Akashiwo*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Cochlodinium*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Gymnodinium*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Gyrodinium*		Mixotrophic	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Myzozoa	*Lepidodinium*	*Lepododinium viride*	Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Nusuttodinium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Testudodinium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Karenia*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Karlodinium*		Autotroph	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Myzozoa	*Takayama*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Nematodinium*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Proterythropsis*		Autotroph	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Myzozoa	*Warnowia*		Autotroph	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Myzozoa	*Woloszynskia*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Levanderina*		Autotroph	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Myzozoa	*Pseudadenoides*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Pyramidodinium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Noctiluca*		Phagotrophic	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Myzozoa	*Oxyrrhis*		Autotroph	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Myzozoa	*Amphidiniopsis*		Autotroph	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Myzozoa	*Archaeperidinium*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Brandtodinium*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Heterocapsa*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Coolia*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Sabulodinium*		Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Myzozoa	*Thecadinium*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Pentapharsodinium*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Peridinium*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Peridiniopsis*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Cryptoperidiniopsis*		Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Myzozoa	*Pseudopfiesteria*		Autotroph	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Myzozoa	*Blepharocysta*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0

Myzozoa	*Podolampas*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Protoperidinium*	*Protoperidinium punctulatum*	Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Scrippsiella*		Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Myzozoa	*Thoracosphaera*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Spiniferodinium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Plagiodinium*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Pyrocystis*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Biecheleria*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Pelagodinium*		Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Myzozoa	*Yihiella*		Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Amoebophrya*		Parasites	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Myzozoa	*Duboscquella*		Parasites	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Myzozoa	*Torodinium*		Autotroph	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Myzozoa	*Parvilucifera*		Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Myzozoa	*Perkinsus*		Parasites	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Nematoda	*Pellioditis*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Nematoda	*Calomicrolaimus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Nematoda	*Molgolaimus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Nematoda	*Halomonhystera*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Nemertea	*Cerebratulus*		Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ochrophyta	*Actinoptychus*	*Actinopthychus octonarius*	Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ochrophyta	*Ditylum*	*Ditylum sp.* (NGS)	Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ochrophyta	*Bacillaria*	*Bacillaria paxillifera*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Pseudo-nitzchia*	*Pseudo-nitzschia delicatissima*	Autotroph	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Ochrophyta	*Odontella*		Autotroph	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Attheya*		Autotroph	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chaetoceros affiinis*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chatoceros curvisetus*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chaetoceros debilis*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chaetoceros decipiens*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chaetoceros densus*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chaetoceros diadema*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Chaetoceros*	*Chaetoceros sp.*	Autotroph	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Ochrophyta	*Bacteriastrum*	*Bacteriastrum hyalinum*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Coscinodiscus*	*Coscinodiscus sp.*	Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Ochrophyta	*Asterionellopsis*		Autotroph	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Eucampia*	*Eucampia zodiacus*	Autotroph	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Leptocylindrus*		Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Ditylum*	*Ditylum brightwelli*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Stephanopyxis*	*Stephanopyxis sp*	Autotroph	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Ochrophyta	*Rhizosolenia*	*Rhizosolenia setigea*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Ochrophyta	*Thalassionema*	*Thalassionema*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0

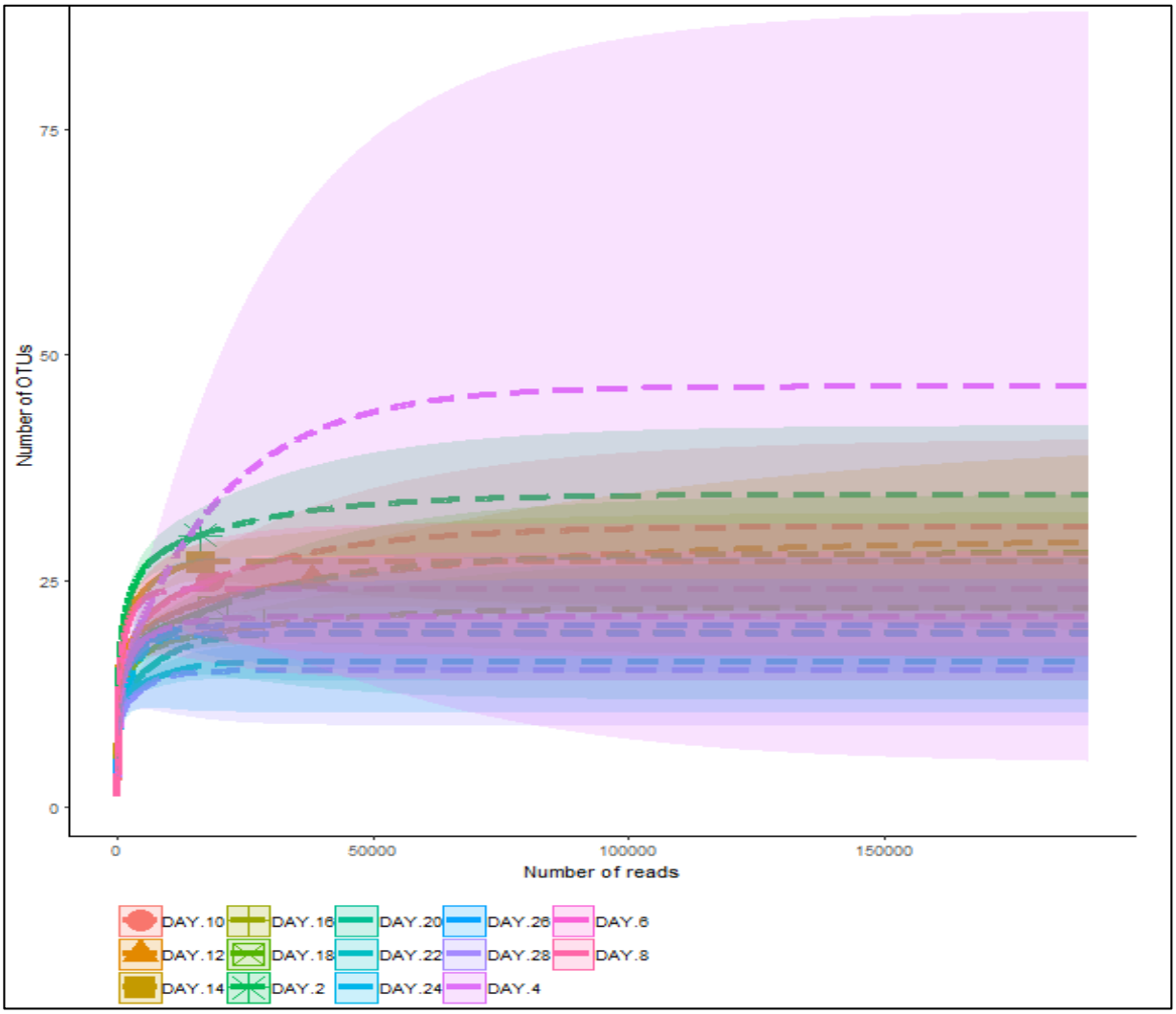
		frauenfeldii																	
Ochrophyta	*Thalassiosira*	*Thalassiosira punctigera*	Autotroph	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Ochrophyta	*Dictyocha*	*Octactis speculum*	Autotroph	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Phaeophyceae	*Undaria*		Autotroph	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Phoronida	*Phoronis*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Phoronida	*Phoronopsis*		Phagotrophic	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Picozoa	*Picomonas*		Saprotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Porifera	*Clathrina*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Porifera	*Leucascus*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Porifera	*Soleneiscus*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Porifera	*Swartschewskia*		Phagotrophic	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Protozoa incertae sedis	*Ebria*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Protozoa incertae sedis	*Ancyromonas*		Phagotrophic	1	1	1	1	1	1	1	1	1	0	0	0	0	0
Protozoa incertae sedis	*Kiitoksia*		Phagotrophic	1	1	1	1	0	0	1	0	0	0	0	0	0	0
Radiozoa	*Phyllostaurus*	*Phyllostaurus sp.*	Phagotrophic	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Rhizaria	*Globigerina cf.*		Phagotrophic	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Rotifera	*Rotaria*		Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Rotifera	*Brachionus*		Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Rotifera	*Keratella*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Rotifera	*Encentrum*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Rotifera	*Epiphanes*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Rotifera	*Cephalodella*		Phagotrophic	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Rotifera	*Notommata*		Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Rotifera	*Proales*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Rotifera	*Polyarthra*		Phagotrophic	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Rotifera	*Macrochaetus*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Rotifera	*Trichotria*		Phagotrophic	1	1	1	1	1	1	1	0	0	0	0	0	0	0
Xenacoelomorpha	*Nemertinoides*		Phagotrophic	1	1	1	1	0	0	0	0	0	0	0	0	0	0
			Total	33	32	29	28	26	24	20	17	15	12	11	98	88	37
				39	23	83	68	44	45	77	70	22	10				

Supplementary Table 4. Correlation matrix showing pairwise τ values between variables. Significant values applying Bonferroni correction are marked with *.

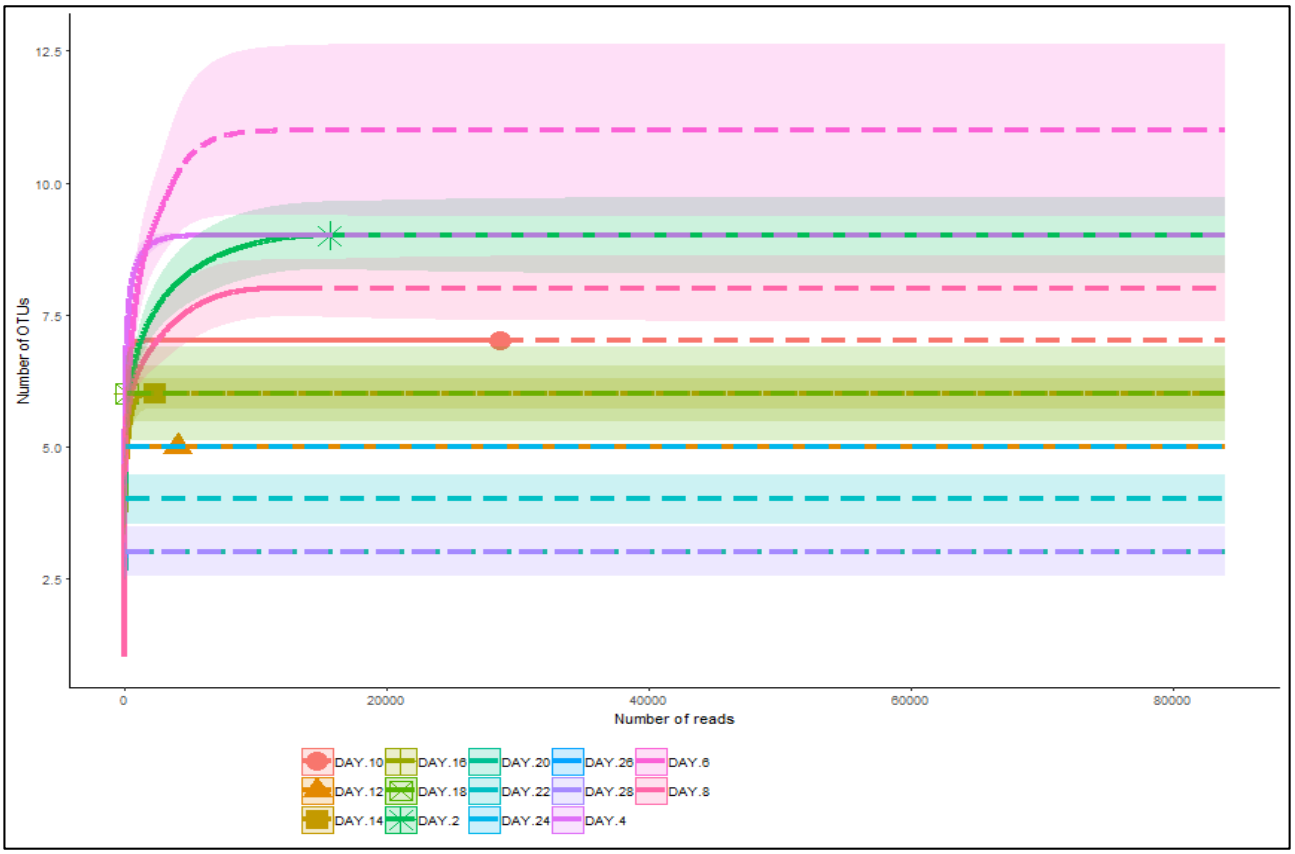
	Temperature	Oxygen	Salinity	pH	NH4
Autotroph	-0.209	0.693*	-0.216	0.767*	-0,978*
Mixotrophic	-0.188	0.715*	-0.173	0.723*	-0.938*
Other/Unknown	-0.203	0.717*	-0.206	0.761*	-0.896*
Parasite	-0.173	0.667*	-0.227	0.768*	-0.955*
Phagotrophic	-0.209	0.693*	-0.217	0.768*	-0.978*
Saprotrophic	-0.396	0.819*	-0.252	0.814*	-0.805*
Symbiont	-0.282	0.699*	-0.347	0.818*	-0.895*

Supplementary Figure 1. A and B, sample size-based rarefaction (solid line segments) and extrapolation (dotted line) curves for species richness (q = 0) with 95% confidence intervals (shaded areas) in the samples analysed high-throughput sequencing. Curves were constructed plotting the number of reads by the number of OTUs (A: 18S rDNA, and B: COI metabarcodes). C and D, coverage-based rarefaction (solid line) and extrapolation (dotted line) curves for species richness (q = 0) with 95% confidence intervals (shaded areas) (A, 18S rDNA, and B, COI metabarcodes). Data correspond to samples obtained on even voyage days (2 to 28).

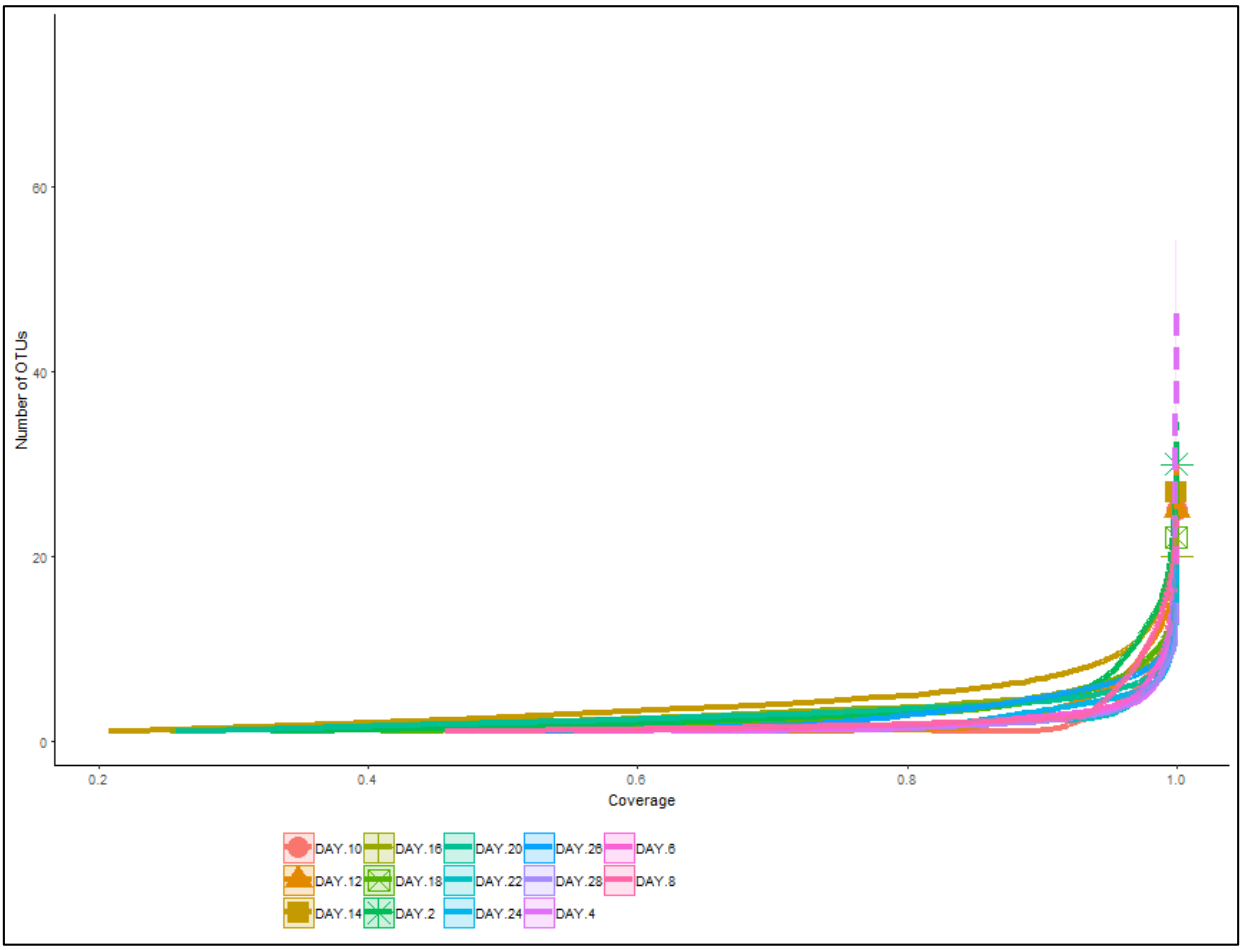
A)



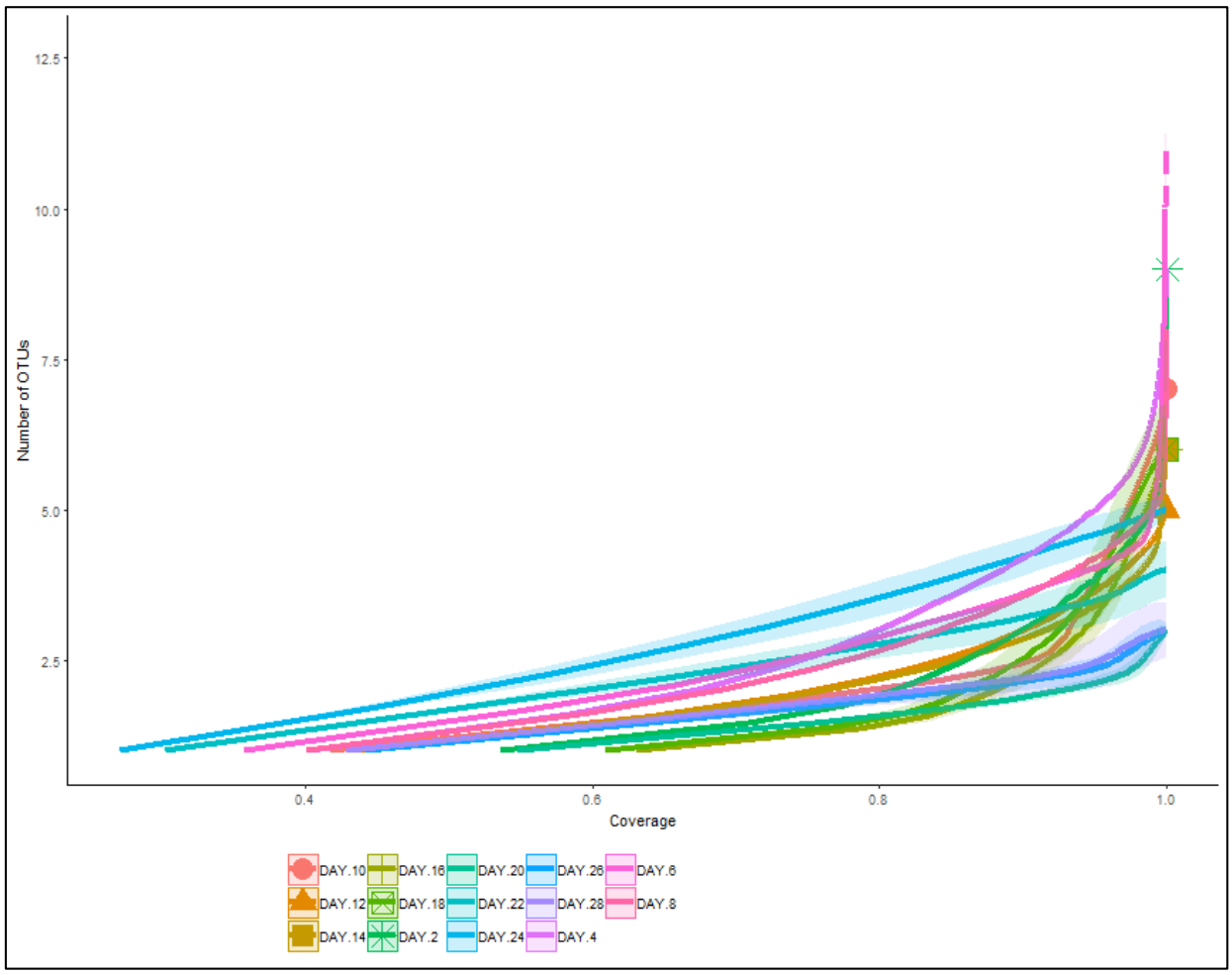
B)


12.5
10.0
7.5
5.0
2.5
Number of OTUs
0
20000
40000
60000
80000
Number of reads
DAY.10
DAY.12
DAY.14
DAY.16
DAY.18
DAY.2
DAY.20
DAY.22
DAY.24
DAY.26
DAY.28
DAY.4
DAY.6
DAY.8

C)


Number of OTUs
60
40
20
0
0.2
0.4
0.6
0.8
1.0
Coverage
DAY.10
DAY.12
DAY.14
DAY.16
DAY.18
DAY.2
DAY.20
DAY.22
DAY.24
DAY.26
DAY.28
DAY.4
DAY.6
DAY.8

D)


12.5
10.0
7.5
5.0
2.5
Number of OTUs
0.4
0.6
0.8
1.0
Coverage
DAY.10
DAY.12
DAY.14
DAY.16
DAY.18
DAY.2
DAY.20
DAY.22
DAY.24
DAY.26
DAY.28
DAY.4
DAY.6
DAY.8