The following supplements accompany the article

Short larval exposure to low level of copper has long-lasting latent effects on juvenile performance in the sea urchin *Evechinus chloroticus*

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Supplement 1: Dissolved copper in coastal water and in experimental setting

Table S1.1: Copper (Cu) contamination (μg l^{-1}) in coastal water in areas with low anthropogenic impact, areas with high impact (urban/industrial, boating activity and mining) and water quality guidelines in various countries.

All concentrations reported are the dissolved fraction (< 0.45 μm).

Site	Country	Cu	Reference
Low impact			
Waitemata Harbour	New Zealand	< 1 - 1.3	(Gadd & Cameron 2012)
Seto	Japan	0.79	(Kobayashi & Okamura 2004)
Various locations	Antarctica	0.18 - 1.17	(Honda et al. 1987)
Highimpact, urban/industrial			
Port Phillip Bay	Australia	0.4 - 0.6	(Gorski & Nugegoda 2006)
Wellington	New Zealand	<0.5 – 2.9	(Rouchon 2015)
East London Harbour	South Africa	0.6 - 42.6	(Fatoki & Mathabatha 2001)
Port Elizabeth Harbour	South Africa	0.5 - 11.3	(Fatoki & Mathabatha 2001)
San Francisco Bay	USA	0.4 - 10.8	(SFEI 2015)
Southwest Coast	India	3.9 - 13.1	(Udayakumar et al. 2011)
Pondicherry Coast	India	0.7 - 61.5	(Govindasamy et al. 1998)
San Jorge Bay	Chile	0.62 - 1.96	(Valdés et al. 2011)
High impact, boating			
Auckland's Marinas	New Zealand	< 1 - 20.0	(Gadd & Cameron 2012)
San Diego Bay's Marinas	USA	1.1 - 21.0	(Schiff et al. 2007)
High impact, mining			
Kanayama Cove	Japan	0.1	(Kobayashi & Okamura 2004)
Chanaral Bay	Chile	<1 - 32	(Stauber et al. 2005)
Water quality guidelines			
For 95% species protection	Australia and New Zealand	1.3	(ANZECC 2000)
For 90% species protection	Australia and New Zealand	3	(ANZECC 2000)
	USA	3.1	(US EPA 2007)
	UK	5	(Cole et al. 1999)
	South Africa	5	(Department of Water Affairs and Forestry 1995)

Table S1.2: Copper concentration in experimental treatments. Water samples were collected after the experiment, using the same setup and material and were filtered through a 0.45 µm mesh. Four measurements for control water were taken between 2011 and 2014.

Copper level	Nominal concentration (µg l^{-1})	Measured dissolved concentration (µg l^{-1})
Control	0	<0.5, <0.5, 0.5, 1.3
ANZECC	1.3	2.3
Field	2.9	3.8
Field x 2	5.8	6.1
High	10	10.4

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Supplement 2: Larval survival, development and growth

Larval survival and development

Methods

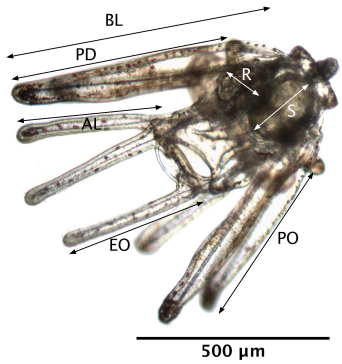
Direct effects of copper treatments on larval stages were evaluated by (1) survival, (2) normal development, (3) delayed development, (4) growth and (5) morphometrics. The baseline for survival was larval density at 4 d rather than initial density to remove natural variation in early survival across jars. Normal development was expressed as the proportion of live larvae rated as normal (Table S2.1). In both survival and normal development analysis, 13 d was removed as only partial sampling was done (Late exposure group only) and 25 d and 27 d were considered as the same level of treatment to allow comparison between the two timing groups. Delayed development was measured as the proportion of normal larvae at the final stage of 8-armed plutei during last sampling (25 d post-fertilisation for Early group and 27 d for Late group).

Larval survival and normal development was analysed using a three-ways split-plot factorial Repeated Measures ANOVA with copper level and timing of exposure as 'between' factors and larval age (in days post-fertilisation) as 'within' factor. The effect of copper level on delayed development was analysed using a one-way ANOVA separately for Early and Late groups as sampling was done two days later in Late group, which may affect the proportion of 8-armed plutei. All three variables were arcsin square-root transformed to meet ANOVA assumptions. F-ratios values were calculated from Type II SS. When copper level factor was significant ($p < 0.05$) a post-hoc Dunnett's comparison test was used to compare all treatments against their control.

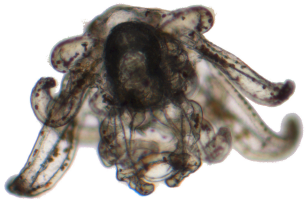
Jars 10 (Late ANZECC treatment), 19 (Late High treatment) and 30 (Early Field treatment) were lost at 11 d, 6 d and 25 d respectively. Jar 15 (Early ANZECC treatment) was not sampled at 25 d and removed from the survival and normal development analyses to have a balanced design.

Table S2.1: Developmental categories of *Evechinus chloroticus* larvae.

Arrows on 8-armed pluteus show morphometric measurments. BL: total body length, PO: postoral arms, AL: anterolateral arms, PD: posterodorsal arms, EO: preoral arms, S: stomach, R: rudiment.

Category	Description	Photo	Rating
8-armed pluteus	All 4 pairs of arms well developed		Normal

Category	Description	Photo	Rating
6-armed pluteus	Well developed PD arms, EO arms absent or budding (i.e. no skeletal rod visible under dissecting microscope).		Normal
4-armed pluteus	Well developed AL arms, PD arms absent or budding		Normal: 4 d and 11 d Delayed: 25 d and 27 d
2-armed pluteus	Well developed PO arms, AL arms absent or budding		Normal: 4 d Delayed: 11 d, 25 d and 27 d
Missing arm	One or more arm missing. Only if the other arm of the pair is well developed.		Abnormal
Broken	Broken arm or skeletal rod in body		Abnormal

Category	Description	Photo	Rating
Stunted	PO arms < half BL		Abnormal
Abnormal	Larvae with more than one abnormality or with severe abnormality not fitting any other categories.		Abnormal

Results

Larval survival and normal development were not affected by copper exposure or timing of exposure (Table S2.2). Only larval age had a significant effect, with survival and proportion of normal larvae declining with time.

Table S2.2: Repeated measures ANOVA of *Evechinus chloroticus* larval survival rate (A) and larval normal development rate (B) to test effects of copper exposure (copper level) and timing of exposure (timing) during 3 sampling (Age) throughout larval stage. Significant effects ($p < 0.05$) are highlighted in bold.

Source of variation	df	SS	*F*	p
(A) Larval survival				
Timing	1, 16	0.0392	0.708	0.413
Copper level	4, 16	0.628	2.838	0.059
Age	2, 32	12.49	178.5	**<0.001**
Timing x Copper level	4, 16	0.209	0.942	0.465
Timing x Age	2, 32	0.032	0.464	0.633
Copper level x Age	8, 32	0.470	1.678	0.142
(B) Larval normal development				
Timing	1, 16	0.097	2.532	0.131
Copper level	4, 16	0.153	0.999	0.437
Age	2, 32	3.922	80.18	**<0.001**
Timing x Copper level	4, 16	0.151	0.985	0.443
Timing x Age	2, 32	0.134	2.739	0.080
Copper level x Age	8, 32	0.265	1.356	0.253

However, there was a trend of delayed development (Figure S2.1) with a lower proportion of 8-arms larvae in the High copper level in both Early and Late exposure

groups (Early exposure: 0.53 ± 0.46 in High level *vs* 0.79 ± 0.17 in control; Late exposure: 0.52 ± 0.22 in High level *vs* 0.95 ± 0.03 controls; mean ± standard deviation). However, this difference was significant only in the Late exposure group (Dunnett's contrasts, Early: t = -1.26, p = 0.57; Late: t = -5.06, p = 0.002).

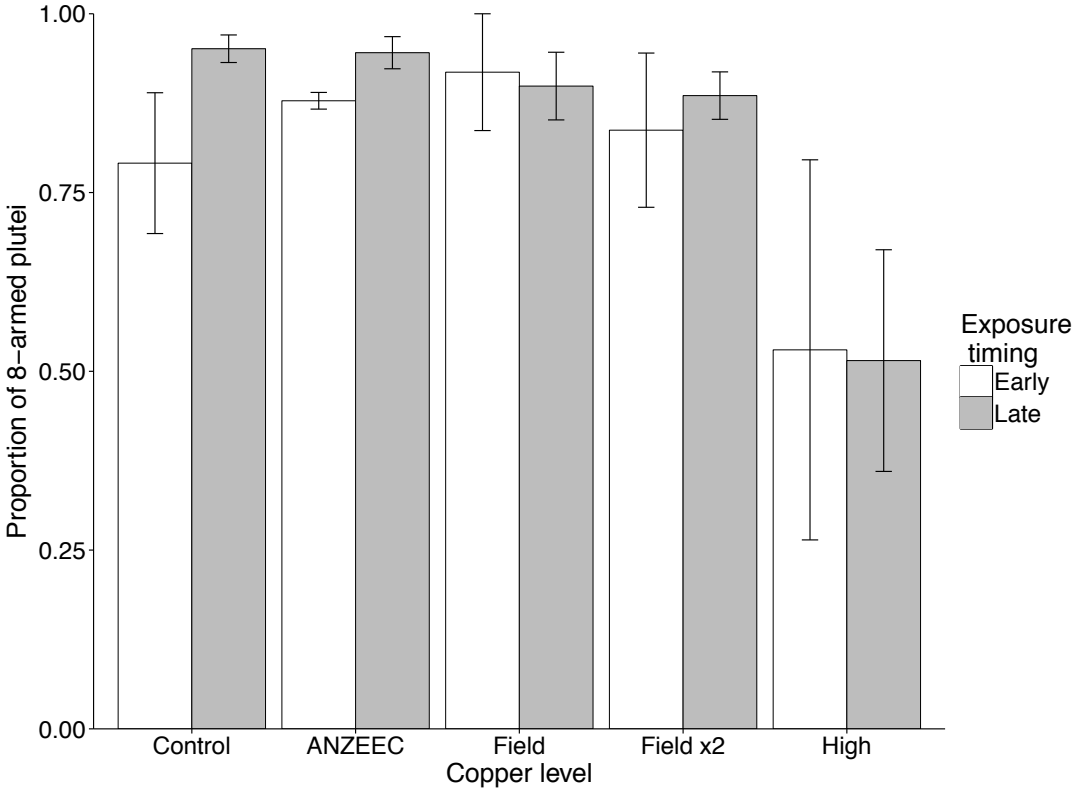


Figure S2.1: Delayed development of *Evechinus chloroticus* larvae exposed to copper expressed as proportion of normal larvae having reached the 8-armed pluteus stage at day 25 post-fertilisation for Early exposure group (white bars) and day 27 for Late exposure group (grey bars). Copper levels: Control, i.e. no added copper; ANZECC, 2 µg l^{-1}; Field, 3 µg l^{-1}; Field x2, 6 µg l^{-1}; and High, 10 µg l^{-1}. Error bars represent the standard error of the mean (N = 3).

Larval growth and morphometrics

Methods

For traits present from mid-larval stages: body length (BL), postoral arms (PO) and anterolateral arms (AL), average growth rate *per* jar was measured between 11 d and 25 d post-fertilisation in Early exposure group and between 13 d and 27 d post-fertilisation. Time interval was 14 days in both timing groups. All response variables were analysed separately for both exposure-timing groups to account for difference in timing of sampling using one-way ANOVAs. Assumptions were met without transformation and F-ratios were calculated from Type II SS.

For traits present only in late larval stages: posterodorsal arms (PD), preoral arms (EO) and rudiment size (R), or for which growth is of no interest (i.e. stomach size),

measures during last sampling (25 d in Early exposure group and 27 d in Late exposure group) were analysed separately for both exposure-timing groups. A nested mixed effect ANOVA was used with jar (random effect) nested within copper level (fixed effect) as fixed effect and individual larvae as residuals. Assumptions were were met without transformation. F-ratios were calculated from Type I SS.

Rudiment presence was expressed as the proportion of 8-armed pluteus having a rudiment visible under compound microscope *per* jar. Rudiment presence was arcsin square-root transformed and analysed using a separate one-way ANOVA for both exposure timing groups. F-ratios were calculated from Type I SS.

Results

Larval growth for all three variables: body length (BL), postoral arms (PO) and anterolateral arms (AL), was significantly lower in the Early exposure group with and average of 23% growth in BL, 18% in PO and 51% in AL against a growth rate of respectively 31%, 27% and 95% in Late exposure group (Table S2.3). However, copper level was not significant and controls for each variable were also slightly lower in Early treatment than in Late treatment, although the difference was significant only for AL (t-test; t = -3.45, df = 4, p = 0.03; Figure S2.2). This suggests that the difference between Early and Late treatment is due to stage-dependent growth rather than timing of copper exposure. Indeed growth rate in Late groups was measured slightly later (13 – 27 d post-fertilisation) than in Early groups (11 – 25 d post-fertilisation). Interestingly, growth rate was higher in Late groups for all variables and copper levels except for arm growth (both PO and AL) in High copper level. There might be a trend of decreasing arm growth in High copper level but it is masked by the difference in sampling timing.

Table S2.3: Effect of copper exposure on *Evechinus chloroticus* growth rate in larvae exposed to copper early (4 - 6 days post-fertilisation) or late (11 - 13 days post-fertilisation) during larval development. Growth was measured between 11 – 25 d post-fertilisation in the Early exposure group and 13 – 27 d post-fertilisation in the Late exposure group.

One-way ANOVA model, d.f. = 4 on 7 (Early group) and 4 on 8 (Late group).

Variable	Copper level in Early group *F* (p)	Copper level in Late group *F* (p)
Body growth (BL)	1.21 (0.39)	0.33 (0.85)
Postoral arm growth (PO)	0.52 (0.72)	3.71 (0.05)
Antelateral arm growth (AL)	0.16 (0.95)	2.42 (0.13)

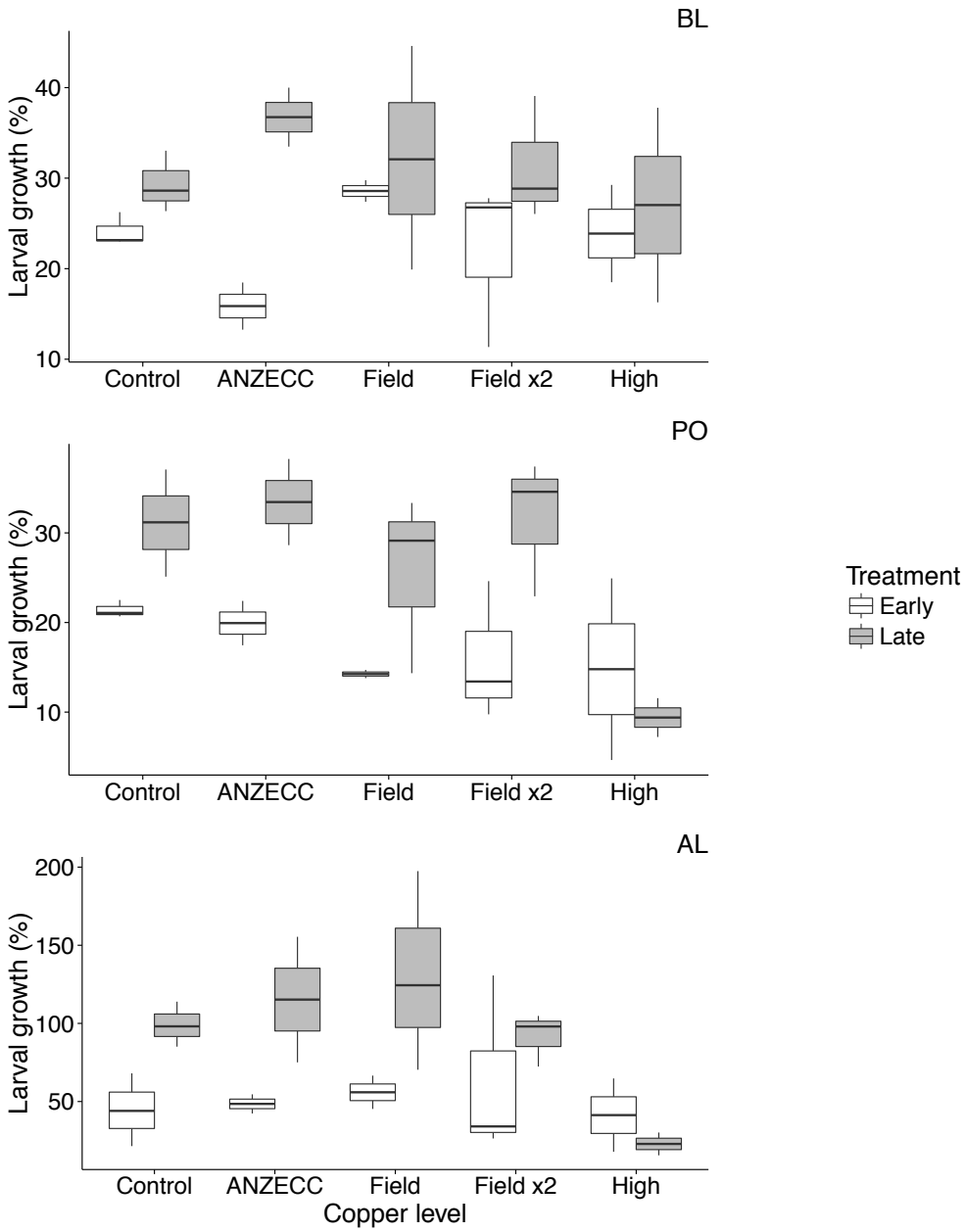


Figure S2.2: Proportion of growth of *Evechinus chloroticus* larvae body length (BL), postoral arms (PO) and anterolateral arms (AL) during the last 2 weeks of larval stage.

Larvae where exposed to different levels of copper either Early (4 - 6 days post-fertilisation) or Late (11 - 13 days post-fertilisation) in larval stage. Copper levels: Control, i.e. no added copper; ANZECC, 2 µg l^{-1}; Field, 3 µg l^{-1}; Field x2, 6 µg l^{-1}; and High, 10 µg l^{-1}. Boxes represent the median and quartiles.

Copper exposure had no significant effect on larval morphometric measures at the end of the larval stage (PD, EO and R) except for EO in Early exposure group (Table S2.4). However, the significance in EO was driven by a difference between Field and High copper level (Tukey comparisons: $p = 0.02$) and not between controls and copper treatments. Rudiment presence was not affected by copper exposure in both timing groups (one-way ANOVA, Early group: $F = 4.28$, $p = 0.06$; Late group: $F =$

0.25, p = 0.90). The difference between Early and Late groups could not be evaluated, as measurements were not taken on the same day for both groups.

Table S2.4: Effect of copper exposure (copper level) on *Evechinus chloroticus* morphometric measurements in late larval stage

(25 d post-fertilisation for Early exposure group and 27 d for Late exposure group) analysed using a mixted effect nested ANOVA. Significant effects ($p < 0.05$) are highlighted in bold.

Variable	Copper level in Early group *F* (p)	Copper level in Late group *F* (p)
Posterodorsal arm length (PD)	1.05 (0.45)	1.39 (0.31)
Preoral arm length (EO)	**5.81 (0.02)**	0.44 (0.78)
Stomach size	1.11 (0.42)	1.96 (0.19)
Rudiment size	0.37 (0.77)	0.62 (0.66)