

1 **Poorer diversity but tougher species in old ballast water: biosecurity challenges**
2 **explored from visual and molecular techniques.**

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14 **Abstract**

15 Millions of tons of water cross the oceans inside ships' ballast tanks every day. Planktonic
16 species hitch-hike with water and some may pose risks to ecosystems and economies if
17 get released and establish outside their native range. We monitored ballast water in
18 different trans-equatorial travels, visually and using molecular techniques, and found
19 significant increases of potential nuisance taxa over travel duration, despite evident
20 diversity depletion. Thus, less diverse but more resistant and potentially more harmful
21 communities persist in ballast water over long voyages. If we consider the enormous
22 volume transported every day, the persistence of resistant species in ballast water would
23 be threatening the global marine biodiversity. This should be taken into account when
24 modeling and assessing the bioinvasion risks associated with the ballast water and transfer
25 considered in the future research.

26
27 **Key words:**

28 Ballast water age, molecular techniques, taxonomical identification, nuisance species,
29 risk assessment.
30

31 Introduction

32 The estimated amount of water transferred in ballast tanks of cargo ships on a daily basis
33 exceeds 27 million tons, with about 7,000 species entrapped and hitchhiking in that water
34 (GloBallast Partnerships, 2017). This shipping-mediated worldwide circulation of species
35 contributes to the dispersal of organisms considered harmful to humans, like pathogens
36 (Gollasch et al. 2015), toxic algae (Hallegraeff 2015, Ardura et al. 2020a) and biological
37 invaders (e.g. Carlton & Geller 1993, Molnar et al. 2008, Hewitt et al. 2009). Under the
38 application of the Ballast Water Management Convention (BWMC) of 2004
39 ([https://www.imo.org/en/OurWork/Environment/Pages/BWMConventionandGuidelines](https://www.imo.org/en/OurWork/Environment/Pages/BWMConventionandGuidelines.aspx)
40 [.aspx](https://www.imo.org/en/OurWork/Environment/Pages/BWMConventionandGuidelines.aspx), accessed April 2021) that entered into force in September 2017, upon discharge
41 ballast water (BW) must comply with the standards stipulating allowable number of
42 organisms. In the current situation and for successful enforcement of the BWMC, an
43 efficient compliance monitoring should be implemented. This requires robust risk
44 assessment and prioritisation of the vessels to be checked-up. The Regulation D-2 Ballast
45 Water Performance Standard requires BW to contain less than 10 viable organisms per
46 $\text{m}^3 \geq 50 \mu\text{m}$ (typically zooplankton) and less than 10 viable organisms per ml between 10
47 and $50 \mu\text{m}$ (where most phytoplankton species fit); plus, limited colony forming units of
48 the pathogens *Vibrio cholera*, *Escherichia coli* and intestinal Enterococci. These
49 standards should be accomplished via onboard BW treatment or offshore BW exchange,
50 that can be effective management method for ballast water. However, most of the current
51 treatments are effective only for larger organisms of the size $>50 \mu\text{m}$, and the effective
52 treatments for smaller organisms produce toxic products, use high energy, space and have
53 high operation costs (Lakshmi et al., 2021). The filtration together with strong hydroxyl
54 radical treatment could be an effective option for the treatment of physicochemical
55 parameters and inactivation of smaller organisms (Lakshmi et al., 2021). In any case,
56 further research is necessary to identify the scope and effectiveness of the existing
57 systems and developing innovative methods for ballast water treatment, something that
58 should have been implemented, as was marked by the D-2 compliance, in all the ships, in
59 the end of year 2020. Besides, sometimes BW treatment systems fail to effectively kill
60 all the entrapped biota (Werschkun et al. 2014, Batista et al. 2017, Kang et al. 2020) and
61 BW exchange is not 100% efficient to prevent the arrival of new exotic propagules (e.g.
62 Casas-Monroy et al. 2016; Darling et al. 2018). Thus, zooplankton species like cyclopoid
63 copepods and phytoplankton have demonstrated ability to survive diverse treatments like
64 UVC, H_2O_2 , TiO_2 , and more (Garcia-Garay et al. 2018). Ballast water risk assessment is
65 required for granting exemptions under Regulation A-4
66 (<https://academic.oup.com/icesjms/article/65/2/121/735809>). Therefore, and since all
67 ships must meet D2 standards by 8 September 2024, better evidence-based understanding
68 of BW-related risk factors is needed to inform more targeted (and thus cost-effective)
69 enforcement measures, being urgent to prioritize research efforts especially for the most
70 dangerous BW.

71 Ship routes are very variable in duration and paths, thus BW communities arriving at
72 destination ports are very diverse, varying in composition, abundance and proportion of

73 potentially risky species (e.g. Carlton and Geller, 1993; Smith et al., 1999; Verling et al.
74 2005; Cordell et al. 2009; Zaiko et al., 2015a; Ardura et al., 2020a). Inside the tank, BW
75 experiences oxygen depletion and large ammonium and temperature changes, sometimes
76 over a few days of travel depending on the route (e.g., Zaiko et al. 2015b, 2020).
77 Consequently, the abundance of organisms decreases rapidly along the voyage. Studies
78 have demonstrated that, the biggest declines in species density happen frequently during
79 the first five days of travel (Gollasch et al. 2000a; Gollasch et al. 2000b; Olenin et al.
80 2000; Cordell et al. 2009). Species viability is thus negatively correlated with BW age
81 (time elapsed between BW uptake and discharge), and BW >10 days old is considered of
82 low risk in risk assessments (e.g. Brisky et al., 2014; Chan et al., 2014; Verna et al. 2016
83 and references therein).

84 In this study we postulate a counterintuitive hypothesis that the risk of introduction of
85 invasive species might increase with the BW residence time due to selective survival of
86 highly tolerant and resistant taxa, i.e. those with broad ecological niches and higher
87 invasion potential (e.g. Keller et al. 2011). From this perspective, the species managed to
88 survive in BW over longer voyages might be also less susceptible to BW treatment and
89 upon discharge in a new environment would establish and thrive more easily, thus posing
90 a higher biosecurity risk. To test this hypothesis, we analyzed the evolution of BW
91 communities over two long trans equatorial travels focusing on potentially harmful
92 species, combining morphological and DNA tools to characterize the BW community.

93

94 **Methods**

95 We have reanalyzed data obtained from BW communities sampled during the two RV
96 Polarstern cruises: ANT XXIX-1 EUROPA (2012) and PS102 (2016) from Bremerhaven
97 to Cape Town (Fig. 1). At each cruise, a dedicated aft ballast tank was filled with coastal
98 water out of Bremerhaven port with no treatment applied to the BW upon loading or
99 during the experiment. The experiment was performed over 20 and 28 days of navigation
100 in 2012 and 2016 respectively, as described in Zaiko et al. (2015b), and Ardura et al.
101 (2020b).

102 Briefly, BW samples (100 L) (twenty in 2012 and fourteen in 2016, Supplementary Table
103 1) were collected via the sounding pipe (20 mm diameter) using the in-built electric pump
104 and pre-concentrated with plankton net (50 and 20 μm , in 2012 and 2016 respectively).
105 Despite the difference in pore size, in both cases the fractions composed of eukaryotic
106 protists and metazoan populations were recovered, leaving aside the fractions of small
107 Phytoplankton and Picoplankton, formed mainly by bacteria and viruses (Omori and
108 Ikeda, 1992). For visual analysis, the sample was examined under microscope (50x
109 magnification) and identified taxonomically by experts, through taxonomical guides. The
110 living (moving or externally intact) organisms were quantified. Individuals of more
111 abundant species were isolated, identified to the lowest possible taxonomic level *de visu*
112 and/or DNA barcoded using Zhan et al. (2013) 18S rRNA gene (18S) primers for
113 zooplankton and Rivera et al. (2017) RuBisCo (rbcL) gene primers for phytoplankton.

114 For metabarcoding analyses, three 2 L BW from the same pipe were collected per day
115 and vacuum-filtered through a 0.2µm Nucleopore™ membrane, then preserved in 96%
116 ethanol until eDNA extraction.

117 DNA metabarcoding was done using partial *rbcL* and cytochrome oxidase subunit I (COI)
118 genes as metabarcodes in 2012 (Zaiko et al. 2015b), and COI (Leray et al. 2013 primers)
119 and V4 region of 18S (primers described in Zhan et al. 2013) in 2016, taking strict
120 measures to avoid cross-sample contamination (negative PCR controls with pure water,
121 sterile sample manipulation inside a laminar flow PCR-cabinet). DNA extraction, library
122 preparation, high-throughput sequencing and bioinformatics analysis with strict quality
123 filters and the pipeline explained in Ardura et al. (2020b).

124 Taxonomy followed the World Register of Marine Species (WORMS)
125 (<http://www.marinespecies.org/>) and AlgaeBase (<http://www.algaebase.org/>). Potential
126 risk species were considered those listed as non-indigenous to North European waters in
127 WORMS and relevant literature; invasive species catalogued in GISD (Global Invasive
128 Species Database at <http://www.iucngisd.org/gisd/>, Pagad et al., 2015) and CABI
129 (Invasive Species Compendium at <https://www.cabi.org/ISC>, CABI, 2020); toxic algae
130 (HABs) from <http://www.marinespecies.org/hab> (Moestrup et al., 2009).

131 Metabarcoding data were analysed as presence (1) or absence (0) of each Molecular
132 Operational Taxonomic Unit (MOTU = putative species). Functional richness and
133 Shannon functional diversity were calculated from the number of genera (as not all the
134 assignments reached the species level), under seven functional groups: autotrophs,
135 mixotrophic, phagotrophic, parasites, saprophytes, symbiotic, other (Ortiz-Alvarez et al.
136 2018). Differences among communities were checked with contingency Chi-square tests.
137 Best-fit trends (linear, exponential, quadratic, polynomial) were chosen using Akaike
138 information criteria. Statistical analyses and visualizations were performed with the free
139 PAST software (Hammer et al., 2001).

140 Physical-chemical parameters of BW were measured at each sampling time using YSI
141 Professional Plus Multimeter and were reported in Zaiko et al. (2015b, 2020) and Ardura
142 et al. (2020). Tables containing the organisms identified de visu and the Operational
143 Taxonomic Units (putative species) found from metabarcoding can be found in Ardura et
144 al. (2015) and Zaiko et al. (2015b) for 2012 voyage, and Ardura et al. (2020b) for 2016
145 voyage. The results from both voyages are summarized together in the Supplementary
146 Table 2, highlighting the coincident taxa identified from visual and molecular
147 methodologies.

148

149 **Results**

150 The largest decline in the abundance of living organisms occurred within the first days of
151 travel in both expeditions, with only a few plankton individuals detected towards the end
152 of observation (Figure 2). The proportion of phytoplankton decreased sharply and almost

153 disappeared in 2012 (1% the last day of voyage); however, in the less hypoxic travel of
154 2016 the decline of individuals occurred much later, and the proportion of phytoplankton
155 remained quite high (Figure 2). The density of individuals increased the last days of
156 travels due to zooplankton. Zooplankton species persistently occurring en route were
157 rotifers in 2012 (identified as *Brachionus* sp. from DNA analysis), and copepods (*Nitokra*
158 *spinipes*, some of them juveniles (copepodites), and *Paracalanus parvus*) in 2016. In both
159 travels, the persistence of different zooplankton species was supported by the appearance
160 of females with eggs and copepodites from the day 5 and day 12 in 2012 and 2016,
161 respectively, and their presence until the end of both travels.

162 In 2012, 165 MOTU obtained from metabarcoding, from both markers, were assigned to
163 137 genera, and the decline was much softer than that observed *de visu* (Figure 3). In
164 2016, 631 MOTU (337 genera) were recovered from both markers. Although the
165 reduction of the species richness found from DNA was highly significant in the two
166 travels (Figure 3), the functional Shannon index did not change so much until travel day
167 #20. The initial functional community structure (detected from DNA) was significantly
168 different between 2012 and 2016 studies: 65% autotrophs, 25% phagotrophic and 10% of
169 other groups in 2012, while 28% autotrophs, 59% phagotrophic and 13% of others in
170 2016 ($\chi^2 = 90.684$, $p=1.69 \times 10^{-21}$; Monte Carlo $p = 0.0001$). All the functional groups
171 decreased more or less proportionally over time and at the end of the observations the
172 communities were similar to those of the beginning although comprised of fewer species
173 (initial *versus* final functional community structure $\chi^2 = 0.088$, $p=0.766$ and $\chi^2 = 0.746$,
174 $p=0.388$ in 2012 and 2016 respectively). Notably, the proportion of autotrophs also did
175 not differ substantially at the beginning and the end of the experiments (Fig. 2).

176 The potentially resistant communities (those persistently detected until the end of the
177 experiment) were very different in 2012 and 2016, sharing only 32% of phyla at the end
178 of the travel, but their functional evolution was quite similar especially for nuisance
179 species. In the two travels, BW communities were enriched in potential nuisance species
180 (harmful algae and known invasive species), of both phytoplankton and zooplankton,
181 with significantly increasing trends (Fig. 4; r^2 values of the slope and their significance
182 are given in the graph). A closer look at the resistant, potentially harmful species present
183 on day#20 (the last day of BW observations in 2012) showed that near one half were
184 autotrophs (Supplementary Tables 1A for 2012 and 1B for 2016), several of them with
185 symbiotic species.

186

187 **Discussion**

188 In this study, we have found evidence of the proportion of potentially harmful species
189 increasing in BW during long voyages, and at least some resistant species surviving much
190 longer than 10 days, even reproducing. Although the overall decline in BW biodiversity
191 observed visually in these two voyages was rather consistent with trends reported
192 elsewhere (Gollasch et al. 2000a; Gollasch et al. 2000b; Olenin et al. 2000; Cordell et al.
193 2009), the results support the hypothesis of risk of introduction of invasive species

194 increasing with BW residence time. Although the abiotic conditions were different and
195 the diversity was not the same in the two voyages examined, they shared the same trend:
196 old BW –at the end of the voyage- contained a higher proportion of species potentially
197 invasive than at the beginning of the voyage; even resistant to current BW treatments,
198 that are effective only for larger organisms (Lakshmi et al., 2021).

199 The species seen here to reproduce until the end of the voyage are indeed potentially
200 harmful and are known to be highly resistant. Briski et al. (2010, 2011) reported
201 diapausing eggs of *Brachionus* and *Keratella* from ballast sediments of transoceanic
202 ships. It is possible that the increase of small ciliated zooplankton observed *de visu* the
203 last survey day in 2012 (already described by Zaiko et al. 2015b) was due to the hatching
204 of resting eggs entrapped in ballast tank sediments. On the other hand, the two copepod
205 species found persistently in visually analyzed samples till the end of 2016 cruise are
206 known for their wide environmental tolerances. Thus, *Paracalanus parvus* is nearly
207 cosmopolitan, found in the oceans worldwide except in the Arctic -some records in the
208 Indian Ocean and the Pacific to be confirmed (www.marinespecies.org). *Nitokra spinipes*
209 has been found from many latitudes in the Northern Hemisphere, at large temperature
210 (from 0 to 26°C) and salinity (from 0 to 30‰) ranges (Dahl & Breitholz 2008), and is an
211 invasive species to Lake Ladoga, Russia (Dudakova 2012). Although there is not one
212 specific trait or a specific set of traits common to all invasive species, there is a suite of
213 traits that invasive species often have. Not all invasive species will have all the traits, but
214 most of them seem to have one or more of these traits; amongst them are: a broad native
215 range, tolerance to a wide range of conditions, and a generalist habitat (Williams and
216 Meffe, 1998). Therefore, considering the golden rule of invasive species “the more
217 tolerant the more dangerous” (Ardura et al. 2015), these species might be assumed of high
218 bioinvasion risk, especially in the light of their survival in the harsh BW conditions over
219 quite extreme transequatorial transit, and their more than likely resistance to currently
220 available BW treatments effective for the larger organisms (Lakshmi et al., 2021).

221 This new view of old BW could have an indirect support from bacteria. In recent studies
222 on BW bacterial community, Khandeparker et al. (2020) and Wang et al. (2020)
223 discovered that voyage duration or, more generally, BW holding time influence in
224 different ways the pathogenic community. In Khandeparker et al. (2020) study, the
225 diversity of pathogenic bacteria was greater mid-voyage than at the end, while Wang et
226 al. (2020) found a greater diversity of bacterial pathogens in long holding time BW.
227 Khandeparker et al. (2020) attributed the increase of viable bacterial diversity during the
228 voyage to the decline of phytoplankton and zooplankton, because phytoplankton and
229 zooplankton mortality could release the bacteria associated with them, which could
230 influence the bacterial diversity; therefore, it is probably that the increase of some
231 eukaryotic species that reproduce in old BW, found in our study as *Brachionus* sp. in
232 2012 and *N. spinipes* and *P. parvus* in 2016, contributes to control pathogenic bacteria in
233 BW after several days.

234 This study was built upon a combination of visual observation, DNA barcoding and
235 metabarcoding. We have seen living individuals after 20 and 28 days of voyage and

236 observed an increase in their abundance (rotifers) or juveniles (copepods), so we can be
237 reasonably sure they are reproducing. Metabarcoding alone would not be sufficient,
238 because the mere presence of DNA in water samples does not ensure that the species is
239 alive (e.g., Kelly et al. 2014, Von Ammon et al. 2018). The bigger proportion of species
240 whose DNA appeared until the end of the travel of 2012 (versus 2016) could be explained
241 from a longer preservation of DNA in anoxic conditions (Borin et al. 2008, Corinaldesi
242 et al. 2011). This would explain also the apparent discrepancy between the rapid decline
243 of individuals but slow reduction of MOTU that year. Anoxic conditions were shown to
244 slow marine eDNA decay (Weltz et al., 2017), and may affect inferences made from
245 eDNA regarding contemporary species presence. This effect is very strong. In ponds,
246 anoxic conditions enhance preservation of sediments and the communities that live there
247 so dramatically, that historical pond biodiversity can be assessed from sediments' eDNA
248 (Alderton et al., 2017; Emson et al., 2017; Harper et al., 2018).

249 In conclusion, the increase in the proportion of potentially harmful species along the
250 travel suggests that the BW age should not be directly employed in risk analysis as
251 currently done. Although the density of organisms declined and was quite low mid-travel,
252 it increased towards the end in both 2012 and 2016 cruises. All together the results
253 indicate a selection for resistant species that will be a function of the travel duration. Final
254 communities will be poorer in diversity but richer in problematic species that may survive
255 and adapt better in the recipient ecosystem, perhaps outcompeting local species that
256 occupy the same niche. On the other hand, some species were found in BW sediments in
257 other cruises (Briski et al. 2010, 2011). This should be taken as an additional call for
258 caution, because species occurring in tank wall biofilms and sediments resist treatments
259 and current ballast water exchange (Baier et al. 2014). Moreover, if we consider that a
260 very big amount of BW is daily transported and there is an important variation between
261 communities transported by ships within the same route, we are exponentially multiplying
262 the risks. From our results, recommendations would be: a) to concentrate on species
263 contained in old BW for the design of biocide water treatments; b) to design future ballast
264 tanks where sediment deposition is minimized.

265

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273 **Author Contributions**

274 E.G.V., A.A. and A.Z. conception and design; E.G.V. and A.Z. sampling; E.G.V. and
275 A.Z. onboard samples analysis; A.A. laboratory analysis; A.A. and J.L. NGS analysis;

276 E.G.V. and A.A. statistical analysis; A.A. and E.G.V. writing the article and finally, all
277 authors revised the manuscript.

278 **Accessibility of molecular data**

279 The individual barcodes are available in GenBank
280 (<https://www.ncbi.nlm.nih.gov/genbank/>) with the reference numbers MK295012-
281 MK295026 for 18S and MK314115-16 for rbcL. The Fastaq files are available in
282 GenBank, reference PRJNAS510002 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA510002>)
283 with the references SAMN10592608-SAMN10592648 (18S rDNA) and
284 SAMN10592566-SAMN10592607 (COI gene).

285 **Additional Information**

286 Competing Interests: The authors declare no competing interests.

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478 **Figure legends**

479 **Fig. 1.** Ship map with maritime routes and their densities (<https://www.kiln.digital/>).
480 Colour lines and ship cargo correspondence: yellow, containers; blue, dry bulk; red,
481 tankers; green, gas bulk; purple, vehicles. The orange line marks the equator and the line
482 with blue dots the trans-equatorial route of RV Polarstern. Vertical heat colour scales
483 (dark red for higher values to dark blue for lower values) show changes in main
484 environmental factors along the travels: % of dissolved oxygen (mg/L), NH₄ (mg/L) and
485 Temperature (°C) as T. ND: no data available.

486 **Fig. 2.** Organisms observed visually in ballast water in ANT XXIX-1 (PA) and PS102
487 (PB) expeditions along the voyage. Density of organisms per 100 L (presented in relation
488 to the density of the first voyage day) and proportion of autotroph individuals.

489 **Fig. 3.** Evolution of functional diversity of the analysed ballast water communities on RV
490 Polarstern's ANT XXIX-1 (PA) and PS102 (PB) expeditions. Proportion of autotroph
491 species detected from DNA; functional richness (as proportion of species over the
492 maximum detected); Shannon diversity, estimated from the number of MOTU assigned
493 to each functional group (autotrophs, mixotrophic, phagotrophic, parasites, saprophytes,
494 symbiotic, other).

495 **Fig. 4.** Evolution of risk species in Polarstern ballast water along trans-equatorial travels,
496 presented as odds of risk species (identified from DNA) in phytoplankton and
497 zooplankton. Best-fit equations of trend lines and their r^2 are shown. Risk species are
498 harmful algae and invasive species recognized in scientific literature and international
499 databases. PA: ANT XXIX-1 expedition; PB: PS102 expedition. Significance of slopes
500 is given: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

501

Supplementary Table 1. Functional analysis of the taxa found in RV Polarstern ballast water along travel days across the Equator. A: ANT XXIX-1 EUROPA cruise nuisance species; B: PS102-2016 nuisance species. Nuisance species are harmful algae and invasive species recognized by IUCN and relevant literature. Results are presented as presence 1, absence 0. Densities (individuals/100L) of >20µm organisms observed at x50 magnification, classified as phytoplankton or zooplankton, are presented by sampling day.

Supplementary Table 2. Taxa found in RV Polarstern ballast water along travel days across the Equator, showing the genera identified from de visu, barcoding and/or NGS methodology in: A) ANT XXIX-1 EUROPA cruise of 2012; B: PS102 cruise of 2016. Results are presented as presence 1, absence 0. Coincident taxa identified from visual and molecular methodologies are marked with *.