EXCELLENT PUBLISHERS

*International Journal of Current Research and Academic Review*

**ISSN: 2347-3215 Special Issue-2 (January-2015) pp. 182-189**

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# An experimental study of short term hypoxia and anoxia on phosphate concentrations and alkaline phosphatase activity in estuarine sediment

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**KEYWORDS**

Hypoxia, Anoxia, Phosphate concentrations, Alkaline Phosphatase activity

**A B S T R A C T**

Hypoxia (low oxygen) and anoxia (no oxygen) are rapidly expanding in estuaries and coastal waters, which have a significant impact on water quality and results in eutrophication. This study investigates the effect of overlying water oxygen on the exchange of phosphate between the surface sediment and the overlying waters using laboratory microcosms for 7 days. Short term effects of hypoxia and anoxia were also examined on alkaline phosphatase activity in the sediment. Oxygen were manipulated into five different treatments; oxic (96% saturation in the overlying water), hypoxia (25% saturation), one day of anoxia (24 hours without oxygen followed by re-aeration of up to 6 days), four days of anoxia (4 days without oxygen and subsequent re-aeration for 3 days) and seven days of anoxia (0% saturation). With decreasing oxygen supply, the concentrations of phosphate rapidly increased and alkaline phosphatase activities in the sediment were reduced. When phosphate concentration is low, alkaline phosphatase is produced by bacteria and phytoplankton to derive their phosphorus requirement from the dissolved organic phosphorus component. During anoxia, phosphorus is not limited due to phosphate release from iron immobilisation. So, less alkaline phosphatase activity is required in anoxia. This study suggests the importance of the duration of hypoxia and anoxia in regulating the balance between phosphate concentration and alkaline phosphatase activity in estuaries.

## Introduction

Phosphorus (P) is known as a limiting nutrient in the coastal and estuarine ecosystem, which gives an impact to the primary production (Paytan and McLaughlin, 2007). P is almost exclusively present as phosphate in natural environment (2). The main source of P in estuaries is through river runoff and the sink is through deposition and burial in the sediment (Slomp, 2011). Under different

environmental conditions, P can be released from the sediment into the overlying water. Oxygen is one of the important factors mediating phosphorus cycle in the estuaries. Sediments from anoxic condition release phosphate from the sediment exceeding the adsorption capacity. In oxic condition, released phosphate is rapidly readsorbed by iron oxyhydroxides or precipitated as apatite (Middelburg and Levin, 2009). At present, the coastal and estuarine nutrient biogeochemical cycle is constantly changing, with continuous persistence of anthropogenic impacts by increasing pollutions, wastes, and causing imbalance to the ecosystem functions (Madsen, 2008). Human intervention has also affected marine phosphorus cycle by doubling the source of phosphorus through sewage discharges and fertiliser runoff (Paytan and McLaughlin, 2007). Phosphate accumulation in the system may generate eutrophication, causing algal bloom (Howarth *et al.*, 2011) and eventually affect the food chain, which will in turn affect the environmental health of the ecosystem.

Several papers have investigated the effect of overlying water aeration on enzyme activities (Chene t al., 2011) and short term anoxia on enzyme activities and microbial activy (Bartoli *et al.*, 2009), but to my knowledge the effect of short term anoxia and reaeration on phosphate and alkaline phosphatase (AP) activity was not investigated. In this paper, we aim to contribute to the understanding of ecosystem functioning by relating the exchange of phosphate concentrations between the sediment and the overlying water with alkaline phosphatase activity in the sediment.

## Materials and Methods

Samples were collected during low tide in Breydon Water, Great Yarmouth, an inland tidal estuary in June, 2010 using 8.4 cm diameter and 30 cm high Plexiglas cylindrical cores. Sediment samples were filled up to 10 cm with the sediment, leaving 20 cm for the overlying water column. After sampling, the cores were brought back to the laboratory where they were left overnight and the incubations were started the following day according to the specification of the NICE (Nitrogen Cycling in Estuary) protocol handbook by (Dalsgaard *et al.*, 2000). The cores were covered with a plastic lid with two holes; the first one stoppered with a rubber stopper for water sampling and the second one with a tube for air or nitrogen bubbling. The cores were maintained in the absence of light to quantify benthic respiration (dark fluxes). The cores were kept at a constant temperature of 12 °C in a cold room, approximately equivalent to the *in-situ* temperature in the study area.

The experimental setup consisted of five treatments, 1) oxic (95% oxygen saturation) 2) hypoxic (25% oxygen saturation) 3) One day anoxic (1DA) (bubbling with oxygen free nitrogen, followed by subsequent re-aeration for another 6 days with air pump) 4) Four days anoxic (4DA) (bubbling with oxygen free nitrogen, followed by re-aeration with air pump for 3 days) and 5) anoxic (0% oxygen saturation). The oxygen saturation in oxic treatment was bubbled with air, the oxygen saturation in hypoxia manipulated by 3% of nitrogen, 97% of oxygen gas mixtures and the anoxic treatment was bubbled with nitrogen until 0% saturation was achieved.

Four day and one day anoxic treatment was carried out to determine how fast the system can recover from short term anoxic events followed by re-aeration over 3 days and 6 days, respectively. The 7 day experiment was selected in order to capture the consequences of short term hypoxia

(episodic hypoxia), which often lasts from several days up to a week (Sagasti *et al.*, 2003).

Twenty ml of overlying water were sampled daily for 7 days to measure phosphate concentrations in the overlying seawater. The water samples were filtered using 0.45 µm cellulose filters. The 20 ml nutrient samples were transferred to 30 ml universal bottles and kept at -20 °C and were analysed by the standard autoanalyser method using SKALAR autoanalyser. Porewater phosphate concentration was obtained by centrifugation for 10 min at 3000 rpm in 50 ml sterile polypropylene centrifuge tubes. The porewater was then filtered using a 0.2 µm disposable filter into 15 ml sterile polypropylene tubes. Before and after the 7 day incubation period, vertical profiles of porewater phosphate concentrations were determined from 1-9 cm depths using the SKALAR autoanalyser.

AP activity was determined fluorometrically using a microplate based enzyme assays according to the method of (Saiya-Cork *et al.*, 2002). One g of sediment suspensions was mixed with 125 ml 50 mM acetate buffer at a pH of 7.8 in a 250 ml beaker using an ultrasonicator water bath for 15 minutes. 200 µl of buffer was dispensed in wells that served as the blank, reference and substrate control. Next, 50 µl of buffer was dispensed in columns that served as the sample controls for the sediment. Then, 200 µl of sediment samples was dispensed into wells that served as the quench, sample control and sample assay.

Fifty microliters of a 10 µM 4 methylumbelliferone solution was dispensed into wells that served as reference standard and quench standard. Lastly, 50 µl of a 200 µM 4-methylumbelliferone phosphate was dispensed into plates that served as a substrate control and the sample assays. The samples to be assayed, buffer, substrates and standard concentrations of 250 µl are pipetted into 96 well, 300 µl black microwell plates (Nunclon Inc.) and fluorescence measured with a microplate reader (Spectramax M2). The fluorescence intensity was measured with 365 nm-excitation and 450 nm emission filters.

Differences between treatments and day of phosphate concentration, porewater phosphate concentrations and alkaline phosphatase activities between treatments and depths were tested using two-way ANOVA. Post hoc Bonferroni test was conducted to identify between which treatments, day and depth, significant differences occurred. Non-linear regressions between alkaline phosphatase activities with phosphate were also assessed. Statistical analyses were performed with software package SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 software (GraphPad Prism Software, San Diego, USA).

## Result and Discussion

Oxygen saturation in the overlying water was maintained daily at 95% in oxic, 25% in hypoxic and 0% in anoxic for 7 days (Figure 1a). Oxygen saturation in one day anoxia decreases from 95% to 0% and increased from 0% to 93% oxygen saturation after re-aeration for 6 days with air pump. Four day anoxia was maintained at 0% oxygen saturation for 4 days and re-aerated for 3 days with air pump, increasing the oxygen saturation to 22% saturation.

The concentration of phosphate remains low in the oxic treatment, but continues to increase in the hypoxic and anoxic treatment (Figure 1b). Phosphate concentration is low in the 1DA treatments and decreases after

day 4. In the 4DA treatments, phosphate concentration increased until day 4 and decreased from day 5 until day 7.

Porewater phosphate concentrations increased particularly below 5 cm depth in the hypoxic, 4DA and anoxic treatments. Porewater phosphate concentrations remained fairly constant in the oxic and 1DA treatments (Figure 2a). AP decreased significantly with depth and were influenced by treatments (two-way ANOVA, $p < 0.001$, for both depth and treatment effect, Figure 2b). Significant interactions between treatment and depth were found for AP (two-way ANOVA, $p < 0.001$). This results from higher AP activities at the surface of oxic and 1DA treatments relative to those at depths compared with the initial pattern.

AP was strongly correlated with porewater phosphate concentrations (Figure 3a–b). This relationship might be due to a response to porewater phosphate concentrations or to overall bacterial activity, which in turn is controlled by the availability of oxygen. Anoxia elevates porewater phosphate concentrations as observed in the deepest part of the anoxic treatment. AP is also low near the surface of the anoxic treatment and decreased with depth. Hence, it is more likely that this significant inverse relationship is due to a response to porewater phosphate concentrations as oxygen concentration is low throughout the anoxic treatment.

Phosphate concentrations for initial in Breydon Water, Great Yarmouth increase with depth as a response to low dissolved oxygen in deeper sediment (Conley *et al.*, 2009). With low oxygen, Fe (III) is reduced to Fe (II) releasing phosphate ions in the deeper sediment (Belias *et al.*, 2007). Alkaline phosphatase activity (AP) in the sediments ranges from 32.17 to 86.40 nmol $g^{-1}$ $h^{-1}$ within the range reported for intertidal mudflats of the German Wadden Sea by (Coolen and Overmann, 2000). AP was inversely related to porewater phosphate concentrations potentially because autrotroph need less AP when a free phosphate concentration is high (Jones, 2002).

In the oxic treatments, the low phosphate concentration over time may be attributed to the high removal of phosphate at the oxic sediment-water interface, which can be bound to the surface of carbonate grains of the sediments (Rasheed, 2004). Re-aeration after 1 day of anoxia decrease phosphate concentrations in the water column was probably due to the capture of released phosphate back to the sediment surface.

Similar sorption of phosphate from the water column to the sediment during a positive redox-shift in lab experiments has been described by Hupfer and Lewandowski (2008). After the shift from anoxic to oxic conditions, the sediment started to oxidise and bind phosphate more efficiently (Jordan *et al.*, 2008). In anoxic conditions, phosphate is continuously released due to iron mobilisation at the sediment-water interface (Middelburg and Levin, 2009).

Increasing AP was observed in the oxic treatments relative to that in the initial treatments. The inverse relationship of AP to porewater phosphate concentrations showed that AP is high when porewater phosphate concentration is low. There are two possible mechanisms that may cause this.

Firstly, high phosphate concentration is known to act as an inhibitor of AP, based on the catalysis of AP to produce phosphate from organic phosphorus.

Secondly, at high phosphate concentrations, there is a reduced requirement for the microorganisms to produce AP (Zhou *et al.*, 2008). In this study, increasing phosphate concentrations were observed in the deepest layers of anoxic sediment and presumably anaerobic microorganisms are not experiencing phosphate limitation, so the expression of AP was repressed (Steenbergh *et al.*, 2011).

When phosphate concentration is low in the water, microorganisms or plankton may produce phosphatase to hydrolyse organic phosphorus (Chen *et al.* 2011). Phosphate is immobilised by co-precipitation of Fe (III) in oxic condition, so AP must be released to allow microorganisms to overcome the resulting lower phosphate concentrations. AP is lower in the hypoxic, 4DA and anoxic treatments that may result from AP inhibition in the presence of phosphate concentrations (Jones, 2002) as well as by inhibition of the reduced metal from Fe (III) to Fe (II) in the sediment (Jordan *et al.*, 2008).

## Conclusions

Phosphate is released into the overlying water in low oxygen conditions. In oxygenated conditions, phosphate is directed into the sediments, driven by the redox chemistry of iron. So, overall, the research between the link of oxygen and phosphorus biogeochemistry is important from the perspective of ecosystem functions. A preliminary threshold of the severity of short term effects was also examined, which may offer beneficial values in terms of ecosystem management.

Hypoxia and anoxia up to 7 days alter the phosphate concentrations but short term anoxia up to 24 hours has only mild biogeochemical effect. Therefore, the duration of hypoxic and anoxic events on sediment biogeochemistry should be incorporated as an important factor influencing estuarine ecosystem studies if we are to further our understanding and predictive capabilities of the response of estuarine ecosystem to perturbations such as eutrophication or climate change.

a) b)

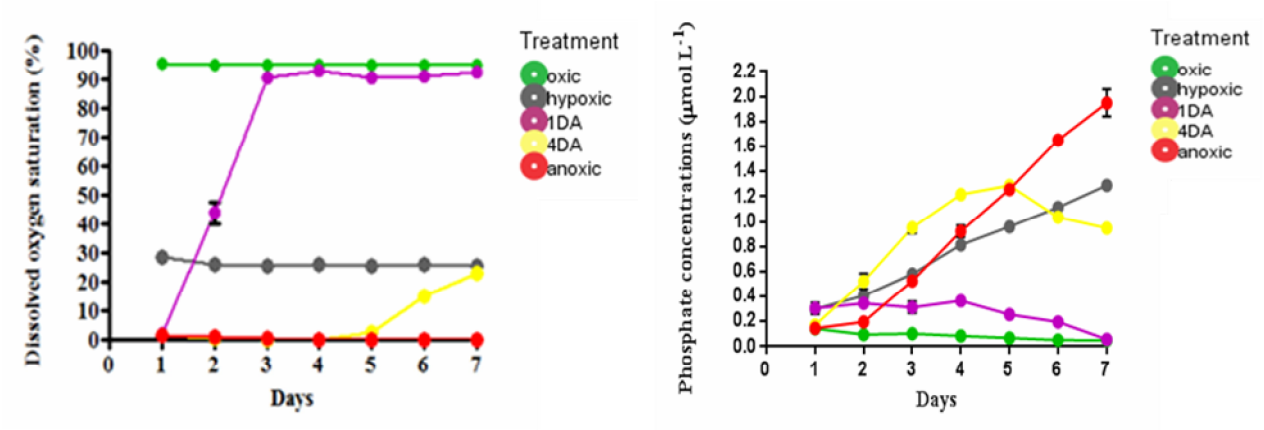


**Figure.1** Evolution of daily a) oxygen saturation and b) phosphate concentrations exposed to different oxygen treatments during the 7 day experiment. Values are reported as mean ± standard error of mean of 5 replicates. No vertical bar indicates standard error is smaller than the symbol size. Abbreviations, 1DA = 1 day anoxic, 4DA = 4 day anoxic.

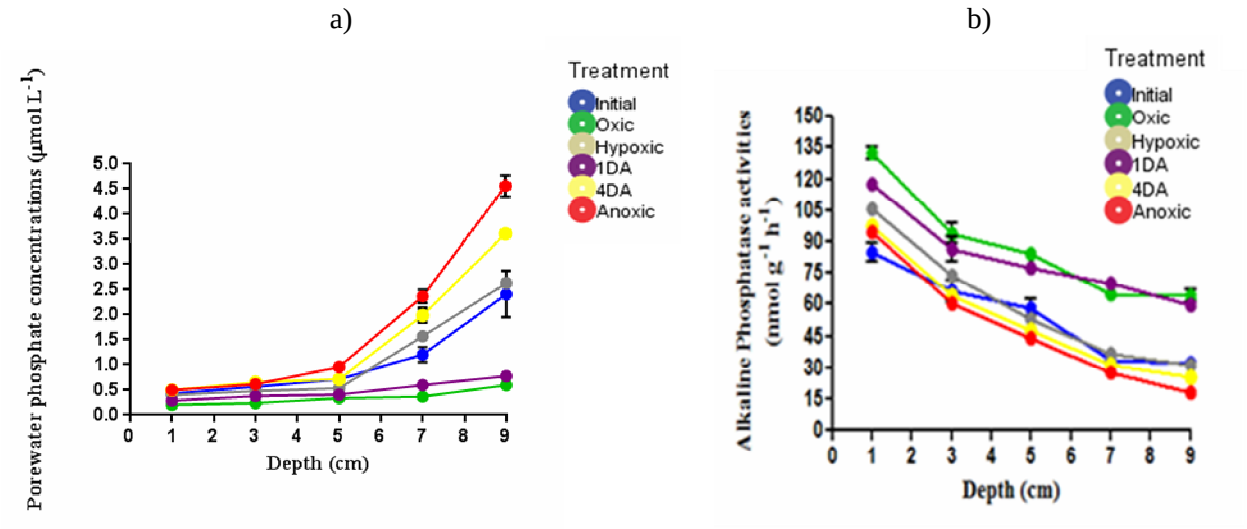


**Figure.2 a)**Porewater phosphate concentrations and **b)** vertical profiles of potential alkaline phosphatase activities in the sediment taken at the start and on day 7 of laboratory incubations from five different treatments, oxic, hypoxic, 1DA, 4DA and anoxic, reported as mean ± SE, n=5.

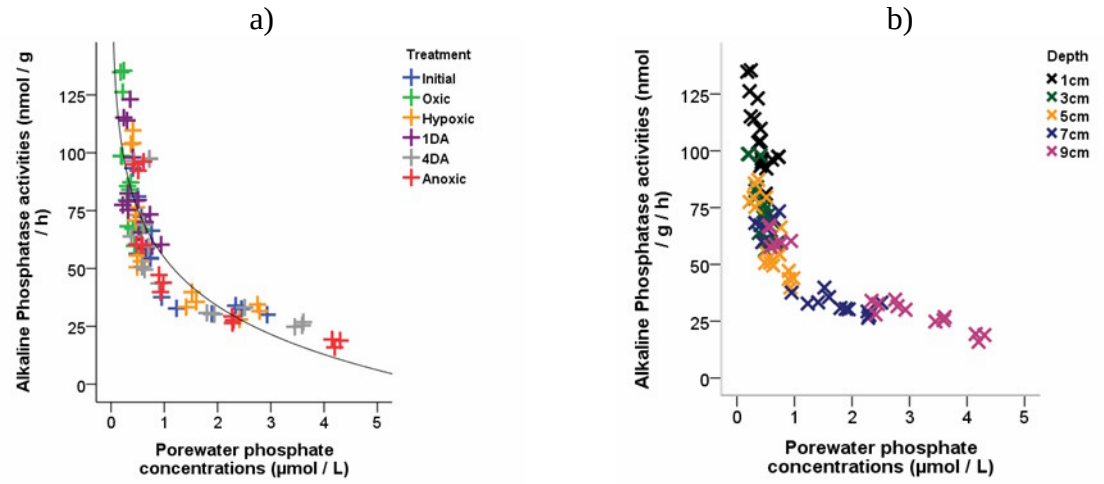


**Figure.3** Scatterplots of AP and pore water phosphate concentrations according to different **a)** treatments and **b)** depths

## Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or fal-sification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

## Acknowledgments

We would like to thank Jenny Stevenson and Simon Ellis for their assistance during field sampling, Kimberly Goodey for her technical expertise in using the SKALAR autoanalyser and Emily Sear for her assistance in setting up the experiments. The

authors declare that there is no conflict of interest.

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