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SUZANNE C. MILLS & JOHN D. REYNOLDS

Influence of eutrophication on freshwater mussels and the survival of endangered European bitterling, *Rhodeus sericeus*

MILLS & REYNOLDS: EUTROPHICATION EFFECTS ON FISH AND THEIR HOSTS

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S. C. Mills, J. D. Reynolds
School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK
Tel: +44-1603-592056
Fax: +44-1603-592250

e-mail: s.c.mills@uea.ac.uk
e-mail: reynolds@uea.ac.uk

Corresponding author: S. C. Mills

21 **Abstract**

22

23 Species conservation may be complicated in host-parasite interactions. The sub-lethal
24 impacts of pollution on one species may be lethal to interacting species. Agricultural
25 run-off, domestic and industrial waste are the major polluters of freshwaters, causing
26 eutrophication in waterbodies and declines in populations of many species of
27 freshwater organisms. We investigated two effects of eutrophication, oxygen and algae,
28 on the interaction between a freshwater fish that is endangered in several countries, the
29 European bitterling, *Rhodeus sericeus*, and unionid mussel hosts that they require for
30 spawning. In low concentrations of either oxygen or algae, a greater proportion of
31 bitterling were ejected prematurely from the mussel host. Furthermore, low algae and
32 oxygen reduced mussel ventilation rates and increased valve closure. The mussels may
33 have ejected bitterling eggs from their gills in order to increase the surface area of their
34 gills for oxygen uptake and filter feeding. Alternatively, the mussel responses to
35 adverse conditions may have created an environment inside their shells that bitterling
36 embryos could not survive. The mussels' ability to withstand anoxia in their mantle
37 cavity may have further exacerbated this situation. Our study shows that the sub-lethal
38 effects of pollution on one species can have lethal effects on another species with
39 which it interacts. Such interactions need to be considered in conservation
40 programmes.

INTRODUCTION

Direct impacts of contaminants on the health and survival of aquatic organisms are well-known (e.g. Mason, 1996a). There are also examples of indirect impacts of contaminants on species through, for example, effects on their predators, prey or parasites (Lafferty & Kuris, 1999). Therefore, restoration of habitats using recommendations based solely on lethal toxicity tests of contaminants may not be the most effective conservation method either for one species or for interacting species.

Eutrophication, caused by an excess input of nutrients, is a widespread and growing problem in lakes, rivers, estuaries and coastal oceans (Clark, 1992; Mason, 1996a; Smith, 1998). Most of the excess nutrient input to waterbodies is caused by runoff from agricultural lands and as domestic and industrial waste (Mason, 1996a; Carpenter *et al.*, 1998). Pollution is recognised as one of the most significant factors causing major declines in the populations of freshwater species in many parts of the world (Clark, 1992; Moyle & Leidy, 1992; Winfield, 1992; Lawton & May, 1995; Maitland, 1995; Mason, 1996a). Eutrophication affects freshwater species either by deleterious affects of oxygen depletion from the degradation of organic material or by monospecific algal blooms (Moss, 1988; Freedman, 1995).

The reproductive success of an endangered freshwater fish, the European bitterling, *Rhodeus sericeus,* depends upon the unionid mussel hosts in which the fish spawn (Reynolds *et al.*, 1997; Smith *et al.*, 2000; Mills & Reynolds, in press). Male bitterling guard a territory containing at least one mussel and court females towards the mussels. Female bitterling use their long ovipositors to lay 2-4 eggs into water tubes

64 within the gills of a mussel at each spawning. Males release sperm over the inhalant
65 siphon which is carried in the mussel's inhalant water current over the eggs and
66 fertilises them. The eggs are incubated within the mussel's gills for up to 6 weeks and
67 once they have absorbed their yolk sac the larvae leave the mussel via the exhalant
68 siphon (Reynolds *et al.*, 1997). Bitterling cannot reproduce without mussels.
69 European bitterling have been classified as rare and vulnerable over much of
70 the western part of their European range, and have disappeared completely in the most
71 polluted areas (e.g. Rhine-Main basin) (Lelek, 1980). Widespread concern has resulted
72 in *R. sericeus* being listed as a protected species in Appendix III of the Bern
73 Convention with legislation in five countries (Maitland, 1994). The IUCN placed *R.*
74 *sericeus* as vulnerable in France (IUCN, 1995), endangered in Slovenia (Povž, 1992)
75 and it is one of six of the most endangered species in Belgium and protected by law
76 (Bervoets *et al.*, 1990). While the bitterling's unionid hosts have often undergone local
77 declines, they are not protected by the Bern Convention, however some species are
78 protected under country legislation (Wells & Chatfield, 1992). Eutrophication has
79 caused the decline of *Anodonta cygnea* and *Unio pictorum* in Poland which are Red
80 List classified as endangered and vulnerable respectively (Dyduch-Falniowska, 1992).
81 In Germany, *R. sericeus* and its four mussel hosts, *A. anatina*, *A. cygnea*, *U. pictorum*
82 and *U. tumidus* are all highly vulnerable (Blab *et al.*, 1984; Schmidt, 1990).
83 Furthermore, the Red Data List in Switzerland places *A. anatina*, *U. pictorum* and *U.*
84 *tumidus* as vulnerable but *R. sericeus* as strongly vulnerable (Duelli, 1994; Kirchhofer
85 & Hefti, 1996). In the European areas where mussels survive and bitterling have

86 disappeared it is not known at which life stage these fish are most sensitive to industrial
87 pollution (Lelek, 1980).

88 In this study we investigate two potential effects of eutrophication on the
89 reproductive success of the bitterling. The process of eutrophication drastically reduces
90 the dissolved oxygen concentration of many systems and stimulates growth of green
91 algae (*Chlorophyta*) (Mason, 1996b; Diaz, 2001). Eutrophic lakes and rivers may also
92 have low chlorophyll *a* concentrations if high densities of herbivorous *Daphnia magna*
93 are present, as these graze on green algae (Carvalho, 1994; Carpenter *et al.*, 1998). Our
94 study focused on the effects of reduced oxygen and varying algae concentrations on the
95 survival of bitterling embryos in mussel hosts. Bitterling show no parental care and
96 their offspring survival depends on the mussels' reaction to changes in environmental
97 conditions. Our aim was to investigate the indirect effects of eutrophication on the
98 developing bitterling embryos through reactions of their mussel hosts and to use this
99 information to make recommendations about the conservation of bitterling across
100 Europe.

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102

103 **METHODS**

104

105 **Study species**

106 One hundred and fifty individuals of the mussel species *Anodonta anatina* and
107 bitterling of mixed sex were collected from Reach Lode, a tributary of the River Cam,
108 Cambridgeshire, Southern England, at the point of confluence with Wicken Lode,

N.G.R.: TL 545 696 during March and April 2000. The mussels were collected by hand and the fish were collected with high frequency (600 Hz) pulsed DC Electracatch WFC 12 electrofishing equipment. The mussels were maintained in outdoor pools and fed daily three litres of a live algal suspension derived from an outdoor pool that had been seeded with *Chlorella vulgaris*. The bitterling were kept in stock aquaria that were aerated continuously with a TETRA*tec*® IN 1000 internal aquarium filter and illuminated by an Aqua-Glow 40W fluorescent aquarium lamp on a 16L:8D photoperiod. The fish were fed a mixed diet of live *Daphnia pulex*, *Chaoburus* pupae, *Culex* and *Chironomid* larvae, frozen *D. pulex*, *Tubifex*, *Artemia salina*, dried protein mix and trout pellets.

Effect of treatments on bitterling survival

Four mussels were placed in sand-filled round glass containers (10 x 6 cm) and arranged in a square formation within a 300 litre aquarium. A bitterling of each sex in reproductive condition was added to the aquarium. The bitterling were observed until they had spawned twice in each mussel. We performed 24 replicates of this experiment. The mussels were transferred to a 300 litre aquarium that had been separated into four quarters and one mussel was placed in each compartment. After 12 hours of acclimation at high oxygen and high algae conditions, the treatment oxygen and algae concentrations were created gradually. Four treatments were distributed randomly within each of the four compartments: high oxygen and high algae, low oxygen and high algae, high oxygen and low algae, and low oxygen and low algae.

Mussels were observed twice daily for 6 weeks, and at each observation the number of embryos or larvae ejected were noted.

Oxygen conditions

The high dissolved oxygen concentration used in our experiments was 130 % air saturation. This was based on the mean oxygen concentration for March, April and May measured from 1993 to 1998 by the UK Environment Agency 2.1 km upstream from our study site (Reach Lode, Hallards Fen Rd, Cambridgeshire, UK).

The low dissolved oxygen concentration used in our experiments ranged from 55 to 75 % of air saturation. This was based on the mean concentration from 1990 to 1999 at Bramerton Woods End (River Yare) which is downstream of a sewage treatment works (UK Environment Agency data). The aim of our experiment was to investigate bitterling survival due to the reactions of their mussel hosts to changes in the environment. We therefore used low dissolved oxygen concentrations that would not cause direct harm to freshwater fish embryos (Dourdoroff & Shumway, 1970; USEPA, 1986; Dean & Richardson, 1999).

The treatment oxygen concentrations were created using pre-determined ratios of nitrogen and oxygen bubbled into the tanks so that oxygen levels fell to the desired level within 2-3 hr. A transparent plastic lid was placed over the aquarium to help maintain control. The oxygen concentration in the compartments was measured twice daily using a dissolved oxygen meter for the duration of the experiment.

Algae conditions

Mean chlorophyll *a* concentrations < 4 μg l^{-1} represent oligotrophic conditions (Mason, 1996b; Soto & Mena, 1999), and 8.2 μg l^{-1} represents the average chlorophyll *a* concentration for Trowse Mill (River Yare) upstream of a sewage treatment works (UK Environment Agency data). We therefore used 6-9 μg l^{-1} of *Chlorella vulgaris* for the low algae treatment.

Mean chlorophyll *a* concentrations of 25 μg l^{-1} represent eutrophic conditions in UK rivers (Anonymous, 1994), and 39.6 μg l^{-1} represents the average concentration for Bramerton Woods End (River Yare) downstream of a sewage treatment works (UK Environment Agency data). We therefore used 38-40 μg l^{-1} of *C. vulgaris* for the high algae treatment.

The treatment algal concentrations were created using pre-determined quantities of a live algal suspension derived from an outdoor pool that had been seeded with *C. vulgaris*. Algal concentrations were calculated using the concentration of chlorophyll *a* pigment in water extracted in acetone every 48 hours following the method of (Mackereth *et al.*, 1978).

Effect of treatments on mussel ventilation rates

Following the main experiment, four *A. anatina* mussels that had not been accessible to bitterling were distributed among the four compartments at high oxygen and high algae conditions. We performed 11 replicates of this experiment. The velocity of water in the exhalant streams (water flow speed) and the ventilation rate of the mussels were measured at two separate intervals over 12 hours before the oxygen and

177 algal treatments were created and then afterwards every 48 hr for 5 weeks. The valve
178 position of each individual was also daily registered: (1) active, when siphons were
179 visible and valves gaping and (2) inactive, when the valves were closed.

180 The water flow speed (cm s^{-1}) was determined using a small thermistor probe
181 based on the principle described by La Barbara (1976) and Vogel (1981) following the
182 methods described by Meyhofer (1985) and Tankersley & Dimock (1993a). The
183 thermistor probe is electrically heated and cools at a rate proportional to the speed of
184 the mussel's exhalant water flow. The temperature change is recorded as a voltage. The
185 tip of the thermistor probe (3 mm diameter) was manoeuvred in front of the exhalant
186 siphon of the mussel using a micro-manipulator and the flow speeds measured. Ten
187 measurements of voltage output were taken manually from the voltmeter over 10 min
188 for each mussel while ventilating (i.e. valves open and siphons extended). Mean rates
189 at which mussels process the ambient water (ventilation rate, l h^{-1}) were calculated
190 from flow speeds by simultaneously determining the cross sectional area of the
191 exhalant siphon from a grid (0.5 x 0.5 mm) held next to the mussel's siphon. To
192 calibrate the flow probe, voltage outputs were measured over a range of known flow
193 speeds and temperatures (16 - 22°C) using pipe tubing over a range of cross sectional
194 areas (0.71, 0.126, 0.283, 0.385, 0.503 cm^2).
195
196

RESULTS

Effects of treatments on bitterling survival

As expected the chlorophyll *a* concentrations in the high algae treatments were higher than those in the low algae treatments (Kruskal-Wallis test: $H_{96} = 47.1$, $P < 0.001$; Table 1), and the oxygen concentrations in the high oxygen treatments were higher than those in the low oxygen treatments (Kruskal-Wallis test: $H_{96} = 65.5$, $P < 0.001$; Table 1).

insert Table 1

The proportion of bitterling eggs that were ejected prematurely was significantly different between the four treatments (univariate model, $F_{3,93} = 9.57$, $P < 0.001$; Fig. 1). Tukey Post Hoc tests showed that more eggs were ejected in the low O_2 low algae treatment than in the high O_2 high algae ($P < 0.001$) and than in the low O_2 high algae treatment ($P < 0.02$). More eggs were also ejected at high O_2 low algae than at high O_2 high algae ($P < 0.01$). The proportion of eggs ejected was not affected by the order of spawning preference (univariate model, $F_{3,93} = 0.15$, $P = 0.93$).

insert Figure 1

Bitterling eggs were ejected significantly more quickly from mussels in the high algae treatments than those ejected from mussels in the two low algae treatments (days to ejection: high O_2 high algae = 5.9 ± 0.5; low O_2 high algae = 5.0 ± 0.5; high O_2 low

220 algae = 7.0 ± 0.5; low O_2 low algae = 6.9 ± 0.5; univariate model $F_{3,92} = 4.19$, $P < 0.01$;
221 Tukey Post Hoc tests, $P < 0.05$).
222
223 **Effect of treatments on mussel ventilation rates**
224 As with the previous experiment, we confirmed that the chlorophyll *a* concentrations in
225 the high algae treatments were higher than those in the low algae treatments (Kruskal-
226 Wallis test: $H_{40} = 27.8$, $P < 0.001$; Table 1), and the oxygen concentrations in the high
227 oxygen treatments were higher than those in the low oxygen treatments (Kruskal-
228 Wallis test: $H_{40} = 29.6$, $P < 0.001$; Table 1).
229 As expected there was no difference in mussel ventilation rates among the four
230 treatments before the conditions were applied (one-way ANOVA: $F_{3,37} = 0.07$, $P =$
231 0.98; Fig. 2). Also as expected, there was no difference in ventilation rates of mussels
232 in the high oxygen and high algae treatment before and after the conditions were
233 applied (Paired *t* test: $t_{11} = 1.45$, $P = 0.18$; Fig. 2). However, the ventilation rates of
234 mussels in all of the other treatments were reduced after the conditions were applied
235 (Paired *t* test: low O_2 high algae: $t_{10} = 2.9$, $P < 0.02$; high O_2 low algae: $t_9 = 5.1$, $P <$
236 0.001; low O_2 low algae: $t_{10} = 4.8$, $P <$ 0.001; Fig. 2). The ventilation rates of mussels
237 in the high oxygen and high algae treatment were significantly higher than the rates of
238 mussels in both of the low algae treatments (ANOVA: $F_{3,37} = 4.56$, $P < 0.01$; Tukey
239 Post Hoc: low O_2 low algae: $P < 0.01$; high O_2 low algae: $P <$ 0.05; Fig. 2).
240
insert Figure 2
241

what about low DO with algae?

242 The proportion of observations during which mussels were active (valves open,
243 siphons extended and mussels ventilating) was significantly different between the four
244 treatments (univariate model: $F_{3,37} = 17.1$, $P < 0.001$; Fig. 3). The mussels were more
245 active in the high O_2 high algae treatment than mussels in both of the low algal
246 treatments (Tukey Post Hoc: $P < 0.001$). Mussels in the low O_2 high algae treatment
247 were also more active than mussels in both of the low algae treatments (Tukey Post
248 Hoc: high O_2 low algae: $P < 0.001$; low O_2 low algae: $P < 0.01$).
249
insert Figure 3
250
251
252 **DISCUSSION**
253
254 This study has shown that as the concentrations of oxygen and algae decrease a greater
255 proportion of bitterling are ejected from mussel hosts (Fig. 1), and that these conditions
256 match decreased mussel ventilation rates (Fig. 2) and valve activity (Fig. 3).
257 It seems likely that bitterling were affected indirectly by the oxygen and algae
258 conditions through the reactions of their mussel hosts. Bitterling embryos are 2-3 mm
259 in diameter, and on average mussel would have contained 6-8 of them based on two
260 spawnings and 3-4 eggs deposited per spawning (Mills & Reynolds, in press).
261 Therefore, the presence of bitterling embryos in a mussel's water tubes would almost
262 certainly obstruct water flow, and in a similar way to glochidia in gravid gills
263 (Tankersley & Dimock, 1993b). Therefore mussels may have benefited from ejecting
264 bitterling from their gills through increased surface area of the gills for oxygen uptake

and filter feeding. This behaviour may become more frequent when oxygen and algae conditions decline, as gravid female unionid mussels also abort their own larvae (glochidia) prematurely under conditions of low oxygen (Tankersley & Dimock, 1993a). Alternatively, bitterling embryos may have been ejected as a side-effect of other mussel behavioural reactions to declining algae and oxygen conditions.

It is unlikely that the observed bitterling mortality was due to direct effects of the oxygen and algae conditions in the aquaria. Although mortality increased under low oxygen, the concentrations were above those known to cause impairment to freshwater fish embryos (Dean & Richardson, 1999). Additionally, bitterling mortalities were highest in the low algae treatments and considering bitterling embryos obtain their nutrients from the yolk sac it is hard to see how low algae concentrations could have caused the mortalities directly. Instead, bitterling may have died from the adverse conditions inside their hosts created by the environmental changes. Mussels reduce their ventilation rates in low oxygen and algae conditions (Fig. 2). If the gill oxygen concentration fell well below 50 %, bitterling embryos may have died. Our observations of reduced ventilation rates in low oxygen and starvation agree with other studies on freshwater mussels (Badman, 1975; Famme & Kofoed, 1980; Sobral & Widdows, 1997).

The increased closure of mussel valves in low algae conditions (Fig. 3) may be responsible for the higher bitterling mortality observed in the two low algae treatments and for the longer time taken for the eggs to be ejected. Valve closure stops water transport and oxygen delivery and accumulates anaerobic and excretory products which are all detrimental to embryonic survival (Famme & Kofoed, 1980; Herreid,

288 1980; Bayne & Newell, 1983; Massabuau *et al.*, 1991). Valve closure is considered a
289 general response by bivalves to environmental stressors (Sloof *et al.*, 1983; Kramer *et*
290 *al.*, 1989; Reynolds & Guillaume, 1998), and studies have shown that freshwater
291 mussels escape food deprivation and low oxygen with shell closure (Salánki, 1964;
292 Badman, 1974; Heinonen *et al.*, 1997; Sobral & Widdows, 1997). Mussels also remain
293 closed in metabolic dormancy under prolonged adverse conditions (Storey & Storey,
294 1990; Hand, 1991) due to their ability to tolerate environmental anoxia (Holopainen &
295 Penttinen, 1993). Unionid mussels in particular are highly tolerant compared with other
296 freshwater bivalves (Matthews & McMahon, 1999). These behavioural adaptations of
297 mussels may further compromise the survival of developing bitterling. Whereas most
298 fish species with parental care increase fanning under adverse conditions such as low
299 oxygen (e.g. Jones & Reynolds, 1999), mussels close their valves and tolerate anoxia
300 until conditions improve. We suggest that this behaviour may be one of the reasons
301 why bitterling have disappeared from some polluted European areas in which mussels
302 are still present (Lelek, 1980).

303 The excess of nutrients such as phosphates, that cause eutrophication, may
304 themselves have a negative impact on the survival of hosts and their parasites.
305 Reynolds & Guillaume (1998) have shown that high phosphate concentrations lower
306 mussel ventilation rates and increase bitterling mortality. There may be a synergistic
307 effect of the cause (high phosphates) and the result of eutrophication (low O_2 and low
308 algae) on mussel behaviour and bitterling survival. We suggest that eutrophication in
309 the wild, such as in rivers downstream of sewage treatment outlets, may be even more
310 damaging to bitterling survival than has been shown by this study.

311 Our study presents a case whereby a species in symbiosis is particularly
312 vulnerable to environmental changes through impacts on their hosts and their hosts'
313 behavioural responses. Such interactions between species complicate conservation
314 management as a sub-lethal effect of a contaminant on one species can be translated
315 into lethal impact on another. For example, toxic chemicals and trace metals are more
316 toxic to parasites, such as intestinal helminths and tapeworms, than to their hosts due to
317 the hosts' physiological concentrating effects (Riggs *et al.*, 1987; Lafferty & Kuris,
318 1999). Thus, we suggest that habitat restoration programmes in the bitterling's native
319 European range do more than conserve mussels: they should also aim for the high
320 levels of algae and oxygen shown in this study.
321 In conclusion, our study shows that bitterling are vulnerable to adverse
322 environmental conditions indirectly through the reactions of their mussel hosts.
323 Mussels reduce their ventilation rates and close their valves in low algae and also in
324 low oxygen conditions. The resultant oxygen depletion inside the mantle cavity may
325 cause death of the developing embryos. The ability of unionid mussels to tolerate long
326 periods of anoxia in their gills may exacerbate this situation. Alternatively, mussels
327 may actively eject live bitterling from their gills in order to increase the gill area
328 available for oxygen absorption and filter feeding. Regardless of the mechanism, our
329 study highlights the necessity of also considering the impacts of pollution on the
330 behaviour of hosts as well as the target species for understanding conservation.
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Table 1 Concentrations of chlorophyll *a* ($\mu g\ l^{-1}$) and dissolved oxygen (% saturation) among the four treatments in the two experiments. N = 24 for each treatment in the experiment on effects on bitterling. N = 11, 10, 9 and 10 respectively for the treatments in the experiment on effects on mussels .

Treatment	Effects on bitterling experiment		Effects on mussels experiment	
	Chl *a* ($\mu g\ l^{-1}$) $\overline{X} \pm$ SE	O_2 (%) $\overline{X} \pm$ SE	Chl *a* ($\mu g\ l^{-1}$) $\overline{X} \pm$ SE	O_2 (%) $\overline{X} \pm$ SE
High algae high oxygen	40.8 ± 2.1	136 ± 1.3	38.4 ± 3.4	132 ± 1.2
High algae low oxygen	40.9 ± 2.1	56 ± 1.0	38.7 ± 5.5	73 ± 1.1
Low algae high oxygen	8.3 ± 2.2	133 ± 5.3	6.0 ± 1.3	133 ± 1.7
Low algae low oxygen	9.5 ± 2.1	57 ± 0.6	8.0 ± 1.1	72 ± 0.9

491 **Figure legends**

492

493 **Fig. 1** Mean (+ 1 SE) proportion of eggs ejected by mussels in the four treatment

494 conditions. N = 24 in all cases.

495

496 **Fig. 2** Mean (+ 1 SE) ventilation rate of mussels in the four treatment conditions.

497 Numbers above bars refer to sample size. □ = before and ■ = after treatments were

498 applied.

499

500 **Fig. 3** Mean (+ 1 SE) proportion of observations that mussels' valves were found to be

501 open and the mussels ventilating. Numbers above bars refer to sample size

Figure 1. *Mills and Reynolds*

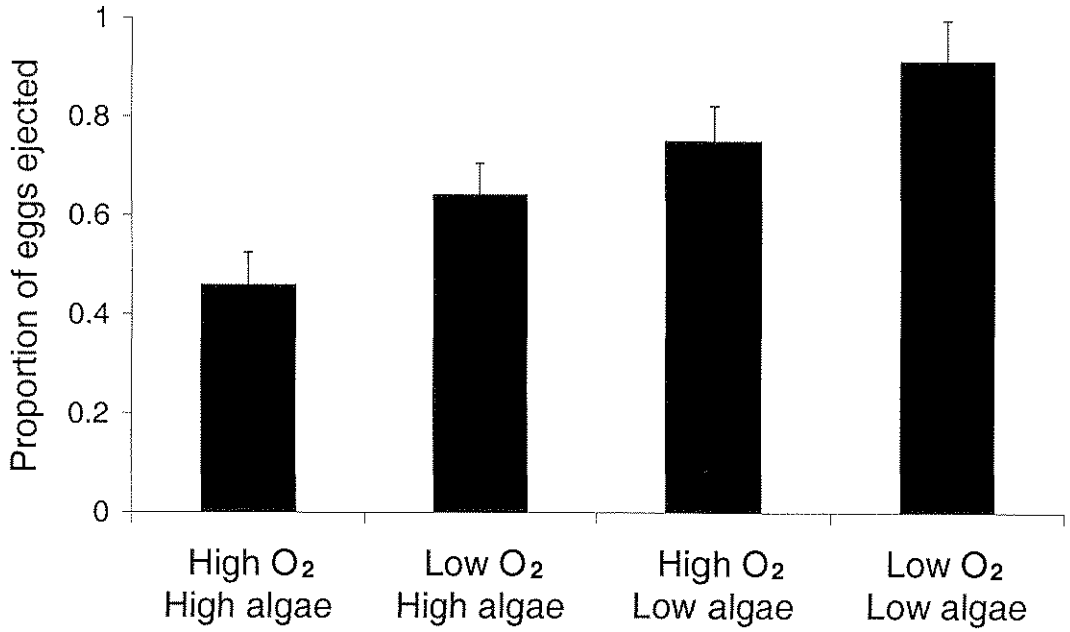

Figure 2. *Mills and Reynolds*

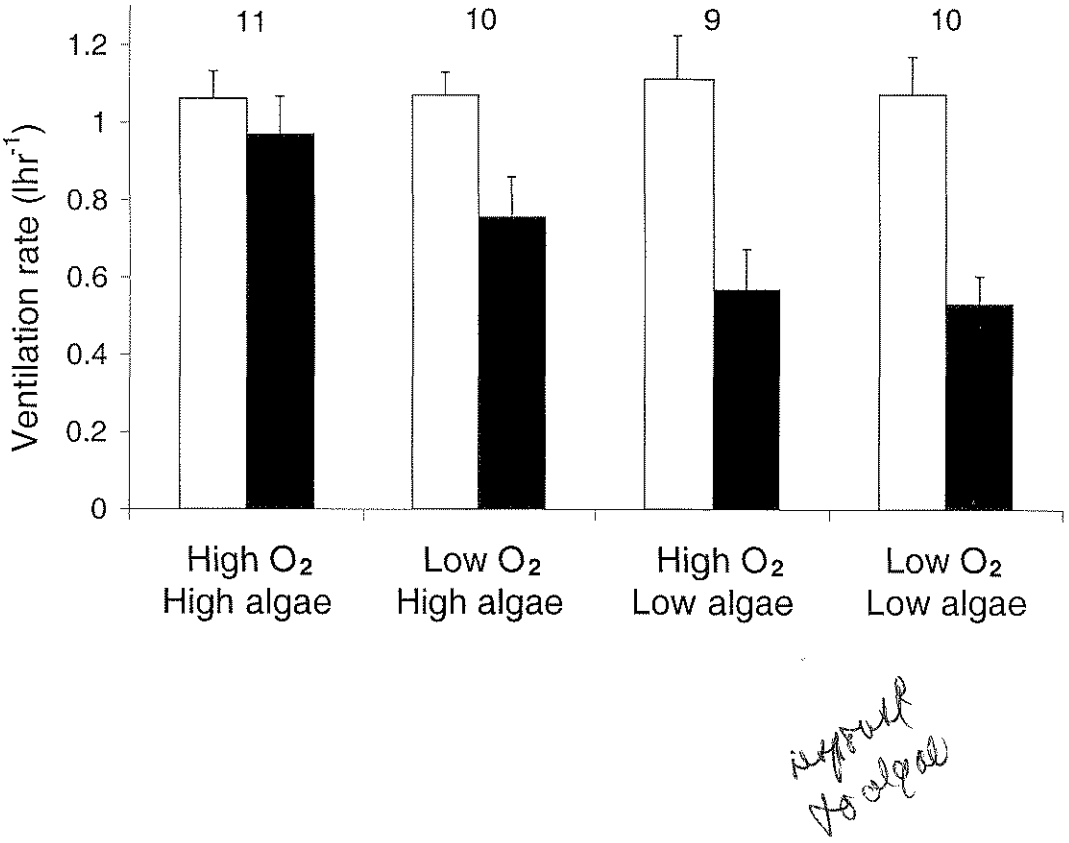

Figure 3. *Mills and Reynolds*