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Effects of silver nanocolloids on early life stages of the scleractinian coral *Acropora japonica*

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Abstract

In this study, the effects of silver nanocolloids (SNC) on the early life stages of the reef-building coral *Acropora japonica* were investigated. The tolerance of this species to SNC contamination was estimated by exposing gametes, larvae, and primary polyps to a range of SNC concentrations (0, 0.5, 5, 50, and 500 μg l^{-1}). Pure SNCs were immediately ionized to Ag^{+} in seawater and concentrations of ≥50 μg l^{-1} SNC had a significant detrimental effect on fertilization, larval metamorphosis, and primary polyp growth. Exposure to 50 μg l^{-1} SNC did not significantly affect larval survival; however, the larvae were deformed and lost their ability to metamorphose. At the highest concentration (500 μg l^{-1} SNC), all gametes, larvae, and primary polyps died. These experiments provide the first data on the effects of silver-nanomaterial-contaminated seawater on cnidarians, and suggest that silver nanomaterials can influence the early development of corals through anthropogenic wastewater inputs.

1. Introduction

Nanotechnology is rapidly developing in a variety of industries. In recent years, silver

37 nanomaterials, including nanocolloids and nanoparticles, have been widely used in hygiene
38 products and industry for their antibacterial activity. Such uses of silver nanomaterials carry a
39 high risk of impacting aquatic environments through anthropogenic wastewater inputs
40 (Wijnhoven et al., 2009). Several reports describe the impacts of silver nanoparticles on marine
41 organisms, such as shellfish (e.g. Ringwood et al., 2010; Gomes et al., 2013) and sea urchins
42 (e.g. Gambardella et al., 2013; Šiller et al., 2013). However, the effects of seawater
43 contaminated with silver nanomaterials on cnidarians remain unexplored. Scleractinian corals
44 (Cnidaria: Anthozoa) play important roles as primary producers and providers of structural
45 habitat for other marine organisms in ecosystems. Because coral live in shallow areas that
46 permit the penetration of light for photosynthesis, they may be influenced by nanomaterials
47 from anthropogenic wastewater inputs. There are few studies about the effects of nanomaterials
48 on cnidarians. The behavior of freshwater hydra *Hydra vulgaris* is reportedly disrupted by
49 rod-shaped semiconductor nanoparticles (Malvindi et al 2008), and the scleractinian coral
50 *Montastraea faveolata* expelled algal symbionts when exposed to titanium dioxide (TiO_2)
51 nanoparticles (Jovanović and Guzmán 2014). Corals have been used as the test animal for
52 investigating the effects of environmental perturbations such as high and low temperature
53 (Suwa et al. 2008), hypo-osmosis (Kerswell and Jones, 2003), ocean acidification (e.g. Suwa et
54 al., 2010), biocides (e.g. Watanabe et al., 2007), herbicides (e.g. Jones et al., 2003), cyanide

55 (Jones and Hoegh-Guldberg, 1999), oils (e.g. Negri and Heyward, 2000), and metals (e.g.
56 Harland and Brown, 1989). The genus *Acropora* is one of the most widespread, abundant, and
57 species-rich (113–180 species) coral genera in Pacific coral reefs (Veron, 2000; Wallace, 1999).
58 The early life stages of these corals have frequently been used in eco-toxicological studies (e.g.
59 Watanabe et al., 2007; Negri et al., 2007; Morita et al., 2009) because it is easy to obtain
60 *Acropora* gametes. In this study, it is hypothesized that silver nanocolloids (SNCs) may have an
61 impact on the early life stages of *Acropora japonica.* To test this hypothesis, the tolerance of
62 this species to SNC contamination was estimated by exposing gametes, larvae, and primary
63 polyps to a range of initial SNC concentrations (0, 0.5, 5, 50, and 500 $\mu g\ l^{-1}$).
64
65 **2. Materials and Methods**
66 **2-1. Coral sampling**
67 Gravid colonies of *A. japonica* were collected from Okinoshima, Tanabe Bay, Wakayama, Japan
68 (33°71′N, 135°3′E) 6 d before spawning. The colonies were maintained in a running seawater
69 tank under natural light conditions at the Seto Marine Biological Laboratory, Field Science
70 Education and Research Center, Kyoto University, Wakayama, Japan. Coral spawning took
71 place at night after the full moon in July 2012. Gametes were collected after spawning in
72 accordance with Morita et al., 2006.

73

74 **2-2. Silver Nanocolloids**

75 Silver nanocolloidal solution (25.7 mg l^{-1}, as measured by inductively coupled plasma mass
76 spectrometry (ICP-MS; Thermo Scientific X Series 2, Thermo Scientific, PA, USA) was
77 purchased from Utopia (TX, USA). The diameter of the silver nanocolloids (SNC) was
78 determined using an ultra-high resolution scanning electron microscope SU8000 series
79 (HITACHI, Tokyo, Japan) operated at 120 kV. Particle size was confirmed to be 57.2 ± 3.6 nm
80 (n =3, mean ± SD) in ultrapure water using a Delsa Nano Zeta Potential and Submicrometer
81 Particle Size Analyzer (Beckman Coulter, Inc., Fullerton, CA). The Zeta potential of the SNC
82 was -45.1 ± 1.9 mV in ultrapure water and but could not be measured in seawater due to the
83 presence of salt. The SNC solution was diluted to nominal concentrations of 0.5, 5, 50, and 500
84 μg l^{-1} with 0.22-μm membrane-filtered seawater (MFSW). MFSW served as a control. The
85 volume of purified water was adjusted between the four SNC solutions and the control
86 condition because the amount of purified water added as part of the SNC stock solution ranged
87 from 0 v/v% in the control to 1.95 v/v% in the 500 μg l^{-1} SNC condition. A 1-ml sample of all
88 experimental seawater was collected immediately before and after each experiment and was
89 preserved in a freezer at -30 ºC for Ag analysis. The total amount of Ag from SNC and Ag^+ in
90 each water sample was measured by ICP-MS. To isolate Ag^+ from the SNC solution (a mixture

of silver colloids and Ag^+), 0.5 ml of test solution was filtered through a 3-kDa membrane filter (0.5-ml centrifugal-type filter, EMD Millipore Corporation, Billerica, MA, USA) at 14,000 × *g* and 4°C for 10 min; this filter size was chosen because the mean diameters of the SNCs and Ag^+ were 57.2 nm and 0.162 nm (Shannon 1976), respectively, and the 3-kDa membrane excludes particles ≥2 nm. The Ag^+ concentration in the filtered solution was measured using ICP-MS. Two milliliters of ultrapure nitric acid (Ultrapur-100, specific gravity 1.42, Kanto Chemical Co., Tokyo, Japan) was added to 100-µl water samples in a 50-ml Teflon beaker (Sanplatec Co., Osaka, Japan). The mixture was heated to 110ºC until almost all of the liquid had evaporated. Two milliliters of ultrapure nitric acid and 0.5 ml of hydrogen peroxide (for atomic absorption spectrometry, Kanto Chemical Co., Tokyo, Japan) were then added to the beaker and heated until the mixture was nearly dry. The residue was dissolved with 1.0% ultrapure nitric acid solution to a volume of 12.0 ml and then subjected to ICP-MS analysis. Measurements were conducted in triplicate and the data were averaged. All exposure experiments were conducted in a thermostatic room maintained at 27.0 ± 0.5°C for the fertilization experiment and 27.0 ± 0.3°C for other experiments. The water temperature was logged every 15 min throughout the experiments using data loggers (Thermochron iButtons DS1922; Maxim Integrated Products, Sunnyvale, CA, USA).

2-3. Fertilization experiment

Four crosses using gametes from four spawned colonies of *A. japonica* were performed. Each sperm-egg combination was considered to be a separate cross. All crosses were performed in a plastic cup filled with 200 ml of SNC solution and crosses were replicated three times at each SNC concentration. Approximately 200 eggs were combined with sperm at a final concentration of 10^5 sperm ml^{-1}. Fertilized eggs were fixed with 5% formalin 2 h after the addition of sperm, and the number of unfertilized eggs and developing embryos were counted under a dissecting microscope to calculate the rate of fertilization.

2-4. Larval experiment

Planula larvae of *A. japonica* were prepared by mixing gametes from all of the spawned colonies. Planula larvae were maintained in a container with 0.10-μm cartridge filtered seawater until the experiment started. Water was exchanged twice per day. Individual 5-day-old larvae were added to the wells of 24-well plastic culture plates (Iwaki Glass, Tokyo, Japan). Each well contained 2 ml experimental SNC seawater. Four plates containing 20 larvae (20 larvae per plate × 4 plates) were prepared for each SNC treatment. Surviving larvae were counted every 2 d during the 10-day culture experiments. SNC-contaminated MFSW was exchanged once per day during the experiment.

127

128 **2-5. Larval metamorphosis experiment**

129 The ability of the coral larvae to metamorphose after 24 h of exposure to SNC was examined
130 using the coral metamorphosis-inducer peptide Hym-248 (Iwao et al., 2002). We added 4 ml
131 peptide solution (1×10^{-6} M, dissolved in MFSW) to each well of a 24-well plastic culture plate.
132 One larva that had been pre-exposed to SNC for 24 h was added to the peptide solution in each
133 well. Four plates containing 20 larvae (20 larvae per plate × 4 plates) were prepared for each
134 SNC treatment. Thus, metamorphosis of 80 larvae was observed for each SNC condition. The
135 number of metamorphosed larvae was counted after 12 h of exposure to the peptide. Larvae
136 were considered to have metamorphosed normally when they had developed septa (Iwao et al.
137 2002) and had become bilaterally symmetric in appearance.

138

139 **2-6. Polyp experiment**

140 Primary polyps were prepared according to Suwa et al. 2010. Primary polyps were prepared by
141 inducing the settlement of 7-day-old *A. japonica* larvae using Hym-248. A 20-μl aliquot of $2 \times$
142 10^{-4} M Hym-248 in MFSW was added to each well of a 6-well plastic culture plate (Iwaki Glass,
143 Tokyo, Japan). A peptide solution was created by mixing individual drops containing four larvae
144 in 20 μl MFSW with individual 20-μl drops of peptide. Seven drops of this peptide solution was

added to each well, for a total of 28 larvae and 280 μl of peptide. After the induction of metamorphosis, 10 ml of MFSW was added to each well of the plate. Larvae that settled on the seawater surface and on the sides of the plastic culture plates were removed, whereas those that settled at the bottom of the wells were used for the experiment. In each treatment, five 6-well culture plates, each containing approximately 25 settled polyps were prepared and maintained with a daily change of experimental seawater for 10 d. After 2 and 10 d, polyp size was evaluated by measuring the projected areas occupied using a digital camera (E-330; Olympus, Tokyo, Japan) connected to a dissecting microscope (SMZ 645; Nikon, Tokyo, Japan) and the ImageJ 1.38 program (National Institutes of Health, Bethesda, MD, USA).

2-7. Statistical analysis

The rates of fertilization, larval survivorship, and metamorphosis did not conform to parametric assumptions, and thus differences between treatments were assessed using Kruskal–Wallis ANOVA followed by Steel's *post hoc* pairwise comparison with the control. Differences in the growth of polyps were analysed using nested ANOVA followed by Dunnett's pairwise comparison with the control. All statistical analyses were performed using JMP 10.0.2 software (SAS Institute, Cary, NC, USA).

3. Results

Almost all SNC in all treatments was ionized to Ag^+ regardless of the amount SNC added (Table 1). The concentrations of total Ag (SNC and Ag^+) and Ag^+ in the control condition were below the quantification limit.

The fertilization rate of *A. japonica* was significantly lower for gametes exposed to 50 and 500 μg l^{-1} SNC than for the controls (Fig. 1, Kruskal–Wallis $\chi^2 = 15.9$, df = 4, $p < 0.05$; paired comparisons using Steel's test, both $p < 0.05$). No fertilization success was observed for gametes exposed to 500 μg l^{-1} SNC. Larval survivorship was significantly decreased relative to the controls after 2 d of exposure (Fig. 2, Kruskal–Wallis $\chi^2 = 14.6$, df = 4, $p < 0.05$; paired comparisons using Steel's test, each $p < 0.05$), and all larvae died after 4 d of exposure to 500 μg l^{-1} SNC. The survival rates of larvae exposed to 0.5, 5 and 50 μg l^{-1} SNC were not significantly different from that of the controls (Fig. 2, Steel's test, each $p > 0.05$). However, all larvae exposed to 50 μg l^{-1} SNC stopped swimming and were malformed after 2 d of exposure (Fig. 3B), whereas control larvae were rod-shaped and continued swimming (Fig. 3A). Larvae exposed to 0.5 and 5 μg l^{-1} SNC showed same morphology and behavior to those in the control condition. The metamorphosis rate of larvae exposed to 50 and 500 μg l^{-1} SNC for 24 h was significantly lower than that of the controls (Fig. 4, Kruskal–Wallis $\chi^2 = 23.8$, df = 4, $p < 0.05$; paired comparisons using Steel's test, each $p < 0.05$). All larvae metamorphosed normally under

control conditions, whereas 14.0% and 0% of larvae successfully metamorphosed in 50 and 500 μg l^{-1} SNC, respectively.

Polyps were significantly smaller in 50 μg l^{-1} SNC (0.49 ± 0.02 mm^2, means ± SD) than in the control condition (Figs. 3C and 5, 0.94 ± 0.02 mm^2, means ± SD, nested-ANOVA, $F_{3,666}$ = 728, $p < 0.05$; paired comparisons by Dunnett's test, $p < 0.05$) after 2 d of exposure. All polyps exposed to 50 μg l^{-1} SNC were malformed (Fig. 3D) and all polyps exposed to 500 μg l^{-1} SNC died after 2 d of exposure. Polyps exposed to 50 μg l^{-1} SNC remained malformed and the projected area of polyps exposed to 5 μg l^{-1} SNC was not significantly different from that of controls even after 10 d of exposure (Fig. 5B, Dunnett's test, $p > 0.05$).

4. Discussion

Silver nanomaterials are widely used in hygiene products and industry for their antibacterial activity and have a potentially high risk of negative impacts on aquatic environments through anthropogenic wastewater inputs (Wijnhoven et al., 2009). Marine animals in nearshore and marine areas around estuaries are at particular risk of harm from silver nanomaterials. However, the effects of silver nanomaterials on cnidarians, including corals, remain unexplored. In this study, the effects of seawater contaminated with SNC on the early life stages of the coral *A. japonica* were investigated.

199 This is the first study of the effects of silver nanomaterials in corals and cnidarians. Exposure
200 of the coral *A. japonica* to SNC-contaminated seawater had negative impacts on fertilization,
201 larval survival, larval metamorphosis, and primary polyp growth at concentrations of ≥50 μg l^{-1}.
202 SNC at concentrations of 0.1–1000 μg l^{-1} do not affect the fertilization of sea urchins, although
203 developmental delay and anomalies were induced by 72 h of exposure to 0.1 μg l^{-1} SNC
204 (Gambardella et al., 2013). In the present study, exposure to 50 μg l^{-1} SNC did not significantly
205 decrease larval survival, but the larvae were deformed and lost their ability to metamorphose.
206 This deformation of larvae has also been reported for oysters after exposure to 0.16 μg l^{-1} SNC
207 (Ringwood et al., 2010) and sea urchins after exposure to 300 μg l^{-1} SNC (Šiller et al., 2013).
208 The difference in the effective concentrations found in these studies may be due to the species
209 under investigation or the experimental conditions. The degree of ionization and size of particles,
210 in addition to the concentration of the particles, influence the toxicity of silver nanomaterials
211 (Keneddy et al., 2010). For example, Šiller et al. reported that Ag^+ ions are more toxic to sea
212 urchin larvae than citrate-capped SNC, of which less than 1% is ionized (Šiller et al., 2013).
213 Almost all of the SNC used in the present study was ionized to Ag^+ ions. There have been no
214 reports detailing the toxicity of Ag^+ ions to corals. In a study of the effects of metal ions on
215 coral fertilization, copper ions were reported to have the highest level of toxicity among lead,
216 zinc, cadmium and nickel ions and the lowest effective concentrations of copper on the

217 fertilization success of *A. tenuis* and *A. longicyathus*, were 66.6 and 23.6 μg l^{-1}
218 (Reichelt-Brushett and Harrison, 2005). Larval settlement success of *A. tenuis* is also
219 significantly decreased by 42.0 μg l^{-1} of ionic copper (Reichelt-Brushett and Harrison, 2000).
220 These values for the lowest effective copper dose are similar to that of the lowest effective ionic
221 silver concentrations of 46.2 to 68.4 μg l^{-1} found in the present study. This suggests high toxicity
222 of silver ions to coral in the early stages of development.
223 In addition to the degree of ionization, internal bioaccumulation of SNC should also be
224 considered. Bioaccumulation of SNC has been reported in some marine molluscs (Zuykov et al.,
225 2011; Al-Sid-Cheikh et al., 2013; Li et al., 2013). In the scallop *Chlamys islandica*, larger silver
226 nanoparticles accumulated in the digestive system over a longer period, and had a different
227 distribution, than smaller particles (Al-Sid-Cheikh et al., 2013). In adult corals, metal ion
228 bioaccumulation was investigated both in the field (Reichelt-Brushett and McOrist, 2003) and in
229 indoor exposure experiments (Bastidas and García, 2004; Bielmyer et al., 2010). These studies
230 show that symbiotic algae, *Symbiodinium* spp. (zooxanthellae), accumulate more metal ions
231 than their coral host. This suggests that the expulsion of algae is a detoxifying mechanism for
232 corals. Although there is still no evidence for bioaccumulation of SNC or other nanomaterials in
233 corals, increased expulsion of zooxanthellae from coral after exposure to TiO_2 nanoparticles has
234 been reported (Jovanović and Guzmán 2014). Nonetheless, internally accumulated particulate

235 contaminants may damage corals chronically, even after the contaminants have been removed
236 from the surrounding water column.
237 The physiological mechanism underlying the effects of SNC on marine organisms is still not
238 well understood. In sea urchin embryos, cholinesterase activity is inhibited by metal
239 nanomaterials, including SNC (Gambardella et al., 2013). In adult coral colonies of
240 *Montastraea franksi*, DNA is damaged and the expression pattern of oxidative stress genes is
241 altered by copper ions (Schwarz et al., 2013). The expression of oxidative stress gene HSP 70 is
242 increased by TiO_2 nanoparticles in the adult colonies of *Montastraea faveolata* (Jovanović and
243 Guzmán 2014). It is hypothesized that SNC induces DNA damage and alterations of gene
244 expression patterns in corals.
245 In conclusion, pure SNC is immediately ionized to Ag^+ and this may influence multiple early
246 life stages of corals. However, knowledge concerning the effects of SNC on coral and other
247 marine organisms is still poor. Studies investigating the relationship between toxicity and level
248 of SNC ionization, the effects of internal SNC bioaccumulation, the physiological mechanism
249 underlying the effects of SNC, the effects of SNC on multiple life stages, synergistic effects of
250 SNC and other environmental factors, and effects of long-term exposure to low levels of SNC
251 are necessary to understand the toxicity of SNC to marine organisms.
252

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260

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343 Figure legends
344 Fig. 1. Fertilization rate 2 h after mixing sperm with the eggs of *Acropora japonica* subjected to
345 various concentrations of silver nanocolloids (SNC). In each repetition, the fertilization success
346 of 200 eggs was recorded. Asterisks indicate the statistical significance compared with the
347 control condition ($P < 0.05$, Kruskal–Wallis ANOVA/Steel's pair-wise comparison). Error bars

348 = SD (n = 4)

349

350 Fig. 2. Survivorship of *Acropora japonica* larva after a 10-d exposure to various concentrations
351 of silver nanocolloids (SNC). In each repetition, the survivorship of 20 larvae was recorded.
352 Asterisks indicate the statistical significance compared with the control condition ($P < 0.05$,
353 Kruskal–Wallis ANOVA/Steel's pair-wise comparison). Error bars = SD (n = 5)

354

355 Fig. 3. Representative images of *Acropora japonica* larvae (A, B) and primary polyps (C, D)
356 under different conditions of silver nanocolloid exposure. A larva and primary polyp in the
357 control condition (A, C) and exposed to 50 μg l^{-1} silver nanocolloid (SNC)-contaminated
358 seawater for 2 d (B, D). Scale bar = 200 μm

359

360 Fig. 4. Metamorphosis rate of *Acropora japonica* larvae that were pre-exposed to different
361 silver nanocolloid (SNC) concentrations for 24 h. In each repetition, the metamorphosis of 20
362 larvae was recorded. Asterisks indicate the statistical significance compared with the control
363 condition ($P < 0.05$, Kruskal–Wallis ANOVA/Steel's pair-wise comparison). Error bars = SD (n
364 = 5).

365

366 Fig. 5. Areas of occupation by primary polyps of *Acropora japonica* after 2 d (A) and 10 d (B)
367 of incubation with different concentrations of silver nanocolloids (SNC). In each repetition, the
368 occupied areas of approximately 40 primary polyps were recorded. Asterisks indicate the
369 statistical significance compared with the control condition ($P < 0.05$, nested
370 ANOVA/Dunnett's pair-wise comparison). Error bars = SD (n = 5).

Table 1. Conditions of Ag during experiments. Summary of chemical Ag conditions in each experiment. Seawater sampling was conducted before and after each experiment, except for the fertilization experiment, for which sampling was conducted only before starting the experiment.

Life stage	Nominal Ag (μg l^{-1})	Timing of sampling	SNC and Ag^+ (μg l^{-1})	Ag^+ (μg l^{-1})	Quantitation limit (μg l^{-1})	Temperature (^oC)
Fertilization	0	before experiment	nd	nd	0.92	27.1 ± 0.6
Fertilization	0.5	before experiment	1.83 ± 2.09	2.61 ± 1.25	0.92	27.1 ± 0.6
Fertilization	5	before experiment	7.50 ± 2.80	8.28 ± 1.59	0.92	27.1 ± 0.6
Fertilization	50	before experiment	61.4 ± 2.57	68.4 ± 2.16	0.92	27.1 ± 0.6
Fertilization	500	before experiment	548 ± 10.2	545 ± 16.3	0.92	27.1 ± 0.6
Metamorphosis	0	before experiment	nd	nd	2.75	26.6 ± 0.3
Metamorphosis	0.5	before experiment	4.16 ± 1.96	6.11 ± 3.40	2.75	26.6 ± 0.3
Metamorphosis	5	before experiment	10.2 ± 3.62	9.46 ± 1.24	2.75	26.6 ± 0.3
Metamorphosis	50	before experiment	75.7 ± 3.42	76.8 ± 1.90	2.75	26.6 ± 0.3
Metamorphosis	500	before experiment	621 ± 5.57	638 ± 7.07	2.75	26.6 ± 0.3
Metamorphosis	0	after experiment	nd	nd	0.92	26.6 ± 0.3
Metamorphosis	0.5	after experiment	1.40 ± 0.77	2.19 ± 0.74	0.92	26.6 ± 0.3
Metamorphosis	5	after experiment	7.84 ± 0.83	7.99 ± 1.11	0.92	26.6 ± 0.3
Metamorphosis	50	after experiment	63.6 ± 3.19	62.5 ± 0.62	0.92	26.6 ± 0.3
Metamorphosis	500	after experiment	656 ± 12.0	591 ± 8.99	0.92	26.6 ± 0.3
Larvae	0	before experiment	nd	nd	1.24	26.6 ± 0.3
Larvae	0.5	before experiment	5.40 ± 2.77	4.60 ± 1.44	1.24	26.6 ± 0.3
Larvae	5	before experiment	17.6 ± 10.5	14.2 ± 2.87	1.24	26.6 ± 0.3
Larvae	50	before experiment	37.7 ± 9.00	46.2 ± 1.32	1.24	26.6 ± 0.3
Larvae	500	before experiment	346 ± 44.5	385 ± 32.0	1.24	26.6 ± 0.3
Larvae	0	after experiment	nd	nd	1.36	26.6 ± 0.3
Larvae	0.5	after experiment	2.26 ± 0.93	1.82 ± 0.54	1.36	26.6 ± 0.3
Larvae	5	after experiment	7.77 ± 1.72	11.7 ± 4.94	1.36	26.6 ± 0.3
Larvae	50	after experiment	67.5 ± 4.54	69.8 ± 3.72	1.36	26.6 ± 0.3
Larvae	500	after experiment	303 ± 68.6	348 ± 15.5	1.36	26.6 ± 0.3
Primary polyp	0	before experiment	nd	nd	1.24	26.6 ± 0.3
Primary polyp	0.5	before experiment	1.82 ± 0.87	2.80 ± 2.28	1.24	26.6 ± 0.3

Primary polyp	5	before experiment	15.0 ± 6.82	10.6 ± 1.00	1.24	26.6 ± 0.3
Primary polyp	50	before experiment	93.3 ± 6.84	99.2 ± 1.83	1.24	26.6 ± 0.3
Primary polyp	500	before experiment	757 ± 18.2	785 ± 2.25	1.24	26.6 ± 0.3
Primary polyp	0	after experiment	nd	nd	1.36	26.6 ± 0.3
Primary polyp	0.5	after experiment	0.63 ± 0.57	3.53 ± 1.38	1.36	26.6 ± 0.3
Primary polyp	5	after experiment	0.72 ± 0.60	1.58 ± 0.79	1.36	26.6 ± 0.3
Primary polyp	50	after experiment	39.0 ± 2.04	39.5 ± 1.11	1.36	26.6 ± 0.3
Primary polyp	500	after experiment	360 ± 13.9	438 ± 6.13	1.36	26.6 ± 0.3

The limit of quantitation is 3.3 times the limit of detection.

Background values of Ag^+ in seawater were measured and subtracted from the data of samples.

nd: not detected, means ± SD, n = 3

5

Fig. 1

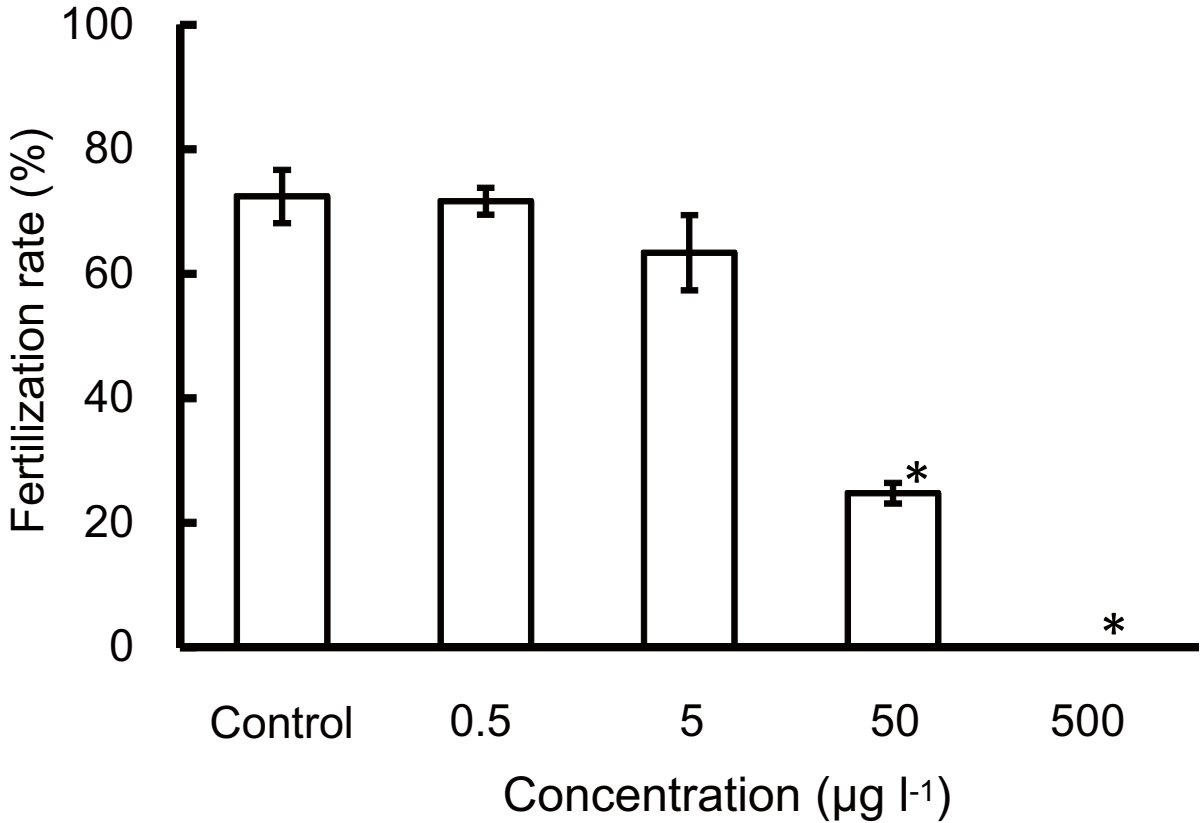

100
80
60
40
20
0
Fertilization rate (%)
*
*
Control
0.5
5
50
500
Concentration (μg l-1)

Fig. 2

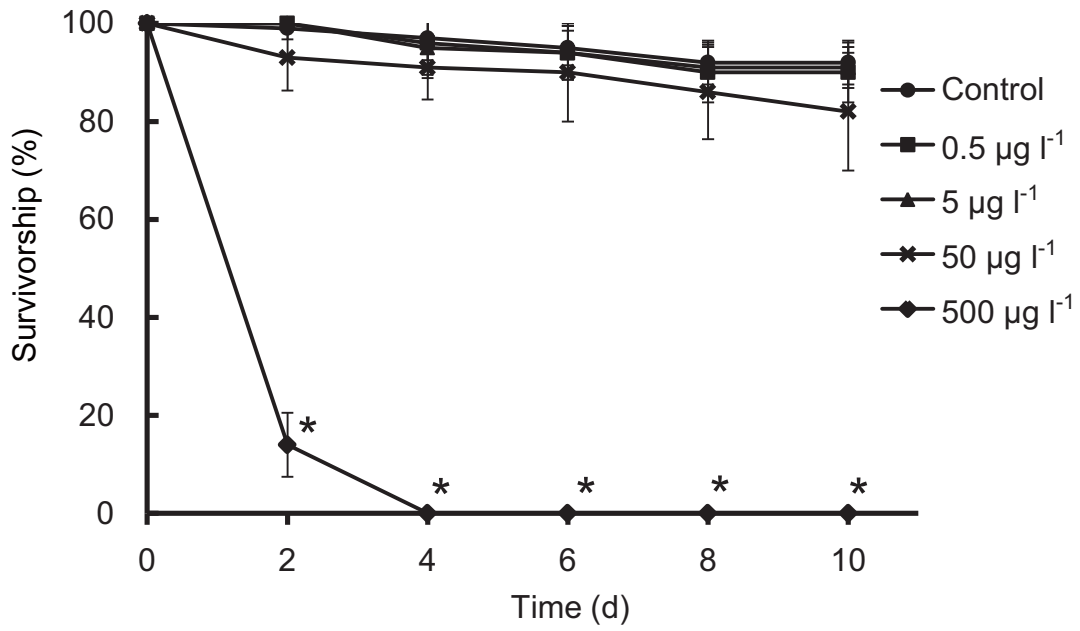

100
80
60
40
20
0
Survivorship (%)
0
2
4
6
8
10
Time (d)
*
*
*
*
*
Control
0.5 µg l-1
5 µg l-1
50 µg l-1
500 µg l-1

Fig. 3

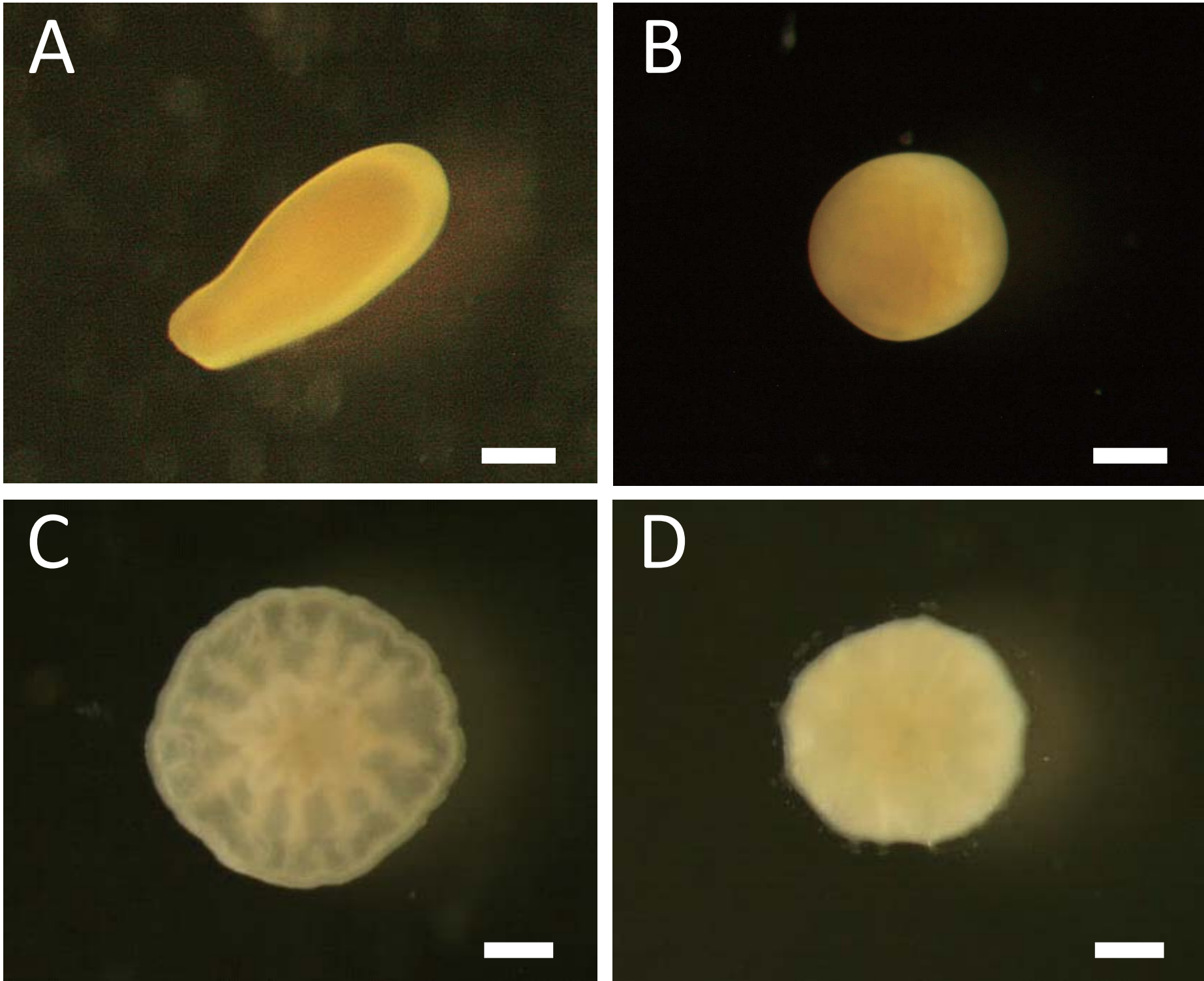


Fig. 4

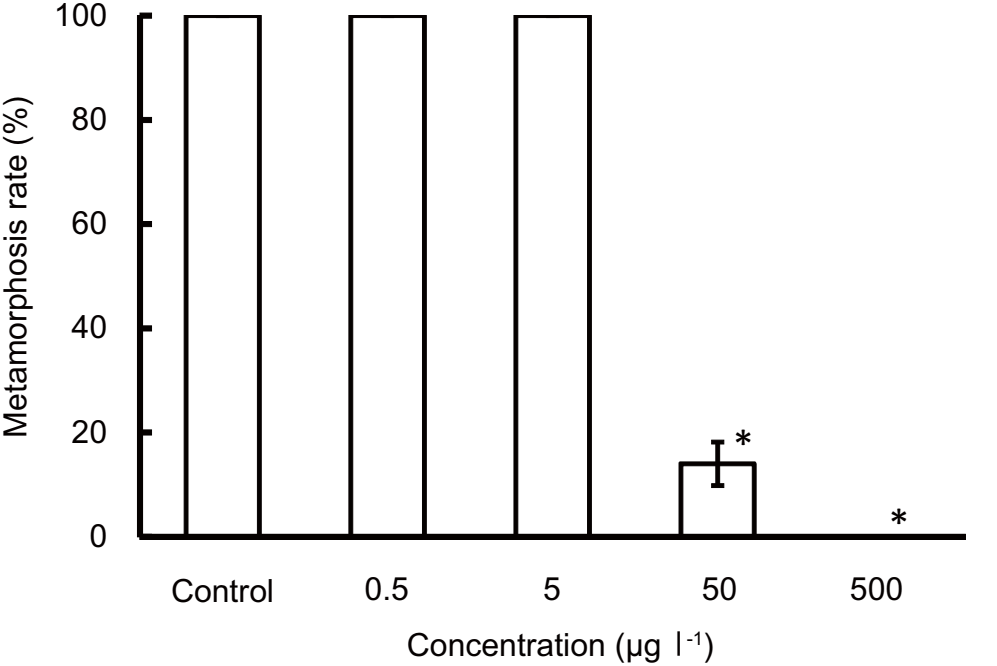
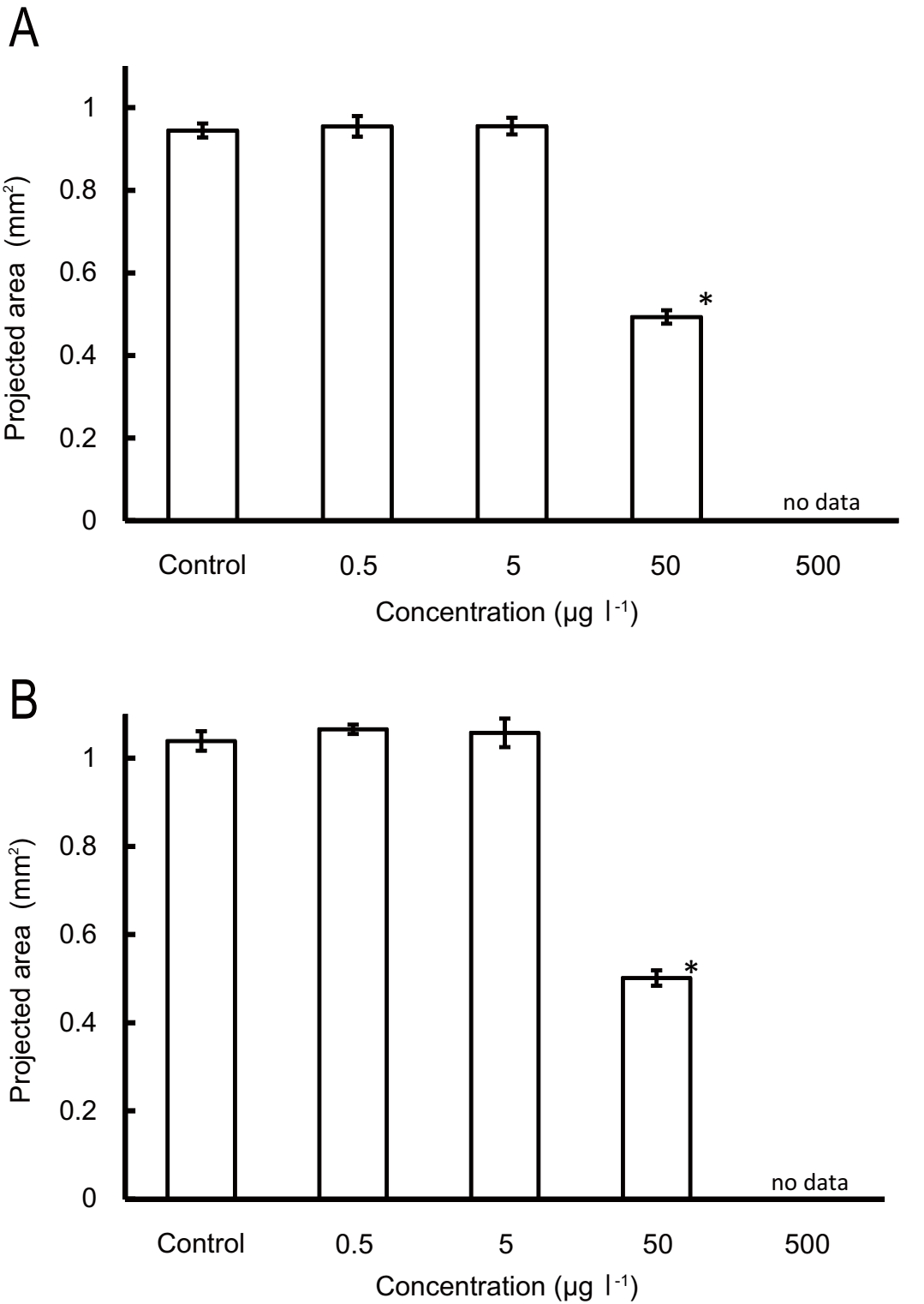

100
80
60
40
20
0
Metamorphosis rate (%)
*
*
Control
0.5
5
50
500
Concentration (μg l-1)

Fig. 5


A
Projected area (mm²)
1
0.8
0.6
0.4
0.2
0
*
no data
Control
0.5
5
50
500
Concentration (µg l⁻¹)
B
Projected area (mm²)
1
0.8
0.6
0.4
0.2
0
*
no data
Control
0.5
5
50
500
Concentration (µg l⁻¹)