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1 Extraction of metazoan meiofauna from muddy deep-sea samples:
2 operator and taxon effects on efficiency

3

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19

20

21 Abstract

22 Deep-sea metazoan meiofaunal specimens are usually
23 extracted from muddy samples by centrifugation in a fluid in
24 which meiofauna tend to float and sediment particles tend to
25 sink. Although the procedure is in common use, its efficiency
26 has seldom been examined. The study reported here showed that
27 well-trained operators extracted metazoan meiofauna with
28 efficiencies that were different enough to be a concern in
29 quantitative studies. Therefore, samples should be assigned to
30 operators in a stratified-random manner. In the course of these
31 studies, both operators extracted individuals of the common
32 nematode family Desmoscolecidae significantly less efficiently
33 than other nematode families, a bias that could interfere with
34 studies that compared relative abundances of nematode families.

35

36 Keywords: benthos; Desmoscolecidae; Harpacticoida; Ludox®;

37 Nematoda

38

39 Highlights

- 40 • Most deep-sea meiofaunal samples are extracted by
41 floatation, e.g., in Ludox®.
- 42 • Operators differed significantly in extraction efficiency.
- 43 • Operators extracted desmoscolecids significantly less well
44 than nondesmoscolecids.

45 1. Introduction

46 Ideally, when an investigator takes a sediment sample for
47 the study of an ecological question, no individuals are lost
48 during collection and sample processing. The possibility of
49 loss is of particular concern for students of sediment-dwelling,
50 metazoan meiofauna (hereafter meiofauna), which are difficult to
51 see with the naked eye. The most accurate method of counting
52 meiofauna in a preserved sediment sample is to examine the
53 entire sample, aliquot by aliquot, through a dissection
54 microscope (Nichols, 1979), but this procedure consumes so much
55 time that many investigators (e.g., Jenkins, 1964; Heip et al.,
56 1974) have proposed methods to speed the process. Because the
57 buoyancies of most sediment particles are less than those of
58 most meiofaunal individuals (ostracods are an exception), most
59 of the meiofauna will float in the upper portion of the
60 supernatant in a fluid of appropriate density. Most of the
61 sediment particles will sink and constitute the "sediment
62 fraction" or "pellet." Some workers (e.g., Bowen et al., 1972;
63 de Jonge and Bouwman, 1977) allowed gravity to do the
64 separation. Others (e.g., Nichols, 1979; Schwinghamer, 1981)
65 used centrifugation to speed the process. Workers have used
66 several fluids of appropriate density, in particular, colloidal
67 silicas with the brand names Ludox® (du Pont) and Levasil® (H. C.
68 Starck, now available from Akzo Nobel Chemicals GmbH as Levasil

69 CS40-316P). Centrifugation-in-a-fluid-of-appropriate-density
70 (CFAD) methods can extract most shallow-water meiofaunal groups
71 with efficiencies approaching 100% (see, e.g., Burgess, 2001; Du
72 et al., 2009).

73 Although CFAD methods have been used to extract the
74 meiofauna of muddy deep-sea sediments for more than a decade
75 (see, e.g., Vanreusel et al., 2000; Tselepidis and Lampadariou,
76 2004), the present authors know of only two published studies of
77 extraction efficiency for such samples. Escobar-Briones et al.
78 (2008) removed all the meiofauna from each sample by hand,
79 counted the specimens of each group, returned the specimens to
80 the appropriate sample, and extracted each sample once using a
81 CFAD method based on Ludox-AM[®]. Their method extracted only 27%
82 of the meiofauna from their continental-slope samples and only
83 20% from their abyssal-plain samples, extraction rates too low
84 for quantitative ecological studies. Kitahashi et al. (2014)
85 extracted each sample three times using a CFAD method based on
86 Ludox HS40[®]. They quantified efficiency by searching the pellet
87 by hand for unextracted specimens. Their extraction efficiency
88 for harpacticoids was 100%; they did not report efficiencies for
89 other taxa.

90 Because the extraction of meiofauna from a sample by hand
91 takes an inordinate amount of time, students of the muddy deep
92 sea will certainly continue to take advantage of the greater

93 efficiencies of CFAD methods. At the same time, more
94 information is needed about the problems of the CFAD approach
95 and their potential effects on quantitative ecological studies.
96 The study reported here showed that two carefully trained
97 operators extracted some groups with significantly different
98 efficiencies. Given the likelihood that extraction efficiency
99 is affected by sediment properties, we looked for effects of
100 differences in sediment grain-size distributions and for effects
101 of differences in the concentrations of chloroplastic pigment
102 equivalents (hereafter CPE).

103 **2. Materials and methods**

104 *2.1. Core collection*

105 We planned to sampled each of four latitudes once at ~2700
106 m and once at ~3700 m, but bad weather made this impossible for
107 stations 1 and 2 (see Table 1 and Fig. 1). Samples were
108 collected with a MC 800 Multi Core (Ocean Instruments, San
109 Diego) that had eight tubes of 10-cm inner diameter. Three of
110 the deployments from each station were chosen at random. From
111 each, one of the high-quality cores was selected at random for
112 the analysis of meiofauna. One of the remaining high-quality
113 cores was selected at random from each deployment for
114 determination of the grain-size distribution of the sediment and
115 the concentration of CPE.

116

117 2.2. *On-board core processing*

118 For each core used for meiofauna, the water overlying the
119 sediment was siphoned off and poured onto a sieve with 30- μ m
120 apertures. The top 1 cm of sediment was sliced off and placed
121 in a sample jar, the sieve contents were added, and the jar was
122 filled with 95% ethanol.

123 For sediment grade and CPE, cut-off syringes were used as
124 subcores. Samples for sediment grade were preserved in a 10%
125 solution of formalin and artificial salt water (salinity = 35)
126 and buffered to neutrality with sodium bicarbonate. Those for
127 CPE were stored in the dark at -20°C .

128 2.3. *Laboratory processing*

129 2.3.1. *Extraction of meiofauna*

130 Operator 1 extracted 14 samples; operator 2 extracted 10
131 samples. Each sample was washed with deionized water on a 300-
132 μ m-aperture sieve, and the material that passed through was
133 caught on a 30- μ m-aperture sieve. The meiofauna were removed
134 from the 30- μ m fraction by a method inspired by that of Burgess
135 (2001); see also Lebreton et al. (2012). Thirty ml of Ludox[®] HS-
136 40 (specific gravity = 1.30) and 10-15 ml of the 30- μ m fraction
137 were added to a 50-ml centrifuge tube, and the process was
138 repeated until the entire sample was divided among centrifuge
139 tubes. The contents of each tube were homogenized on a vortex
140 mixer for 5 min and centrifuged at $900 \times g$ for 5 min. The

141 supernatant was washed onto a 30- μ m sieve, and the pellet was
142 retained in the centrifuge tube. The homogenization and
143 centrifugation steps were repeated on the pellet. The
144 supernatants from the two extractions were combined, and the
145 combined supernatant and the final pellet were stored in
146 separate jars of 95% ethanol.

147 2.3.2. *Hand sorting*

148 The supernatant and the pellet samples were stained with
149 rose bengal, and all were searched for meiofauna under a
150 dissection microscope at 25 \times by a third operator. Six groups
151 were counted and removed: copepods, nematodes of the family
152 Desmoscolecidae (hereafter desmoscolecids), kinorhynchs,
153 nauplii, nematodes other than desmoscolecids (hereafter
154 nondesmoscolecids), and ostracods.

155 2.3.3. *Particle-size distribution*

156 The sample was first poured onto a 30- μ m sieve, and the
157 sieve was gently moved up and down for 5 min in a container
158 filled with enough deionized water to cover the sediment in the
159 sieve. The sediment was rinsed on the 30- μ m sieve through 500-,
160 350-, 250-, 177-, 125-, 88-, 62.5-, 45-, and 30- μ m-aperture
161 sieves. The content of each sieve was vacuum filtered onto a
162 dried and tared Whatman GF/D filter. The loaded filters were
163 dried overnight at 60°C, allowed to cool in a desiccator for one
164 hour, and weighed. The less-than-30- μ m fraction of a sample was

165 filtered onto a stack consisting of a dried and tared Whatman
166 GF/D filter on top of a dried and tared 0.1- μm Whatman
167 polycarbonate membrane filter. The stack was dried overnight at
168 60°C, allowed to cool for one hour in a desiccator, and weighed.
169 Note that in the text the term "mud" is used in the sense of the
170 Wentworth scale, i.e., to mean the fraction that passes through
171 a 62.5- μm sieve.

172 2.3.4. *Chloroplastic-pigment equivalents*

173 Each sample for CPE was extracted in the dark and
174 transferred to a tared microcentrifuge tube. It was weighed,
175 freeze-dried, and reweighed. Six hundred μl of 90% acetone was
176 then added, and the contents were vortexed for 15-30 s and
177 frozen overnight. The following day, the sample was vortexed
178 long enough to resuspend the sediment and centrifuged at 1500 \times
179 **g** for 2 min, long enough to remove particles from the
180 supernatant. The fluorescence of chlorophyll a and
181 phaeopigments was measured with a Varian Cary Eclipse
182 Fluorescence Spectrophotometer set at 5 nm excitation and
183 emission slits, excitation at 425 nm, and emission from 600 to
184 880 nm. Peak emission (659-674 nm) and emission at 750 nm were
185 recorded for each sample. After addition of two drops of 10%
186 HCl, the fluorescence was remeasured. The methods of Parsons et
187 al. (1984) were used for calculation of CPE concentrations.

188 2.4. *Statistical analysis*

189 The individual-test significance level was 0.05 and was not
190 corrected for multiple testing. A resampling procedure (Simon,
191 1999) written in the Statistics101 (www.statistics101.net)
192 programming language was used to test for a difference in means
193 (two-tailed). Ninety-five percent confidence limits were
194 calculated of means by resampling; the code can be found in
195 Simon (1999). The Spearman rank-difference method (Tate and
196 Clelland, 1957) as implemented in Statistix 10[®] (Analytical
197 Software) was used to test for correlations.

198 Desmoscolecids and nondesmoscolecids were analyzed
199 separately because preliminary data suggested that the
200 extraction efficiencies of these groups differed.

201 2.5. *Extraction efficiency*

202 2.5.1. Approach

203 Extraction efficiencies are reported in the usual way,
204 i.e., the number in the supernatant divided by the number in the
205 pellet plus the number in the supernatant. Significance tests
206 followed Atchley et al. (1976), Prothero (1986), and Berges
207 (1997) and operated on the ratio of the number of individuals
208 found in the pellet to the number found in the supernatant, so
209 that the numerator and denominator would be independent.

210 2.5.2. Operator extraction efficiency per group

211 For each of the six groups, we asked whether the average
212 extraction efficiency differed between operators 1 and 2.

213 2.5.3. Operator extraction efficiency: correlations with
214 percentage mud and amount of CPE

215 For each of the six groups, Spearman rank correlation was
216 used to determine whether operator efficiency for a given group
217 was correlated with the percentage of mud in the sample and with
218 the amount of CPE in the sample.

219 **3. Results and discussion**

220

221 3.1. Differences in efficiency between operators

222 One would expect that operators trained in exactly the same
223 way using the same equipment would differ very little in their
224 ability to extract meiofauna. Dr. Jeffery Baguley (pers. comm.)
225 made us aware that this assumption can be false. Because no
226 test of this assumption appears in the literature, the
227 performances of our operators were compared. The proportion of
228 the total individuals originally present in the sample that
229 remained in the pellet was less for operator 1 than for operator
230 2 for all six groups (Tables 3 and 4 and Figs. 2 to 4). The
231 differences were significant for desmoscolecids (64.0% versus
232 84.6%), nauplii (95.4% versus 99.5%), and nondesmoscolecids
233 (83.6% versus 96.9%). These differences in extraction
234 efficiency could make real patterns more difficult to perceive.
235 To minimize this problem, investigators should assign samples to
236 operators in a stratified random manner; i.e., each operator

237 should process approximately equal numbers of samples from each
238 station.

239 We sought a property of the samples that could explain the
240 differences in operator performance. The efficiency of neither
241 operator was correlated with CPE for any group. The efficiency
242 of operator 2 (the better operator) was not correlated with
243 percentage mud. In contrast, the efficiency of operator 1 was
244 significantly negatively correlated with the percentage mud in
245 the sample for copepods ($p < 0.034$), nondesmoscolecids ($p <$
246 0.007), and nauplii ($p < 0.001$). The statistic for the test was
247 the number in the pellet divided by the number in the
248 supernatant, so the negative correlation indicates that operator
249 1's extraction efficiency increases with increasing percentage
250 mud. In other words, operator 1 was less efficient when
251 extracting sandier samples. The reason for this result is not
252 obvious, but during the vortexing step, the operators might have
253 achieved different intensities of mixing. If so, and if the
254 sample becomes harder to mix as the degree of sandiness
255 increases, the observed pattern could arise. A more detailed
256 protocol for vortexing the sample may be necessary to minimize
257 differences in technique among operators. Other aspects of the
258 extraction procedure may be involved. For example, at several
259 steps the samples is transferred with the aid of a jet of water.
260 Water will dilute the CFAD and decreases its density.

261 Differences in extraction efficiency between operators could
262 arise from differences in the amount of water they used in
263 transfers. Finally, the efficiency of the procedures, in
264 general, may be subject to improvement. For example, given that
265 meiofaunal individuals must be reach the surface of the sediment
266 to be extracted, it might be worth exploring whether extraction
267 efficiency increases as the depth of the sediment in the
268 extraction vessel decreases.

269 *3.2. Absolute extraction efficiency*

270 Because we planned to sort the pellet, our samples were
271 only extracted twice. Because three extractions are commonly
272 used (see, e.g., Rose et al. 2005, Ingles et al. 2011, Miljutin
273 et al. 2011), our study does not assess the extraction
274 efficiency of the de facto standard method. Still, each
275 extraction requires time and creates opportunities for loss, so
276 we report our results for two extractions (Table 4) to help
277 future workers decide whether two extractions might be
278 sufficient.

279 *3.3. Extraction efficiency of desmoscolecids*

280 In the course of our work, we discovered that
281 desmoscolecids were extracted much less efficiently than
282 nondesmoscolecids. Because the extraction efficiency of the two
283 groups has apparently not been compared, we give our results
284 here. For operator 1, average extraction for the former (64.0%)

285 was significantly less ($p < 0.005$) than that of the latter
286 (83.6%). For operator 2, average extraction for the former
287 (84.6%) was significantly less ($p < 0.005$) than that of the
288 latter (96.9%). Although operator 2 extracted a higher
289 proportion of desmoscolecids than did operator 1, her average
290 efficiency was still only 84.6%.

291 Substantial proportions of the desmoscolecids in a sample
292 can fail to be extracted. In some cases, more desmoscolecids
293 were found in the pellet than in the supernatant. Such
294 inefficiencies could make certain inquiries difficult, for
295 example, comparing abundances of nematode families.

296 Why extraction of desmoscolecids was less efficient than
297 that of nondesmoscolecids is unknown. One explanation could be
298 that two extractions were not enough, and desmoscolecids and
299 nondesmoscolecids would have been extracted with comparable
300 efficiencies if three extractions had been done. At least one
301 other explanation is possible. Unlike other nematodes, most
302 desmoscolecids have conspicuous rings around their bodies
303 composed of foreign particles (including sediment grains) and
304 secretions from the worm (Decraemer and Smol, 2006). The
305 composition of the rings of some individuals may decrease their
306 buoyancy such that they sink below the level in the supernatant
307 that is decanted. If so, an increase in the density of the
308 extraction fluid should increase the extraction efficiency of

309 desmoscolecids. Unfortunately, any increase in the density of
310 the extraction fluid will increase the amount of sediment
311 retained in the supernatant. Preliminary research will be
312 needed to find a compromise appropriate to the questions to be
313 addressed by a study.

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323

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405 nematode community structure. Deep-Sea Res. I 47, 1855-
406 1879.
407

408 Table 1. Station information. The positions and depths are the
409 averages of those of the multiple-corer deployments from a given
410 station.

411

Station	Position	Depth (m)
1	44.0012°N 130.3946°W	3242
2	42.5594°N 131.9228°W	3591
3	39.9917°N 125.8781°W	3676
4	40.0011°N 125.4447°W	2694
5	36.7975°N 123.6998°W	3676
6	36.6806°N 122.8213°W	2720
7	32.8739°N 120.6151°W	3852
8	32.7977°N 120.3709°W	2704

412

413
414 Table 2. For each sample, the percentage of mud (= mud) and the
415 concentration of chloroplastic pigment equivalents (= CPE) in
416 nanograms per gram sediment dry weight are given. The first
417 digit in the sample number is the station number.
418

Sample	Mud	CPE
1181	92.4	52.54
1331	91.8	113.00
1421	91.1	202.03
2341	96.4	154.89
2431	97.5	124.32
2661	97.1	108.91
3271	96.9	360.34
3331	93.8	279.89
3421	94.4	364.25
4111	72.3	1067.80
4271	70.8	1342.91
4561	92.0	1650.62
5231	95.4	2206.25
5351	95.1	3373.32
5521	95.2	2310.17

6111	90.6	3373.06
6261	95.4	4312.13
6311	91.4	2915.08
7161	93.7	867.51
7381	96.2	498.54
7451	94.5	378.87
8151	86.7	1794.04
8481	76.5	341.40
8521	78.8	1051.40

420

421

422 Table 3. Operator identity and abundances of meiofaunal groups in the supernatant (=

423 super) and pellet fractions of the 0-1 cm layer of 78.5 cm² samples. Desmos = nematodes

424 of the Desmoscolecidae. Kinos = kinorhynchs. Nondesmos = all other nematodes.

425

Station	Operator	Sample	Fraction	Copepods	Desmo	Kinos	Nondesmos	Nauplii	Ostracods
1	2	1181	Super	84	152	16	1664	99	13
			Pellet	2	68	1	102	2	2
1	1	1331	Super	69	104	7	1134	48	8
			Pellet	1	48	3	254	3	7
1	1	1421	Super	120	107	9	638	113	11
			Pellet	5	83	2	157	12	10
2	2	2341	Super	73	134	7	1591	109	5
			Pellet	0	25	1	16	0	5
2	1	2431	Super	123	56	7	1357	102	3
			Pellet	5	67	0	298	1	6
2	2	2661	Super	109	137	21	1105	177	12
			Pellet	3	19	0	14	0	4
3	2	3271	Super	117	247	31	2196	159	11
			Pellet	1	97	2	65	2	6

3	1	3331	Super	147	82	16	924	222	6
			Pellet	1	145	1	253	9	12
3	1	3421	Super	151	210	26	1427	509	17
			Pellet	5	116	0	122	9	6
4	1	4111	Super	194	232	21	714	176	8
			Pellet	46	206	8	588	32	14
4	2	4271	Super	204	594	31	2471	226	11
			Pellet	1	58	6	101	1	14
4	2	4561	Super	230	808	77	4382	265	17
			Pellet	0	83	1	24	0	8
5	1	5231	Super	176	732	91	2369	324	5
			Pellet	2	214	1	201	7	5
5	1	5351	Super	148	932	114	2131	285	4
			Pellet	2	97	1	62	0	4
5	2	5521	Super	118	833	52	1922	129	5
			Pellet	0	41	2	27	0	0
6	1	6111	Super	286	314	68	1522	425	15
			Pellet	16	255	0	356	38	7
6	1	6261	Super	270	546	102	1915	327	7
			Pellet	1	96	3	140	7	5
6	2	6311	Super	274	608	202	3658	353	8

			Pellet	2	97	4	60	2	7
7	1	7161	Super Pellet	149 0	158 45	22 0	1490 170	191 5	6 11
7	2	7381	Super Pellet	197 4	217 17	11 0	1623 48	201 0	5 12
7	1	7451	Super Pellet	143 2	117 77	15 0	1398 203	189 3	1 11
8	1	8151	Super Pellet	110 10	373 176	21 4	3023 1022	201 19	2 4
8	1	8481	Super Pellet	215 4	161 114	31 0	2128 348	247 6	13 11
8	2	8521	Super Pellet	260 1	231 69	33 2	2444 253	312 3	9 17

426

427

428 Table 4. Extraction efficiencies (the ratio of the number in
 429 pellet divided by the number in supernatant and pellet combined)
 430 as percentages and the results of tests for differences between
 431 operators (as assessed by the ratio of the number in the pellet
 432 to the number in the supernatant).

433

Group	Average extraction efficiency (%)		Test for difference
	Operator 1	Operator 2	Probability
Copepods	96.3	99.1	> 0.109
Desmoscolecids	64.0	84.6	< 0.008
Kinorhynchs	92.7	94.7	> 0.465
Nauplii	95.4	99.5	< 0.014
Nondesmoscolecids	83.6	96.9	< 0.013
Ostracods	45.7	60.6	> 0.227

434

435 Figure captions

436 Fig. 1. Chart showing the locations of the stations and the
437 2700-m and 3700-m isobaths. The insert shows the position of
438 the chart relative to the west coast of the United States.

439 Fig. 2. Extraction efficiency of Copepoda and Kinorhyncha for
440 operator 1 (circles) and operator 2 (triangles).

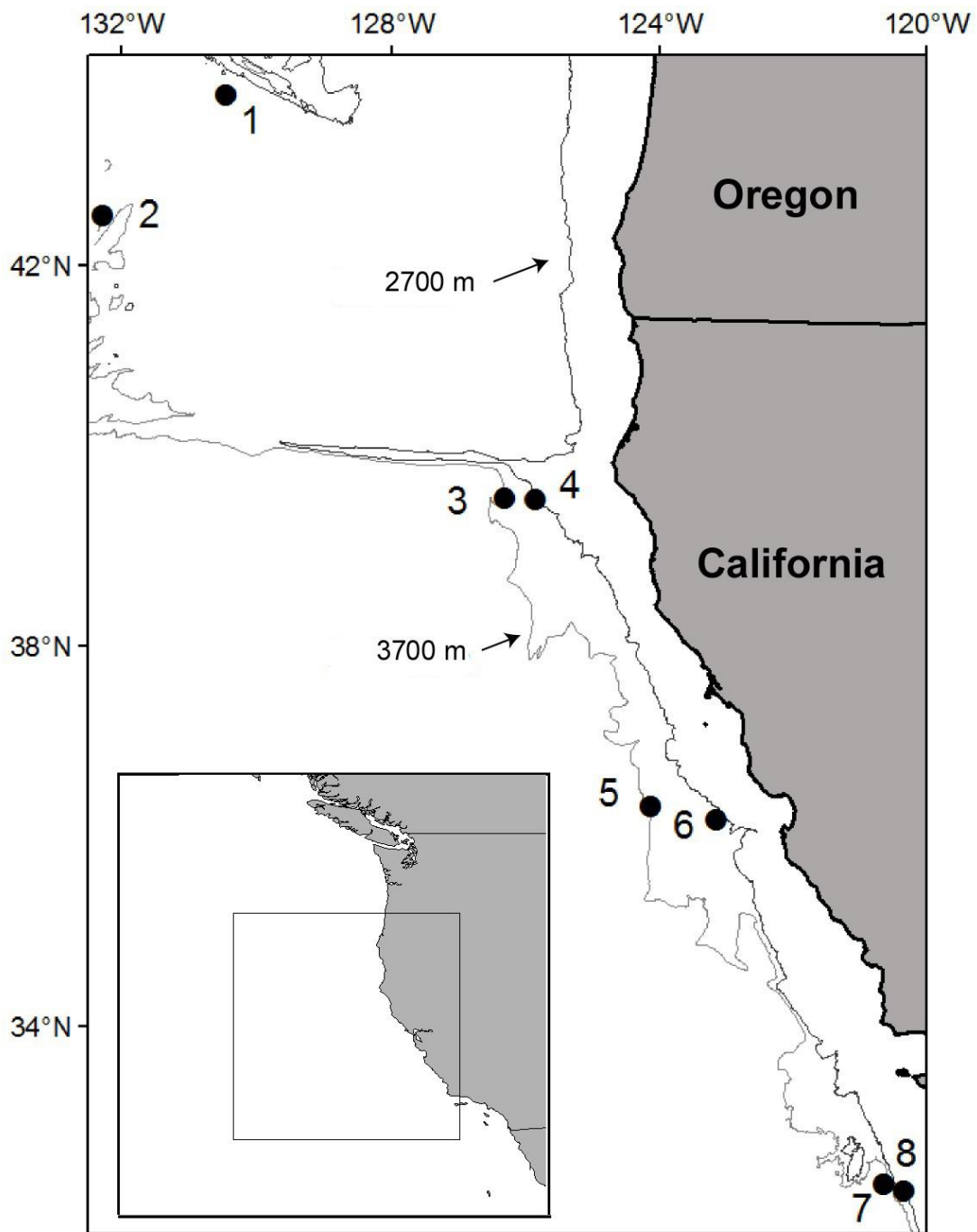
441 Fig. 3. Extraction efficiency of Desmoscolecidae and
442 nondesmoscolecid nematodes for operator 1 (circles) and operator
443 2 (triangles).

444 Fig. 4. Extraction efficiency of nauplii and Ostracoda for
445 operator 1 (circles) and operator 2 (triangles).

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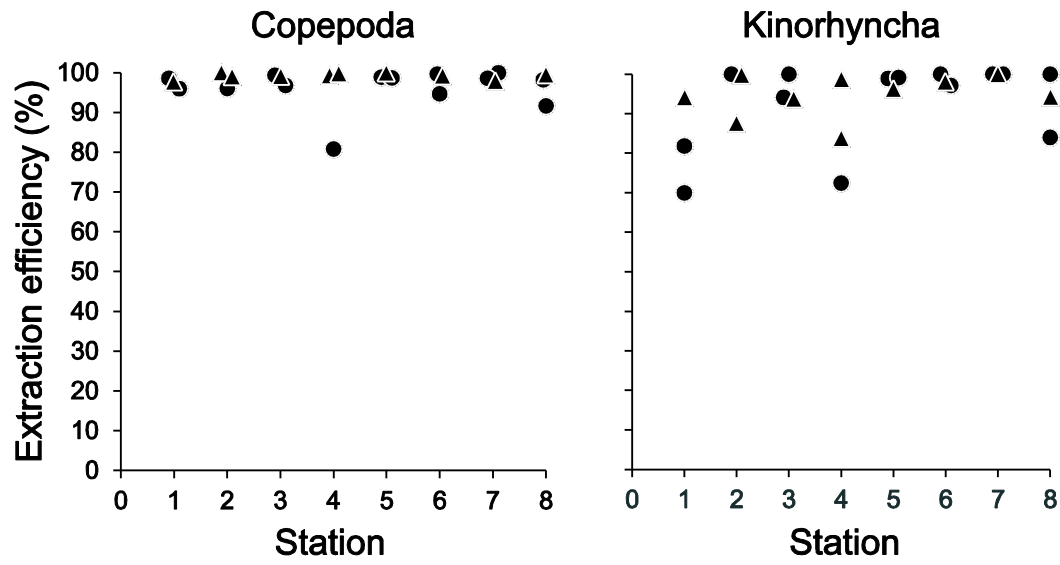
448



449

450 Fig. 1.

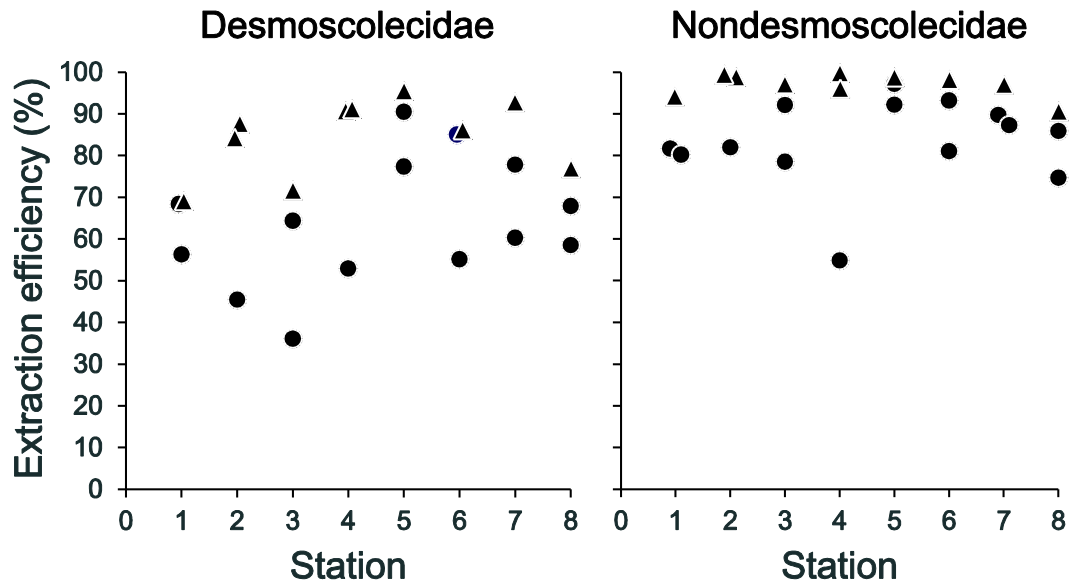
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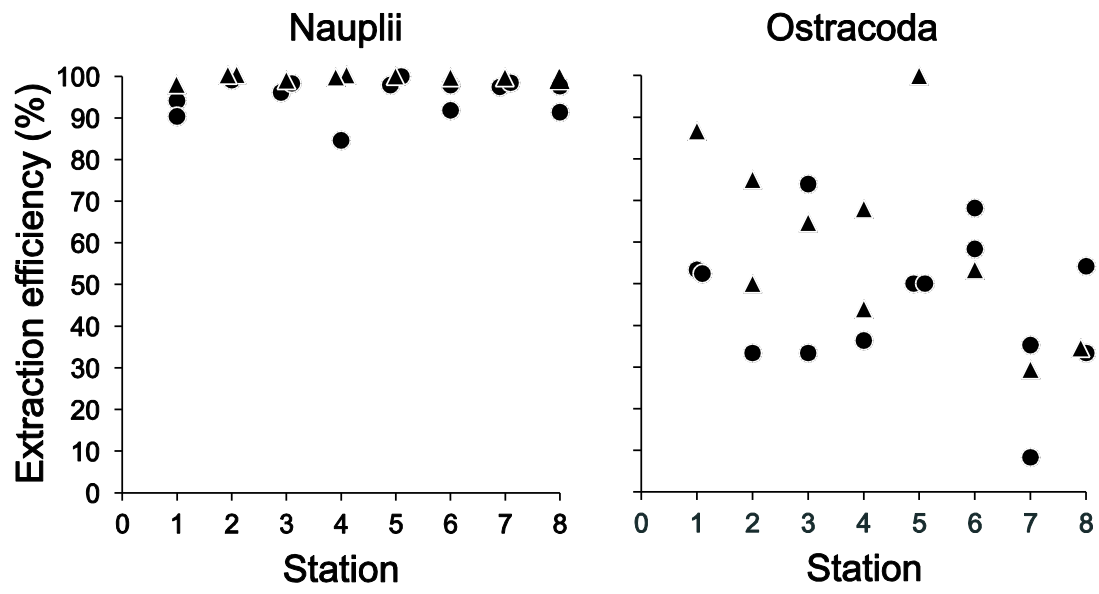
453 Fig. 2.

454



455

456 Fig. 3.



457

458 Fig. 4.

