Experimental assessment of copepod survival in response to the harmful dinoflagellate Karenia selliformis from the southeastern coast of Hokkaido, Japan

Experimental assessment of copepod survival in response to the harmful dinoflagellate *Karenia selliformis* from the southeastern coast of Hokkaido, Japan

TAKUYA OHNISHI[1,*], YUKIKO TANIUCHI[1], TSUYOSHI WATANABE[1], TOMOYUKI SHIKATA[2], HIROMI KASAI[1]

[1] *Kushiro Field Station, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Kushiro, Hokkaido 085–0802, Japan*

[2] *Goto Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Goto, Nagasaki 853–0508, Japan*

* Corresponding author: Takuya Ohnishi; E-mail, oonisi_takuya92@fra.go.jp

Running page head: Effect of *K. selliformis* on copepod survival

18 **Abstract**

19 In the autumn of 2021, a large-scale harmful algal bloom (HAB) emerged along the
20 southeast coast of Hokkaido, Japan, and this was predominantly composed of the dinoflagellate
21 *Karenia selliformis*. The emergence of *K. selliformis*-dominated HABs was the first observation
22 in Japan; therefore, no previous reports exist on the impact of HABs on zooplankton in the
23 region. This study investigated the effects of *K. selliformis* on the survival of copepods—a
24 critical component of the zooplankton community. The results indicated that the survival rates
25 of all six copepod species examined were markedly reduced, particularly at elevated *K.*
26 *selliformis* concentrations or during extended exposure. The copepod survival rate decline
27 occurred even in the absence of direct contact or ingestion of *K. selliformis*, thus implying that
28 *K. selliformis* may excrete harmful substances extracellularly. Feeding experiments revealed
29 that *Neocalanus plumchrus* consumes *K. selliformis* cells; however, the ingestion rate
30 diminished with increased concentrations of *K. selliformis* cells. The results suggest that larger
31 copepods with smaller surface-to-volume ratios may possess greater resilience to the harmful
32 substances compared to smaller species. Further, the HAB in southeast Hokkaido likely exerted
33 deleterious effects on lower trophic levels within the marine ecosystem by disrupting copepod
34 survival and feeding activity.

35 **Key words:** copepod survival, harmful algae, ingestion rate, *Karenia selliformis*, marine
36 ecosystem

Introduction

The emergence of a significant harmful algal bloom (HAB) was observed along the Pacific coast of southeastern Hokkaido, Japan, in 2021. The dinoflagellate *Karenia selliformis* Haywood was identified as the dominant species in the HAB, marking the first instance of a *K. selliformis*-dominated bloom in Japan (Iwataki et al. 2022). The HAB persisted from mid-September to late November (Kuroda et al. 2021) and extended >300 km along the southeastern coast of Hokkaido, affecting areas with a depth of <300 m (Kuroda et al. 2022, Takagi et al. 2022). High-density blooms were noted on the continental shelf, sometimes exceeding 10^4 cells mL^{-1} (Kuroda et al. 2022). The prolonged and extensive HAB severely impacted marine life and fisheries, with substantial mortalities in species such as salmon, sea urchins, and mussels (Hasegawa et al. 2022, Iwataki et al. 2022). However, research on these effects has been primarily focused on the mass mortalities of commercially important fishes and larger, visually observable organisms.

HABs are increasingly reported in previously unaffected regions, yet their impacts on marine ecosystems in these areas are poorly understood (Gobler 2020). The rising frequency, intensity, and geographical spread of HABs in marine ecosystems are a growing global environmental concern. To better comprehend how species involved in HABs disrupt marine ecosystems and form dense blooms, it is essential to clarify the interactions between HAB species and their potential predators. Phytoplankton, including HAB species, are predominantly

56 consumed by zooplankton, with copepods being the primary herbivorous zooplankton group.
57 According to reviews by Turner & Tester (1997) and Turner (2014), the impact of HAB species
58 on copepods varies; some studies have indicated that various copepods can graze on HAB
59 species without noticeable adverse effects, while other findings link exposure to or ingestion of
60 HAB species to negative impacts on copepods, including reduced feeding, growth,
61 development, egg production, and survival. Turner & Tester (1989) found that certain species
62 with in the genus *Karenia* were harmless to specific copepod species. Conversely, other studies
63 have found *Karenia* spp. detrimental to different copepod species (e.g., Huntley et al. 1986,
64 Uye & Takamatsu 1990, Breier & Buskey 2007). Variations between studies might be
65 attributable to differences in harmful algal species and their concentrations, fluctuating toxin
66 levels, and varying tolerance to specific toxins among copepod species and populations (Dam
67 2013, Turner 2014). The relationship between HAB species and herbivorous zooplankton is
68 inconsistent, highlighting the need for experiments with coexisting copepods and HAB species
69 to understand their trophic interactions (Turner 2014).

70 To investigate the effects of *K. selliformis* on copepods, this study conducted survival
71 and grazing experiments with copepods found in the southeastern region of Hokkaido, utilizing
72 *K. selliformis* isolated from the 2021 HAB in the same area. Our experimental design and
73 methodologies include:

74 - **Survival experiments:**

We structured these into control and experimental groups to monitor copepod mortality, using the Kaplan–Meier method and Generalized Linear Models to assess the impact of *K. selliformis*.

- **Grazing experiments:**

These were designed to measure ingestion rates and explore the relationship between copepod grazing behavior and algal cell concentrations, contrasting *K. selliformis* with a non-toxic diatom.

Materials and Methods

Karenia selliformis culture conditions

Karenia selliformis strain Ks-1 was isolated from seawater samples collected from the southeastern coast of Hokkaido, Japan, on 28 September 2021 (42° 56.65′N, 144°26.88′E). The clonal strain was not axenic. All references to *K. selliformis* within this paper pertain exclusively to this strain. The isolate was cultured in a growth chamber under specific conditions: a temperature of 13℃, a 12:12 hour light:dark photoperiod, salinity of 33.0, and cool white fluorescent lighting at an intensity of 150 μmol photons m^{-2} s^{-1}. The culture medium used was a modified seawater medium (modified SWM-3; hereafter referred to as SWM), as detailed by Yamasaki et al. (2007). For the experiments, *K. selliformis* cells in their logarithmic growth phase were used. Algal cell concentrations were measured using an optical plastic

94 plankton counter (Matsunami Glass Ind., Ltd., Osaka, Japan). In situations where algal cell
95 concentrations were low (≤100 cells mL^{-1}), a boundary slide glass (Matsunami Glass Ind., Ltd.,
96 Osaka, Japan) was utilized.
97
98 **Survival experiments**
99 To assess the impact of *K. selliformis* on copepod survival, we conducted experiments
100 with six copepod species from different taxonomic families: *Acartia tumida* Willey, 1920,
101 *Centropages abdominalis* Sato, 1913, *Eurytemora herdmani* Thompson & Scott, 1897,
102 *Metridia pacifica* Brodsky, 1950, *Neocalanus plumchrus* Marukawa, 1921, and *Paracalanus*
103 *parvus* Claus, 1863. These species are significant prey for juvenile fishes important to fisheries,
104 including salmon and walleye pollock (Okada & Taniguchi 1971, Nishiyama & Hirano 1985,
105 Nagata et al. 2007, Hirakawa et al. 2019). Zooplankton specimens were collected in 2022 using
106 a plankton net with a 45-cm mouth opening, 335-μm mesh, and 1-L cod-end. Off the
107 southeastern coast of Hokkaido aboard the R.V. "Hokko-Maru", the net was towed vertically
108 from a depth of 150 m, while at Kushiro Harbor, Hokkaido, the vertical tow was conducted just
109 above the seafloor at ca. 6 m (Fig. 1, Table 1). After collection, target copepod species were
110 immediately sorted from specimens with a large-bore pipette by naked eye or under a
111 stereoscopic microscope, and provided for the subsequent survival experiments.

112 The survival experiments included two control conditions and four experimental
113 conditions. The control group involved copepods reared in 0.8-μm filtered seawater (FSW) or
114 the SWM (see "*Karenia selliformis* culture conditions"); experimental group exposed copepods
115 to three different *K. selliformis* cell concentrations (10, 100, and 1000 cells mL^{-1}; hereafter
116 referred to as KS10, KS100, and KS1000, respectively), which reflected the densities observed
117 in the field during the 2021 HAB in southeast Hokkaido (Kuroda et al. 2021). Additionally,
118 copepods were reared in a *K. selliformis* elution (KSE) to examine the effect of extracellular
119 chemical compounds from *K. selliformis*. KSE was obtained by filtering KS1000 through a 0.2-
120 μm syringe filter (DISMIC-25CS; Advantec, Tokyo, Japan). We used 50-mL plastic culture
121 flasks (Corning, NY, USA) for the experiments, each containing one or more copepod
122 individuals (Table 1). The flasks were incubated in the dark. The incubation temperature was
123 set to the surface temperature when each copepod species was collected (Table 1). Dead
124 copepods were counted at 3, 6, 12, and 24 hours, and then every 24 hours for up to 8 days. A
125 copepod was considered dead if it showed no movement, even when prodded with a soft plastic
126 needle. No water replacements were conducted during the experimental period.
127 Survival curves were derived using the Kaplan–Meier method, with statistical
128 differences analyzed using log-rank pairwise tests (Kaplan & Meier 1958). A generalized linear
129 model (GLM) analysis was conducted to assess the relationship between the median lethal time
130 (LT_{50}) and conditions, including incubation temperature, copepod species, and treatments with

131 *K. selliformis*. Incubation temperature was used as a numerical explanatory variable, whereas
132 copepod species and treatments with *K. selliformis* were categorical explanatory variables. The
133 'bestglm()' function in the *bestglm* package v. 0.37.3 (McLeod et al. 2020) determined the best
134 subset of variables based on the Akaike Information Criterion (AIC). The GLM assessed the
135 effects of the best subset of variables on LT_{50}, using a Gaussian error distribution with the base
136 R function 'glm()'. All statistical analyses were performed in R v. 4.1.2 (R Core Team 2023)
137 using R Studio v. 2022.02.0+443.
138 To explore grazing on *K. selliformis*, fecal pellet production was monitored. The
139 presence of fecal pellets was checked almost daily, and species producing fecal pellets were
140 subjected to grazing experiments.
141
142 **Grazing experiments**
143 Grazing experiments were conducted specifically on *N. plumchrus*, as it was the only
144 species among the copepods that produced fecal pellets during the survival experiments. The
145 objective was to determine the relationship between their ingestion rate and algal cell
146 concentration. The non-toxic diatom *Bacterosira* sp. was used as the control prey. *Bacterosira*
147 sp. was isolated off Mombetsu in Hokkaido, Japan, in March 2021 and cultured at 4°C, under
148 a 12:12 hour light:dark photoperiod with cool white fluorescent lighting at an intensity of 80

149 μmol photons m^{-2} s^{-1} using f/2 medium with a salinity of 33.0. The culture condition for *K.*
150 *selliformis* was the same as described in the section "*Karenia selliformis* culture conditions".
151 Zooplankton specimens were collected from the southern area of the Sea of Okhotsk
152 (45°24.98′N, 145°10.02′E) on 24 August 2022 using the same plankton net as in the survival
153 experiment, aboard the R.V. "Wakataka-Maru". The net was towed vertically from a depth of
154 150 m. Post-collection, *N. plumchrus* were sorted and reared in a 3-L bottle filled with 0.8-μm
155 filtered seawater. The *N. plumchrus* sorted for this study were checked to be actively swimming.
156 During the cruise, the bottle was stored in an incubator at 5°C in the dark. In the laboratory,
157 each copepod was individually transferred to its own 50-mL plastic culture flask (Corning, NY,
158 USA), ensuring that each flask contained only one copepod. These flasks were then
159 supplemented with algae at concentrations ranging from 100 to 2000 cells mL^{-1}, with each flask
160 dedicated to a single type of algae — either *Bacterosira* sp. or *K. selliformis*. For *Bacterosira*
161 sp., 18 flasks were prepared, and for *K. selliformis*, 79 flasks were used. In addition to these
162 experimental flasks, ten control flasks without any copepods were also established for each
163 algae type. The experiment was conducted on a plankton wheel, which rotated at ca. 1 rpm at
164 5°C in the dark to promote uniform algal distribution. Due to the plankton wheel's capacity to
165 hold only 20 flasks at a time, the experiment was carried out in six separate runs. Algal
166 concentrations in each flask were measured at the beginning and end of the 24-hour grazing

experiment to calculate the ingestion rates, which were determined following the formula proposed by Frost (1972):

$$k = \frac{\ln C_t - \ln C_0}{T}$$

$$g = k_{AVG} - \frac{\ln E_t - \ln E_0}{T}$$

$$I = \frac{E_0 - E_t}{\ln E_0 - \ln E_t} \times \frac{Vg}{N}$$

where k is the algal growth coefficient; g is the grazing coefficient; I is the ingestion rate (cells copepod^{-1} hour^{-1}); C_0 and C_t are cell concentrations (cells mL^{-1}) in the control flask at the beginning and after 24 hours of the experiment, respectively; k_{AVG} represents the average algal growth coefficient, calculated as the mean of k values obtained from the control flasks; E_0 and E_t are cell concentrations (cells mL^{-1}) in the experimental flask at the beginning and end of the 24-hour grazing experiment; T is the duration of the grazing experiment (hours); V is the volume of seawater in the flask (mL); and N is the number of copepods in the flask.

Based on the calculated ingestion rates, a simple linear regression analysis was conducted to investigate the relationship between ingestion rate (I) and initial cell concentration (E_0) for each algal species, *Bacterosira* sp. and *K. selliformis*, using Microsoft Excel for Mac v. 16.81.

Results

Effects of *Karenia selliformis* on copepod survival

In the survival experiment, the control group exhibited either a 100% survival rate or a gradual decline for all copepod species, except for *Paracalanus parvus* (Fig. 1). The survival rate of *P. parvus* in the control group was below 50% within 24 hours in the SWM condition and within 48 hours in the FSW condition. No significant differences were observed between the survival curves of SWM and FSW for *P. parvus* (Table 2, Fig. 2). For *Centropages abdominalis*, *Eurytemora herdmani*, and *Metridia pacifica*, the survival rate in all experimental conditions decreased with exposure time, showing significant differences from the control group. For the remaining species, *Acartia tumida*, *Neocalanus plumchrus*, and *P. parvus*, some experimental conditions did not show significant differences in the survival curve from that of the control group. In *A. tumida* and *P. parvus*, the survival curve did not differ significantly between the KS10 condition and control group. In *N. plumchrus*, neither KS10 nor KS100 treatments had lethal effects on all individuals, and there were no significant differences in the survival curve from that of the control group. In the KS1000 condition, significant differences in the survival curve were observed for all species compared to that of the control group. *Acartia tumida*, *C. abdominalis*, and *P. parvus* experienced a rapid decline in survival, dropping below 10% within the first 24 hours. Conversely, *E. herdmani* and *N. plumchrus* showed no immediate impact on survival within the first 24 hours, with a progressive decrease in survival rates thereafter. In the KSE condition, all species experienced reduced survival rates, with survival curves significantly differing from those of the control group.

205 The LT_{50} for each experimental condition was calculated from the survival experiment
206 results (Table 3). The LT_{50} varied widely among species and treatments, ranging from 3 to 218
207 hours. As the concentration of *K. selliformis* increased, there was a notable decrease in LT_{50}
208 (e.g., for *E. herdmani*, 218, 171, and 113 hours for KS10, KS100, and KS1000, respectively).
209 The LT_{50} of KSE was slightly higher than that of KS1000. The 'bestglm()' function selected
210 both copepod species and treatments with *K. selliformis* as the best subset of variables,
211 underlining their significant effects on LT_{50} (Table 4). The incubation temperature was not a
212 significant factor in the model. The GLM analysis using the 'glm()' function analyzed the
213 relationship between LT_{50} and the best subset of variables (Table 5). Several copepod species
214 showed significant associations with LT_{50}, with *E. herdmani* and *N. plumchrus* displaying high
215 significance and substantial coefficients. Among the treatments with *K. selliformis*, KS1000
216 and KSE showed significant negative coefficients.
217 During the survival experiments, only *N. plumchrus* produced fecal pellets in the KS10
218 and KS100 treatments, whereas other species did not produce any fecal pellets.
219
220 **Grazing rate of *Neocalanus plumchrus* on *Karenia selliformis***
221 The grazing experiment focused exclusively on *N. plumchrus*, as it was the only
222 species that produced fecal pellets during the survival experiments. When feeding on the non-
223 toxic diatom *Bacterosira* sp., there was a positive correlation between food concentration and

224 ingestion rate (n = 18, r^2 = 0.642, p<0.001) (Fig. 3). However, this correlation was not observed
225 when *N. plumchrus* fed on *K. selliformis*. Instead, a negative correlation was found between *K.*
226 *selliformis* concentration and ingestion rate (n = 79, r^2 = 0.300, p<0.001). In the experiments
227 with *Bacterosira* sp., all 18 individuals fed. In contrast, 49.4% of the 79 individuals did not feed
228 in the *K. selliformis* experimental group.
229
230 **Discussion**
231 In this study, we examined the survival rates of copepods exposed to the harmful
232 dinoflagellate *Karenia selliformis*. We observed distinct survival patterns between the control
233 group and various experimental conditions, indicating that *K. selliformis* significantly impacts
234 copepod survival (Table 2, Fig. 2). However, this negative effect varied among the six copepod
235 species studied. Specifically, *Acartia tumida*, *Centropages abdominalis*, and *Paracalanus*
236 *parvus* experienced substantial reductions in survival rates in the KS1000 condition, falling
237 below 10% within the first 24 hours of exposure. For *P. parvus*, the survival rate fell below
238 50% in the SWM after 24 hours. The observed reduction in the survival rate in the control group
239 may not be primarily due to incubation temperature and starvation, as a previous study has
240 demonstrated survival under similar conditions (7 days at 18℃ without feeding; Checkley
241 1980). The reduction in survival rates in our study, particularly during the handling of *P. parvus*,
242 could potentially be attributed to stress induced by net sampling and sorting processes.

Although it is difficult to exclude the potential effects of factors other than the influence of *K. selliformis*, the fact that the survival rate maintained 100% in the FSW for up to 12 hours, while it dropped below 10% in the KS1000, clearly indicates a rapid impact of *K. selliformis* on the survival rate of *P. parvus*. In contrast, *Eurytemora herdmani* and *Neocalanus plumchrus* showed no immediate change in survival rates within the first 24 hours but exhibited gradual declines thereafter.

Previous research on copepod exposure to *Karenia* species, such as *Karenia mikimotoi* Hansen & Moestrup and *Karenia brevis* Davis—as reviewed by Turner (2014)—has reported harmful effects on copepod survival, while some studies observed no adverse impacts. The variability in experimental designs, including differences in exposure durations, cell concentrations, and algal strains, makes it challenging to draw conclusive comparisons across studies. Uye & Takamatsu (1990) examined the effects of harmful algae on two copepod species and found different survival patterns between *Pseudodiaptomus marinus* Sato, 1913 and *Acartia omorii* Bradford, 1976 when exposed to *K. mikimotoi*, thus highlighting varying responses among copepod species even under similar experimental conditions.

Moreover, many previous studies on the effects of HABs on copepod survival have employed relatively short exposure periods, often limited to 24 hours. Turner (2014) has emphasized the importance of extending experimental durations to several days or weeks to accurately capture the impacts of HABs, considering their actual occurrence durations. Our

findings demonstrate variability in the tolerance of different copepod species to *K. selliformis* (Fig. 2), suggesting that short-term experiments might not fully capture the extent of the negative impacts on copepod survival. This study contributes to our understanding of the differential responses of copepod species to HABs and highlights the need for species-specific considerations in assessing the impacts of HABs on marine ecosystems. However, it is important to acknowledge that our experimental design did not include water replacements, potentially leading to an overestimation of mortality rates. The absence of water replacements could result in the accumulation of waste products from organisms and a decline in water quality over time, factors that could independently affect copepod survival. Incorporating periodic water replacements and/or providing non-toxic feed to the control group could enable a more accurate assessment of copepod responses to *K. selliformis*.

In addition to observational data, we used a GLM to quantitatively determine the effects of various factors on the LT_{50} (Tables 4, 5). Examining the model summary, the 'bestglm()' function underscored the impact of both the copepod species and treatment with *K. selliformis* as the best subset of variables on determining LT_{50}. The copepod species significantly affected LT_{50}; similarly, the treatment with *K. selliformis* was a critical factor, as indicated by the *F*-value of 4.664 and significant *p*-value (Table 4). The incubation temperature was not included in the best subset of variables, possibly due to multicollinearity. Since the incubation temperature in our experiments was set according to the surface temperature at the

collection sites for each copepod species, the parameters might not be uniquely identifiable due to almost complete collinearity between the incubation temperature and copepod species. Therefore, the potential variability in *K. selliformis* harmfulness at different temperatures cannot be ruled out. *Acartia tumida*, *E. herdmani*, and *N. plumchrus* significantly influenced LT_{50} values; notably, their incubation temperature was set at 5°C, which was lower than that of other species (8–13°C) (Table 1). Takagi et al. (2022) reported that the estimated preferred temperature range for *K. selliformis* growth is 11–17°C, with an optimum temperature of approximately 14°C. This preferred growth temperature range was derived from analyses of chlorophyll *a* concentration and sea surface temperature obtained from satellite imagery during the 2021 HABs. A mismatch between the temperature during the survival experiments and the preferred growth temperature for *K. selliformis* may affect the physiological state of *K. selliformis*, including harmful substance production. Previous studies have found that the toxicity of *Karenia* species changes with different culture conditions (Brown et al. 2006, Medhioub et al. 2009, Wang et al. 2019).

Based on the best subset of explanatory variables (copepod species and treatment with *K. selliformis*), the GLM analysis highlighted the significant influence of specific copepod species on LT_{50} (Table 5). Notably, *N. plumchrus* and *E. herdmani* had highly significant coefficients and *A. tumida* showed a marginally significant coefficient. Coefficient variations between species suggests that there are differences in how susceptible each species is to the

300 effects of *K. selliformis*. Although we cannot rule out the possibility that differences in
301 temperature may affect the harmfulness of *K. selliformis*, the observation of different
302 coefficients even among copepod species reared at the same temperature (*A. tumida*, *E.*
303 *herdmani*, and *N. plumchrus* were reared at 5°C; Table 1) emphasizes the variation in resistance
304 to *K. selliformis* among copepod species. Moreover, the GLM analysis revealed the importance
305 of treatment with *K. selliformis*, with KS1000 and KSE significantly influencing LT_{50}. Focusing
306 on the cell concentration of *K. selliformis*, higher cell concentrations of *K. selliformis* resulted
307 in a stronger impact on LT_{50}. This trend suggests that in the HABs of eastern Hokkaido in 2021,
308 especially in areas where *K. selliformis* cell concentrations were notably high (sometimes
309 exceeding 10^4 cells mL^{-1}; Kuroda et al. 2022), copepod survival may have been severely
310 threatened.

311 In our study, all copepod species exposed to *K. selliformis* elution (KSE) exhibited
312 survival curves that significantly differed from those of the control group (Table 2, Fig. 2). The
313 patterns of decline in survival rates for copepods exposed to KSE were similar to those observed
314 in the KS1000 condition. Since KSE was prepared by just removing *K. selliformis* cells from
315 the KS1000, these findings imply that copepod survival decreased even without direct physical
316 contact or ingestion of *K. selliformis*. This points to the possibility of *K. selliformis* releasing
317 harmful substances extracellularly. The phenomenon of extracellular toxin secretion by related
318 species such as *K. mikimotoi* and *K. brevis* has been well established (Pierce & Henry 2008,

319 Prince et al. 2010, Seger & Hallegraeff 2022). Additionally, Tang et al. (2021) identified the
320 extracellular secretion of gymnodimine by *K. selliformis* isolated in Tunisia.

321 If the *K. selliformis* in our study indeed released harmful substances extracellularly, it
322 is likely that no additional harmful substances accumulated in the cell-free KSE flasks during
323 the survival experiments. The absence of active *K. selliformis* cells could result in a slower
324 decline in survival rates and a slightly higher LT_{50} for KSE compared to those of the KS1000
325 condition (Table 3, Fig. 2). In regions where HABs of *K. selliformis* occur at high
326 concentrations (e.g., near the southeastern coast of Hokkaido) where concentrations sometimes
327 exceed 10^4 cells mL^{-1} (Kuroda et al. 2021), the harmful substances released into the
328 environment by *K. selliformis* could have broader ecological implications. These harmful
329 substances may pose risks for other phytoplankton as competitors and higher trophic level
330 organisms that do not typically feed on them, in addition to organisms directly consuming the
331 algae.

332 Our study did not identify the specific harmful substances involved; notably, different
333 toxin profiles can be produced within the same harmful algal species under varying conditions
334 and strains (Kubis et al. 2023). Therefore, future research should focus on identifying the
335 harmful substances produced by *K. selliformis* isolated from the HAB off the southeastern coast
336 of Hokkaido. Gaining an understanding of these harmful substances and their effects on a range

337 of marine organisms is crucial for a comprehensive assessment of the impact of *K. selliformis*
338 blooms on marine ecosystems.

339 During the survival experiment, *N. plumchrus* was the only copepod species among
340 those tested that did not show a decrease in survival in specific experimental conditions (KS10
341 and KS100) (Fig. 2). In addition, *N. plumchrus* exhibited the second-highest LT_{50} at 108 hours
342 in the KS1000 condition (Table 3). Notably, *N. plumchrus* was the largest copepod in the study,
343 with a prosome length of ~4 mm, followed by *M. pacifica* at ~2 mm. The potentially higher
344 resistance of *N. plumchrus* to *K. selliformis* compared to that of other copepod species might
345 be attributed to its larger body size, which results in a lower surface-to-volume ratio. This ratio
346 is crucial in determining the sensitivity of an organism to external toxins (Christoffersen 1996).

347 During the survival experiments with KS10 and KS100, *N. plumchrus* produced fecal
348 pellets, thus indicating ingestion of *K. selliformis*. To explore this further, we conducted a
349 grazing experiment using *Bacterosira* sp., (a non-toxic diatom) as the control prey to determine
350 the ingestion rate of *N. plumchrus* on *K. selliformis*. Typically, copepod ingestion rate increases
351 with food concentration up to a certain point (Frost 1972). However, the relationship between
352 ingestion rate and algal concentration was different when *N. plumchrus* was fed *K. selliformis*
353 compared to that when fed *Bacterosira* sp. (Fig. 3). With *Bacterosira* sp., the ingestion rate was
354 positively correlated with algal concentration, and all individuals consumed it. In contrast, the
355 ingestion rate of *N. plumchrus* did not increase with algal concentration when fed *K. selliformis*

356 and showed a negative correlation; approximately half of the individuals did not feed on *K.*
357 *selliformis* at all. This suggests that the ingestion rate of *N. plumchrus* is lower when feeding
358 on *K. selliformis*, particularly at higher *K. selliformis* concentrations.

359 Xu & Kiørboe (2018) studied the impact of a Tunisian strain of *K. selliformis* on the
360 feeding behavior of *Temora longicornis* Müller, 1785 from Øresund, Denmark. They found that
361 *T. longicornis* could graze on *K. selliformis* but at a reduced rate compared to that when grazing
362 on non-toxic algae, which supports the findings of our study. *Neocalanus plumchrus* is a
363 dominant herbivorous copepod in the subarctic Pacific Ocean and its marginal seas (Kobari &
364 Ikeda 2001). In summer, *N. plumchrus* (along with other large copepods like *Neocalanus*
365 *cristatus* Krøyer, 1848, *Neocalanus flemingeri* Miller, 1988, and *Eucalanus bungii* Giesbrecht,
366 1893) can constitute 80–95% of the zooplankton biomass in the surface layer (Ikeda et al. 2004).
367 In the western North Pacific, most *N. plumchrus* individuals undergo a large-scale ontogenetic
368 vertical migration and exit the surface waters around August (Tsuda et al. 1999). The absence
369 of these large, toxin-tolerant copepods in the surface layer during autumn may possibly have
370 been advantageous for the bloom development of *K. selliformis* in southeastern Hokkaido.
371 Although zooplankton grazing cannot terminate an established HAB, it has the potential to
372 inhibit initial bloom development (Turner 2010, 2014). Enhancing our understanding of the
373 interactions between harmful algal species and copepods is crucial in comprehending the
374 mechanisms behind the formation and termination of HABs (Turner 2006).

375 This study explores the influence of the harmful dinoflagellate *K. selliformis* on the
376 survival and ingestion rates of copepods. The observed significant differences in survival curves
377 between control and experimental group underscore the detrimental effect of *K. selliformis* on
378 various copepod species. Our findings point to the extracellular release of harmful substances
379 by *K. selliformis*, highlighting its potential widespread impact on marine organisms in affected
380 areas. These results suggest that *K. selliformis* could have a considerable impact on the lower
381 trophic levels of the marine ecosystem, particularly in regions experiencing HABs. To fully
382 comprehend the ecological consequences of *K. selliformis* blooms, further research is necessary
383 to identify the specific harmful substances it produces and to understand their effects on
384 different components of marine ecosystems.
385
386 **Acknowledgments**
387 We would like to express our sincere thanks to the captains, officers, and crew of the
388 R/V Hokko-maru and the R/V Wakataka-maru. This study was supported by funds from the
389 Japan Fisheries Research and Education Agency.
390
391 **References**
392 Breier CF, Buskey EJ (2007) Effects of the red tide dinoflagellate, *Karenia brevis*, on grazing
393 and fecundity in the copepod *Acartia tonsa*. J Plankton Res 29: 115–126.

394 Brown AFM, Dortch Q, Dolah FMV, Leighfield TA, Morrison W, Thessen AE, Steidinger K,
395 Richardson B, Moncreiff CA, Pennock JR (2006) Effect of salinity on the distribution,
396 growth, and toxicity of *Karenia* spp. Harmful Algae 5: 199–212.
397 Checkley DM (1980) Food limitation of egg production by a marine, planktonic copepod in
398 the sea off southern California. Limnol Oceanogr 25: 991–998.
399 Christoffersen K (1996) Ecological implications of cyanobacterial toxins in aquatic food
400 webs. Phycologia 35: 42–50.
401 Dam HG (2013) Evolutionary adaptation of marine zooplankton to global change. Annu Rev
402 Mar Sci 5: 349–370.
403 Frost BW (1972) Effects of size and concentration of food particles on the feeding behavior
404 of the marine planktonic copepod *Calanus pacificus*. Limnol Oceanogr 17: 805–815.
405 Gobler CJ (2020) Climate change and harmful algal blooms: insights and perspective.
406 Harmful Algae 91: 101731.
407 Hasegawa N, Watanabe T, Unuma T, Yokota T, Izumida D, Nakagawa T, Kurokawa T,
408 Takagi S, Azumaya T, Taniuchi Y, Kuroda H, Kitatsuji S, Abe K (2022) Repeated
409 reaching of the harmful algal bloom of *Karenia* spp. around the Pacific shoreline of
410 Kushiro, eastern Hokkaido, Japan, during autumn 2021. Fish Sci 88: 787–803.
411 Hirakawa K, Kaga T, Sato T, Kasai H (2019) Regional characteristics of zooplankton as food
412 for juvenile chum salmon *Oncorhynchus keta* in the coastal waters around Hokkaido,

413 with special reference to copepod community structure. Bull Plankton Soc Japan 66: 72–
414 85.
415 Huntley M, Sykes P, Rohan S, Marin V (1986) Chemically-mediated rejection of
416 dinoflagellate prey by the copepods *Calanus pacificus* and *Paracalanus parvus*:
417 mechanism, occurrence and significance. Mar Ecol Prog Ser 28: 105–120.
418 Ikeda T, Kanno Y, Ozaki K, Shinada A (2004) Metabolic rates of epipelagic marine copepods
419 as a function of body mass and temperature. Mar Biol 139: 587–596.
420 Iwataki M, Lum WM, Kuwata K, Takahashi K, Arima D, Kuribayashi T, Kosaka Y,
421 Hasegawa N, Watanabe T, Shikata T, Isada T, Orlova TY, Sakamoto S (2022)
422 Morphological variation and phylogeny of *Karenia selliformis* (Gymnodiniales,
423 Dinophyceae) in an intensive cold-water algal bloom in eastern Hokkaido, Japan.
424 Harmful Algae 114: 102204.
425 Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Am
426 Stat Assoc 53: 457–481.
427 Kobari T, Ikeda T (2001) Ontogenetic vertical migration and life cycle of *Neocalanus*
428 *plumchrus* (Crustacea: Copepoda) in the Oyashio region, with notes on regional
429 variations in body sizes. J Plankton Res 23: 287–302.

Kubis JAT, Rodríguez F, Rossignoli AE, Riobó P, Sar EA, Sunesen I (2023) Morphological, phylogenetic and toxinological characterization of potentially harmful algal species from the marine coastal waters of Buenos Aires Province (Argentina). Phycol 3: 79–105.

Kuroda H, Azumaya T, Setou T, Hasegawa N (2021) Unprecedented outbreak of harmful algae in Pacific coastal waters off southeast Hokkaido, Japan, during late summer 2021 after record-breaking marine heatwaves. J Mar Sci Eng 9: 1335.

Kuroda H, Taniuchi Y, Watanabe T, Azumaya T, Hasegawa N (2022) Distribution of harmful algae (*Karenia* spp.) in October 2021 off southeast Hokkaido, Japan. Front Mar Sci 9: 841364.

McLeod A, Xu C, Lai Y (2020) bestglm: Best subset GLM and regression utilities. R package version 0.37.3, URL https://CRAN.R-project.org/package=bestglm.

Medhioub A, Medhioub W, Amzil Z, Sibat M, Bardouil M, Ben Neila I, Mezghani S, Hamza A, Lassus P (2009) Influence of environmental parameters on *Karenia selliformis* toxin content in culture. Cah Biol Mar 50: 333–342.

Nagata M, Miyakoshi Y, Ando D, Fujiwara M, Sawada M, Shimada H, Asami H (2007). Influence of coastal seawater temperature on the distribution and growth of juvenile chum salmon, with recommendations for altered release strategies. Nor Pac Anadr Fish Comm Bull 4: 223–235.

448 Nishiyama T, Hirano K (1985) Prey size and weight relations in larval walleye pollock
449 (*Theragra chalcogramina*). Bull Plankton Soc Japan 32: 45–59.
450 Okada S, Taniguchi A (1971) Size relationship between salmon juveniles in shore waters and
451 their prey animals. Bull Fac Fish Hokkaido Univ 22: 30–36.
452 Pierce RH, Henry MS (2008) Harmful algal toxins of the Florida red tide (*Karenia brevis*):
453 natural chemical stressors in South Florida coastal ecosystems. Ecotoxicology 17: 623–
454 631.
455 Prince EK, Poulson KL, Myers TL, Sieg RD, Kubanek J (2010) Characterization of
456 allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. Harmful Algae
457 10: 39–48.
458 R Core Team (2023) R: A language and environment for statistical computing. R
459 Foundationfor Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
460 Seger A, Hallegraeff G (2022) Application of clay minerals to remove extracellular
461 ichthyotoxins produced by the dinoflagellates *Karlodinium veneficum* and *Karenia*
462 *mikimotoi*. Harmful Algae 111: 102151.
463 Takagi S, Kuroda H, Hasegawa N, Watanabe T, Unuma T, Taniuchi Y, Yokota T, Izumida D,
464 Nakagawa T, Kurokawa T, Azumaya T (2022) Controlling factors of large-scale harmful
465 algal blooms with *Karenia selliformis* after record-breaking marine heatwaves. Front Mar
466 Sci 9: 939393.

467 Tang Z, Qiu J, Wang G, Ji Y, Hess P, Li A (2021) Development of an efficient extraction
468 method for harvesting gymnodimine-A from large-scale cultures of *Karenia selliformis*.
469 Toxins 13: 793.
470 Tsuda A, Saito H, Kasai H (1999) Life histories of *Neocalanus flemingeri* and *Neocalanus*
471 *plumchrus* (Calanoida: Copepoda) in the western subarctic Pacific. Mar Biol 135: 533–
472 544.
473 Turner JT, Tester PA (1989) Zooplankton feeding ecology: copepod during an expatriate red
474 tide. In: Novel phytoplankton blooms: causes and impacts of recurrent brown tides and
475 other unusual blooms (eds Cosper EM, Bricelj VM, Carpenter EJ). Springer-Verlag,
476 Berlin, pp. 359–374.
477 Turner JT, Tester PA (1997) Toxic marine phytoplankton, zooplankton grazers, and pelagic
478 food webs. Limnol Oceanogr 42: 1203–1213.
479 Turner JT (2006) Harmful algae interactions with marine planktonic grazers. In: Ecology of
480 harmful algae (eds Granéli E, Turner JT). Springer-Verlag, Berlin, pp. 259–270.
481 Turner JT (2010) Zooplankton community grazing impact on a bloom of *Alexandrium*
482 *fundyense* in the Gulf of Maine. Harmful Algae 9: 578–589.
483 Turner JT (2014) Planktonic marine copepods and harmful algae. Harmful Algae 32: 81–93.
484 Uye S, Takamatsu K (1990) Feeding interactions between planktonic copepods and red-tide
485 flagellates from Japanese coastal waters. Mar Ecol Prog Ser 59: 97–107.

Wang H, Niu X, Feng X, Gonçalves RJ, Guan W (2019) Effects of ocean acidification and phosphate limitation on physiology and toxicity of the dinoflagellate *Karenia mikimotoi*. Harmful Algae 87: 101621.

Xu J, Kiørboe T (2018) Toxic dinoflagellates produce true grazer deterrents. Ecology 99: 2240–2249.

Yamasaki Y, Nagasoe S, Matsubara T, Shikata T, Shimasaki Y, Oshima Y, Honjo T (2007) Allelopathic interactions between the bacillariophyte *Skeletonema costatum* and the raphidophyte *Heterosigma akashiwo*. Mar Ecol Prog Ser 339: 83–92.

Figures

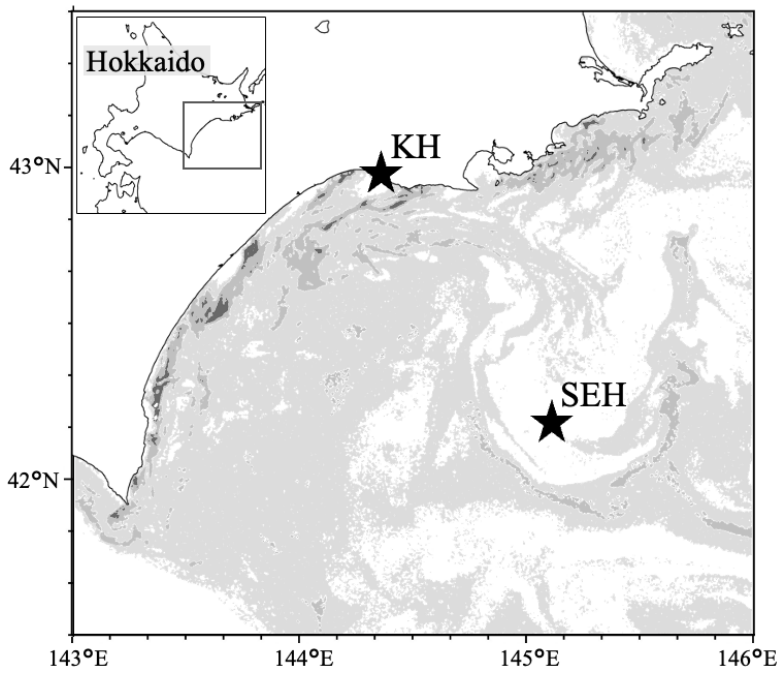
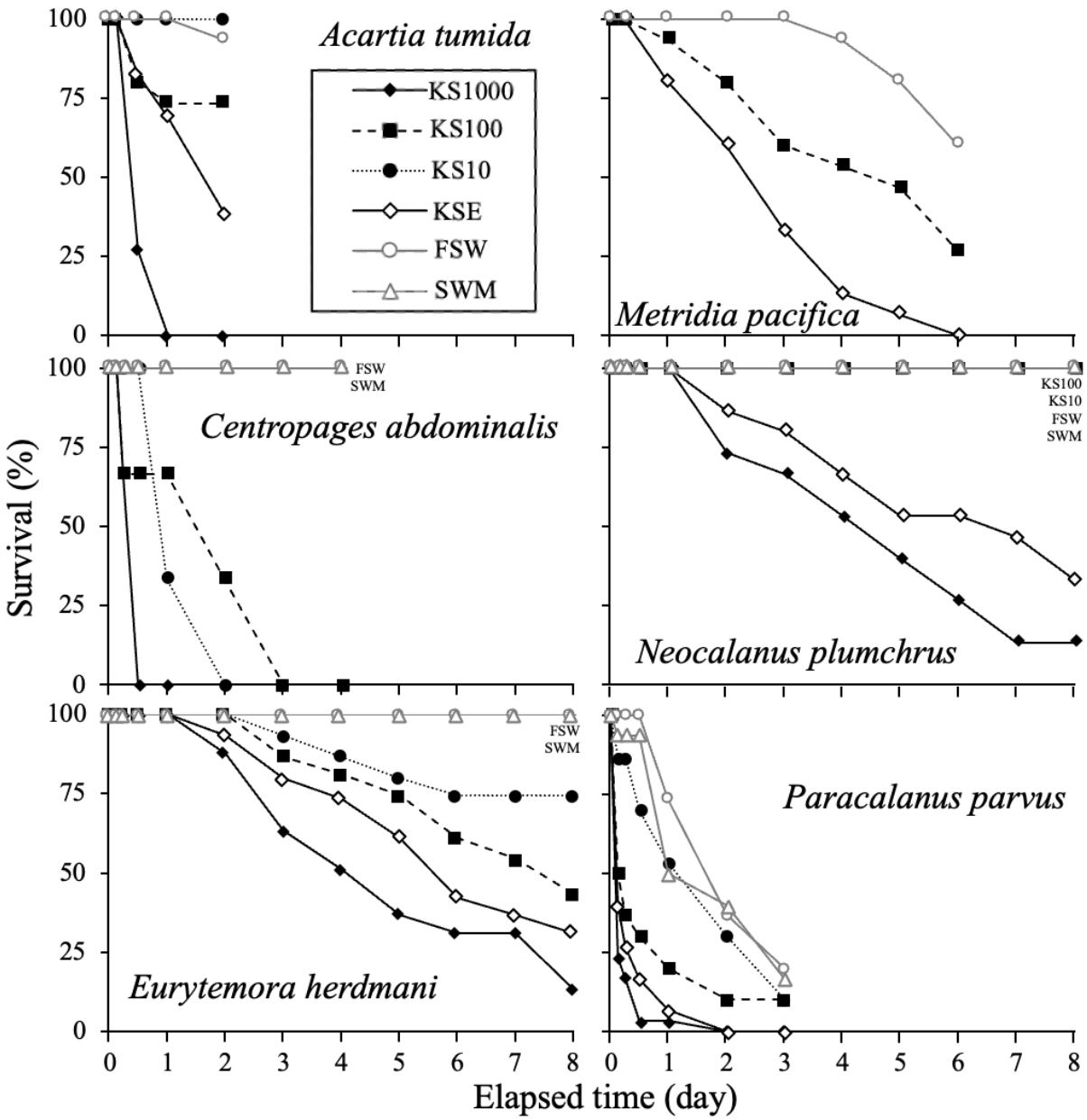


Fig. 1. Locations of zooplankton sampling sites for the survival experiment. The abbreviation "KH" marks the site at Kushiro Harbor (42°58.52′N, 144°22.23′E), while "SEH" denotes the location off the southeastern coast of Hokkaido (42°15.00′N, 145°07.50′E). Zooplankton specimens were collected in 2022 (see Table 1 for details). The gradient colors on the map schematically represent the distribution of *Karenia* spp. in southeastern Hokkaido as of 9 October 2021, according to estimates by Kuroda et al. (2022). Light gray indicates cell concentrations ranging from 10 to 100 cells mL^{-1}, medium gray signifies 100 to 1000 cells mL^{-1}, and dark gray marks concentrations exceeding 1000 cells mL^{-1}. Note that zooplankton specimens were not collected during the harmful algal bloom event.

506



507

508 **Fig. 2.** Survivorship of copepods exposed to three *Karenia selliformis* (KS) suspensions (10,

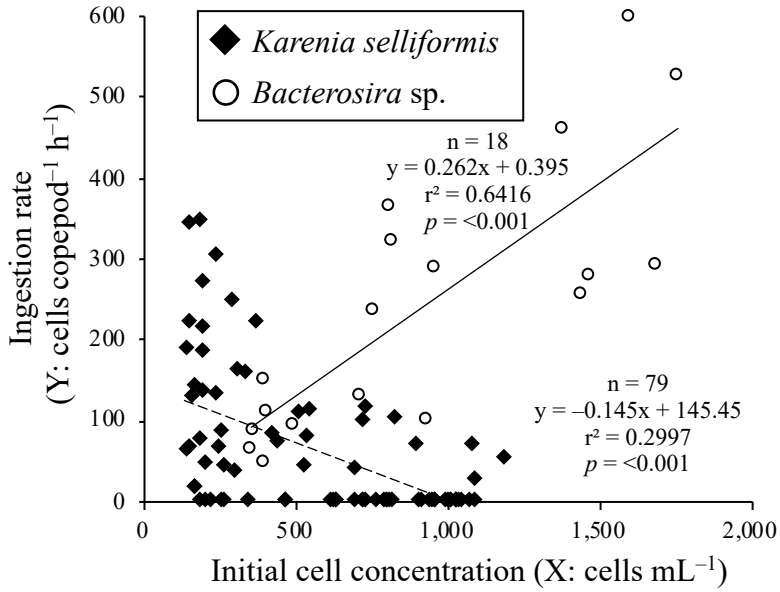
509 100, and 1000 cells mL^{-1}), KS elution (KSE) reflecting extracellular compounds, artificial

510 seawater (SWM), and 0.8-μm filtered seawater (FSW). Labels are provided at the end points

511 of plots that overlap throughout to aid in distinguishing these lines.

512

513



514

515 **Fig. 3**. Ingestion rates of *Neocalanus plumchrus* in grazing experiments conducted with

516 *Bacterosira* sp. and *Karenia selliformis*.

517

518 **Tables**

519

Table 1. Sampling dates and locations for copepods used in the survival experiment and experimental designs.

Species	Copepodite stage	Sampling		Survival experiment			
		Date	Location*	Temperature (°C)	No. of conditions	No. of flasks per condition	Copepods per flask
Acartia tumida	C6F	22 Apr.	KH	5	5	3	5–6
Centropages abdominalis	C6F	2 Nov.	KH	12	5	3	1
Eurytemora herdmani	C6F	24 Mar.	KH	5	6	3	5–7
Metridia pacifica	C6F	14 Jan.	SEH	8	3	3	5–6
Neocalanus plunchrus	C5	14 May	SEH	5	6	5	3
Paracalanus parvus	C6F	13 Oct.	KH	13	6	3	10

All sampling was conducted in 2022.

*SEH: off the southeastern coast of Hokkaido (R.V. Hokko-Maru)

KH: Kushiro Harbor, Hokkaido

520

521

Table 2. Kaplan-Meier survival curve comparisons using log-rank pairwise tests.

Species	Group comparisons	Pairwise comparisons					
		KS1000	KS100	KS10	KSE	SWM	FSW
Acartia tumida	**	a	b	bc	b	–	c
Centropages abdominalis	*	a	ab	b	–	c	c
Eurytemora herdmani	**	a	b	b	ab	c	c
Metridia pacifica	**	–	a	–	b	c	–
Neocalanus plumchrus	**	a	b	b	a	b	b
Paracalanus parvus	**	a	b	c	ab	c	c

*p<0.05; **p<0.01.

Different letters in the same row indicate significantly different results, with p-values adjusted for multiple testing using the Benjamini-Hochberg method (p<0.05). The symbol '–' indicates no data available for the respective conditions.

522

Table 3. Median lethal times (LT_{50}, hours) across copepod species and experimental conditions.

	KS10	KS100	KS1000	KSE
Acartia tumida	n. d.	63	12	39
Centropages abdominalis	24	34	6	–
Eurytemora herdmani	218	171	113	145
Metridia pacifica	–	105	–	59
Neocalanus plumchrus	n. d.	n. d.	108	148
Paracalanus parvus	32	8	3	4

'n.d.' indicates that all individuals survived (no dead), and '–' indicates that no data were collected for the corresponding condition.

523

524

Table 4. Best subset of variables determined by 'bestglm()' function based on AIC.

Source	Df	Sum Sq	Mean Sq	*F*-value	*p*-value	
Copepod species	5	117349	23470	6.692	0.00338	**
Treatment with *K. selliformis*	3	49075	16358	4.664	0.02205	*
Residuals	12	42088	3507	–	–	–

*$p<0.05$; **$p<0.01$

525

526

Table 5. Summary of GLM coefficients for best subset of variables effects on median lethal time.

Variable	Coefficient	±SE	*t*-value	*p*-value
Intercept (*Paracalanus parvus*, KS10)	86.838	37.645	2.307	0.0397*
Neocalanus plumchrus	202.063	41.877	4.825	0.0004***
Eurytemora herdmani	150.22	41.877	3.587	0.0037**
Acartia tumida	91.468	41.877	2.184	0.0495*
Metridia pacifica	81.863	53.104	1.542	0.1491
Centropages abdominalis	–3.385	45.925	–0.074	0.9425
KS100	–60.314	36.689	–1.644	0.1261
KSE	–112.884	39.187	–2.881	0.0138*
KS1000	–126.452	37.456	–3.376	0.0055**

*p<0.05; **p<0.01; ***p<0.001

527