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COMMENT

Causes of the disappearance of the aquatic plant *Egeria densa* and black-necked swans in a Ramsar sanctuary: comment on Mulsow & Grandjean (2006)

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In an attempt to demonstrate the ultimate causes of the massive mortality and emigration of black-necked swans at the Ramsar site Carlos Anwandter Nature Sanctuary, Mulsow & Grandjean (2006, available at: www.int-res.com/articles/esep/2006/E66.pdf) used field data, microcosm experiments and basic chemical theory, to postulate that calcium bicarbonate is removed from water, due to the increase in sulphate by the effluent of the pulp mill of CELCO, Valdivia, Chile. This process is deemed to affect the photosynthetic rate of the plant *Egeria densa,* the main food of the black-necked swans, which in turn provokes the known mortality and emigration of these birds from this site. We critically analyzed the study of Mulsow & Grandjean (2006), and we found a serious lack of consistent arguments that precludes any fundamental statement about the effects of sulfate and bicarbonate on *E. densa*. Here we discuss our concerns.

FIELD DATA

Mulsow & Grandjean (2006) described that 6 replicated samples were collected from 11 stations along the Cruces river; 4 and 7 stations were considered as affected and not affected by the effluents of CELCO, respectively. Concentrations of SO_4^{2-} and HCO_3^- were measured at each station. Since these replicates were subsamples of the sites or stations (i.e. within nested design), the most appropriate analysis is a nested ANOVA. However, this configuration was not followed, and the ANOVA used by Mulsow & Grandjean (2006) results in pseudoreplication when using subsamples as replicates (Hurlbert 1984). This problem increases erroneously the degrees of freedom in the error source of variation and induces an increase in the probability of Type I Error (rejection of the null hypothesis being true). When averaged across subsamples at each station (Mulsow & Grandjean 2006, their Table 1), concentrations of SO_4^{2-} and HCO_3^- can be correctly compared using ANOVA.

After repeating the analyses presented by Mulsow & Grandjean (2006), we found (as they reported) a statistically significant difference in the concentration of HCO_3^- at unaffected sites with respect to those in areas affected by the effluents of CELCO (ANOVA, $p = 0.001$, $F = 22.68$; MS = 326.8; error df = 9). However, in contrast to results reported by Mulsow & Grandjean (2006), concentrations of SO_4^{2-} in affected and unaffected sites were not statistically significantly different (ANOVA, $p = 0.084$, $F = 3.78$; MS = 4.65; error df = 9). Significant differences in SO_4^{2-} and HCO_3^- (p-values <0.05) are found exclusively by the effects of pseudoreplication.

These statistical problems show that the field data of Mulsow & Grandjean (2006) do not provide a fundamental basis upon which to postulate an effect of SO_4^{2-} on the HCO_3^- concentration in the water of the Cruce river. A robust assessment of spatial variation of SO_4^{2-} and HCO_3^- is critical because Mulsow & Grandjean (2006) postulate that the SO_4^{2-} discharged by CELCO into the river is the main cause of the changes observed in HCO_3^- across the Sanctuary. This is

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claimed by Mulsow & Grandjean (2006) when they mention a 'strong negative correlation' between SO_4^{2-} and HCO_3^-. However, the authors present a confused description of their results and associated analyses; they report a determination coefficient (r^2), which implies the use of linear regression instead of correlation analysis. Using data from Mulsow & Grandjean (2006), we ran a correlation analysis which indicated a negative relationship between SO_4^{2-} and HCO_3^- (*r*-Pearson = –0.687, p = 0.001), but the regression analysis showed that only 48.6% of the variation in HCO_3^- may be explained by variation in SO_4^{2-} ($r^2 = 48.6$, p = 0.017, n = 11; and $r^2 = 47.2$, p = 0.001, n = 18, including all the averaged subsamples in the analysis). Regrettably, the authors did not include any information on the number of observations used in the analysis and, of more importance, they did not describe the exact statistical procedure that was applied. Thus, any attempt to interpret these results is inappropriate.

We conclude that the incorrect analyses of Mulsow & Grandjean (2006) and their confusing report of their field evidence weakly support the hypothesis being tested in their laboratory experiment: the effect of SO_4^{2-} on *Egeria densa*.

EXPERIMENTAL DESIGN

The laboratory experiment of Mulsow & Grandjean (2006) consisted of a 'bottle experiment' (Diamond 1986) where plants of *Egeria densa* were cultivated in replicated compartments and oxygen production was measured. After these trials, some plants remained as controls; the rest were randomly assigned to the treatments, consisting of several doses of SO_4^{2-} (K_2SO_4).

The unsolvable problem here is that the amounts of SO_4^{2-} used were several orders of magnitude higher than concentrations ever observed in the field (up to nearly 2200). There is nothing to conclude from such experiments regarding what occurs in the field, especially in an experiment designed to describe the 'phenomenon that must have occurred in the Sanctuary after the CELCO-ARAUCO pulp mill began operations' (Mulsow & Grandjean 2006, p. 9). To explain why O_2-production experiments were performed with up to 9800 mg l^{-1} K_2SO_4, when the maximum SO_4^{2-} concentration reported in the river was 4.52 mg l^{-1}, Mulsow & Grandjean (2006) argued that these high concentrations were equivalent to the SO_4^{2-} concentration accumulated during 15 to 60 d. However, there is absolutely no guarantee that this was the case in the Cruces river, and no data are presented to support this. Also, Mulsow & Grandjean (2006) described these concentrations as 'equivalent doses', while this term represents a radioactive definition, not a concentration of a formal chemical compound. It is highly unlikely that any plant of *Egeria densa* has ever been exposed to such high concentrations of SO_4^{2-}, and therefore all conclusions based on the results of these experiments are fairly unrealistic.

Mulsow & Grandjean (2006) tested for differences among treatments using ANCOVA, comparing the slope of the relationship between O_2 and time (continuous variables) in different experimental treatments (discrete variable). This analysis also suffers from serious uncertainties both in presentation and interpretation. For instance, Mulsow & Grandjean (2006) stated, 'the rates of photosynthetic production of oxygen… were *not* statistically different', and the reported p-value suggested the contrary (i.e. significant differences, $p < 0.05$). The authors also state, 'when the same plants were exposed to a dose of SO_4 … photosynthetic rates decreased… and the differences were statistically significant', but there is no indication of the analysis applied (ANCOVA as well?) and its statistics (*F*- and p-values). Confusion reaches a maximum when, in the last sentence of the abstract, the authors reported the use of an ANOVA and $p < 0.05$, while in the text they indicated the use of an ANCOVA.

In summary, the conditions of the laboratory experiment have a reduced power to conclude the causes of disappearance of *Egeria densa* in the Sanctuary, and the inconsistent statistical analyses and interpretations raise serious concerns about the experimental results.

PLANT PHYSIOLOGICAL CONSIDERATIONS

Mulsow & Grandjean (2006) used volumetric measurements of O_2 release to quantify the photosynthetic rate of *Egeria densa*. This technique is somewhat obsolete (actually used only for teaching purposes) because it is not reliable, does not include other important parameters and only provides a rough estimation of photosynthetic activity. Thus, it cannot be used for comparison between treatments and never in a peer-reviewed research. The most useful and up-to-date system is the Clark electrode for O_2 evolution, which, in the liquid phase, is used to measure simultaneously temperature, light and pH within the leaf chamber (Mommer et al. 2005).

More doubts about the experimental setup of Mulsow & Grandjean (2006) arise from their Fig. 1: photosynthetic rates in pre-treatment plants showed large differences between the respective groups (from 0.5 to nearly 4.0 ml O_2 g^{-1}). A continuous increase in photosynthetic rate versus time appears in Figs. 1 & 2 (Mulsow & Grandjean 2006); however, under controlled conditions, this rate should be more or less constant, which suggest that some conditions in the experiments changed or that the values were cumulative in time (which is wrong: cumulative measurements are not good indicators of instantaneous rates). It should be noted that *Egeria densa* is not a C_4 vascular plant as Mulsow & Grandjean (2006) stated: this species, like other submerged plants (which are C_3-photosynthetic type, with a Kranz-less C_4-acid metabolism during light conditions), is able to concentrate CO_2 (using a mechanism similar to those of C_4 species) or uptake HCO_3^-, characteristics which are induced under stress conditions of low CO_2 level (Holaday & Bowes 1980, Casati et al. 2000, Leegood 2002).

Mulsow & Grandjean (2006) concluded that *Egeria densa* died in the Sanctuary after high input of SO_4^{2-} by the pulp mill of CELCO, that resulted in precipitation of calcium bicarbonate, causing a HCO_3^- deficit which induced a decrease in photosynthesis in *E. densa*. These authors found low photosynthesis, with an increase in photorespiration and resultant loss of turgidity and a change in colour (plants turning brownish). It is well known in plant physiology, however, that even when photosynthesis is equal to zero (with an increase in photorespiration or respiration), most plants usually remain alive (Larcher 1995). Moreover, aquatic species are able to use the CO_2 released from photorespiration or respiration (Leegood 2002). Considering the large concentrations of SO_4^{2-} used in the experiments, we could easily conclude that plants were probably subjected to osmotic stress (high sulphate salt concentration in the solution) or to the toxic effect of sulphate salt, which could induce an imbalance in the nutrient solution (or in the membrane transport process).

CHEMICAL MECHANISMS

The title of the paper by Mulsow & Grandjean (2006) is misleading in stating, 'Incompatibility of sulphate compounds and soluble bicarbonate…': SO_4^{2-} is compatible with HCO_3^- at the pH of the Cruces river (ca. 6.0 to 7.4) (UACh 2005). The sentence 'Calcium bicarbonate in water can be easily disassociated by a stronger anion such as SO_4^{2-}.' (Mulsow & Grandjean 2006, p. 6) is not correct because the authors refer to the following reaction:

$$HCO_3^- + SO_4^{2-} = CO_3^{2-} + HSO_4^-$$

with an equilibrium constant of 4×10^{-9} (Harris 1999). In other words, the former reaction does not occur as written, because it goes back to the left (to reactant). Furthermore, the same authors declared that 'After losing the ionic equilibrium, addition of SO_4^{2-} resulted in the loss of $(HCO_3)^-$ according to…' (see second equation of Mulsow & Grandjean 2006, p. 10), which is wrong because, as indicated above, there is no such loss. These authors also indicated that the addition of SO_4^{2-} results in the loss of HCO_3^- and then present the equation with H_2SO_4 instead of SO_4^{2-}. Finally, they write, 'Thus, a strong negative correlation results between SO_4 and $(HCO_3)^-$ as demonstrated here. At near neutral pH, this relationship can be summarized as…:' (see third equation of Mulsow & Grandjean 2006. p. 10). This equation is incorrectly presented, since concentrations must be written in individual form (i.e. $2[Ca^{2+} + Mg^{2+}]$ must be $2[Ca^{2+}] + 2[Mg^{2+}]$). It seems to us, that with this equation the authors are saying that when SO_4^{2-} increases the concentration of HCO_3^- decreases. However, it is not a traditional algebraic equation, but a charge balance. Since all solutions are electrically neutral, the addition of an extra amount of SO_4^{2-} also introduces the same amount of positive charge (i.e. cations; in the study by Mulsow & Grandjean [2006] these were K^+ ions from their use of K_2SO_4). The corresponding equation of the new charge balance of that dissolution is as follows: $[HCO_3^-] = 2[Ca^{2+}] + 2[Mg^{2+}] + [K^+] + 2[SO_4^{2-}]$. This shows that the concentration of HCO_3^- is not affected by the presence of SO_4^{2-}. The only factors that could affect the equilibrium of HCO_3^- in the Cruces river would be water temperature and, especially, pH. However, pH values in the river fluctuate between ca. 6.0 and ca. 7.4 (see UACh 2005).This is far lower than the value needed to change HCO_3^- into CO_3^- (i.e. nearly 10 to 11).

CONCLUSIONS

Using data from Mulsow & Grandjean (2006) we have demonstrated here that there is not a significant spatial variation in sulphate concentrations across the Cruces river. Furthermore, in our comment on the chemistry we have shown that sulphate and bicarbonate are compatible and not negatively related as proposed by Mulsow & Grandjean (2006). Both observations allow us to conclude that the unrealistic and reduced repeatability of their experimental design performed in order to test the effect of SO_4^{2-} on photosynthesis in *Egeria densa* has no empirical or theoretical basis. Thus, the question about the causes of the disappearance of this plant cannot be related to the interaction between sulphate and bicarbonate. The social, political and scientific confrontations derived from the Cruces river ecological disaster require precise and objective analyses to discuss its ultimate causes. Consequently, our best efforts are focused upon setting up a constructive dialogue among scientists, using scientific language and replicable methods. Also, as scientists, we are committed to sharing our results based on solid evidence with the public and the government, independently of which interests they support or threaten. This commitment includes the criticism of peer reviewed research, such as this paper.

Disclosure: The authors have no personal, financial and/or other conflict of interest with the pulp mill of CELCO, other timber industries or governmental agencies linked to the study area. Our main motivation is to use scientific methods to produce high-quality information to understand the causes of environmental degradation occurring in the Cruces river wetland ecosystem.

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