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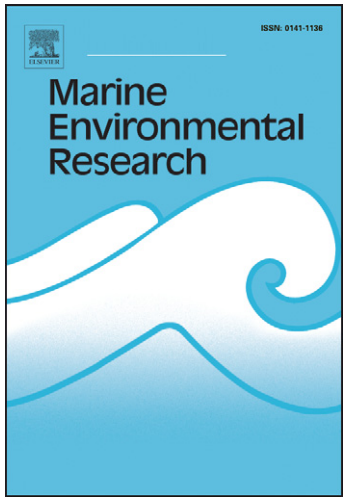

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Sea urchin fertilization in a warm, acidified and high pCO_2 ocean across a range of sperm densities

Running title: Urchin fertilization in a warm acidic ocean

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ABSTRACT

Marine invertebrate gametes are being spawned into an ocean simultaneously warming, acidifying and increasing in pCO_2. Decreased pH/increased pCO_2 narcotizes sperm indicating that acidification may impair fertilization, exacerbating problems of sperm limitation, with dire implications for marine life. In contrast, increased temperature may have a stimulatory effect, enhancing fertilization. We investigated effects of ocean change on sea urchin fertilization across a range of sperm densities. We address two predictions: 1) low pH/increased pCO_2 reduces fertilization at low sperm density and 2) increased temperature enhances fertilization, buffering negative effects of acidification and increased pCO_2. Neither prediction was supported. Fertilization was only affected by sperm density. Increased acidification and pCO_2 did not reduce fertilization even at low sperm density and increased temperature did not enhance fertilization. It is important to identify where vulnerabilities lie across life histories and our results indicate that sea urchin fertilization is robust to climate change stressors. However, developmental stages may be vulnerable to ocean change.

Keywords: climate change; ocean warming; ocean acidification; sperm concentration; sea urchin; fertilization; pH/pCO_2

1. Introduction

The global oceans are on a trajectory of change as they simultaneously warm, acidify and increase in pCO_2 (=hypercapnia) due to anthropogenic alteration of the Earth's climate (Caldeira and Wickett, 2003). These climate change stressors exert negative effects on marine biota as single stressors and are also likely to have

interactive effects that are difficult to predict (Fabry et al., 2008, Pörtner et al., 2005; Pörtner 2008; Przeslawski et al., 2008; Widdicombe and Spicer, 2008). Temperature dictates all physiological processes and influences marine faunal distributions (Clarke, 2003; Mueter and Litzow, 2009; Richardson, 2008). Climate change driven high sea temperatures cause mass mortality in benthic communities and a plethora of other negative effects including disease increase and bleaching in corals (Coma et al., 2009; Garrabou et al., 2009; Przeslawski et al., 2008). Hypercapnia and acidification exert negative effects on the metabolism, biochemistry and skeleton formation of marine species (Pörtner et al., 2005; Pörtner 2008; Widdicombe and Spicer, 2008). Tolerance to climate change and other anthropogenic stressors varies greatly among species making it difficult to make broad extrapolations on how marine communities will respond to climate change (Dupont and Thorndyke, 2009; Kurihara, 2008; Pörtner, 2008; Przeslawski et al., 2008).

Marine environmental research has a new challenge, to determine the impact of climate change stressors on marine biota placed within the setting of projected environmental change. Due to differences in ocean chemistry, circulation patterns and the inverse relationship between temperature and CO_2 uptake, ocean regions will differ in the extent and rate at which they warm or acidify (Blackford and Gilbert, 2007; Levitus et al., 2005; Poloczanska et al., 2007). Empirical data on the integrative effects of climate change stressors, in a climate and regionally-relevant setting, are urgently needed to test predictions on the fate of local biota in the face of climate change (Fabry et al., 2008, Pörtner, 2008; Przeslawski et al., 2008; Widdicombe and Spicer, 2008).

Reproduction in the sea often involves biphasic life histories with benthic and planktonic life stages that differ in their sensitivities to environmental perturbations. Early planktonic stages are considered the most vulnerable. Failure of the planktonic

stages of some species (eg. calcifiers) and facilitation of others (eg. jellyfish, ascidians) in response to climate change indicates potential for major anthropogenic modification of marine ecosystems (Dupont and Thorndyke, 2009; Fabry et al., 2008; Richardson, 2008). Disruption of fertilization by climate change stressors would have dire consequences for marine life and recent studies indicate that this fundamental life process is impaired by acidification (Havenhand et al., 2008; Kurihara and Shirayama, 2004; Parker et al., 2009).

For free spawning marine invertebrates the chance of egg and sperm meeting can be negatively impacted by gamete dilution and sperm limitation conditions (Denny and Shibata 1989; Levitan et al., 1991; Levitan, 1995). Seawater chemistry influences fertilization kinetics as seen in cases where toxicants exert a disproportionate negative effect at low sperm densities (Dinnell, et al., 1987; Hollows et al., 2007). If climate change stressors impair fertilization at low sperm densities this would exacerbate the problems of sperm limitation. On the other hand, ocean warming may enhance fertilization due to positive effects on sperm swimming speeds and heightened sperm-egg collisions as documented for sea urchin fertilization (Hagström and Hagström, 1959; Mita et al., 1984). Ocean warming may ameliorate the problems of sperm limitation and help to buffer the potential negative effects of acidification. The interactive effects of ocean warming and acidification on fertilization are difficult to predict.

Sea urchin gametes and embryos have long been used as a model system for environmental monitoring and ecotoxicology and their biology is well characterised (Bay et al., 1993; Brokow 1990; Byrne et al., 2008; Carr et al., 2006). We used gametes of the sea urchin *Heliocidaris erythrogramma* to assess the impacts of ocean warming and acidification on fertilization in the low sperm conditions that eggs may encounter in the sea (Levitan, 1995). A number of studies investigating impacts of

low pH (eg. pore water, acid leachates) and thermal pollution (eg. powerplant effluent) indicate that marine fertilization is robust to these stressors (Bay et al., 1993; Carr et al., 2006; Greenwood and Bennett, 1981; Wilson and Hyne, 1997), but recent studies indicate that acidification has a negative effect on fertilization (Havenhand et al., 2008; Kurihara and Shirayama, 2004; Parker et al., 2009). For *H. erythrogramma* we sought to explain the contrasting results where low pH impaired fertilization in one study (potentially due to reduced sperm motility) (Havenhand et al., 2008), but not in another (Byrne et al., 2009).

H. erythrogramma resides in the southeast Australia global change hot spot, where sea surface temperatures (SST) are warming three times faster than the global average (Poloczanska et al., 2007; Figueira and Booth, 2009). We do not know if simultaneous exposure to ocean warming, acidification and hypercapnia will have a negative impact on fertilization particularly in context of the low sperm concentrations that may occur in nature. It is essential to determine where vulnerabilities lie for marine life histories and so we asked the question: How is fertilization affected in a warm and high pCO_2/acidic ocean? With simultaneous exposure to near future projected ocean pH/pCO_2 and temperature conditions (2100, IPCC 2007) we examined the interactive effects of acidification and warming on fertilization in *H. erythrogramma* across a range of sperm densities. The experiments addressed two predictions: 1) low pH reduces fertilization success with disproportionate negative effects at low sperm density and 2) increased temperature enhances fertilization thereby buffering potential negative effects of acidification.

2. Materials and Methods

(Please place Tables 1 and 2 near the beginning of the Materials and Methods)

2.1 Study organism and exposure to stressors

Heliocidaris erythrogramma (65-75 mm test diameter) were collected at low tide from the shallow subtidal (0.5-1.0 m depth) at Little Bay (33°59'S: 151°15'E) and Clovelly Bay (33°55'S: 151°15'E), Sydney, Australia, in February 2009. The urchins were placed in aquaria in a constant temperature room (22°C). This temperature was similar to recent ambient SST as determined from a local reference station (www.mhl.nsw.gov.au). Within 2 days of collection the urchins were induced to spawn by injection of 1-2 ml of 0.5 M KCl. Gametes for each experiment were pooled from multiple males and females, at least 4 of each sex, to represent a population of spawners as might occur in the field. This was also done to avoid the marked variation among experiments encountered in single male-female crosses with *H. erythrogramma* (Evans and Marshall, 2005). Gametes were collected from the gonopores using glass pipettes. Sperm were kept dry until used. The eggs were placed in fresh filtered seawater (FSW, 1.0 µm), mean salinity 37.4 ppt (SE 0.2, n = 3) and mean pH 8.17 (SE= 0.02, n = 3).

Experimental pH was achieved by bubbling seawater with CO_2 gas. Dissolved oxygen (DO) levels (> 90%) were maintained by simultaneous bubbling of air in all water used. Seawater variables (pH, DO, temperature) were measured using a WTW Multiline F/Set-3 multimeter. Total alkalinity (TA) was determined by potentiometric titration. Experimental water parameters in the 9 temperature-pH/pCO_2 combinations used are detailed in Table 1. Aragonite (ΩAr) and calcite (ΩCa) saturation values and pCO_2 were determined from TA, pH and salinity data using the CO_2 System Calculation Program (Lewis and Wallace, 1998) (parameters: K1,K2 from Mehrbach et al., 1973 refit by Dickson and Millero, 1987; $KHSO_4$, Dickson; pH: NBS scale mol/kg-H_2O – cited in Lewis and Wallace, 1998).

We investigated the effects of near-future ocean warming and acidification for southeast Australia (2070-2100: ca. SST + 2-4 °C; pH - 0.2-0.4 units, Figueira and Booth, 2009; IPCC 2007; Poloczanska et al., 2007) on fertilization of *H. erythrogramma* in multifactorial experiments incorporating a titration of sperm density (10-10^3 sperm ml^{-1}) across a range of sperm to egg ratios (10:1-1500:1). For clarity we subsequently refer to sperm to egg ratios, but this relates directly to sperm density (see Table 2). The three factors examined, temperature (22 °C, 24 °C, 26 °C), pH (7.6, 7.8, 8.17) pCO_2 (range 354-1892 ppm) and sperm concentration/sperm to egg ratio (Table 1,2) were tested in all combinations with 3 independent sources of gametes. Experimental temperature (24 °C, 26 °C, + 2-4 °C above ambient), pH (pH 7.6, 7.8: 0.4-0.6 pH units below ambient) and pCO_2 (367-1892 ppm) conditions represent the 2070-2100 'business as usual' climate change scenario and beyond (IPCC 2007).

2.2 Fertilization

Before use, the quality of each gamete source was checked microscopically, eggs were checked for shape and appearance and sperm were checked for motility. The total number of eggs for each experiment was measured from a 50 ml suspension determined through counts of 100 µl aliquots. For each experiment 750 eggs were placed into each of 36 beakers (3 temperatures X 3 pH/ pCO_2 levels X 4 sperm concentrations) containing 250 ml of experimental FSW 15 min prior to fertilization. Egg density (3 ml^{-1}) was kept constant across all 3 experiments each of which used an independent source of gametes. Separate water baths were used for each temperature treatment to maintain constant temperature. The number of sperm present in 1 µl of the semen sample combined from all males used per experimental run was determined

using a haemocytometer and this calculation was used to determine how much sperm to add to 10 ml of experimental seawater to create a stock solution. After brief activation (ca. 1-2 sec) of sperm in experimental water, the appropriate amount of the sperm solution was added to the beakers containing eggs to obtain the required sperm concentration/sperm to egg ratio (Table 2). After 15 min the FSW was removed by gentle aspiration to remove excess sperm and the eggs were resuspended in fresh experimental FSW. The beakers were covered in Parafilm to minimise evaporation. At 2 hr fertilization success was determined as the proportion of eggs that had a fertilization envelope or exhibited cleavage, out of 3 samples of 50 eggs taken from each egg suspension. The mean of these counts was used as the data point for statistical analysis. The pH of each beaker was measured with the meter at the end of the experiment (2h). For the control pH 8.17 and pH 7.8 treatments there was no change over the 2 h. For the pH 7.6 treatment there was a slight increase 0.01-0.03 pH units in the beakers after 2 h.

2.3 Statistical analyses

Data on percentage fertilization were analysed by 3-way ANOVA with temperature, pH and sperm concentration (sperm:egg ratio) as fixed factors. The percentage data were arcsine transformed prior to ANOVA and assumptions of the analysis were checked before proceeding. Levene's test indicated homogeneity of variance. Where significant differences were evident, Tukey Multiple-Comparisons Test was used for post-hoc analyses. Data analyses were run on SPSS software (version 17.0).

3. Results

Sperm concentration had a dramatic effect on fertilization success (figure 1, Table 3). High rates of fertilization (> 90%) were reliably obtained in controls with the two highest sperm to egg ratios used (500 and 1500 sperm:egg). A sperm to egg ratio of at least 100:1 was required to obtain high fertilization rates (mean 85% SE= 6.6%, n=3). At the lower sperm density (10:1 sperm:egg) percent fertilization dropped sharply (mean 31.7%, SE = 6.1%, n=3).

Across all treatments there was a highly significant effect of sperm density, but no significant effect of temperature or interaction between factors (Table 3). Therefore low pH did not reduce the percentage of fertilization even at the lowest sperm densities used and increased temperature did not enhance fertilization at any sperm density.

Post hoc tests indicated that the percentage of fertilization at the two highest sperm:egg ratios used 500:1 and 1500:1 were not significantly different. However the percentage of fertilization in the two lower sperm:egg ratios 10:1 and 100:1 differed to all other sperm density treatments.

4. Discussion

The relationship between sperm density and fertilization in *H. erythrogramma*, follows the well-documented pattern for sea urchin fertilization kinetics (Denny and Shibata 1989; Levitan et al., 1991; Pennington, 1985). However, neither of our predictions in relation to climate change stressors was borne out.

Although a plethora of factors (eg. turbulence, boundary layer effects) influence fertilization in the field, our experiments were specifically designed to determine the effect of climate change stressors on fertilization at the sperm density

conditions that eggs may encounter in nature. The data are similar to those obtained in a previous study of *H. erythrogramma* using a single sperm concentration (10^3 sperm ml^{-1}) (Byrne et al., 2009). With regard to prediction #1, the data indicated that near future ocean acidification (to pH 7.6) and increased pCO2 (to 1892 ppm) does not reduce fertilization success even at very low sperm densities (10 sperm per egg). Similarly, a number of ecotoxicology and climate change studies show that sea urchin fertilization is robust to a broad pH range with impairment only at extreme levels well below projections for ocean acidification by 2100 (pH 7.1- 7.4, 2000-10,000 ppm CO_2), (Bay et al. 1993; Carr et al., 2006; Kurihara and Shirayama, 2004; but see Havenhand et al., 2008). This is also the case for bivalve fertilization (Byrne, 2009; Havenhand and Shlegal, 2009; but see Parker et al., 2009).

With regard to prediction #2, our data indicated that fertilization was not enhanced by a +4 °C increase above ambient SST. Increased temperature did not positively or negatively affect fertilization in *H. erythrogramma*. Temperature enhancement of fertilization in echinoids in previous studies involved treatments (+7-12 °C above ambient) (Hagström and Hagström, 1959; Mita et al., 1984) more extreme than those used here. Fertilization in many sea urchin species and other invertebrates (eg. asteroids, gastropods, corals) is known to be robust to increased temperature (Chen and Chen, 1992; Davis, 2000; Fujisawa, 1989; Lee et al., 2004; Mita et al., 1984; Negri et al., 2007; O'Connor and Mulley, 1977; Rupp, 1973; Sewell and Young 1999). This may be associated with a temperature-independent period immediately post fertilization (Yamada and Mihashi, 1998).

A sperm to egg ratio of 100:1 (sperm density of 10^2 sperm ml^{-1}) produced high fertilization rates (ca. 85%) in *H. erythrogramma* with a sharp decline at lower density indicating cut off levels for fertilization test sensitivity with this species. This underscores the importance of in determining sperm to egg ratios for these tests as

highlighted elsewhere (Dinnel et al., 1987). Previous studies of fertilization in *H. erythrogramma* used 10^3-10^5 sperm ml^{-1} to achieve fertilization rates > 60% (Evans and Marshall, 2005; Havenhand et al., 2008; Marshall et al., 2004) higher sperm densities than used here. These studies differed from this present study in the use of single male by female crosses. Polyandry is well known to enhance fertilization in *H. erythrogramma* (Evans and Marshall, 2005). We used gametes from multiple males and females to avoid strong variance caused by maternal (environmental) effects, paternal (genetic) effects, and male x female interactions seen in *H. erythrogramma* in single dam-sire crosses (Evans and Marshall, 2005). Variable experimental conditions (eg. gamete source, gamete age, concentration, vessel size) are well known to influence fertilization rates (eg. Hollows et al., 2007; Song et al., 2009) and would also contribute to differences among studies. Our data provide an indication of the amount of sperm required for high fertilization rates in the multiple individual spawning events observed in the field for *H. erythrogramma* and other sea urchins (Himmelman et al., 2008; Pennington, 1985).

Where comparable data from fertilization assays using a similar approach to the one used here are available, the high rates (> 90%) of fertilization obtained for the large eggs (400 µm diam) of *H. erythrogramma* at sperm densities of 10^3 sperm m^{-1} differs from that reported for the small eggs (< 130 µm diam) of *Strongylocentrotus* spp. where high rates of fertilization require 10^6 – 10^7 sperm ml^{-1} (Levitan, et al., 1991; Pennington, 1985). Due to the higher probability of sperm encountering eggs of larger size (egg-target model), the lower sperm density required to obtain high fertilization in rates *H. erythrogramma* is in accord with expectations (Levitan, 2006).

As shown for *H. erythrogramma* (Havenhand et al., 2008) low pH/increased pCO_2 is well known to have an inhibitory effect (CO_2 narcosis) on sperm motility. Low pH (ca. pH 7.0-7.5) is used as a mechanism to reduce sperm respiration in the

gonad and seminal fluid with the internal pH (pHi) of sperm being pH ca. 6.8 (Chia and Bickell, 1983; Mita and Nakamura, 2001; Mohri and Yasumasu, 1963). The inhibitory effect of low pH on sperm motility is over ridden in nature by the respiratory dilution effect of seawater (increased oxygen tension) and by the egg jelly peptides that promote sperm motility at low pH (Brokaw, 1990; Chia and Bickell, 1983; Darszon et al., 2008; Ward et al., 1985). This mechanism appears to be common in marine invertebrates and likely to involve similar cGMP messenger systems (Darszon et al., 2008; Hoshi et al., 1994; Matsumoto et al., 2003). The eggs of *H. erythrogramma* provide a considerable egg jelly surface area to provide sperm activating peptide. Although pre-treatment of the sperm of *H. erythrogramma* in acidic/high CO_2 seawater would have had a narcotic effect on sperm motility, in our experiments, this effect appears to have been overcome by seawater respiratory and stimulatory egg jelly peptide effects. Our results contrast with the decreased fertilization in *H. erythrogramma* at pH 7.7 (pH 8.1: 62% fertilization; pH 7.7: 51% fertilization) (Havenhand *et al.,* 2008). Different experimental conditions, polyandry (this study) –vs- single male-female crosses (Havenhand et al., 2008) are likely to have influenced the contrasting results. Our use of a multiple male sperm and female egg source would have reduced variability among experimental runs (Evans and Marshall, 2005).

Most of our knowledge of the effects of climate change stressors on fertilization in the sea is based on studies of robust species used as model organisms for laboratory studies. We have poor data for the diversity of marine invertebrates. Negative effects of ocean change on fertilization may be identified as more taxa are investigated and thresholds for deleterious effects are likely to vary among species. It is crucial to determine the interactive effects of ocean warming, acidification and hypercapnia on marine biota within the context of current conditions and projections

for regional change, thereby allowing identification of vulnerabilities across complex marine life histories. Interactive effects with other anthropogenic stressors also need to be considered (Crain et al., 2008; Przeslawski et al., 2005, 2008).

Understanding the vulnerabilities of the gametes and propagules integral to the persistence of marine populations is crucial as we endeavour to predict how marine populations and ecosystems will fare in the face of climate change. Ocean acidification has dire consequences for larvae due to impaired larval calcification and hypercapnia (Clark et al., 2009; Dupont et al., 2008; Kurihara, 2008). However, in areas of strong warming such as southeast Australia, embryos of species like *H. erythrogramma* that are sensitive to thermal effects (Byrne, 2009; Byrne et al., 2009) may not reach the larval stage in a warm ocean regardless of pH. Developmental failure regardless of the stage affected or stressor(s) involved will cause recruitment failure with flow-on effects to marine ecosystems (Przeslawski et al., 2008). Relatively small perturbations in planktonic and recruitment phases can translate to large alterations of adult populations (Uthicke et al., 2009).

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Table 1 Experimental water conditions. Carbonate and pCO_2 parameters were determined using the CO_2 System Calculation Program (Lewis and Wallace, 1998) (see methods). Data are means of values determined for the 3 sources of FSW used, standard error in parentheses. Temp: temperature (°C), TA: total alkalinity (μmol/kgSW), CO_2 : partial pressure of carbon dioxide (μatm), Ca: calcite saturation, Ar: aragonite saturation.

Temp	pH	TA	ρCO2	ΩCa	ΩAr
22	8.17	2398 (10)	367 (26)	5.4 (0.2)	3.5 (0.13)
22	7.8	2398 (10)	1105 (5)	2.4 (0.01)	1.6 (0.01)
22	7.6	2398 (10)	1823 (8)	1.6 (0.01)	1.1 (0.01)
24	8.17	2398 (10)	370 (26)	5.7 (0.21)	3.7 (0.14)
24	7.8	2398 (10)	1124 (5)	2.6 (0.01)	1.7 (0.01)
24	7.6	2398 (10)	1858 (8)	1.7 (0.01)	1.1 (0.01)
26	8.17	2398 (10)	373 (27)	5.9 (0.22)	3.9 (0.14)
26	7.8	2398 (10)	1142 (5)	2.7 (0.02)	1.8 (0.01)
26	7.6	2398 (10)	1892 (8)	1.8 (0.01)	1.2 (0.01)

Table 2 Relationship between sperm concentration and sperm:egg ratios.

Relationship between sperm concentration and sperm:egg ratios in the four concentrations used in our experiments. Egg numbers in 250 ml beakers were constant at 3 eggs ml^{-1}.

Sperm: egg	Sperm/ml
1500: 1	4.5×10^3
500: 1	1.5×10^3
100: 1	3.0×10^2
10: 1	3.0×10

Table 3. Results of 3-way ANOVA examining the effect of temperature, pH and sperm concentration on fertilization in *Heliocidaris erythrogramma*. Significance is indicated by *. SS: sum of squares, df: degrees of freedom, MS: mean square, Temp: temperature, Sperm: sperm concentration.

Source	SS	df	MS	F	p
Temp	0.03	2	0.016	0.7	0.48
pH	0.02	2	0.011	0.5	0.62
Sperm	16.15	3	5.383	241.8	0.00*
Temp × pH	0.03	4	0.006	0.3	0.89
Temp × Sperm	0.03	6	0.006	0.2	0.96
pH × Sperm	0.03	6	0.005	0.2	0.96
Temp (pH × Sperm)	0.06	12	0.005	0.2	0.99
Error	1.60	72	0.022		
Total	104.90	108			

Fig. 1 Effect of temperature and pH on the percentage of fertilization in *H. erythrogramma* across the range of sperm to egg ratios and sperm concentrations indicated in the inset key. Values are means ± SE. The 22°C and pH 8.17 treatment represented ambient conditions. The lower densities of sperm are likely to be most realistic with regard to fertilization conditions in the field.

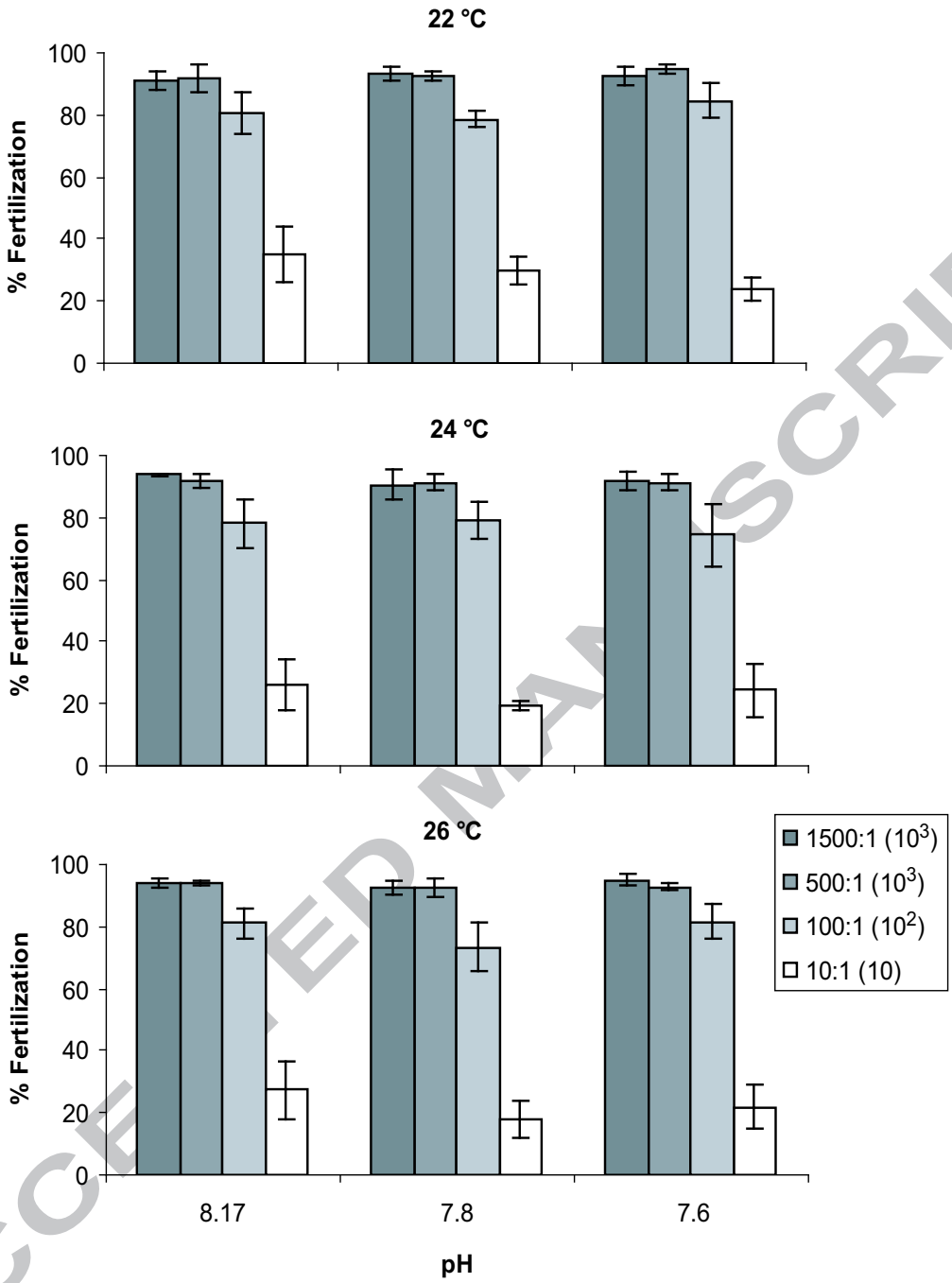

Fig1